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(54) Title: CONJUGATED VIRUS-LIKE PARTICLES AND USES THEREOF AS ANTI-TUMOR IMMUNE REDIRECTORS

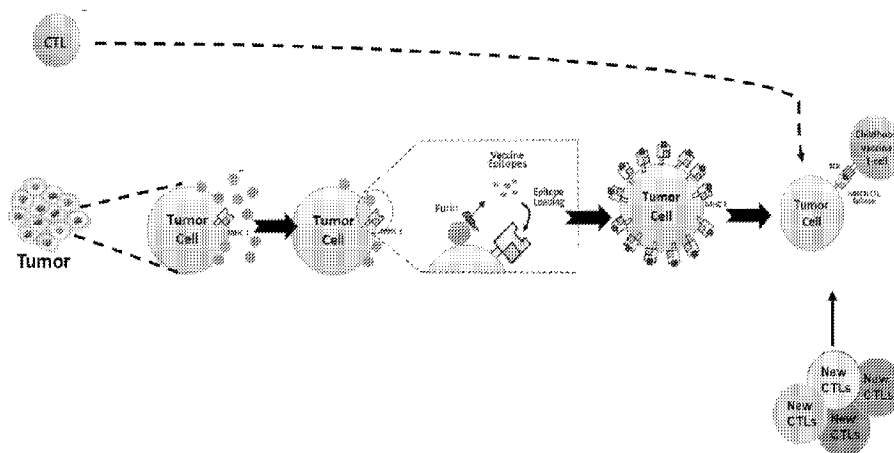


FIGURE 1

(57) **Abrégé/Abstract:**

Disclosed is a new class of conjugated virus-like particles (VLPs). These conjugated VLPs bind a wide variety of tumors and comprise epitopes recognized by a prior T cell immune response already existing in a host. These epitopes are derived from pathogens or previous vaccinations (such as early childhood vaccines). This provokes the body's pre-existing cytotoxic immunity obtained through previous infection or previous childhood vaccination to be redirected to the tumor cells for tire elimination of cancer, and form long-term anti-tumor immunity. The described conjugated VLPs are useful for tailoring a broad range of tumors towards a response from existing immunity circumventing the need to identify tumor antigens or generate tumor-specific immune responses, importantly, the compositions and methods described herein broadens opportunities for treatment for ail cancer types in subjects who previously had un-targetable cancers due to various technological and biological limitations of currently available immuno-therapeutic drugs.

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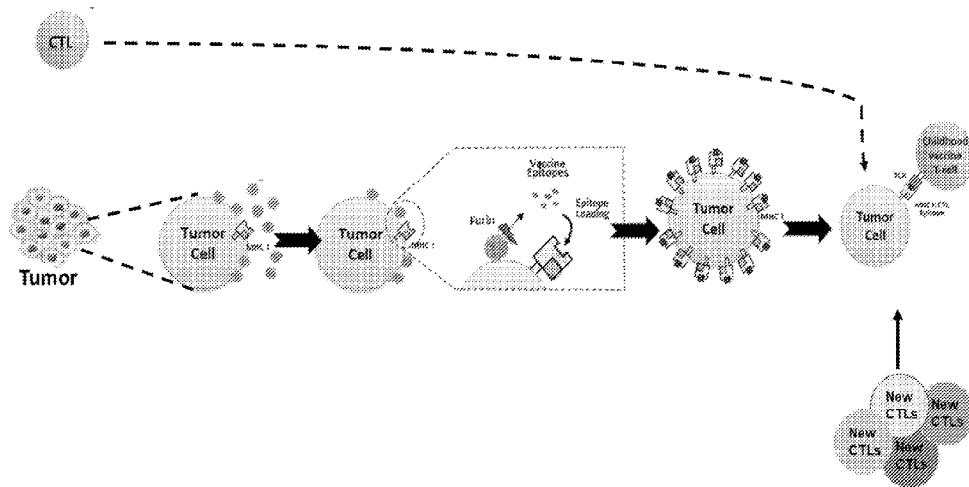


FIGURE 1

(57) Abstract: Disclosed is a new class of conjugated virus-like particles (VLPs). These conjugated VLPs bind a wide variety of tumors and comprise epitopes recognized by a prior T cell immune response already existing in a host. These epitopes are derived from pathogens or previous vaccinations (such as early childhood vaccines). This provokes the body's pre-existing cytotoxic immunity obtained through previous infection or previous childhood vaccination to be redirected to the tumor cells for tire elimination of cancer, and form long-term anti-tumor immunity. The described conjugated VLPs are useful for tailoring a broad range of tumors towards a response from existing immunity circumventing the need to identify tumor antigens or generate tumor-specific immune responses, importantly, the compositions and methods described herein broadens opportunities for treatment for ail cancer types in subjects who previously had un-targetable cancers due to various technological and biological limitations of currently available immuno-therapeutic

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CONJUGATED VIRUS-LIKE PARTICLES AND USES THEREOF AS ANTI-TUMOR IMMUNE REDIRECTORS

PRIORITY

[0001] This application claims priority to, and the benefit of, United States Provisional Patent Application Serial Number 62/785,502, filed December 27, 2018, which is hereby incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0002] Pursuant to the EFS-Web legal framework and 37 CFR §§ 1.821-825 (see MPEP § 2442.03(a)), a Sequence Listing in the form of an ASCII-compliant text file (entitled “8002PCT_ST25.txt” created on December 26, 2019, and 28,572 bytes in size) is submitted concurrently with the instant application, and the entire contents of the Sequence Listing are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The invention relates to conjugated virus-like particles (VLPs) that preferentially bind tumors. The VLPs of the invention include a recall protein capable of eliciting a T cell response using preexisting recall protein-specific T cells that selectively attack the tumor to which the VLP is bound.

BACKGROUND

[0004] According to the National Cancer Institute, the overall rate of cancer deaths continue to decrease: the overall cancer incidence rates have declined in men and have stabilized in women. The five-year survival has also improved for most but not all common cancers. And yet it is estimated that in 2017 there will be an additional 1,688,780 new cancer cases diagnosed and 600,920 cancer deaths in the US alone. Typical cancer treatment includes chemotherapy, radiation, and surgery. However, surgery is highly invasive and often fails—especially after metastasis. Chemotherapy and radiation can be effective, but result in harsh side-effects that drastically reduce quality of life. Despite these treatments, many cancers remain refractory to treatment and the treatments can be ineffective in combating metastatic cancers even when successful in reducing or eliminating the primary tumor. Targeted delivery

has become one of the most promising, but also most challenging, opportunities for improving the treatment of these diseases. The first attempts at developing delivery vehicles were antibody-drug conjugates. In almost all cases, the goal is to deliver cytotoxic T cells (CTLs) to the site of cancer cells to achieve selective killing of cancer cells. More recently, attempts have been made to use such immunotherapy to stimulate the immune system and specifically target proteins preferentially present on the surface of the cancer cell, resulting in targeted elimination of the cancer cells. Such therapies are attractive in that they are target specific, and potentially less toxic without nonspecific autoimmunity. They are also considered less invasive or traumatic compared to surgery, radiation or chemotherapy. However, cancer vaccines based on cancer-associated antigens can have limited success due to poor clinical immunogenicity, immune tolerance, and off target effects, for example. Moreover, such methods typically require identifying a cancer-associated antigen specific to a given patient's cancer to achieve effective targeting of the cancer.

[0005] Hence, this approach has failed on multiple occasions because most cancer-associated antigens are self-antigens that are tolerated by the immune system, resulting in poor immune responses. Importantly, successes demonstrated by these specific cancer antigen-specific immunotherapeutics in gold standard animal models have not been always translatable to humans. Last but not least, not all the patients suffering from cancer will express the same antigens on tumors, thus there is a limitation with respect to broad applicability. Recently, this field has now switched to focusing on individual tumor mutations within a patient which might result in a cancer-specific neoepitopes. Using these neoepitopes as antigens, termed "neo-antigens" has revived the cancer vaccine space. However, this is a highly personalized therapy that involves a lengthy development time that is both costly and difficult for widespread implementation. Similar limitations are also seen in conjugated antigen receptor T cell-based (CAR-T cell) strategies although curative

[0006] One class of therapeutics, which has been gaining favor is the immune checkpoint blockade therapies. This mode of treatment is based on the premise that tumor growth and progression is driven by its ability to prevent specific targeting and destruction by the immune system. In light of this, checkpoint inhibition therapies act to reverse such "immune-suppression", thereby reinitiating proper and effective antitumor function of the immune system. Indeed, these therapies have shown significant benefit for a variety of cancers (melanoma, colorectal cancer) that previously had very poor prognosis. However, it is well established that the tumor microenvironment dampens the ability of endogenous anti-tumor immune cells to eradicate cancers. As such, drugs that target the checkpoint pathway members

programmed cell death – 1 (PD-1) or programmed death ligand 1 (PD-L1) work to block immunosuppressive pathways, and have been shown to assist the body's immune system in fighting cancer. Unfortunately, checkpoint inhibitor drugs do not work in the majority of patients, with a dismal 70% non-responder rate. This is primarily due to the lack of a pre-existing anti-tumor CD8+ T cell immune response that can infiltrate the tumor. Hence, checkpoint inhibitors are generally considered ineffective in treating cancers that frequently lack a significant anti-tumor immune infiltrate (also sometimes known as “cold tumors” or “non-immunogenic”), especially during early phases of development when the tumor develops at the site of origin.

[0007] As a result of this, current treatments retain the same recognized limitations of toxicity, limited responder population (not applicable to a broad variety of cancers) and most importantly, they also possess a cost-prohibitive development pathway resulting in higher medical costs for the patient. It is therefore important to consider an alternative immunotherapeutic strategy that can circumvent the issues of immune tolerance as well as lack of immune infiltrates and thus there remains a need for compositions and methods that produce strong, durable, cancer-specific T cell responses to inhibit tumor growth, progression, and metastasis without undue characterization of individual patient cancer types.

SUMMARY OF THE INVENTION

[0008] In various embodiments a conjugated Virus Like Particle (“VLP”) is provided wherein the VLP comprises a capsid protein, wherein said capsid protein is capable of binding, or binds to, a cancer cell; and a fusion protein comprising at least a protein cleavage sequence, wherein the protein cleavage sequence is preferentially cleaved in the presence of a tumor; and at least one recall protein, wherein said recall protein is a protein or fragment thereof having a sequence that is an epitope capable of being bound by, or is bound by, an existing T cell in a patient.

[0009] In various embodiments, the capsid protein is from a papillomavirus. In various embodiments, the papillomavirus comprises an L1 protein. In various embodiments, the recall protein comprises the epitope of a pathogen. In various embodiments, the VLP comprises at least one fusion protein comprising at least two recall proteins. In various embodiments, the VLP comprises at least two fusion proteins each comprising a distinct recall protein. In various embodiments, at least two of the recall proteins are proteins or fragments thereof having a sequence from different T cell epitopes from the same pathogen. In various

embodiments, at least two of the recall proteins are proteins or fragments thereof having the sequence from different T cell epitopes for different pathogens.

[0010] In various embodiments, the pathogen is a virus, a bacterium, a fungus, or a parasite. In various embodiments the epitope is derived from, or comprised of a sequence or subset of the sequence of, an epitope of a human vaccine. In various embodiments the vaccine is an early childhood vaccine or a vaccine approved for adults. In various embodiments, the fusion protein is conjugated to a cysteine, a lysine, or an arginine of the capsid protein via a disulfide linkage, a maleimide linkage, or an amide linkage. In various embodiments, the capsid protein and the fusion protein are expressed contiguously as a fusion protein. In various embodiments, the vaccine is a vaccine for a pathogen. In various embodiments, the pathogen is a virus, a bacterium, a fungus, or a parasite. In various embodiments, the vaccine elicits immunity to a vaccinia virus, varicella zoster virus, a Herpes zoster virus, rubella, a hepatitis virus, e.g., hepatitis A virus, or hepatitis B virus, or hepatitis C virus, an influenza virus type A or type B, a measles virus, a mumps virus, a poliovirus, a variola (smallpox) virus, a rabies virus, dengue virus, Ebola virus, West Nile virus, a yellow fever virus, or a zika virus, or cytomegalovirus, or Epstein–Barr virus. In various embodiments the vaccine elicits an immunity to a bacterial infection, and wherein the bacterium is *Bordetella pertussis*, *Clostridium tetani*, *Chlamydia trachomatis*, diphtheria, *Hemophilus influenza*, *Meningococcus*, e.g., *Meningococcal ACWY*, *Pneumococcus*, *Vibrio cholera*, *Mycobacterium tuberculosis*, Bacille Calmette Guerin (BCG), typhoid, *E. coli*, *Salmonella*, *Legionella pneumophila*, *Rickettsia*, *Treponema pallidum pallidum*, *Streptococcus* group A or group B, *Streptococcus pneumonia*, *Bacillus anthracis*, *Clostridium botulinum*, or *Yersinia sp.* In various embodiments, the parasite is *Entamoeba histolytica*, *Toxoplasma gondii*, a *Trichinella sp.*, e.g., *Trichinella spiralis*, a *Trichomonas sp.*, e.g., *Trichomonas vaginalis*, a *Trypanosoma sp.*, e.g., *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, or a *Trypanosoma cruzi*, or a *Plasmodium*, e.g., *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

[0011] In various embodiments, the VLP is capable of eliciting a T cell response of a threshold of at least 2-fold above baseline of total CD8+ T cells. In various embodiments, the CD8+ T cells are also CD69+. In various embodiments, the CD8+ T cells are also CD69+CD103+. In various embodiments, the CD8+ T cells are Tissue Resident Memory T cells (TRM cells) originating from non-lymphoid organs. In various embodiments the capsid protein is capable of selectively binding a heparin sulfate proteoglycan (“HSPG”).

[0012] In various embodiments, a method is provided for treating a subject with a cancer, comprising administering a conjugated virus-like particle (VLP), wherein the VLP comprises: (a) a capsid protein, wherein said capsid protein is capable of binding a cancer cell; and (b) a fusion protein comprising at least: i) a cleavage sequence, wherein the cleavage sequence is preferentially cleaved in the presence of a tumor; and ii) at least one recall protein, wherein said recall protein is a protein or fragment thereof having a sequence that is an epitope capable of being bound by an existing T cell in a patient wherein the cleavage sequence is bound to the capsid protein. In various embodiments the method further comprises a second administration of the recall protein. In various embodiments, the second administration of the recall protein is delivered as a conjugated VLP. In various embodiments, the second administration of the recall protein is delivered as a vaccine. In various embodiments, the second administration of the recall protein is delivered as an isolated peptide in an adjuvant.

[0013] In various embodiments a method is provided for providing a conjugated virus-like particle (VLP) to a patient in need thereof, comprising: (i) measuring preexisting immunity in a patient; and (ii) selecting an appropriate conjugated VLP for administration to a patient in need thereof, said VLP comprising: (a) a capsid protein, wherein said capsid protein is capable of binding a cancer cell; and (b) a fusion protein comprising at least: i) a cleavage sequence, wherein the cleavage sequence is preferentially cleaved in the presence of a tumor; and ii) at least one recall protein, wherein said recall protein is a protein or fragment thereof having a sequence that is an epitope capable of being bound by an existing T cell in a patient, wherein the cleavage sequence is bound to the capsid protein; wherein the T cell epitope can elicit a T cell the baseline of total CD8+ CD69+ positive T cells.

[0014] In various embodiments, a boosting vaccine is delivered to a patient. In various embodiments, the boosting vaccine is delivered at least two weeks following the administration of the conjugated VLP. In various embodiments, the boosting vaccine is delivered two weeks before the administration of the conjugated VLP. In various embodiments, the vaccine is shingles vaccine, a PREVNAR13® vaccine, a HEPLISAV-B® vaccine, an MMR-II vaccine, ZOSTAVAX®, or ENGERIX-B®. In various embodiments the vaccine is for shingles. In various embodiments, the vaccine is PREVNAR13®. In various embodiments, the vaccine is HEPLISAV-B®. In various embodiments the subject is a human patient at least 50 years old. In various embodiments, the patient was previously treated with a CAR-T, therapeutic vaccine, check point inhibitor, oncolytic virus, neo-antigen vaccine, neo-adjuvant, chemo-therapy, radiation, surgery, but was previously not shown to have an anti-tumor effect.

In various embodiments, the recall protein is not a tumor associated antigen. In various embodiments, the method inhibits the growth, progression or metastasis of a tumor.

[0015] In various embodiments, the tumor is a small lung cell cancer, hepatocellular carcinoma, liver cancer, hepatocellular carcinoma, melanoma, metastatic melanoma, adrenal cancer, anal cancer, aplastic anemia, bile duct cancer, bladder cancer, bone cancer, brain or central nervous system (CNS) cancer, breast cancer, cancer of unknown primary origin, Castleman disease, cervical cancer, colon/rectum cancer, endometrial cancer, esophagus cancer, Ewing family of tumors, eye cancer, gallbladder cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (GIST), gestational trophoblastic disease, Hodgkin disease, Kaposi sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, leukemia, liver cancer, lung cancer, lymphoma, malignant mesothelioma, multiple myeloma, myelodysplastic syndrome, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, penile cancer, pituitary tumors, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma, skin cancer, stomach cancer, testicular cancer, thymus cancer, thyroid cancer, uterine sarcoma, vaginal cancer, vulvar cancer, Waldenstrom macroglobulinemia, Wilms tumor, non-Hodgkin lymphoma, Hodgkin lymphoma, Burkitt's lymphoma, lymphoblastic lymphomas, mantle cell lymphoma (MCL), multiple myeloma (MM), small lymphocytic lymphoma (SLL), splenic marginal zone lymphoma, marginal zone lymphoma (extra-nodal or nodal), mixed cell type diffuse aggressive lymphomas of adults, large cell type diffuse aggressive lymphomas of adults, large cell immunoblastic diffuse aggressive lymphomas of adults, small non-cleaved cell diffuse aggressive lymphomas of adults, or follicular lymphoma, head and neck cancer, endometrial or uterine carcinoma, non-small cell lung cancer, osteosarcoma, glioblastoma, or metastatic cancer. In one embodiment, the cancer is a breast cancer, a cervical cancer, an ovarian cancer, a pancreatic cancer, or melanoma,

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 illustrates the mechanism of action of the disclosed conjugated VLPs.

[0017] FIG. 2A shows mouse TC-1 tumor growth over time for C57BL/6 mice where the TC-1 tumors express both green fluorescent protein (GFP) and luciferase. FIG. 2B shows a graphical representation of fluorescence activated flow cell sorting cytometry (FACS) data generated from analysis of the TC-1 tumors excised from the C57BL/6 mice either treated

with VLP (lighter shaded peak) or not treated with VLP (darker shaded peak). Left panel is gated for GFP the right panel is gated to VLP detected by way of an phycoerythrin (PE)-conjugated anti-mouse antibody specific for VLP.

[0018] FIG. 3 illustrates the E7 T cell-mediated cytotoxic effects of HPV16 E7 conjugated VLP in a B16 luciferase and ID8 luciferase tumor cell model *in vitro* in a time dependent manner. Particularly, FIG. 3A and FIG. 3B show B16-LUC cells incubated with varying concentrations of E7 peptide alone or E7 peptide conjugated to VLP at 5.5 hrs and 8.5hrs, respectively. Likewise, FIG. 3C and FIG. 3D show ID8 cells incubated with E7 peptide alone or E7 peptide conjugated to VLP at 5.5 hrs and 8.5 hrs, respectively.

[0019] FIG. 4 illustrates the E7 T cell-mediated cytotoxic effects of HPV16 E7 conjugated VLP in a B16 luciferase and ID8 luciferase tumor cell model *in vitro* in a time dependent manner. Particularly, FIG. 4A and 4B show that cytotoxicity is improved between 8.5 hrs and 22.5 hrs, respectively, for E7-conjugated VLP incubated with B16-LUC cells and murine CD8+ HPV16 E7-specific T cells. Similarly, FIG. 4C and 4D show that cytotoxicity is improved between 8.5 hrs and 22.5 hrs, respectively, for E7-conjugated VLP incubated with ID8 cells and murine CD8+ HPV16 E7-specific T cells.

[0020] FIG. 5 illustrates batch consistency of E7 T cell-mediated cytotoxic effects of HPV16 E7 conjugated VLP (batches F1-F3) on B16 luciferase and ID8 luciferase cells after two separate studies ending at 23hrs and 23.5 hrs time points, respectively. FIG. 5A and FIG. 5B show two separate studies ending at time points, 23 hrs and 23.5 hrs, respectively, of E7 T-cell-mediated cytotoxic effects of three independently created batches of conjugated VLP conjugated to E7 peptide in B16-LUC cells. FIG. 5A and FIG. 5B show two separate studies ending at time points, 23 hrs and 23.5 hrs, respectively, of E7 T-cell-mediated cytotoxic effects of three independently created batches of conjugated VLP conjugated to E7 peptide in ID8 cells.

[0021] FIG. 6 illustrates batch consistency of E7 T cell-mediated cytotoxic effects of HPV16 E7-conjugated VLP on MC38 cells after two separate studies ending at 23 or 23.5 hrs, time points respectively. For three different batches of conjugated E7-conjugated VLPs. FIG. 6A represents results from the 23 hr timepoint study and FIG. 6B represents results from the 23.5 hr timepoint study.

[0022] FIG. 7 provides *in vivo* efficacy data as measured by bioluminescence in a C57BL/6 mouse model injected with either MHC-restricted H-2D^b HPV16 E7 peptide, buffer control, VLP-conjugated to the E7, or unconjugated VLP alone. In each instance the mice were thereafter injected with murine specific H-2D^b HPV16 E7 T cells. FIG 7A shows results for all

controls and test mice. Individual lines in FIG 7A represent average tumor growth for all control and test mice respectively FIG. 7B provides buffer control results. FIG. 7C provides results for unconjugated VLP. FIG. 7D provides results for E7-conjugated VLP. FIG. 7E provides results for E7 peptide alone. Individual lines in FIG. 7B represent separate mice in each group.

[0023] FIG. 8 illustrates the *in vivo* efficacy of E7-conjugated VLP anti-tumor immune redirection in B16.F10 mice possessing an acute immune response, i.e. mice were treated 2 weeks post peptide RAHYNIVTF (SEQ ID NO: 1) vaccination, to the HPV16 E7 peptide RAHYNIVTF (SEQ ID NO: 1), by assessment of tumor volume. FIG. 8A provides a graphical representation of tumor size data vs. days post-injection of various samples including control VLP, i.e. unconjugated VLP, and E7-conjugated VLP. FIG. 8B provides graphical representation of mouse survival rate post B16.F10 tumor cell injection for various samples, including control VLP, i.e. unconjugated VLP alone, and E7-conjugated VLP. FIG. 8C and FIG. 8D are similar to Figures 8A and 8B, except that the HPV16 E7 immune response was in memory phase, i.e. mice were treated 6 weeks post peptide RAHYNIVTF (SEQ ID NO: 1) vaccination,; and that the tumor volumes prior to treatment was between 50-100 mm³. The mice were then challenged with tumors and E7-conjugated VLP injected into the mice and survival rates quantitated.

[0024] FIG. 9 shows SIINFEKL/K^b (SEQ ID NO: 2) FACS analysis of mouse tumor cells incubated *in vitro* with OVA-conjugated VLP. FIG. 9A shows FACS data resulting from incubation of ID8-LUC (top panel), B16-LUC (middle panel), and MC38 (lower panel) cells with OVA peptide alone or OVA-conjugated VLP. FIG. 9B shows the same cells, ID8-LUC (top panel), B16-LUC (middle panel), and MC38 (lower panel), stained by binding of an H-2D^b antibody specific for MHC-I complexed with OVA peptide.

[0025] FIG. 10 provides bar graph data for CMV-conjugated VLP triggered immune redirection cytotoxicity of various immortalized human tumor cell lines including HLA.A2 positive HCT116 cells (FIG. 10A), OVCAR3 cells (FIG. 10B), and MCF7 cells (FIG. 10C). The bars in each graph, from left to right, represent tests performed with no antigen, CMV pp65 HLA-A*0201 (NLVPMVATV)(SEQ ID NO: 3) peptide (henceforth "CMV peptide") alone at 1 µg/mL, VLP alone, i.e. non-conjugated VLP at 2.5 1 µg/mL, CMV peptide conjugated to VLP at 2.5 µg/mL, and CMV peptide alone at 14 pg/mL.

[0026] FIG. 11 is a photograph of an SDS-PAGE analysis of reducing conditions for VLP conjugation. The left lane is a molecular weight standards lane. The next lanes in the

gel are various conjugation reaction conditions utilizing tris(2-carboxyethyl)phosphine (TCEP) in various ratios to RG1 VLP.

[0027] FIG. 12 is a photomicrograph generated by transmission electron microscopy (TEM) of conjugated VLPs post conjugation reaction utilizing varying ratios of TCEP to RG1 VLP. The upper left panel shows the results of conjugation following reaction of TCEP with RG1 at a 10:1 ratio. The upper right panel shows the same reaction at ratio 3:1, bottom left at 5:1, and bottom right at 1:1.

[0028] FIG. 13 is a photograph of an SDS-PAGE analysis of the level of conjugation on RG1 VLP, reduced at various ratios with TCEP during the VLP conjugation reaction as indicated in the gel.

[0029] FIG. 14 provides photographs of SDS-PAGE analysis of WT VLP (FIG. 14A) and RG1 (FIG. 14B) conjugated to various vaccine epitopes.

DETAILED DESCRIPTION

Definitions

[0030] This specification describes exemplary embodiments and applications of the disclosure. The invention, however, is not limited to these exemplary embodiments and applications or to the manner in which the exemplary embodiments and applications operate or are described herein. Various embodiments, features, objects, and advantages of the present teachings will be apparent from the description and accompanying drawings, and from the claims. As used herein, the terms "comprise", "comprises", "comprising", "contain", "contains", "containing", "have", "having" "include", "includes", and "including" and their variants are not intended to be limiting, are inclusive or open-ended and do not exclude additional, unrecited additives, components, integers, elements or method steps. For example, a process, method, system, composition, kit, or apparatus that comprises a list of features is not necessarily limited only to those features but may include other features not expressly listed or inherent to such process, method, system, composition, kit, or apparatus.

[0031] "About" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0032] "Conjugated virus-like particle" or "conjugated VLP" as used herein is a VLP that also comprises a fusion protein bound to the capsid proteins of the VLP.

[0033] "Cleavage sequence" as used herein may include specific peptide sequences, or more often, peptide motifs at which site-specific proteases cleave or cut the

protein. Cleavage sites can be used, for example, to cleave off an affinity tag thereby restoring the natural protein sequence or to inactivate a protein or to activate proteins. In the present invention the cleavage is referring to proteolytic cleavage. In various embodiments, the proteolytic cleavage is carried out by peptidases, proteases or proteolytic cleavage enzymes before the final maturation of the protein. Proteins can also be cleaved as a result of intracellular processing of, for example, misfolded proteins. Another example of proteolytic processing of proteins is secretory proteins or proteins targeted to organelles, which have their signal peptide removed by specific signal peptidases before release to the extracellular environment or specific organelle. In one embodiment of the present invention, the cleavage sequence is specifically recognized by the furin which cleaves and releases the recall proteins from the conjugated VLP, making them available for loading onto the tumor surface. In various embodiments, the cleavage sequence is comprised of cysteine, lysine and/or arginine residues, which not only allow the recall protein to be cleaved from the VLP, but also serve as anchors to conjugate the recall protein to the capsid protein until release by cleavage protein, such as furin which are selectively present at the site of the tumor.

[0034] "Epitope" is a set of amino acid residues that create recognition by or are recognized by a particular immunoglobulin or, in the context of T cells, those residues necessary for recognition by T cell receptor proteins and/or major histocompatibility (MHC) receptors. The amino acid residues of an epitope need not be contiguous/consecutive. In an immune system setting, *in vivo* or *in vitro*, an epitope may be a composite of the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a three dimensional structure recognized by an immunoglobulin, T cell receptor, and/or HLA molecule.

[0035] "Fusion Proteins of the invention comprise at least: (1) a cleavage sequence, wherein the cleavage sequence is preferentially cleaved at the site of the surface of a tumor; and (2) at least one recall protein wherein said recall protein is a protein or fragment thereof having a sequence that is an epitope capable of being bound by an existing T cell in a patient); and (3) is capable of being covalently linked to the VLP. Conjugated VLPs preferentially bind to tumor cells.(If it is only conjugated that bind remove edit above for VLP, otherwise remove preceding).

[0036] "HPV" and "human papillomavirus" refer to the members of the family Papillomavirus that are capable of infecting humans. There are two major groups of HPVs defined by their tropism (genital/mucosal and cutaneous groups), each of which contains multiple virus "types" or "strains/genotypes" (e.g., HPV 16, HPV 18, HPV 31, HPV 32, etc.).

[0037] "Human vaccine" as used herein means a biological preparation that improves immunity to a particular disease in a human. A vaccine typically contains an antigenic agent(s) that resembles a disease-causing agent (pathogen), and is often made from weakened or killed forms of the microbe, its toxins or one or multiple immunogenetic surface proteins of the disease causing agent. The antigenic agent stimulates the body's immune system to recognize the disease causing as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these pathogens should actual infection/exposure occur. Human vaccines include vaccines against viral diseases and bacterial diseases. In various embodiments, vaccines against viral diseases include hepatitis A, B, E virus, human papillomavirus, influenza virus, Japanese encephalitis virus, measles virus, mumps virus, polio virus, rabies virus, rotavirus, rubella virus, tick-borne encephalitis virus, varicella zoster virus, variola virus, and yellow fever virus. Human vaccines against viral diseases those are under development include dengue vaccine, eastern equine encephalitis virus, HTLV-1 T lymphocyte leukemia vaccine, and respiratory syncytial virus vaccine. Such a vaccine include, in some embodiments, current vaccines in development or currently United States Food and Drug Administration (FDA)-approved vaccinations. Examples of vaccines that are compatible are listed in Table 2. The embodiments of the invention are not limited to the listed vaccines, and could apply to any vaccine developed to provide immunity in a human subject.

[0038] "Inhibiting," "reducing," or "prevention," or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition/reduction or elimination to achieve a desired result, such as inhibiting, reducing, or preventing or reducing tumor mass, progression and/or metastasis.

[0039] "MHC" or "major histocompatibility complex" is a group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. MHC proteins are found in all higher vertebrates. There are two main types of MHC molecules, MHC class I and MHC class II. In humans there are three different genetic loci that encode MHC class I molecules (the MHC-molecules of the human are also designated human leukocyte antigens (HLA)): HLA-A, HLA-B, and HLA-C. HLA-A*01, HLA-A *02, and HLA-A * 11 are examples of different MHC class I alleles that can be expressed from these loci.

[0040] "Papillomavirus" refers to all members of the papillomavirus family (*Papillomaviridae*). An extensive list of papillomavirus types and the ability to make the respective VLPs can be referenced using this publication: "Classification of papillomaviruses

(PVs) based on 189 PV types and proposal of taxonomic amendments,” de Villers et al., 401(1):70-79, 2010, PMID: 20206957 (all the tables).

[0041] “Preferentially cleaved protein” as used herein means that the fusion protein is preferentially cleaved from the capsid protein at the site of a tumor. This preferential tumor-site cleavage may be due to: (1) the unique cleavage sequence on the fusion protein, and/or (2) the unique tumor microenvironment. For example, in one embodiment, the fusion protein comprises a cleavage sequence that is preferentially cleaved by the enzyme furin, which is known to be expressed in high concentrations in tumor cells.

[0042] “Protein,” “polypeptide,” and “peptide,” as used herein, are not restricted to any particular number of amino acids; these terms are sometimes used interchangeably herein. The properties and amino acid sequences of the proteins of the invention, and of the nucleic acids encoding them, are well-known and can be determined routinely, as well as downloaded from various known databases. (See, e.g., the NCBI GenBank databases). Some sequences are provided herein. However, some sequence information is routinely updated (e.g. to correct mistakes in the previous entries), so updated (corrected) information about the proteins and nucleic acids encoding them is included in this application. Information provided in the sequence databases discussed herein is incorporated by reference.

[0043] “Recall protein” as used herein refers to a protein or fragment thereof derived from a vaccine or pathogen to which the patient or subject has been previously exposed, this previous exposure having resulted in durable T cells that recognize and are specific for the recall protein. A recall protein is distinguished from a tumor-specific antigen.

[0044] A “recall response” is an immune response in which an antigen-primed cytotoxic T cell, Th1 T cell, Th2 T cell, and/or B cells primed by a vaccine or other pathogen present in the patient binds the recall protein.

[0045] A “subject,” or “subject in need thereof” as used herein, includes any animal that has a tumor/cancer or has had a tumor/cancer or has a precancerous medical condition or cell. Suitable subjects (patients) include laboratory animals, such as mouse, rat, rabbit, guinea pig, or pig, farm animals, such as cattle, sporting animals, such as dogs or horses, domesticated animals or pets, such as a horse, dog, or cat, nonhuman primates, and humans.

[0046] “T cell response” as used herein refers to the immune response elicited by T cells as they encounter antigens. Naïve mature T cells are activated upon encountering antigen presented by B cells, macrophages, and dendritic cells, and produce armed effector T cells. Effector T cells are either CD8+ T cells that differentiate into cytotoxic T cells, or CD4+ T cells that primarily induce the humoral immune response. The T cell immune response

further generates immunological memory that gives protection from the subsequent challenge by the pathogen. In various embodiments, the T cell response is at a threshold of at least 2-fold above the baseline of total CD8+ T cells. In various embodiments, the CD8+ T cells are CD69+ as well.

[0047] "Therapeutic compositions" are compositions that are designed and administered to patients. Therapeutic compositions, e.g., therapeutic conjugated VLP-containing compositions, are used to treat benign or malignant tumors or patients/subjects at risk for such tumors. In some embodiments, the conjugated VLPs are administered to a subject who previously had a tumor and is currently apparently tumor/cancer free, in an effort to enhance the inhibition or the recurrence of the tumor/cancer.

[0048] "Virus-like particle" or "VLP" refers to a multi-protein structure comprised of viral structural proteins, such as envelop or capsid proteins that can self-assemble into a particle that resembles a virus but lacks the viral genetic material. VLPs are non-infectious and non-replicating, yet morphologically similar to viruses. The VLPs disclosed herein bind to or have an inherent tropism for tumor cells.

[0049] "VLP vaccine" refers to a formulation, which contains 1, 2, 3, 4, 5, or more conjugated VLPs described herein. In one embodiment, compositions comprising the conjugated VLP described herein are in a form that is administered to a subject in order to redirect immunity already existing in the subject and to thereby inhibit the proliferation, growth, and/or metastasis of a tumor in the subject. Typically, a VLP vaccine comprises a conventional saline or buffered aqueous solution medium in which the compositions described herein are suspended or dissolved, although administration of dry powder, for example by inhalation, and even formulation with an additional adjuvant, such as alum, is also contemplated. The composition of the present invention can be used to inhibit the proliferation, growth, and/or metastasis of a tumor. Upon introduction into a host, a conjugated VLP-containing composition of the invention (e.g., a VLP vaccine) is able to provoke an immune response including, but not limited to, the production of cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses.

Conjugated Virus-Like Particles

[0050] In various embodiments, disclosed are conjugated VLPs for treating cancer in a subject. In various embodiments, the compositions described herein comprise a capsid

protein, wherein said capsid protein binds a cancer cell. In various embodiments the compositions described herein further comprise a fusion protein comprising at least: (1) a cleavage sequence, wherein the cleavage sequence is preferentially cleaved in the presence of a tumor; and (2) at least one recall protein, wherein said recall protein is a protein or fragment thereof having a sequence that is an epitope that is bound by an existing T cell in a patient. In various embodiments the cleavage sequence is bound to the capsid protein.

[0051] Capsid Proteins. In various embodiments, a VLP is provided. VLPs are comprised of viral structural particles, i.e. capsid proteins that self-assemble into a particle that resembles a virus but lacks the viral genetic material of a virus. VLPs are excellent delivery molecules because they are non-infectious and can be re-engineered to specifically target or bind to tumor cells.

[0052] It has been reported that HPV capsids (VLP and pseudovirions (PsV)) have tumor tropism and directly bind and infect tumor cells, including, e.g., ovarian and lung cancer cells. In various embodiments the conjugated VLPs described herein preferentially bind to tumor cells, e.g., they bind more to tumor cells than to non-tumor cells, and the presence of conjugated VLPs may result in a positive pro-inflammatory tumor microenvironment that attracts infiltrating CD8+ T cells. At the same time, the VLPs may stimulate a response by adaptive memory T cells that resulted from a previous vaccination or infection and that recognize the recall protein's CD8+ T cell epitope. In various embodiments these strong responses are able to bypass immune tolerance, and render the tumor cells susceptible to this preexisting immunity thereby inhibiting the growth, progression and metastasis of the tumor.

[0053] Any VLP that preferentially binds to tumor cells relative to normal cells may be used with the present invention. In various embodiments, the VLP is an animal virus-based VLP. In various embodiments, the animal virus-based VLP is derived from HBVc or HPV. In various embodiments, the VLP is a bacteriophage-based VLP, such as MS2, Q β , or P22. In various embodiments, the VLP is a plant virus-based VLP such as cowpea chlorotic mottle virus (CCMV) or cowpea mosaic virus (CPMV). In various embodiments, the VLP is a currently commercialized prophylactic VLP-based vaccine, including GlaxoSmithKline's ENGERIX® (hepatitis B virus) and CERVARIX® (human papillomavirus), and Merck and Co., Inc.'s RECOMBIVAX HB® (hepatitis B virus) and GARDASIL® (human papillomavirus). Other embodiments of the VLP include VLP-based vaccine candidates in clinical trials or preclinical evaluation, such as, influenza virus, parvovirus, and Norwalk virus. In various embodiments, the VLP is a hamster polyoma virus, *Macrobrachium Rosenbergii* *Nodavirus*, HBsAg, HCV, retrovirus, or an HBc.

[0054] In one embodiment, the VLP is comprised of structural proteins derived from a papillomavirus. The papillomavirus virion contains 72 pentamers (capsomeres) of L1 protein. The L1 protein is capable of self-assembly into capsid-like structures that are morphologically indistinguishable from native virions when expressed in eukaryotic cells. The L1 monomer contains 12 b-strands, 6 loops (BC, CD, DE, EF, FG, HI), and 5 helices (H1-H5). Most of the loops are highly exposed towards the outer surface of the capsid, attachment of a fusion protein to one of these loops, by, e.g., disulfide linkage, maleimide linkage, by “click” chemistry or by binding to a polyionic docking site, as described herein, in these areas will result in the recall protein being displayed on the outer surface of VLPs.

[0055] **Papillomavirus VLPs.** In various embodiments, a conjugated VLP is provided comprising a papilloma (PV) L1 protein, or an PVL1 and PV L2 protein. The VLP in some embodiments comprises both papilloma L1 and L2 proteins.

[0056] In an embodiment, the conjugated papillomavirus VLP comprises an L1 capsid protein and a fusion protein. In other embodiments, the conjugated VLP comprises an L1 capsid protein, an L2 capsid protein, and a fusion protein. The L1 polypeptide can be full length L1 protein or an L1 polypeptide fragment. In specific embodiments, the full-length L1 protein or L1 polypeptide fragment is VLP assembly-competent; that is, the L1 polypeptide will self-assemble to form capsomeres that are competent for self-assembly into higher-order assemblies, thereby forming a VLP. In more specific embodiments, the VLPs comprise a fully assembled papillomavirus capsid, a structure of about 50 nm and composed of 72 capsomeres or 360 copies of L1 protein.

[0057] The L1 sequences are known for substantially all papillomavirus genotypes identified to date, and any of these L1 sequences or fragments are contemplated as being employed in the present compositions. Examples of L1 polypeptides include, without limitation, full-length L1 polypeptides, e.g., HPV16 L1 polypeptide, SEQ ID NO: , L1 truncations that lack the native C-terminus, L1 truncations that lack the native N-terminus, and L1 truncations that lack an internal domain. The L1 protein may be for example a modified L1 protein, e.g., a modified HPV16 L1 protein wherein the HPV16 L2 amino acids 17-36 (RG1 epitope) are inserted within the DE-surface loop of HPV16 L1. (See, Schellenbacher et al., 2013, *J. Invest Dermatol*; 133(12):2706-2713; Slupetzky et al., 2007, *Vaccine*, 25:2001-2010; Kondo et al., 2008, *J. Med. Virol.*, 80:841-6; Schellenbacher et al., 2009, *J. Virol.*, 83:10085-10095; and Caldeira et al., 2010, *Vaccine*, 28:4384-93).

[0058] The L2 polypeptide can be full-length L2 protein or an L2 polypeptide fragment. The L2 sequences are known for substantially all papillomavirus genotypes

identified to date, and any of these L2 sequences or fragments can be employed in the present invention. Examples of L2 polypeptides include, without limitation, full-length L2 polypeptides, e.g., HPV16 L2 polypeptide, SEQ ID NO: , L2 truncations that lack the native C-terminus, L2 truncations that lack the native N-terminus, and L2 truncations that lack an internal domain.

[0059] The papillomavirus VLPs can be formed using the L1 and optionally L2 polypeptides from any animal papillomavirus, or derivatives or fragments thereof. Thus, any known (or hereafter identified) L1 and optionally L2 sequences of human, bovine, equine, ovine, porcine, deer, canine, feline, rodent, rabbit, etc., papillomaviruses can be employed to prepare the VLPs or capsomeres of the present invention. (See de Villiers et al., *Virology*, 324:17-27, 2004, for a near complete listing of papillomavirus genotypes and their relatedness, incorporated herein by reference).

[0060] In various embodiments, the VLP is comprised of a papilloma virus (PV) L1 protein, or a PV L1 and PV L2 protein. Papillomaviruses are small, double-stranded, circular DNA tumor viruses. The papilloma virion shells contain the L1 major capsid protein and the L2 minor capsid proteins. Expression of the L1 protein alone or in combination with the L2 protein in eukaryotic or prokaryotic expression systems is known to result in the assembly of capsomeres and VLPs. As used herein, the term "capsomere" is intended to mean a pentameric assembly of papillomavirus L1 polypeptides, including full-length L1 protein or fragments thereof. Native L1 capsid proteins may self-assemble via intermolecular disulfide bonds to form pentamers (capsomeres).

[0061] The papillomavirus virion may contain 72 pentamers (capsomeres) of L1 protein. (See, Trus et al., *Nat. Struct. Biol.*, 4:413-420, 1997). The L1 protein is capable of self-assembly into capsid-like structures that are morphologically indistinguishable from native virions when expressed in eukaryotic cells. (See, Buck et al., *J. Virol.*, 5190-97, 2008; and Roy et al., *Hum. Vaccin.*, 5-12, 2008, both incorporated herein by reference). The L1 monomer contains about 12 strands, 6 loops (BC, CD, DE, EF, FG, HI), and 5 helices (H1-H5). Most of the loops are highly exposed towards the outer surface of the capsid, attachment of a fusion protein to one of these loops, by e.g., disulfide linkage, maleimide linkage, by "click" chemistry or by binding to a polyionic docking site, as described herein, in these areas will result in the fusion protein being displayed on the outer surface of VLPs.

[0062] In certain embodiments, the L1 and optionally L2 polypeptides that are used to form the VLPs are from a non-human papillomavirus or a human papillomavirus genotype other than HPV-6, HPV-11, HPV-16, and HPV-18. For example, the L1 and/or L2 proteins

may be from HPV 1, 2, 3, 4, 5, 6, 8, 9, 15, 17, 23, 27, 31, 33, 35, 38, 39, 45, 51, 52, 58, 66, 68, 70, 76, or 92.

[0063] In various embodiments, the conjugated VLP presented herein bind to one or more cancer cells. This is in part due to the VLP's selectivity for proteins and/or molecules specific to tumor cells. In various embodiments, the VLP binds to heparin sulfate proteoglycan (HSPG), which is preferentially expressed on tumor cells. As used herein, "binding to a cancer cell" refers to the formation of non-covalent interactions between the capsid protein of the conjugated VLP and the tumor cell such that the conjugated VLP may come into close proximity to the tumor cell and the fusion protein may be cleaved from the VLP, and the recall protein may bind to the MHC receptor present on the tumor cell.

[0064] Animal Virus-based VLPs. In addition to the VLPs described elsewhere herein the VLP may be derived from a hepatitis B virus. The hepatitis B virus is comprised of an internal protein capsid and a lipid envelope containing other proteins. Two different VLPs can be produced from the virus, using either the core antigen that forms the internal capsid or the surface antigen that spontaneously combines with lipids to form nanoparticles (NP)s. The hepatitis B core (HBc) antigen is formed from 240 copies of a single protein. These proteins first form dimers, which then assemble with pentameric or pseudo-hexameric junctions in a T54 icosahedral geometry. The VLP has been produced using multiple technologies including *Escherichia coli* cytosolic accumulation and cell-free protein synthesis. The assembled VLPs are typically purified using size-exclusion chromatography or differential centrifugation. Individual coat proteins have been subsequently obtained by disassembling the VLPs with urea, which allows simultaneous cargo loading and VLP re-assembly.

[0065] Bacteriophage virus-based VLPs. In various embodiments, the VLP is derived from a bacteriophage-based VLP. The three bacteriophages, MS2, Qb, and *Salmonella typhimurium* P22, all infect enterobacteria, most notably *E. coli*. Although all three are composed of only a nucleic acid-filled viral capsid, P22 differs greatly from MS2 and Qb. MS2 and Qb are composed of 90 homodimers and require a specific stem-loop hairpin secondary structure in their RNA genome to trigger VLP self-assembly by binding to the coat proteins. P22, on the other hand, is composed of up to 415 coat proteins, 100–300 scaffold proteins, and 12 portal proteins. However, the P22 VLP has been engineered to consist of 420 coat proteins and only the 100–300 scaffold proteins, which can subsequently be removed with guanidine hydrochloride, leaving only the coat proteins. Like the HBV VLP, these VLPs assemble with icosahedral geometry. All three can be produced in *E. coli*, but Qb can also be produced in yeast and both Qb and MS2 can be produced using cell-free protein synthesis. MS2 VLPs have

been purified using size-exclusion chromatography, differential centrifugation, or immobilized metal affinity chromatography (for VLPs containing hexahistidine tags). Acids or urea can be used to disassemble the purified MS2 VLPs to obtain the dimers, which can then be reassembled after removal of the disassembly agent and the addition of the stem-loop RNA. Qb VLPs have been purified using size exclusion chromatography and the dimers can be obtained by disassembling the VLPs using acid, which can then be reassembled similar to MS2. P22 VLPs have been purified using size-exclusion chromatography or differential centrifugation and can also be disassembled using acid to obtain the coat proteins. Addition of scaffold proteins is required to reassemble the P22 VLP, but these can subsequently be removed. These bacteriophage-derived VLPs differ from HBc VLPs mainly in the assembly stimulus, using additional biomolecules (RNA or proteins) to initiate self-assembly instead of increasing the salt concentration.

[0066] Plant virus-based VLPs. In various embodiments, the VLP is a plant virus-based VLP. In various embodiments, the plant virus-based VLP is derived from the cowpea leaf: CCMV and CPMV. Neither virus has a lipid envelope. Both VLPs assemble with icosahedral geometry. The CCMV VLPs are formed from 90 homodimers and can be produced in *E. coli* or yeast. They have been purified using size exclusion chromatography or immobilized metal affinity chromatography, using coat proteins with hexahistidine extensions. 46,62 Dimers can be obtained by dialyzing the assembled VLPs against 0.5 M CaCl₂ or by purifying hexahistidine tagged dimers directly. Combining the dimers with RNA in a 1:6 mass ratio and lowering the pH to 4–5 induces self-assembly. CPMV, on the other hand, is formed from 60 copies of the VP60 protein which must first be proteolyzed into the L and S coat proteins (60 copies of each). Unfortunately, the VLP cannot be produced using *E. coli* or yeast; insect cells or plants must be used. The VLPs have been purified using differential centrifugation, but the coat proteins cannot yet be obtained in usable quantities. The inability to produce the VLP in *E. coli* or obtain purified coat proteins adds another challenge for targeted drug delivery; however, CPMV has been actively evaluated for therapeutic use due to the ability to easily display ligands on its surface and load cargo through association with its genome. In other embodiments, the plant virus-based VLP is a Tobacco Mosaic Virus (TMV).

[0067] Tumor Specificity of VLPs. In various embodiments, the VLP binds preferentially to tumor cells. The VLPs' tumor preference may originate from several sources including the VLP's charge (positive or negative), shape and size (different aspect ratio filaments and diameter spheres), shielding (self-proteins/peptides and polymers of various

sizes and densities), and targeting (ligands for receptors or environmental factors displayed on different linkers at various densities).

[0068] In terms of charge, in various embodiments, the VLP contains a positive surface charge. Positive charged VLPs stay longer in circulation. Due to the abundant presence of proteoglycan in the cell membrane conferring a negative charge and collagen within the tumor interstitial space conferring a positive charge, positively charged particles are more likely to have enhanced binding to mammalian cells and are better able to avoid aggregation and penetrate tumor tissue. Some examples demonstrating these charge-based effects include polyarginine-decorated CPMV found to be taken up eight times more efficiently than native CPMV in a human cervical cancer.

[0069] With regards to shape, the shape and flexibility of the VLP will play an additional role in VLPs ability to diffuse throughout a tumor. E.g. A comparison between the diffusion profiles of a spherical and rod-shaped particle was performed with CPMV and TMV using a spheroid model, and it was shown that whereas CPMV (Sphere) experienced a steady diffusion profile, TMV (rod shape) exhibited a two-phase diffusion behavior that entailed an extremely rapid early loading phase, which could be attributed to its movement axially, acting like a needle. Some other advantageous properties that are conferred by elongated particles include better margination toward the vessel wall and stronger adherence due to greater surface area for interaction, which not only have implications for tumor homing but also for enhanced targeting of cardiovascular disease.

[0070] Besides passive tumor homing properties, natural interactions of viruses with certain cells can also be exploited. CPMV (cowpea mosaic virus) in particular exhibits unique specificity in interacting with surface vimentin, which is found on endothelial, cancer, and inflammatory cells. The native affinity of CPMV for surface vimentin allows for high-resolution imaging of microvasculature up to 500 μm in depth, which cannot be achieved through the use of other nanoparticles, as they tend to aggregate and block the vasculature. This interaction can be utilized for a range of applications, such as delivery to a panel of cancer cells including cervical, breast, and colon cancer cell lines, delineation of atherosclerotic lesions, and intravital imaging of tumor vasculature and angiogenesis. Another example of an existing endogenous association is CPV with transferrin receptor (TfR), an important receptor for iron transport into cells and highly upregulated by numerous cancer cell lines. Even after dye labeling, CPV retains its specificity for TfR and was shown to bind to receptors found on HeLa cervical cancer cells, HT-29 colon cancer cells, and MDA-MB-231 breast cancer cells.

[0071] In various embodiments, the VLP is capable of targeting a protein expressed preferentially on the tumor cell surface. Such proteins are typically overexpressed on the surface of tumor cells, but some if not all may also be found in the blood, i.e. serum. Non-limiting examples of such surface markers include: CEA (carcinoembryonic antigen), E-cadherin, EMA (epithelial membrane antigen; aka MUC-1), vimentin, fibronectin, Her2/neu (human epidermal growth factor receptor type 2, also called Erb b2), $\alpha\beta$ 3 integrin, EpCAM (epithelial cell adhesion molecule), FR- α (folate receptor-alpha), PAR (urokinase-type plasminogen activator receptor), and transferrin receptor (over expressed in tumor cells).

[0072] Peptides are often used to label cancerous cells based on recognition of their transmembrane proteins. The most commonly used peptide is arginylglycylaspartic acid (RGD), composed of L-arginine, glycine, and L-aspartic acid. RGD was first isolated from the cell-binding domain of fibronectin, a glycoprotein that binds to integrins, and is involved in cell-cell and cell-extracellular matrix (ECM) attachment and signaling by binding collagen, fibrin, and proteoglycans. RGD peptides have the highest affinity for a type of cell surface integrins, $\alpha\beta$ which are highly expressed in tumoral endothelial cells, but not in normal endothelial cells. In various embodiments such a peptide sequence is incorporated into the conjugated VLP.

[0073] **Fusion Protein.** In various embodiments the fusion protein comprises a cleavage sequence. The cleavage sequence can be any sequence capable of being preferentially cleaved by or near a tumor cell. The insertion of this cleavage sequence into the fusion protein, allows the protein to remain inactive until it enters the tumor micro-environment. By taking advantage of the elevated activities of particular proteases in cancer tissues, the recall protein is not released from the VLP and able to actively coat MHC receptors until the recall protein enters the tumor microenvironment. Several proteases are known in the art to be active in the tumor microenvironment. For example, several metallo-, cysteine and serine proteases are known. From the standpoint of cancer therapy, an additional attraction is that because the proteases responsible for prodrug cleavage may come not just from cancer cells but also from the stromal components of tumors, release of the active drug direction into the tumor microenvironment does not depend on a target expressed only by the cancer cells. Instead, it is the entire tumor ecosystem that represents the target.

[0074] **Recall Protein.** In various embodiments, the recall protein is an epitope, which is recognized by a T cell or T cell population, which already exists in a subject. In various embodiments, this existing T cell or T cell population exists because of a prior infection or vaccination. In various embodiments of the invention, the recall protein is an epitope that is

capable of being, bound by a T cell. In various embodiments, the recall protein is an epitope capable of being bound by a T cell already present in a subject. In this context, "capable of being bound" means that a "epitope" is presented on the surface of a cell, where it is bound to MHC molecules directly. T cell epitopes presentable by MHC I can be bound by the T cell receptor of cytotoxic CD8 T lymphocytes (CDS T cells or CTLs). T cell epitopes presentable by MHC I are typically peptides of 9 to 12 amino acids in length. In various embodiments, a conjugated VLP is provided which allows release of a T cell response eliciting peptide, which is directly presentable via MHC class I. As the released recall protein does not require delivery to the antigen processing machinery in the cytosol, the T cell response eliciting peptide are presented on the surface of the target cell in a short amount of time. Hence, in one embodiment of the invention, in less than 8.5 hours after administration the target cell the T cell response eliciting peptide is presented on the surface of the target cell via an MHC class I molecule. In another embodiment of the invention, in less than 23.5 hours after introduction of the conjugated VLP to the target cell the T cell response eliciting peptide is presented on the surface of the target cell via an MHC class I molecule. In another embodiment of the invention, the conjugated VLP is capable of mediating T cell cytotoxicity against the target cell within less than 6 hours after administration of the conjugated VLP to the target cell.

[0075] In various embodiments, the fusion protein comprises at least two recall proteins. These recall proteins might be epitopes derived from different proteins, or they may be epitopes of the same protein. In various embodiments, the pathogen is a virus, a bacterium, a fungus, or a parasite.

[0076] In various embodiments, the preexisting T cells are specific to a vaccine epitope. In various embodiments the epitope is derived from an early childhood vaccine. In various embodiments the preexisting immunity is the result of prior administration of a human vaccine.

[0077] Non-limiting examples of a virus includes , a vaccinia virus, a varicella zoster virus, a Herpes zoster virus, rubella, a hepatitis virus, e.g., hepatitis A virus or hepatitis B virus or hepatitis C viruse, influenza, e.g., type A or type B, a measles virus, a mumps virus, a polio virus, a variola (smallpox) virus, a rabies virus, a Dengue virus, an Ebola virus, a West Nile virus, a yellow fever virus, or a zika virus.

[0078] Non-limiting examples of a bacterium include, a *Bordetella pertussis*, *chlamydia*, *trachomatis*, *Clostridium tetani*, diphtheria, *Hemophilus influenza*, *Meningococcus*, *pneumococcus*, vibrio cholera, *Mycobacterium tuberculosis*, BCG, typhoid, *E. coli*, *salmonella*, *Legionella pneumophila*, rickettsia, *Treponema pallidum pallidum*,

Streptococcus group A or group B, *Streptococcus pneumonia*, *Bacillus anthracis*, *Clostridium botulinum*, or a *Yersinia sp* bacteria.

[0079] Non-limiting examples of a parasite include, *Entamoeba histolytica*, *Toxoplasma gondii*, a *Trichinella sp.*, e.g., *Trichinella spiralis*, a *Trichomonas sp.*, e.g., *Trichomonas vaginalis*, a *Trypanosoma sp.*, e.g., *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, or a *Trypanosoma cruzi*, or a plasmodium, e.g., *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

[0080] In various embodiments, the recall protein is selected from the list included in Table 1:

Table 1: Recall Protein Epitopes

Epitope Sequence	SEQ ID NO	Virus Type	MHC allele	Viral Protein
SLPRSRTPI	4	Chicken Pox (VZV)	A*02:01	IE62
SAPLPSNRV	5	Chicken Pox (VZV)	A*02:01	IE62
GSAPLPSNRV	6	Chicken Pox (VZV)	A*02:01	IE62
ALWALPHAA	7	Chicken Pox (VZV)	A*02:01	IE62
SLSGLYVFV	8	Shingles vaccines	A*02:01	Glycoprotein E
YLGVIWNM	9	Shingles vaccines	A*02:01	Glycoprotein E
KIHEAPFDL	10	Shingles vaccines	A*02:01	Glycoprotein E
LLCLVIFLI	11	Shingles vaccines	A*02:01	Glycoprotein E
DLLLEWLYV	12	Shingles vaccines	A*02:01	Glycoprotein E
SMYYAGLPV	13	Shingles vaccines	A*02:01	Glycoprotein E
ILHDGGTTL	14	Shingles vaccines	A*02:01	Glycoprotein E
WLYVPIDPT	15	Shingles vaccines	A*02:01	Glycoprotein E
VLMGFGIIT	16	Shingles vaccines	A*02:01	Glycoprotein E
CLVIFLICT	17	Shingles vaccines	A*02:01	Glycoprotein E
KEADQPWIV	18	Shingles vaccines	A*02:01	Glycoprotein E

VVSTVDHFFV	19	Shingles vaccines	A*02:01	Glycoprotein E
FLICTAKRM	20	Shingles vaccines	A*02:01	Glycoprotein E
VLRTEKQYL	21	Shingles vaccines	A*02:01	Glycoprotein E
HMWNYHSHV	22	Shingles vaccines	A*02:01	Glycoprotein E
TVNKPVVGV	23	Shingles vaccines	A*02:01	Glycoprotein E
FVVFNGHV	24	Shingles vaccines	A*02:01	Glycoprotein E
WIVVNTSTL	25	Shingles vaccines	A*02:01	Glycoprotein E
VAYTVVSTV	26	Shingles vaccines	A*02:01	Glycoprotein E
FMYMSLLGV	27	measles	A*02:01	m50
SLWGSLLML	28	measles	A*02:01	C protein
LLAVIFVMFL	29	measles	A*02:01	H38
SMYRVFEVGV	30	measles	A*02:01	H250-259
ILPGQDLQYV	31	measles	A*02:01	H516-525
KLWCRHFCV	32	measles	A*02:01	H576
KLWCRHFCVL	33	measles	A*02:01	H576
RLSDNGYYTV	34	measles	A*02:01	M164
KLLRYYTEI	35	measles	A*02:01	F205
KLWESPQEI	36	measles	A*02:01	C 84
RLLDRLVRL	37	measles	A*02:01	N50
KLMPNITLL	38	measles	A*02:01	F57
TLLNNCTRV	39	measles	A*02:01	F64
EMLTLATWV	40	Hep B	A*02:01	C64-72
FLPSDFFPSV	41	Hep B	A*02:01	Core 18
FLPADFFPSV	42	Hep B	A*02:01	Core 19
FLPSDFFPSI	43	Hep B	A*02:01	Core 20
WLSLLVPF	44	Hep B	A*02:01	ENV335
FLLTRILTI or FLLTRILTL	45 or 46	Hep B	A*02:01	ENV183
GLSPTVWLSV	47	Hep B	A*02:01	ENV348
LLDYQGMLPV	48	Hep B	A*02:01	ENV260
LLCLIFLLV	49	Hep B	A*02:01	ENV251
SIVSPFIPL	50	Hep B	A*02:01	ENV370
FLLTKILTI	51	Hep B	A*02:01	ENV183
ILSPFLPLL	52	Hep B	A*02:01	ENV371
FLLSLGIHL	53	Hep B	A*02:01	POL 575
GLSRYVARL	54	Hep B	A*02:01	POL 455
SLYADSPSV	55	Hep B	A*02:01	POL 816
YMDDVVLGA	56	Hep B	A*02:01	POL 551
ALMPLYACI	57	Hep B	A*02:01	POL 655
VLHKRTLGL	58	Hep B	A*02:01	HBx 92

CLFKDWEEL	59	Hep B	A*02:01	Hbx115
STLPETTVVRR	60	Hep B	A*03, A*11	Core 141
EYLVSFQVW	61	Hep B	A*31, A*68	core 117
FFPSIRDLL	62	Hep B	A*24	Core 23
SWLSLLVPF	63	Hep B	A*24	Env 334
KYTSFPWLL	64	Hep B	A*24	Pol 756
HLSLRGLFV	65	Hep B	A*02:01	HBx 52-60
CLFKDWEEL	66	Hep B	A*02:01	HBx 115-123
LPSDFFPSV	67	Hep B	B*51	Core 19
GILGFVFTL	68	Influenza	HLA-A2	M1
ILGFVFTLTPSERGLQRRRF	69	Influenza		
LIRHENRMVLASTTAKA	70	Influenza		
LQAYQKRMGVQMQR	71	Influenza		
YVYDHSGEAVK	72			
WLSLLVPFV	73			
(K)GILGFVFTL(T)(V)	74			
KLSTRGVQIASNEN	75			
RGLQRRRFVQNALNGNG	76			
FMYSDFHFI	77			
NLVPMVATV	3			
VAIIEVDNEQPTTRAQKL	78			
Any 9-mer sequence of GACV AIIIEVDNEQPTTRAQKLF AMWRITYKDTVQLRRKL	79			
SVRDRLARL	80			
LLDRVRFMGV	81			
CLGGLLTMV	82			
GLCTLVAML	83			

[0081] In various embodiments the epitope is derived from an epitope of a human vaccine. In various embodiments the vaccine is an early childhood vaccine. Certain non-limiting examples of suitable vaccines are listed in Table 2 below:

Table 2: Approved Vaccines Containing Recall Protein Epitopes

Type	Commercial Name	Form	Source	Responsible National Regulatory Authority
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Hemophilus influenzae type b	Quinvaxem	Liquid: ready to use	Janssen Vaccines Corp.	Ministry of Food and Drug Safety

Diphtheria-Tetanus	Adsorbed DT Vaccine	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Diphtheria-Tetanus-Pertussis (whole cell)	DTP Vaccine	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Hepatitis B	Hepatitis B Vaccine Recombinant	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Polio Vaccine - Oral (OPV) Trivalent	Oral polio	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Polio Vaccine - Oral (OPV) Trivalent	Oral polio	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Tetanus Toxoid	TT vaccine	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Tetanus Toxoid	TT vaccine	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Tetanus Toxoid	TT vaccine	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Measles	Measles vaccine	Lyophilized Lyophilized active component to be reconstituted with excipient diluent before use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Yellow Fever	Yellow Fever	Lyophilized Lyophilized active component to be reconstituted with excipient	Bio-Manguinhos/Fiocruz	Agencia Nacional da Vigilância Sanitária

		diluent before use		
Yellow Fever	Yellow Fever	Lyophilized Lyophilized active component to be reconstitute d with excipient diluent before use	Bio- Manguinho s/Fiocruz	Agencia Nacional da Vigilancia Sanitaria
Yellow Fever	Yellow Fever	Lyophilized Lyophilized active component to be reconstitute d with excipient diluent before use	Bio- Manguinho s/Fiocruz	Agencia Nacional da Vigilancia Sanitaria
Hepatitis B	Heberbiovac HB	Liquid: ready to use	Centro de Ingenieria Genetica y Biotechnolo gia	Centro para el Control Estatal de la Calidad de los Medicamentos
Hepatitis B	Heberbiovac HB	Liquid: ready to use	Centro de Ingenieria Genetica y Biotechnolo gia	Centro para el Control Estatal de la Calidad de los Medicamentos
Rabies	Rabipur	Lyophilized active component to be reconstitute d with excipient diluent before use	Chiron Behring Vaccines Private Ltd.	Central Drugs Standard Control Organization
Rabies	Rabipur	Lyophilized active component to be	GlaxoSmit hKline	Paul-Ehrlich- Institut

		reconstituted with excipient diluent before use	Vaccines GmbH	
Haemophilus influenzae type b	Vaxem HIB	Liquid: ready to use	Novartis Vaccines and Diagnostics S.r.l	Agenzia Italiana del Farmaco
Hepatitis B	Engerix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Hepatitis B	Engerix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Hepatitis B	Engerix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Trivalent	Polio sabin	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Trivalent	Polio sabin	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Measles, Mumps and Rubella	Priorix	Lyophilized active component to be reconstituted with excipient diluent before use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Rotavirus	Rotarix	Liquid: ready to use	GlaxoSmithKline	Federal Agency for Medicines and Health Products

			Biologicals SA	
Polio Vaccine - Oral (OPV) Trivalent	Polioviral vaccine	Liquid: ready to use	Haffkine Bio Pharmaceut ical Corporatio n Ltd	Central Drugs Standard Control Organization
Yellow Fever	Stabilized Yellow Fever Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Institut Pasteur de Dakar	Ministère de la Santé publique
Yellow Fever	Stabilized Yellow Fever Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Institut Pasteur de Dakar	Ministère de la Santé publique
Yellow Fever	Stabilized Yellow Fever Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Institut Pasteur de Dakar	Ministère de la Santé publique
BCG	BCG Freeze Dried Glutamate vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Japan BCG Laboratory	Chiba Local Government

Hepatitis B	Euvax B	Liquid: ready to use	LG Chem Ltd	Ministry of Food and Drug Safety
Hepatitis B	Euvax B	Liquid: ready to use	LG Chem Ltd	Ministry of Food and Drug Safety
BCG	BCG Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
BCG	BCG Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Tetanus Toxoid	Tetatox	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Tetanus Toxoid	Tetatox	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Diphtheria-Tetanus	Diffet	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic	Bulgarian Drug Agency

			Diseases Ltd.	
Diphtheria-Tetanus	Diftet	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Diphtheria-Tetanus (reduced antigen content)	Tetadif	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Diphtheria-Tetanus (reduced antigen content)	Tetadif	Liquid: ready to use	Bul Bio - National Center of Infectious and Parasitic Diseases Ltd.	Bulgarian Drug Agency
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Easyfive-TT	Liquid: ready to use	Panacea Biotec Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	IMOVAX dT adult	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Inactivated (IPV)	IMOVAX POLIO	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Oral (OPV) Trivalent	OPVERO	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé

Polio Vaccine - Oral (OPV) Trivalent	OPVERO	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Oral (OPV) Trivalent	OPVERO	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Tetanus Toxoid	TETAVAX	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Tetanus Toxoid	TETAVAX	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Haemophilus influenzae type b	Act-HIB	Lyophilized active component to be reconstituted with excipient diluent before use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Rabies	VERORAB	Lyophilized active component to be reconstituted with excipient diluent before use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Yellow Fever	STAMARIL	Lyophilized active component to be reconstituted with excipient diluent before use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé

Meningococcal A+C	POLYSACC HARIDE MENINGOC CCAL A+C VACCINE	Lyophilized active component to be reconstitute d with excipient diluent before use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Oral (OPV) Monovalent Type 1	ORAL MONOVAL ENT TYPE 1 POLIOMYE LITIS VACCINE	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
cholera: inactivated oral	Dukoral	Liquid: ready to use	Valneva Sweden AB	Medical Products Agency
BCG	BCG Vaccine	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus	Diphtheria and Tetanus Vaccine Adsorbed (Paediatric)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus	Diphtheria and Tetanus Vaccine Adsorbed (Pediatric)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus	Diphtheria and Tetanus Vaccine Adsorbed (Pediatric)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

Diphtheria-Tetanus (reduced antigen content)	Diphtheria and Tetanus Vaccine Adsorbed for Adults and Adolescents	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	Diphtheria and Tetanus Vaccine Adsorbed for Adults and Adolescents	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	Diphtheria and Tetanus Vaccine Adsorbed for Adults and Adolescents	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)	Diphtheria- Tetanus- Pertussis Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)	Diphtheria- Tetanus- Pertussis Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)	Diphtheria- Tetanus- Pertussis Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B	Diphtheria ,Tetanus, Pertussis and Hepatitis B Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B	Diphtheria ,Tetanus, Pertussis and Hepatitis B	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

	Vaccine Adsorbed			
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B	Diphtheria ,Tetanus, Pertussis and Hepatitis B Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Hepatitis B	Hepatitis B Vaccine (rDNA) (Adult)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Hepatitis B	Hepatitis B Vaccine (rDNA) (Adult)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Hepatitis B	Hepatitis B Vaccine (rDNA) (Paediatric)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Hepatitis B	Hepatitis B Vaccine (rDNA) (Paediatric)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Tetanus Toxoid	Tetanus Toxoid Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Tetanus Toxoid	Tetanus Toxoid Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Tetanus Toxoid	Tetanus Toxoid Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles and Rubella	Measles and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

		excipient diluent before use		
Measles and Rubella	Measles and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles and Rubella	Measles and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles and Rubella	Measles and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles, Mumps and Rubella	Measles, Mumps and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles, Mumps and Rubella	Measles, Mumps and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

		excipient diluent before use		
Measles, Mumps and Rubella	Measles, Mumps and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles, Mumps and Rubella	Measles, Mumps and Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles	Measles Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles	Measles Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Measles	Measles Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

		excipient diluent before use		
Measles	Measles Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Rubella	Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Rubella	Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Rubella	Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Rubella	Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Rubella	Rubella Vaccine, Live, Attenuated	Lyophilized active component to be reconstitute d with	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

		excipient diluent before use		
Tetanus Toxoid	ShanTT	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization
Tetanus Toxoid	ShanTT	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B- Haemophilus influenzae type b	Shan-5	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B- Haemophilus influenzae type b	Shan-5	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization
BCG	BCG Vaccine SSI	Lyophilized active component to be reconstitute d with excipient diluent before use	AJ Vaccines A/S	Danish Medicines Agency
Rotavirus	Rotateq	Liquid: ready to use	Merck Vaccines	CBER/FDA
Measles, Mumps and Rubella	rHA M-M-R II	Lyophilized active component to be reconstitute d with	Merck Vaccines	European Medicines Agency

		excipient diluent before use		
Rotavirus	Rotarix	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Yellow Fever	-	Lyophilized active component to be reconstitute d with excipient diluent before use	Federal State Budgetary Scientific Institution «Chumako v Federal Scientific Center for Reserch & Developme nt of Immune- And Biological Products», Russian Academy of Sciences	Federal Service on Surveillance in Healthcare (ROSZDRAVNAD ZOR) of the Russian Federation
Yellow Fever	-	Lyophilized active component to be reconstitute d with excipient diluent before use	Federal State Budgetary Scientific Institution «Chumako v Federal Scientific Center for Reserch & Developme nt of Immune- And Biological Products», Russian Academy of Sciences	Federal Service on Surveillance in Healthcare (ROSZDRAVNAD ZOR) of the Russian Federation

Yellow Fever	-	Lyophilized active component to be reconstituted with excipient diluent before use	Federal State Budgetary Scientific Institution «Chumakov Federal Scientific Center for Research & Development of Immune- And Biological Products», Russian Academy of Sciences	Federal Service on Surveillance in Healthcare (ROSZDRAVNADZOR) of the Russian Federation
Human Papillomavirus (Quadrivalent)	Gardasil	Liquid: ready to use	Merck Vaccines	European Medicines Agency
Human Papillomavirus (Bivalent)	Cervarix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Human Papillomavirus (Bivalent)	Cervarix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Monovalent Type 1	Polio Sabin Mono T1	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Monovalent Type 1	Polio Sabin Mono T1	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Polio Sabin One and Three	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products

Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Polio Sabin One and Three	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Haemophilus influenzae type b	Haemophilus influenzae type b Conjugate Vaccine	Lyophilized active component to be reconstituted with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Monovalent Type 1	Monovalent type 1 Oral Poliomyelitis vaccine, IP (mOPV1)	Liquid: ready to use	Haffkine Bio Pharmaceutical Corporation Ltd	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Monovalent Type 1	Monovalent Oral Poliomyelitis Vaccine Type 1 (mOPV1)	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Tetanus Toxoid	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name BEtt.	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Pneumococcal (conjugate)	Synflorix	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	European Medicines Agency
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus	Lyophilized active component to be reconstituted with liquid	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

	influenzae type b Conjugate Vaccine	active component before use		
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Bivalent Oral Poliomyelitis Vaccine Type 1&3 (bOPV 1&3)	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Meningococcal A Conjugate 10 µg	Meningococcal A Conjugate MenAfriVac	Lyophilized active component to be reconstituted with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Haemophilus influenzae type b	Quimi-Hib	Liquid: ready to use	Centro de Ingenieria Genetica y Biotecnologia	Centro para el Control Estatal de la Calidad de los Medicamentos
Pneumococcal (conjugate)	Synflorix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	European Medicines Agency
Influenza, seasonal	Fluvirin	Liquid: ready to use	Seqirus Vaccines Limited	CBER/FDA
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Bivalent type 1&3 Oral Poliomyelitis vaccine, IP (bOPV1&3)	Liquid: ready to use	Haffkine Bio Pharmaceutical Corporation Ltd	Central Drugs Standard Control Organization
Influenza, seasonal	Fluzone	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA

Influenza, seasonal	Fluzone	Liquid: ready to use	Sanofi Pasteur- USA	CBER/FDA
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B- Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus influenzae type b Conjugate Vaccine	Lyophilized active component to be reconstitute d with liquid active component before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Influenza, seasonal	GC FLU Multi inj.	Liquid: ready to use	Green Cross Corporatio n	Ministry of Food and Drug Safety
Diphtheria-Tetanus- Pertussis (whole cell)- Hepatitis B- Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus influenzae type b Conjugate Vaccine	Lyophilized active component to be reconstitute d with liquid active component before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Influenza, pandemic H1N1	Panvax	Liquid: ready to use	Seqirus Limited	Therapeutic Goods Administration
Influenza, pandemic H1N1	Green Flu-S	Liquid: ready to use	Green Cross Corporatio n	Ministry of Food and Drug Safety
Influenza, pandemic H1N1	Influenza A (H1N1) 2009 monovalent vaccine	Liquid: ready to use	MedImmun e	CBER/FDA
Influenza, pandemic H1N1	Celtura	Liquid: ready to use	Seqirus GmbH	Paul-Ehrlich- Institut
Influenza, pandemic H1N1	Focetria	Liquid: ready to use	Seqirus Vaccines Limited	

Influenza, pandemic H1N1	Fluvirin-H1N1	Liquid: ready to use	Seqirus Vaccines Limited	CBER/FDA
Influenza, pandemic H1N1	Panenza	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Influenza, pandemic H1N1	Influenza A (H1N1) 2009 monovalent vaccine	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA
Influenza, pandemic H1N1	Influenza A (H1N1) 2009 monovalent vaccine	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA
Diphtheria-Tetanus-Pertussis (whole cell)-Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis and Haemophilus influenzae type b Conjugate Vaccine	Lyophilized active component to be reconstituted with liquid active component before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Inactivated (IPV)	Poliorix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Inactivated (IPV)	Poliorix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Pneumococcal (conjugate)	Prevenar 13	Liquid: ready to use	Pfizer	European Medicines Agency
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus influenzae type b Conjugate	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

	Vaccine Adsorbed			
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus influenzae type b Conjugate Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Diphtheria, Tetanus, Pertussis, Hepatitis B and Haemophilus influenzae type b Conjugate Vaccine Adsorbed	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Monovalent Type 3	Polio Sabin Mono Three (oral)	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Monovalent Type 3	Polio Sabin Mono Three (oral)	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Inactivated (IPV)	Poliomyelitis vaccine	Liquid: ready to use	Bilthoven Biologicals	Medicines Evaluation Board (MEB)
Polio Vaccine - Inactivated (IPV)	IPV Vaccine SSI	Liquid: ready to use	AJ Vaccines A/S	Danish Medicines Agency
Influenza, seasonal	GC FLU inj	Liquid: ready to use	Green Cross Corporation	Ministry of Food and Drug Safety

Polio Vaccine - Oral (OPV) Monovalent Type 2	Polio Sabin Mono Two (oral)	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Polio Vaccine - Oral (OPV) Monovalent Type 2	Polio Sabin Mono Two (oral)	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Typhoid (Polysaccharide)	Typhim-Vi	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Influenza, seasonal	Vaxigrip	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	BIOPOLIO B1/3	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	none	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	none	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Reconstituted).	Lyophilized active component to be reconstituted with liquid active component before use	Biological E. Limited	Central Drugs Standard Control Organization

Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Reconstituted).	Lyophilized active component to be reconstituted with liquid active component before use	Biological E. Limited	Central Drugs Standard Control Organization
cholera: inactivated oral	Shanchol	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization
Measles, Mumps and Rubella	Priorix	Lyophilized active component to be reconstituted with excipient diluent before use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products
Measles	Measles vaccine	Lyophilized active component to be reconstituted with excipient diluent before use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Poliomyelitis Vaccine (Oral), Bivalent types 1 and 3	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Influenza, pandemic H1N1	NASOVAC Influenza Vaccine,	Lyophilized active component	Serum Institute of	Central Drugs Standard Control Organization

	Live Attenuated (Human) Freeze-Dried	to be reconstituted with excipient diluent before use	India Pvt. Ltd.	
Influenza, pandemic H1N1	NASOVAC Influenza Vaccine, Live Attenuated (Human) Freeze-Dried	Lyophilized active component to be reconstituted with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Tetanus Toxoid	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name BEtt.	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Tetanus Toxoid	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name BEtt.	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Japanese Encephalitis Vaccine (Inactivated) 6µg	JEEV®	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Hepatitis A (Human Diploid Cell), Inactivated (Adult)	Havrix 1440 Adult	Liquid: ready to use	GlaxoSmithKline Biologicals SA	Federal Agency for Medicines and Health Products

Hepatitis A (Human Diploid Cell), Inactivated (Paediatric)	Havrix 720 Junior	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Diphtheria-Tetanus-Pertussis (acellular)	Boostrix	Liquid: ready to use	GlaxoSmit hKline Biologicals SA	Federal Agency for Medicines and Health Products
Meningococcal ACYW-135 (conjugate vaccine)	Menveo	Lyophilized active component to be reconstituted with liquid active component before use	GlaxoSmit hKline Vaccines S.r.l.	European Medicines Agency
Meningococcal ACYW-135 (conjugate vaccine)	Menactra	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Easyfive-TT	Liquid: ready to use	Panacea Biotec Ltd.	Central Drugs Standard Control Organization
Japanese Encephalitis Vaccine (live, attenuated)	Japanese Encephalitis Vaccine Live (SA14-14-2)	Lyophilized active component to be reconstituted with excipient diluent before use	Chengdu Institute of Biological Products Co.,Ltd	National Medical Products Administration
Japanese Encephalitis Vaccine (live, attenuated)	Japanese Encephalitis Vaccine Live (SA14-14-2)	Lyophilized active component to be reconstituted with excipient diluent before use	Chengdu Institute of Biological Products Co.,Ltd	National Medical Products Administration

Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Liquid).	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Liquid).	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Japanese Encephalitis Vaccine (live, attenuated)	IMOJEV MD	Lyophilized active component to be reconstituted with excipient diluent before use	GPO-MBP Co., Ltd.	Thai Food and Drug Administration
Diphtheria-Tetanus-Pertussis (whole cell)	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name TRIPVAC	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization

Diphtheria-Tetanus-Pertussis (whole cell)	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name TRIPVAC	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name BE Td	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus (reduced antigen content)	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name BE Td	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Poliomyelitis Vaccine (Oral), Bivalent types 1 and 3	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Inactivated (IPV)	Poliomyelitis vaccine multidose, suspension for injection 2.5 mL	Liquid: ready to use	Bilthoven Biologicals	Medicines Evaluation Board (MEB)
Influenza, seasonal	Nasovac-S Influenza Vaccine,	Lyophilized active component	Serum Institute of	Central Drugs Standard Control Organization

	Live, Attenuated (Human)	to be reconstituted with excipient diluent before use	India Pvt. Ltd.	
Diphtheria-Tetanus-Pertussis (acellular)-Hepatitis B-Haemophilus influenzae type b-Polio (Inactivated)	Hexaxim	Liquid: ready to use	Sanofi Pasteur SA	European Medicines Agency
Meningococcal A Conjugate 5 µg	Meningococcal A Conjugate 5 micrograms MenAfriVac 5µg	Lyophilized active component to be reconstituted with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Liquid).	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	None used on labelling for supply through UN agencies. Also marketed with labelled commercial name ComBE Five (Liquid).	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization

Polio Vaccine - Inactivated (IPV)	Poliomyelitis Vaccine (Inactivated)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Trivalent	BIOPOLIO	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Polio Vaccine - Oral (OPV) Trivalent	BIOPOLIO	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Influenza, seasonal	Influenza Vaccine (Split virion, inactivated)	Liquid: ready to use	Hualan Biological Bacterin Co., Ltd	National Medical Products Administration
Influenza, seasonal	IL-YANG FLU Vaccine INJ.	Liquid: ready to use	IL-YANG PHARMA CEUTICAL CO., LTD.	Ministry of Food and Drug Safety
BCG	BCG vaccine (Freeze Dried) - Intradermal	Lyophilized active component to be reconstituted with excipient diluent before use	GreenSignal Bio Pharma Limited	Central Drugs Standard Control Organization
Influenza, seasonal Quadrivalent	Fluzone Quadrivalent	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA
Influenza, seasonal Quadrivalent	Fluzone Quadrivalent	Liquid: ready to use	Sanofi Pasteur-USA	CBER/FDA
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Bivalent Oral Poliomyelitis Vaccine Type 1&3 (bOPV 1&3)	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia

cholera: inactivated oral	Euvichol	Liquid: ready to use	EuBiologics Co., Ltd.	Ministry of Food and Drug Safety
Polio Vaccine - Oral (OPV) Monovalent Type 2	ORAL MONOVALENT TYPE 2 POLIOMYELITIS VACCINE (mOPV2)	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Polio Vaccine - Oral (OPV) Monovalent Type 3	ORAL MONOVALENT TYPE 3 POLIOMYELITIS VACCINE	Liquid: ready to use	Sanofi Pasteur SA	Agence nationale de sécurité du médicament et des produits de santé
Meningococcal ACYW-135 (conjugate vaccine)	Nimenrix	Lyophilized active component to be reconstituted with excipient diluent before use	Pfizer	European Medicines Agency
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Eupenta	Liquid: ready to use	LG Chem Ltd	Ministry of Food and Drug Safety
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Eupenta	Liquid: ready to use	LG Chem Ltd	Ministry of Food and Drug Safety
Human Papillomavirus (Ninevalent)	Gardasil 9	Liquid: ready to use	Merck Vaccines	European Medicines Agency
Influenza, seasonal Quadrivalent	GCFLU Quadrivalent inj.	Liquid: ready to use	Green Cross Corporation	Ministry of Food and Drug Safety

Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Pentabio	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-Haemophilus influenzae type b	Pentabio	Liquid: ready to use	PT Bio Farma (Persero)	National Agency of Drug and Food Control Indonesia
Hepatitis A (Human Diploid Cell), Inactivated (Adult)	HEALIVE	Liquid: ready to use	Sinovac Biotech Co. Ltd	National Medical Products Administration
Varicella	Varivax	Lyophilized active component to be reconstituted with excipient diluent before use	Merck Vaccines	CBER/FDA
Rotavirus (live, attenuated)	Rotavac	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Diphtheria-Tetanus-Pertussis (acellular)	Adacel	Liquid: ready to use	Sanofi Pasteur Limited	Health Canada - Santé Canada
Influenza, seasonal	AGRIFLU	Liquid: ready to use	Seqirus Vaccines Limited	Health Canada - Santé Canada
Pneumococcal (conjugate)	Prevenar 13 Multidose Vial	Liquid: ready to use	Pfizer	European Medicines Agency
Typhoid (Conjugate)	Typbar-TVC	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization

Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Poliomyelitis Vaccine (live, oral attenuated, human Diploid Cell), type 1 and 3	Liquid: ready to use	Beijing Bio-Institute Biological Products Co.,Ltd	National Medical Products Administration
Japanese Encephalitis Vaccine (Inactivated) (3µg Pediatric)	JEEV®	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Rotavirus (live, attenuated)	ROTASIIL	Lyophilized active component to be reconstituted with excipient diluent before use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Inactivated (IPV)	Poliomyelitis Vaccine (Inactivated)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Polio Vaccine - Inactivated (IPV)	Poliomyelitis Vaccine (Inactivated)	Liquid: ready to use	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization
Influenza, seasonal Quadrivalent	GCFLU Quadrivalent Multi inj.	Liquid: ready to use	Green Cross Corporation	Ministry of Food and Drug Safety
Influenza, seasonal	Serinflu	Liquid: ready to use	Abbott Biologicals BV	Medicines Evaluation Board (MEB)
Polio Vaccine - Inactivated (IPV)	ShanIPV	Liquid: ready to use	Shantha Biotechnics Private Limited (A Sanofi Company)	Central Drugs Standard Control Organization

Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	Bivalent OPV Type 1 and 3 Poliomyelitis Vaccine, Live (Oral)	Liquid: ready to use	Panacea Biotech Ltd.	Central Drugs Standard Control Organization
cholera: inactivated oral	Euvichol-Plus	Liquid: ready to use	EuBiologics Co., Ltd.	Ministry of Food and Drug Safety
Polio Vaccine - Oral (OPV) Bivalent Types 1 and 3	BIOPOLIO B1/3	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
BCG	BCG Freeze Dried Glutamate vaccine	Lyophilized active component to be reconstituted with excipient diluent before use	Japan BCG Laboratory	Chiba Local Government
Pneumococcal (conjugate)	Synflorix	Liquid: ready to use	GlaxoSmithKline Biologicals SA	European Medicines Agency
Rotavirus (live, attenuated)	Rotavac	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Hepatitis A (Human Diploid Cell), Inactivated (Paediatric)	HEALIVE	Liquid: ready to use	Sinovac Biotech Co. Ltd	National Medical Products Administration
Typhoid (Conjugate)	Typhbar-TVC	Liquid: ready to use	Bharat Biotech International Limited	Central Drugs Standard Control Organization
Rotavirus (live, attenuated)	ROTASIIL	Lyophilized active component to be reconstituted with	Serum Institute of India Pvt. Ltd.	Central Drugs Standard Control Organization

		excipient diluent before use		
Japanese Encephalitis Vaccine (Inactivated) 6µg	JEEV®	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization
Japanese Encephalitis Vaccine (Inactivated) (3µg Pediatric)	JEEV®	Liquid: ready to use	Biological E. Limited	Central Drugs Standard Control Organization

[0082] In various embodiments, the recall protein epitope is released following proteolytic cleavage and the epitope binds to an MHC class I molecule. The MHC class I molecule may be from the HLA-A, B, and/or C families. The specific epitope that binds to the MHC class I molecule may be any of those recited in Table 1 or Table 2. The MHC class I molecule itself may be, one or more of the following non-limiting examples: HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, HLA-A*201, HLA-A*020101, HLA-A*0203, HLA-A*0206, HLA-A2, HLA-A2.1, or HLA-A*02.

[0083] In an aspect of the invention the recall protein is about 8 amino acid to about 50 amino acids in length, or about 8 amino acid to about 45 amino acids in length, or about 8 amino acid to about 40 amino acids in length, about 8 amino acid to about 35 amino acids in length, or about 8 amino acid to about 30 amino acids in length, about 8 amino acid to about 25 amino acids in length, about 8 amino acid to about 20 amino acids in length, or is about 8 amino acid to about 15 amino acids in length. In an aspect of the invention the fusion protein is about 13 amino acid to about 50 amino acids in length, or about 13 amino acid to about 45 amino acids in length, or about 13 amino acid to about 40 amino acids in length, about 13 amino acid to about 35 amino acids in length, or about 13 amino acid to about 30 amino acids in length, about 13 amino acid to about 25 amino acids in length, about 13 amino acid to about 20 amino acids in length, or is about 13 amino acid to about 15 amino acids in length. In an aspect of the invention the CD8+ T cell epitope may be, e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17 amino acids in length.

[0084] **Cleavage Sequence.** In various embodiments, a cleavage sequence is provided that allows the recall protein to be released from the VLP so that the recall protein can bind to the MHC on the tumor cell surface. In various embodiments, the VLP must escape the endosome, disassemble, and release their therapeutic cargo to the cytosol in a functional form. In various embodiments the conjugated VLP and/or fusion protein of the conjugated VLP

is susceptible to cleavage by a proteolytic enzyme within the tumor cell and the position of the target cleavage sequence in the VLP or fusion protein is such that the cleavage of the target site releases all or a portion of the recall protein comprising the CD8+ T cell epitope from the conjugated VLP, which complexes with an MHC class I molecule of the tumor cell. Sufficient amounts of conjugated VLP are readily determined by the skilled artisan and it will be appreciated that the amount will depend on, e.g., the characteristics of the subject, e.g., age, weight, gender, and/or medical condition of the subject, and the characteristics of the tumor, e.g., type, volume, and developmental status.

[0085] The cleavage sequence may be recognized by any protease present in a cell. At least 569 known proteases have been described (see, Lopez-Otin, et al., *Nature Reviews Cancer*, 7(10):800–808, 2007). All identified human proteolytic enzymes are classified into five catalytic classes: metalloproteinases, serine, threonine, cysteine and aspartic proteases. A non-limiting list of potential proteases that could be targeted is demonstrated in Table 3, which is a table summarizing the most well studied proteases distributed into five broad classes (in order from greatest to least number): metalloproteinases, serine, cysteine, threonine, and aspartic proteases. Several of these proteases have been found to be over-expressed in cancer cells relative to health cells.

[0086] In various embodiments, the cleavage sequence is recognized by the protease furin, a matrix metalloproteinases (MMPs), e.g., MMP, 1, 2, 3, 7, 8, 9, 11, 13, 14, or 19, an ADAM (a disintegrin and metalloproteinase), e.g., ADAMS 8, 9, 10, 15, 17, or 28, a cathepsin, e.g., cathepsin D, G, H, or N. Elastase, proteinase-3, azurocidin, or ADAMTS-1. In various embodiments, the cleavage sequence is recognized by a furin protease. In various embodiments, the cleavage sequence comprises at least 4 amino acid residues, at least three of which are arginine residues. In various embodiments, the cleavage sequence comprises at least 4 amino acid residues, at least three of which are arginine residues and one of which is either a lysine residue or an arginine residue. In various embodiments, the cleavage sequence is R-X-R/K-R. In various embodiments, the cleavage sequence comprises additional residues. In various embodiments, the cleavage sequence further comprises 1, 2, 3, 4, 5, 6, 7, 8, or 9 additional arginine residues. It is known that arginines are positively charged, and a longer chain of positive charged Arginine residues will bring the peptides closer to the surface of the VLP which is more negatively charged.

Table 3: Proteases and cancers associated with overexpressed proteases

Family	Protease	Location	Cancer	Ref.	Other Diseases	Ref.
Cysteine Cathepsins	General	Intracellular, lysosomes	Most	Table in [121]		
	Cathepsin K	Extracellular, bone	Breast	[178]	Artherosclerosis, osteoporosis	[179-182]
	Cathepsin B	Extracellular and pericellular under pathological conditions	Breast, cervix, colon, colorectal, gastric, head and neck, liver, lung, melanoma, ovarian, pancreatic, prostate, thyroid	[31, 38, 81, 183-196]		
	Cathepsin L		Breast, colorectal	[28]	AD	[197]
Aspartic Cathepsins	Cathepsin E	Endosomal structures, ER, Golgi	Cervical, gastric, lung, pancreas adenocarcinomas	[51-55]		
	Cathepsin D	Lysosome	Breast, colorectal, ovarian	[47-49, 198- 200]	Atherosclerosis	[121]
Kallikreins (hK)	General	Intracellular, secreted	Most	Table in [15, 58]		
	hK1				Hypertension, inflammation	[24]
	PSA (hK 3)		Prostate, ovarian	[201-202]		
	hK10		Colon, ovarian, pancreatic, head and neck	[203-206]		
Serine Proteases	hK15		Ovarian, prostate	[207-208]		
	uPA, nPAR	Membrane, Pericellular	Cervical, colorectal, gastric, prostate	[86, 116, 209-210]		
Caspases		Intracellular			Neurodegenerative disorders	[82]
MMPs	General	Extracellular	Most	Table in [211]		
	MMP-1, -8, -13		Breast	[85, 102-104, 211-212]	Artherosclerosis, RA	[213-214]
	MMP-2, -9		Breast, colorectal, lung, malignant gliomas, ovarian	[91-94] [95- 98]	Bronchiectasis, chronic asthma, COPD, cystic fibrosis, HIV associated dementia, hypertension, stroke	[87, 113- 117]
	MMP-14	Membrane	Breast	[212]		
ADAM		Extracellular			AD	[105, 107, 112]

*Abbreviations: AD: Alzheimer's disease; ADAM: a disintegrin and metalloproteinase domain protease; COPD: chronic obstructive pulmonary disease; ER: endoplasmic reticulum; RA: rheumatoid arthritis

[0087] In various embodiments, the fusion protein must be bound to the capsid proteins of the VLP. There are multiple methods of binding the fusion protein to the capsid protein. In various embodiments of the present invention the cleavage sequence is conjugated through a maleimide linkage or an amide linkage (discussed below). The fusion protein may be linked to any residue on the capsid protein of the VLP; however, disulfide linkages, maleimide linkages, and amide linkages are formed by conjugating the recall protein to cysteine, lysine, or arginine residues.

Surface Functionalization of the VLP

[0088] The VLPs described herein must be functionalized to deliver a recall protein, which must be presented on the surface of the conjugated VLP. In various

embodiments, recall proteins may be conjugated to the VLP through cysteine residues on the capsid protein. Such cysteine molecule can be presented naturally, or by mutation on the surface of the VLP. In various embodiments, the VLP is subjected to reducing conditions sufficient to reduce the sulfhydryl groups of cysteine residues on the surface of the VLP while maintaining the capsid-like icosahedron structures of the VLP. Because of its free sulfhydryl group, cysteine will readily and spontaneously form disulfide bonds with other sulfhydryl-containing ligands under oxidative conditions. Alternatively, a series of compounds add on maleimide readily and irreversibly form thioester linkages with cysteine residues at a pH between 6.5 and 7.5.

[0089] In various embodiments, the fusion protein may also be conjugated to a lysine residue on the VLP. Lysine residues are easily modified because of its primary amine. Using reactions termed n-hydroxysuccinimide (NHS) ester reactions (because NHS is released as part of the reaction), amide bonds are formed at surface-exposed lysine residues. The reaction occurs spontaneously between pH 7.2 and pH 9. This attachment chemistry has been used to display transferrin on MS2, which may allow the VLP to transcytose the blood-brain barrier that could open up a new library of therapies for neurological disorders.

[0090] In various embodiments, the fusion protein may also be conjugated to an aspartate- or glutamate residue. Unlike strategies involving cysteine and lysine, coupling to these residues requires multiple steps. First, the carboxylic acid must be activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Once activated, it will react with NHS to form an NHS ester. Now that the carboxylic acid side-chain has essentially become an NHS ester, a ligand with an exposed primary amine can be used to form a stable amide bond.

[0091] In various embodiments, the VLP comprises a region of negatively charged amino acids on a surface exposed area that is capable of binding to the fusion protein comprising a region of positively charged amino acids. In various embodiments, the region of negatively charged amino acids may be flanked, on one or on both sides, by one or more cysteine residues, referred to as polyanionic: cysteine or more specifically, polyglutamic acid:cysteine or polyaspartic acid:cysteine. In such cases, the conjugation of the VLP and the fusion protein would result from non-covalent binding between the complementary amino acid charges of the VLP and the fusion protein and a disulfide bond between the cysteines. In various embodiments, the cysteine(s) are one or more amino acids away from the region of charged amino acids such that any secondary/tertiary structure would bring the charged amino acid region in close proximity to the cysteine(s). In various embodiments of the invention the

fusion protein comprises at least one recall protein and a polyionic:cysteine for attaching the fusion protein to the VLP comprising a complementary polyionic:cysteine sequence and an enzyme cleavage site positioned between the terminal cysteine and the CD8+ T cell epitope. In various embodiments, the fusion protein comprises, a terminal cysteine, at least one recall protein, and an enzyme cleavage sequence positioned between the terminal cysteine and the recall protein(s).

[0092] Negatively charged amino acids that can be used in producing the conjugated VLP include, e.g., glutamic acid and aspartic acid. These amino acids can be used singly, e.g., polyglutamic acid, or in combination. In a specific embodiment, the region comprises glutamic acid. The number of negatively charged amino acids can vary, and can include about 4 to about 20 amino acids, about 6 to about 18 amino acids, or about 8 to about 16 amino acids, and the like. In a specific embodiment, the region comprises about 8 negatively charged amino acids. In a more specific embodiment, the region comprises EEEEEEEEC (E8C) (SEQ ID NO: 84). In another embodiment, the region comprises CEEEEEEEC (SEQ ID NO: 85). Methods for conjugating fusion proteins to a VLPs via disulfide bonding are known. For instance, the presence of a polyargininecysteine moiety on the fusion protein allows docking of the peptide to the polyanionic site (EEEEEEEC, E8C, SEQ ID NO: 84) present in the various loops of the L1 particles. Covalent cross-linking between the two cysteine residues should render this association irreversible under oxidizing conditions. For the conjugation reactions, purified HPV particles are dialyzed in conjugation buffer (20 mM Tris/HCl pH = 7.5, 150 mM NaCl, 5% glycerol, 0.5 mM CaCl₂) and then the peptide and the oxidizing reagents are added, allowing the reaction to proceed for 16 hrs at 4°C. At the end of the incubation, the reaction mixtures are applied to a size-exclusion column (SEPHADEX® G-100, Pharmacia, New Jersey, US, volume 20 ml, flow rate 1 ml/min, 10 mM Tris/HCl (pH = 7.4), 150 mM NaCl, 0.5 mM CaCl₂) to remove unconjugated peptide and exchange buffer. Conjugated particles that elute in the void volume are identified by the presence of the L1 protein on SDS-PAGE. The conjugated particles are analyzed by electron microscopy. One of ordinary skill in the art can, through routine experimentation, create a VLP that includes a polyionic region in a surface exposed area, e.g., one or more loops, and that is VLP assembly competent.

[0093] In various embodiments, the fusion protein is genetically fused to the coat protein. In various embodiments, the fusion protein may be either covalently or non-covalently linked to the VLP. Rather than attaching the recall protein to the VLP via, e.g., binding of negatively and positively charged amino acids, or via maleimide based conjugation, a nucleic

acid sequence encoding the fusion protein may be inserted into the nucleic acid encoding the capsid protein such that upon expression, a fusion protein is produced wherein the recall protein is inserted into a loop of the capsid protein and displayed on the surface of the VLP.

[0094] In various embodiments, non-natural amino acids may be used to conjugate the fusion protein to the VLP. Beyond the 20 natural amino acids, many non-natural amino acids have been used for site-specific protein conjugation reactions. For example, an azidohomoalanine (AHA) or a p-amino-phenylalanine (pAF) may be incorporated into the VLP coat protein for conjugation. These amino acids are incorporated into proteins in two ways: global methionine replacement and amber stop codon suppression. Because AHA is very similar to methionine, AHA will be incorporated at each AUG codon if the methionine supply is rate limiting, this is termed global methionine replacement. Bacteria auxotrophic for methionine or cell-free protein synthesis can be used to limit-methionine availability. Amber stop codon suppression will incorporate pAF. Amber stop codon suppression uses nonnative synthetases and tRNAs that do not react with the natural amino acids to incorporate the non-natural amino acid at the amber stop codon UAG. AHA, displaying an azide, will participate in in copper(I)-catalyzed azide-alkyne cycloaddition ("click" reaction) and form covalent triazole rings with alkyne-containing ligands.

[0095] In various embodiments, the conjugated VLP comprises, at least one-tenth of the viral coat proteins may display a recall protein. In various embodiments, at least one-fifth of the viral coat proteins may display a recall protein. In various embodiments, about half of the viral coat proteins may display a recall protein. In various embodiments, about two-thirds of the viral coat proteins may display a recall peptide. In various embodiments, nearly all of the viral coat proteins may display a recall protein.

Methods of Treatment

[0096] In various embodiments of the invention, a method for treating a cancer in a subject in need thereof by administering a conjugated VLP to patient in need thereof. The methods of this invention comprise administering the conjugated VLPs of this invention to a subject in need thereof in an amount sufficient to inhibit tumor growth, progression or metastasis. In various embodiments of the invention, the conjugated VLP is administered to a subject in need thereof in amount sufficient to stimulate cytokine production and/or cellular immunity, particularly innate immunity, including stimulating the cytotoxic activity of macrophages and natural killer cells. In various embodiments of this invention a subject in need

thereof is a subject who has been previously treated for a tumor and is currently deemed cancer-free or disease free in accordance with medical standards.

[0097] Briefly, the mechanism of action of the described conjugated VLPs is depicted in Figure 1. The VLPs specifically bind to a tumor cell. (Figure 1, left side, tumor cells). The recall protein epitope on the VLP is then proteolytically cleaved by furin, which is over-expressed in the tumor microenvironment. This in turn leads to release of the epitope from the conjugated VLP and the loading of the epitopes onto the tumor MHC class I molecules (“epitope coating”). (Figure 1, middle exploded view showing block MHI molecule loading with epitope from VLP). The epitope-coated tumor cell is then recognized as a pathogen-infected cell by one or more childhood vaccine T-cells and pre-existing CD8 T cells, yielding a triggered immune redirection response. (Figure 1, far right side of diagram). That is, this recognition event leads to the host’s pre-existing immune memory against pathogens and childhood vaccines activating against the tumor in the tumor microenvironment, attacking and destroying the tumors. Destruction of tumor cells can result in components of the preexisting immune response being exposed to cancer cell antigens. Thus, antigens released from the killed tumor cells will initiate an immune response to recruit additional tumor-specific CD8 T cells, or a “second wave” of T cells that then proceed to attack additional tumor cells in the area. This can result in elicitation of an endogenous immune response against the cancer cell antigens (commonly known as “epitope spreading”), and leads to anti-tumor immune memory.

[0098] Thus, the methods disclosed herein are methods of treating cancer in an individual by utilizing the individual’s own pre-existing adaptive memory immune system to attack cancer cells. The methods described herein make use of the fact that individuals possess preexisting immune responses that were not originally elicited in response to a cancer, but that were elicited instead by routine vaccination scheduling or via microorganisms and pathogens present in the natural environment. Because the cancer cells would not normally express the microbial antigens that elicited the preexisting immune response, it would not be expected that such an immune response would attack a cancer. However, by way of the present methods, such preexisting immune responses can be recruited to attack, kill, and clear a cancer in a subject. This is achieved by introducing into or onto the surface of the cancer one or more antigens known to be recognized by the preexisting immune response in the subject, resulting in cells of the immune response attacking antigen-displaying cancer cells. Further, destruction of cancer cells can result in components of the preexisting immune response being exposed to additional cancer cell antigens. Thus, a general method of the invention can be practiced by recruiting a preexisting microbial or vaccine immune response in an individual to the site of a

cancer, such that the preexisting immune response attacks the cancer. Thus, there are generally four steps involved in the method, including: 1) binding VLPs to the tumor cells, cleavage of the epitope from the VLP leading to MHC binding of the epitopes for display on the tumor cell surface, recognition of the loaded MHC by the subject's pre-existing recalled immunity against the epitope, and triggering of a second wave and longer-term anti-tumoral immunity thereafter.

[0099] Data obtained from cell culture assays and animal studies may be used in formulating a range of dosages for use in humans. The dosages of such compositions lie preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any composition used in the methods of the invention, the therapeutically effective dose may be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (the concentration of the test composition that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information may be used to accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0100] In many instances, it will be desirable to have multiple administrations of the VLP-containing composition, usually at most, at least, or not exceeding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more vaccinations including all ranges there between. The vaccinations will normally be at 1, 2, 3, 4, 5, 6, to 5, 6, 7, 8, 9, 10, 11, to 12 week/month/year intervals, including all values and ranges there between, more usually from three to five week intervals.

[0101] In various embodiments, a method is provided for stimulating the cytotoxic activity of macrophages and natural killer cells by administering to a subject in need thereof an effective amount of a conjugated VLP of this invention. The macrophages and natural killer cells may be those present in the tumor microenvironment. In an aspect of this invention, the conjugated VLPs are administered to the subject in an amount effective to stimulate the cytotoxic activity of macrophages and natural killer cells already present in the tumor microenvironment. In various embodiments of this invention, the conjugated VLPs are administered to the subject in an amount effective to attract macrophages and natural killer cells to the tumor microenvironment.

[0102] In various embodiments of the invention, the conjugated VLPs are administered to the subject in an amount effective to bind sufficient numbers of antibodies to the recall protein to attract and stimulate macrophages, neutrophils and natural killer cells.

[0103] In various embodiments of the invention, a method is provided for redirecting the cytotoxic activity of an existing memory CD8+ T cell to a tumor cell or tumor microenvironment by administering to a subject in need thereof an effective amount of the conjugated VLP of this invention. Preferably, the T cell epitope of the recall protein of the conjugated VLP is from a pathogen for which the subject has been actively vaccinated or from a pathogen that has previously infected the subject and the subject has memory CD8+ T cells that recognize the T cell epitope in complex with an MHC class I molecule on the tumor cells. In an aspect of this invention the effective amount of the conjugated VLP is an amount sufficient to attract the memory CD8+ T cell to the tumor microenvironment. In an aspect of this invention the effective amount of the conjugated VLP is an amount sufficient to stimulate the memory CD8+ T cell present in the tumor microenvironment.

[0104] In various embodiments, the tumor is a small lung cell cancer, hepatocellular carcinoma, liver cancer, hepatocellular carcinoma, melanoma, metastatic melanoma, adrenal cancer, anal cancer, aplastic anemia, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, cancer of unknown primary origin, Castleman disease, cervical cancer, colon/rectum cancer, endometrial cancer, esophagus cancer, Ewing family of tumors, eye cancer, gallbladder cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (gist), gestational trophoblastic disease, Hodgkin disease, Kaposi sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, leukemia, liver cancer, lung cancer, lymphoma, malignant mesothelioma, multiple myeloma, myelodysplastic syndrome, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, penile cancer, pituitary tumors, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma, skin cancer, stomach cancer, testicular cancer, thymus cancer, thyroid cancer, uterine sarcoma, vaginal cancer, vulvar cancer, Waldenstrom macroglobulinemia, Wilms tumor, non-Hodgkin lymphoma, Hodgkin lymphoma, Burkitt's lymphoma, lymphoblastic lymphomas, mantle cell lymphoma (MCL), multiple myeloma (MM), small lymphocytic lymphoma (SLL), splenic marginal zone lymphoma, marginal zone lymphoma (extra-nodal or nodal), mixed cell type diffuse aggressive lymphomas of adults, large cell type diffuse aggressive lymphomas of adults, large cell immunoblastic diffuse aggressive lymphomas of adults, small non-cleaved cell diffuse aggressive lymphomas of adults, or follicular lymphoma, head and neck cancer, endometrial or uterine carcinoma, non-small cell lung cancer, osteosarcoma, glioblastoma, or metastatic cancer. In a preferred embodiment, the cancer is a breast cancer, a cervical cancer, an ovarian cancer, a pancreatic cancer or melanoma,

[0105] The term "cancer" as used herein refers to proliferative diseases, such as lymphomas, lymphocytic leukemias, lung cancer, non-small cell lung (NSCL) cancer, bronchioloalveolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma and Ewing's sarcoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers.

[0106] An aspect of the invention is a method for treating a cancer in a subject in need thereof by administering a conjugated VLP of this invention to the subject wherein the CD8+ epitope of the recall protein is of a failed therapeutic cancer vaccine against a Viral-induced cancer e.g. HPV cervical cancer, HPV+ oral cancer, EBV nasopharyngeal cancer (the "therapeutic vaccine"). The method comprises determining if the subject has been actively vaccinated against the vaccine but did not respond with an anti-tumor effect to the treatment. The patient is then administering to the subject an effective amount of a conjugated VLP of this invention wherein the CD8+ epitope of the recall protein is of the antigenic determinant in the vaccine previously administered to the subject that infected the subject.

[0107] In various embodiments, a method is provided for treating a cancer in a subject in need thereof by administering a conjugated VLP of this invention to the subject wherein the CD8+ epitope of the recall protein is of a failed CAR-T cell therapy against a Viral-induced cancer e.g. HPV cervical cancer, HPV+ oral cancer, EBV nasopharyngeal cancer (the "CAR-T"). The method comprises determining if the subject has been actively treated with the CAR-T but did not respond with an anti-tumor effect to the treatment. The patient is then administering to the subject an effective amount of a conjugated VLP of this invention wherein the CD8+ epitope of the recall protein is of the antigenic determinant in the CAR-T previously administered to the subject that infected the subject.

[0108] In various embodiments, a method is provided for treating a cancer in a subject in need thereof by administering a conjugated VLP of this invention to the subject wherein the CD8+ epitope of the recall protein is of a failed Vaccine or CAR-T cell therapy against a cancer (the "CAR-T"). The method comprises determining if the subject has been actively treated with the CAR-T but did not respond with an anti-tumor effect to the treatment. The patient is then administered an effective amount of a conjugated VLP of this invention wherein the CD8+ epitope of the recall protein is of the antigenic determinant in the CAR-T previously administered to the subject that infected the subject.

[0109] VLPs have adjuvant properties. In some embodiments, the immunogenicity of the conjugated VLP compositions of this invention can be enhanced by the use of additional nonspecific stimulators of the immune response, known as adjuvants. Suitable adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins, or synthetic compositions such as alum.

[0110] Adjuvants include, but are not limited to, oil-in-water emulsions, water-in-oil emulsions, mineral salts, polynucleotides, and natural substances. Specific adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ -interferon, GM-CSF, BCG, aluminum salts, such as aluminum hydroxide or other aluminum compound, MDP compounds, such as thur-MDP and nor-MOP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL), or inactivated microbial agents. RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TOM), and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion. MHC antigens may even be used.

[0111] Various methods of achieving adjuvant affect for the conjugated VLP compositions includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1 % solution in phosphate buffered saline, admixture with synthetic polymers of sugars (CARBOPOL®) used as an about 0.25% solution, aggregation of a protein in the composition by heat treatment with temperatures ranging between about 70°C to about 101 °C. for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin-treated (Fab) antibodies to albumin; mixture with bacterial cells, e.g., *C. parvum*, endotoxins or lipopolysaccharide components of Gram-negative bacteria; emulsion in physiologically acceptable oil vehicles, e.g., mannide monooleate (Aracel A™), or emulsion with a 20% solution of a perfluorocarbon (FLUOSOL-DA®) used as a block substitute may also be employed to produce an adjuvant effect. A typical adjuvant is complete Freund's adjuvant (containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants, and aluminum hydroxide.

[0112] For administration to humans, a variety of suitable adjuvants will be evident to a skilled worker. These include, e.g., Alum-MPL as adjuvant, or the comparable formulation, ASO4, which is used in the approved HPV vaccine CERVARIX®, AS03, AS02, MF59, montanide, saponin-based adjuvants such as GPI-0100, CpG-based adjuvants, or imiquimod. In embodiments of the invention, an adjuvant is physically coupled to the VLP, or encapsulated by the VLP, rather than simply mixed with them. In addition to adjuvants, it may be desirable to co-administer biologic response modifiers (BRM) to enhance immune responses. BRMs have been shown to upregulate T cell immunity or downregulate suppresser cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA, US); or low-dose Cyclophosphamide (CYP; 300 mg/ml) (Johnson/Mead, NJ, US) and cytokines such as γ -interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B-7. In embodiments of the invention, these genes are encapsulated by the VLP to facilitate their delivery into a subject.

[0113] The preparation of compositions that contain polypeptide or peptide sequence(s) as active ingredients is generally well understood in the art. Typically, such compositions are prepared as injectables either as liquid solutions or suspensions: solid forms suitable for solution in or suspension in liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the compositions may contain amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants that enhance the effectiveness of the vaccines. In specific embodiments, vaccines are formulated with a combination of substances.

[0114] The compositions comprising the conjugated VLPs of the present invention are in biologically compatible form suitable for administration *in vivo* to subjects. The pharmaceutical compositions further comprise a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the VLP is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, including but not limited to peanut oil, soybean oil, mineral oil, sesame oil and the like. Water may be a carrier when the pharmaceutical composition is

administered orally. Saline and aqueous dextrose may be carriers when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions may be employed as liquid carriers for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The pharmaceutical composition may also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

[0115] The pharmaceutical compositions comprising the conjugated VLPs of the present invention can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. Oral formulation may include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. In a specific embodiment, a pharmaceutical composition comprises an effective amount of a conjugated VLP of the present invention together with a suitable amount of a pharmaceutically acceptable carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0116] The pharmaceutical compositions of the present invention may be administered by any particular route of administration including, but not limited to intravenous, intramuscular, intraarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracerebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intramyocardial, intraosteal, intraosseous, intrapelvic, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, bolus, oral, parenteral, subcutaneous, vaginal, rectal, buccal, sublingual, intranasal, iontophoretic means, or transdermal means. Most suitable routes are intravenous injection or oral administration. In particular embodiments, the compositions are administered at or near the target area, e.g., intratumoral injection.

[0117] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, intratumoral, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in isotonic NaCl solution and either added to hypodermoclysis fluid or injected at the proposed

site of infusion (see for example, Remington's Pharmaceutical Sciences, 1990). Some variation in dosage will necessarily occur depending on the condition of the subject. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

[0118] The conjugated VLP-containing compositions of this invention may be administered by inhalation. In certain embodiments a composition can be administered as an aerosol. As used herein the term "aerosol" or "aerosolized composition" refers to a suspension of solid or liquid particles in a gas. The terms may be used generally to refer to a composition that has been vaporized, nebulized, or otherwise converted from a solid or liquid form to an inhalable form including suspended solid or liquid drug particles. Such aerosols can be used to deliver a composition via the respiratory system. As used herein, "respiratory system" refers to the system of organs in the body responsible for the intake of oxygen and the expiration of carbon dioxide. The system generally includes all the air passages from the nose to the pulmonary alveoli. In mammals it is generally considered to include the lungs, bronchi, bronchioles, trachea, nasal passages, and diaphragm. For purposes of the present disclosure, delivery of a composition to the respiratory system indicates that a drug is delivered to one or more of the air passages of the respiratory system, in particular to the lungs.

[0119] Additional formulations which are suitable for other modes of administration include suppositories (for anal or vaginal application) and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1 % to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

[0120] The conjugated VLP compositions may be formulated into a vaccine as neutral or salt forms. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the peptide) and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric

hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0121] The pharmaceutical compositions of the present invention can also include an effective amount of an additional adjuvant. As noted herein, papillomavirus VLPs have adjuvant properties. Suitable additional adjuvants include, but are not limited to, Freund's complete or incomplete, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as Bacille Calmette-Guerin (BCG), *Corynebacterium parvum*, and non-toxic cholera toxin.

[0122] Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In all cases the pharmaceutical form must be sterile and must be fluid to the extent that it may be easily injected. It also should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[0123] The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0124] Sterile injectable solutions are prepared by incorporating the conjugated VLPs in the required amount in the appropriate solvent with various ingredients enumerated above, as required may be followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0125] Different aspects of the present invention involve administering an effective amount of a composition comprising the conjugated VLPs to a subject in need thereof. In some embodiments of the present invention, a conjugated VLP comprising a target peptide comprising a CD8+ T cell epitope is administered to the patient to treat a tumor or prevent the recurrence of such tumor. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

Methods of Providing an Appropriate VLP

[0126] In various embodiments, a method for providing a conjugated VLP to a patient in need thereof is provided comprising: (1) measuring the preexisting immunity in a patient, and (2) selecting the appropriate conjugated VLP for administration of a patient in need. The appropriate conjugated VLP to administer to the patient will depend upon the patient's T cell profile. The appropriate conjugated VLP will be one that is capable of eliciting a T cell response that is at least twice the baseline total of CD8+ cells. In various embodiments, the appropriate conjugated VLP will be one that is capable of eliciting a T cell response that is twice the baseline total of CD8+ or total CD8+ CD69+ T cells. The goal is to choose the appropriate conjugated VLP based on the patient's vaccination history or prior exposure to a pathogen. Determining which conjugated VLP is appropriate can be achieved through: (1) patient interviews; (2) review of a patient's medical records; and/or (3) assessing the patient's T cell profile.

[0127] In various embodiments, more than one recall protein might be suitable for eliciting an immune response directed at a tumor. In various embodiments, a conjugated VLP carrying either recall protein will be appropriate. In various embodiments, more than one recall protein is expressed and bound to the VLP. In various embodiments, a single fusion protein will comprise more than one recall protein. In various embodiments, multiple fusion proteins comprising different recall proteins will be conjugated to the VLP. In various embodiments, the invention comprises a population of conjugated VLPs as described herein and a pharmaceutically acceptable excipient. In various embodiments, the conjugated VLPs administered to the subject are identical. In various embodiments, conjugated VLPs carrying different recall protein(s) are administered to a subject.

[0128] **Selection Based on Prior Vaccination.** In various embodiments of the invention, a method of selecting an appropriate conjugated VLP is provided in order to administer to a subject in need thereof. In various embodiments this involves ascertaining if the subject has been actively vaccinated against a given pathogen, e.g., a parasite, a bacterium,

or virus, e.g., measles or polio, and then selecting and administering to the subject a conjugated VLP as disclosed herein wherein the CD8+ T cell epitope of the recall protein is from the pathogen against which the subject has been immunized. In various embodiments, a subject's vaccination history is obtained by reviewing the subject's medical record. In various embodiments, a subject's vaccination history is obtained by interviewing the subject.

[0129] Selection Based on Prior Infection. In various embodiments, the method of selecting an appropriate conjugated VLP for administration to a subject in need thereof involves ascertaining if a subject has been previously infected with a given pathogen, e.g., a parasite, a bacterium, or virus, e.g., measles or polio, and resolved the infection. In various embodiments, the subject is then administered a conjugated VLP comprising a recall protein which comprises said pathogen for which the subject has been previously infected.

[0130] One may ascertain if a subject has been infected with a particular pathogen by reviewing the subjects' medical records or interviewing the subject. Non-limiting examples of CD8+ T cell epitopes that bind to particular MHC class I molecules are set forth in Table 1. The method may also comprise determining which MHC class I determinant(s) the subject's cells express and then administering a conjugated VLP of this invention wherein the CD8+ T cell epitope of the recall protein is a CD8+ T cell epitope of the antigenic component of the pathogen in the vaccine or of the pathogen that previously infected the subject that forms a complex with the subject's MHC class I determinant(s).

[0131] Measuring T cell Responses. In various embodiments, a patient's T cell profile is assessed in order to select an appropriate conjugated VLP using various techniques known in the art. This profile is then used to select the appropriate conjugated VLP to administer. Such techniques including measuring interferon- γ levels, using flow cytometry to isolate Ag-specific CD8+ T cells, and/or cytotoxicity assays. To measure interferon- γ (a marker of T cell activation), intracellular staining of isolated T cells. Alternatively, an enzyme-linked immunosorbent spot (ELISPOT) assay for interferon- γ may be conducted. This technique allows for a high throughput assessment of a patient's T cell profile. This method can potentially detect one in 100,000-300,000 cells. Briefly, a monoclonal antibody for a specific cytokine is pre-coated onto a polyvinylidene difluoride (PVDF)-backed microplate. CD8+ T cells are pipetted into the wells along with dendritic cells and individual peptides and the microplate is placed into a humidified 37°C CO₂ incubator for a period ranging from 24 to 48 h. During incubation, the immobilized antibody binds the cytokine secreted from the cells. After washing a detection antibody specific for the chosen analyte is added to the wells. Following the washes, enzyme conjugated to streptavidin is added and a substrate is added. A

colored precipitate forms, according to the substrate utilized and appears as spot at the sites of cytokine secretion, with each individual spot representing a single producing cell.

[0132] In various embodiments, the invention described here in provides a method of determining the appropriate conjugated VLP to administer to a patient in need thereof, by assessing the patients T cell profile, comprising: (1) collecting PBMCs from patients/participants (pre-vaccination sample), (2) preparing the ELISPOT plates by coating with anti-IFN- γ antibody (incubate overnight), (3) Incubating PBMCs with one of the pool of peptides of interest, ones expected to elicit a T cell response (incubate for 1-2 days), (4) washing the plates, adding a biotinylated secondary antibody (incubating for a few hours), (5) washing the plates, adding avidin conjugated horseradish peroxidase and incubating, (6) washing plates, adding aminoethyl carbazole (AEC) for a few minutes, (7) stopping the reaction (water), and (8) visualizing on an ELISPOT reader. The disclosed method can detect up to one in 100,000-300,000 cells. A two-fold increase in the frequency of antigen-specific T cells should be considered as a signal.

[0133] In various embodiments T cell proliferation can be measured by 3H (tritiated)-thymidine. Such methods are sensitive and can be used for high throughput assays. Such techniques may also include carboxyfluorescein succinimidyl ester (CFSE) and Ki64 intracellular staining.

[0134] **Selecting Recall Proteins based on Tropism.** It is known in the art that some viruses display a tropism for particular type of tissue. For example: viruses that display a tropism for brain tissue include without limitation, JC virus, measles, LCM virus, arbovirus and rabies; viruses that display a tropism for eye tissue include without limitation herpes simplex virus, adenovirus, and cytomegalovirus; viruses that display a tropism for nasal tissue include without limitation, rhinoviruses, parainfluenza viruses, and respiratory syncytial virus; viruses that display a tropism for oral tissue, e.g., oral mucosa, gingiva, salivary glands, pharynx, include without limitation, herpes simplex virus type I and type II, mumps virus, Epstein Barr virus, and cytomegalovirus; viruses that display a tropism for lung tissue include without limitation, influenza virus type A and type B, parainfluenza virus, respiratory syncytial virus, adenovirus, and SARS coronavirus; viruses that display a tropism for nerve tissue, e.g., the spinal cord, include without limitation poliovirus and HTLV-I; viruses that display a tropism for heart tissue, include without limitation, Coxsackie B virus; viruses that display a tropism for liver tissue, include without limitation, hepatitis viruses types A, B, and C; viruses that display a tropism for gastrointestinal tissue, e.g., stomach, and large and small intestine, include without limitation, adenovirus, rotavirus, norovirus, astrovirus, and coronavirus;

viruses that display a tropism for pancreatic tissue, include without limitation, coxsackie B virus; viruses that display a tropism for skin tissue, include without limitation, varicella zoster virus, herpes simplex virus 6, smallpox virus, molluscum contagiosum, papilloma viruses, parvovirus B19, rubella, measles and coxsackie A virus; and viruses that display a tropism for genital tissue, include without limitation, herpes simplex type 2, papillomaviruses, human immunodeficiency virus (HIV).

[0135] In various embodiments, a method for treating a cancer in a subject in need thereof is provided by administering a conjugated VLP of this invention to the subject wherein the recall protein is a CD8+ epitope of a pathogen that has a tropism for the tissue that is the source of the cancer (the “source tissue”). In various embodiments, the appropriate conjugated VLP is selected by first determining the source tissue of the tumor cell and then selecting a recall protein: (1) to which the patient already has existing CD8+ T cells, and (2) that has a tropism for the source tissue of the tumor. The selected conjugated VLP(s) are then administered to the patient in need thereof.

[0136] In various embodiments, a method for treating a lung cancer comprising determining if a subject has been actively vaccinated against a pathogen that infects lung cells, e.g., an influenza virus, e.g., influenza virus type A or type B, then administering an effective amount of a conjugated VLP of this invention wherein the CD8+ T cell epitope of the recall protein is of the antigenic determinants of the pathogen contained in the vaccine and which T cell epitope forms a complex with an MHC molecule class I of the subject. In an aspect of a method of this invention for treating a lung cancer includes determining if a subject has been infected with pathogen that infects lung cells, e.g., an influenza virus, e.g., influenza virus type A or type B, then administering an effective amount of a conjugated VLP of this invention wherein the CD8+ T cell epitope of the recall protein is of that pathogen and which T cell epitope forms a complex with an MHC class I molecule of the subject.

[0137] An aspect of the invention is a method for treating an oral cancer, which are part of the group of cancers commonly referred to as head and neck cancers, by administering a conjugated VLP of this invention wherein the CD8+ epitope of the recall protein is of a pathogen that has a tropism for oral tissue, e.g., a mumps virus, Epstein Barr virus, cytomegalovirus, or a herpes simplex virus type 1. The method comprises determining if a subject in need thereof has been actively vaccinated against, or infected with, e.g., a mumps virus, Epstein Barr virus, cytomegalovirus, or a herpes simplex virus type 1, and if the subject has been vaccinated or infected previously then administering to the subject a conjugated VLP of this invention wherein the CD8+ epitope of the recall protein is of a mumps virus or a

measles virus or of the antigenic component of the vaccine the subject had received, or of the pathogen, i.e., mumps, measles, Epstein Barr virus, cytomegalovirus, or a herpes simplex virus type 1, that had previously infected the subject.

Combination Therapy

[0138] In various embodiments, the conjugated VLP may be co-administered with other cancer therapeutics. Furthermore, in some embodiments, the conjugated VLPs described herein are administered in conjunction with other cancer treatment therapies, e.g., radiotherapy, chemotherapy, surgery, and/or immunotherapy. In some aspects of this invention, the conjugated VLPs described herein are administered in conjunction with checkpoint inhibitors. In various embodiments the chimer VLP may be administered in conjunction with an immune agonist. In various embodiments, the conjugated VLP may be administered in conjunction with treatment with a therapeutic vaccine. In various embodiments, the conjugated VLP may be administered in conjunction with treatment with a conjugated antigen receptor expressing T cell (CAR-T cell). In various embodiments, the conjugated VLP may be administered in conjunction with treatment with another immuno-oncology product. The conjugated VLPs of the present invention and other therapies or therapeutic agents can be administered simultaneously or sequentially by the same or different routes of administration. The determination of the identity and amount of therapeutic agent(s) for use in the methods of the present invention can be readily made by ordinarily skilled medical practitioners using standard techniques known in the art.

EXAMPLES

Example 1

Conjugated VLPs bind to mouse tumors *in vivo*

[0139] The ability of the described conjugated VLPs to bind to tumors is an important first step for the proposed mechanism of action (see Figure 1). Binding of VLP *in vivo* was assessed using a murine cervical cancer model, TC-1. Briefly, unconjugated VLPs are produced in Expi293F™ human cells (ThermoFisher Scientific, Waltham, MA, US) using the Expi293F™ Expression system. In this protocol, 360×10^6 cells in 120 mL of cultured media is prepared in a 250 mL shake flask. Following this, to express the HPV16 L1 VLP, cells were transfected with the HPV16 L1 gene expression vector (Cat #89910, Addgene, Cambridge, MA, US) via the manufacturer's protocols (ThermoFisher Scientific, Waltham, MA, US).

Transfected cells were maintained for 72 hrs and were then harvested. HPV particles were released from Expi293F cells by detergent lysis and the lysate was incubated overnight at 37 °C with a mild detergent, e.g. Brij58 or Triton X-100. The VLP from the overnight incubated lysates were then solubilized by addition of sodium chloride. The lysate was then clarified by low-speed centrifugation. Capsids were separated from cell debris and detergent by high salt extraction followed by ultracentrifugation through an OPTIPREP™ (iodixanol) step gradient according to manufacturer's instructions (Sigma-Aldrich, St Louis, MI, US). Purity of the VLP preparation was determined by SDS- PAGE Coomassie stained gel (4-20% CRITERION™ gel) staining as per manufacturer's protocol (Biorad Laboratories, Hercules, CA, US) and morphology of particles assessed by electron microscopy (EM). (See also, WO 2018/237115).

[0140] The HPV16 L1 wild type protein sequence as reported elsewhere is as follows (SEQ ID NO: 86):

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Met Ser Leu Trp Leu Pro Ser Glu Ala Thr Val Tyr Leu Pro Pro
Val
Pro Val Ser Lys Val Val Ser Thr Asp Glu Tyr Val Ala Arg Thr
Asn
Ile Tyr Tyr His Ala Gly Thr Ser Arg Leu Leu Ala Val Gly His
Pro
Tyr Phe Pro Ile Lys Lys Pro Asn Asn Asn Lys Ile Leu Val Pro
Lys
Val Ser Gly Leu Gln Tyr Arg Val Phe Arg Ile His Leu Pro Asp
Pro
Asn Lys Phe Gly Phe Pro Asp Thr Ser Phe Tyr Asn Pro Asp Thr
Gln
Arg Leu Val Trp Ala Cys Val Gly Val Glu Val Gly Arg Gly Gln
Pro
Leu Gly Val Gly Ile Ser Gly His Pro Leu Leu Asn Lys Leu Asp
Asp
Thr Glu Asn Ala Ser Ala Tyr Ala Ala Asn Ala Gly Val Asp Asn
Arg
Glu Cys Ile Ser Met Asp Tyr Lys Gln Thr Gln Leu Cys Leu Ile
Gly
Cys Lys Pro Pro Ile Gly Glu His Trp Gly Lys Gly Ser Pro Cys
Thr
Asn Val Ala Val Asn Pro Gly Asp Cys Pro Pro Leu Glu Leu Ile
Asn
Thr Val Ile Gln Asp Gly Asp Met Val Asp Thr Gly Phe Gly Ala
Met
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Asp Phe Thr Thr Leu Gln Ala Asn Lys Ser Glu Val Pro Leu Asp
 Ile
 Cys Thr Ser Ile Cys Lys Tyr Pro Asp Tyr Ile Lys Met Val Ser
 Glu
 Pro Tyr Gly Asp Ser Leu Phe Phe Tyr Leu Arg Arg Glu Gln Met
 Phe
 Val Arg His Leu Phe Asn Arg Ala Gly Thr Val Gly Glu Asn Val
 Pro
 Asp Asp Leu Tyr Ile Lys Gly Ser Gly Ser Thr Ala Asn Leu Ala
 Ser
 Ser Asn Tyr Phe Pro Thr Pro Ser Gly Ser Met Val Thr Ser Asp
 Ala
 Gln Ile Phe Asn Lys Pro Tyr Trp Leu Gln Arg Ala Gln Gly His
 Asn
 Asn Gly Ile Cys Trp Gly Asn Gln Leu Phe Val Thr Val Val Asp
 Thr
 Thr Arg Ser Thr Asn Met Ser Leu Cys Ala Ala Ile Ser Thr Ser
 Glu
 Thr Thr Tyr Lys Asn Thr Asn Phe Lys Glu Tyr Leu Arg His Gly
 Glu
 Glu Tyr Asp Leu Gln Phe Ile Phe Gln Leu Cys Lys Ile Thr Leu
 Thr
 Ala Asp Val Met Thr Tyr Ile His Ser Met Asn Ser Thr Ile Leu
 Glu
 Asp Trp Asn Phe Gly Leu Gln Pro Pro Pro Gly Gly Thr Leu Glu
 Asp
 Thr Tyr Arg Phe Val Thr Ser Gln Ala Ile Ala Cys Gln Lys His
 Thr
 Pro Pro Ala Pro Lys Glu Asp Pro Leu Lys Lys Tyr Thr Phe Trp
 Glu
 Val Asn Leu Lys Glu Lys Phe Ser Ala Asp Leu Asp Gln Phe Pro
 Leu
 Gly Arg Lys Phe Leu Leu Gln Ala Gly Leu Lys Ala Lys Pro Lys
 Phe
 Thr Leu Gly Lys Arg Lys Ala Thr Pro Thr Thr Ser Ser Thr Ser
 Thr
 Thr Ala Lys Arg Lys Lys Arg Lys Leu

[0141] For functional assays, live C57BL/6 mice (n=5, The Jackson Laboratory, Bar Harbor, MA, US) were subcutaneously injected with luciferase/green fluorescent protein (GFP)-expressing TC-1 tumor cells (2×10^5 cells). Mice were monitored for approximately 2 weeks to ensure consistency in tumor growth and also until palpable tumors were observed (about 7 mm in diameter). (See, FIG. 2A). Three mice were injected intravenously with 50 μ g

of VLP. Two mice served as untreated controls. After 16-24 hours post injection of the VLP, all 5 mice were sacrificed and the tumors were excised. Tumors were then digested with collagenase before being treated at a dilution of 1:100 at 4 °C in fluorescence activated cell sorting (FACS) staining buffer (phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA)), first with HPV16 anti-V5 anti-mouse antibody specific for VLP (Abcam, Burlingham, CA, US). After one hour, cells were then washed twice with FACS staining buffer and stained for another hour at a dilution of 1:100 at 4 °C with goat phycoerythrin (PE)-conjugated anti-mouse antibody specific for VLP (R&D systems, Minneapolis, MN, US). Cells were then washed twice again with FACS staining buffer before being analyzed by fluorescence activated cell sorting (FACS) cytometry: (1) first, gated for GFP to sort for only tumor cells; and (2) second, to assess the sorted tumor cells for the presence of the VLP (anti-L1 VLP antibody). (See FIG. 2B, right panel and left panel are data each generated from a different mice). FIG. 2B demonstrates that the conjugated VLPs were able to bind to the tumor, as indicated by rightward shift on the histogram plot of both the left and right panels.

Example 2

Conjugated VLP bind to human tumors *in vitro*

[0142] To test for the ability of conjugated VLPs to bind to human tumors *in vitro*, five human tumor cells lines are to be assessed: OVCAR3, MDA-MB231, HCT116, PC-3, and SKOV3 (ATCC, Manassas, VA, US). The respective cell lines will be first seeded in one well within a 6 well plate. After 16 to 24 hours, cells will be dissociated using 0.05% trypsin. Cells will then be counted to achieve 1×10^6 cells, centrifuged, and the supernatant removed before being re-suspended in 100 μ L of FACS buffer (1% FBS in PBS). Next, 0.3 μ g/ml of conjugated VLP will be added to the respective cultures *in vitro* and the cultures incubated for 24 hours under optimal growth conditions. To show that the binding is HSPG-specific, conjugated VLPs will be either pre-incubated with or without heparin (final concentration of 0.1 mg/ml) and incubated at 4°C (to prevent endocytosis) for 1 hr. After 1 hr of binding, cells will be washed twice with 1 mL of FACS buffer before being resuspended in 100 μ L of FACS buffer again. The samples will then be incubated with PE-conjugated anti-mouse antibody specific for VLP at 4°C for 1 hour. After 1 hour, cells will be washed and re-suspended in FACS buffer for analysis by flow cytometry. Binding is then assessed either as a percent positive of total PE+ cells, or of the geometric mean fluorescence, as indicated by a rightward shift on the histogram plot, as in Example 1 and Figures 2A and 2B.

Example 3**Conjugated VLP bind to human tumors *in vivo***

[0143] The described conjugated VLPs will be further tested to assess their ability to bind *in vivo* to human tumors. In a first experiment, mice will be injected with OVCAR3 tumor cells (3×10^6 cells), a human ovarian cancer cell line. At approximately two weeks after injection, the average tumor volume is expected to be approximately 10 mm^3 in volume. Three weeks post injection, the tumor bearing mice will be administered a Cu^{2+} or Zn^{2+} radiolabeled VLPs. Biodistribution and tumor specificity of the VLP is to be assessed at 4, 12, 24, and 48 hours. The tumors are then removed for weighing, radioactive counting, viral micro distribution and cellular colocalization. In addition to tumors, various major organs such as the liver, spleen, kidneys will be assessed for VLPs presence using imaging methods as well as histology to assess the efficacy of VLP homing to the tumor. In further experiments, mice are to be injected with other tumor cell lines such as HCT116, PC-3, or SKOV3 and the same experimental protocol followed.

Example 4**VLP-induced T cell-mediated killing of tumor cells *in vitro* in a time-dependent manner**

[0144] The ability of conjugated VLPs to deliver epitopes to MHC proteins on a tumor cell surface and subsequently redirect existing T cells to subject tumor cells leading to cytotoxic killing of tumor cells was tested *in vitro*. We chose to demonstrate proof-of-concept efficacy using pre-existing murine memory responses to the human papillomavirus HPV16 E7 antigen in a murine tumor model as this would be more clinically relevant. HPV16 E7 is a true human viral antigen, as opposed to often used artificial antigens such as ovalbumin (OVA). It is known that several different E7 antigen-specific CD8^+ T-cells are found in the peripheral blood of healthy human donors with different HLA-I (MHC-I) genes. Therefore, the use of E7 is relevant, provides proof-of-concept showing the redirection of pre-existing CD8^+ T-cell responses to target a non-virally associated malignancy (tumor), and acts as a solid base from which to extend this system to other human viral antigens.

[0145] **Conjugation of VLPs.** To produce conjugated VLPs, recall proteins were synthesized to greater than 85% purity as polycationic 18-mer to 20-mer peptides comprising a maleimide molecule at the N-terminus, followed by a protease cleavage site and ending with a CD8^+ recall epitope, i.e. N-terminal maleimide-RRRRRVKR-epitope (Genscript, New Jersey, US). Samples were sent as lyophilized materials and diluted to a concentration of 1 to 20 mM with dimethyl sulfoxide (DMSO). Conjugated VLP was prepared for conjugation via

the following protocol. First, VLPs at a concentration of at least 1 mg/mL were dialyzed into conjugation reaction buffer (50 mM sodium phosphate, pH 6.5, 500 mM NaCl, 2 mM EDTA, 0.05% Tween 80) with buffer exchanged 3 times (3 + 1 h, 3 + 1 h, and overnight 16 + 3 h at 2 to 8 °C). The next day, VLPs were then treated with TCEP for 1 hour without shaking at room temperature at a TCEP:L1 (monomer) ratio of 10:1 molar ratio, where the concentration of L1 monomer was 0.77 mg/mL. Subsequently, conjugation was performed by adding the 18- to 20-mer peptide at a molar ratio of peptide:L1 monomer of 5:1, bringing the final concentration of L1 monomer to 0.58 mg/mL. The reaction was shaken at 200 rpm for 1 hour. Following conjugation, contents from the reaction were subjected to dialysis (PBS, pH 7.0 + 0.1, 500 mM NaCl, 0.05% Tween 80) using a dialysis cassette (MWCO = 1000 kDa) for about 3 + 1 hours in a cold room (2 to 8 °C). Next, the contents were subjected to density gradient ultracentrifugation (UC) OPTIPREP™ gradient (in DPBS, 0.8M NaCl) at 40,000 rpm, for 16 hours at 16 °C. Fractions (1-3) starting from the interface between 33 - 39% OPTIPREP™ were collected and then subjected to a final dialysis (MWCO = 1000 kDa) for 3 + 1, 3 + 1, and 16 + 3 hours in a cold room. Following purification, samples were analyzed under gel electrophoresis/Coomassie staining to determine % conjugation via the shift in L1 band beyond about 55 kDa.

[0146] Quantification of conjugated VLPs. Product concentrations, as well as % fusion protein conjugation, was determined by reducing SDS-PAGE (4 – 20% CRITERION™ TGX Stain-Free™ Precast Gels, 18 Well Comb, 30 µL, 1.0 mm, Bio-Rad, Hercules, CA, US). In addition, an unconjugated VLP control as well as five known BSA standards (Sigma Life Science, St. Louis, MO, US) of quantities between 5 µg to 2.5 µg will always be included as controls. Gels were run per manufacturer's protocols and then subsequently subjected to Coomassie gel stain as per manufacturer's protocols (Coomassie Brilliant Blue R-250 dye, Bio-Rad, Hercules, CA, US). Amount and/or percent conjugation was determined via densitometry analysis utilizing BioRad software imaging analysis as per the manufacturer's protocols.

[0147] Design of peptides. Peptides are designed to include an N-terminal maleimide-RRRRRVKR-epitope, as described above. The peptides therefore contain a maleimide molecule at the N-terminus of the recall protein followed by a furin cleavage sequence: R X R/K R (SEQ ID NO: 89) upstream of the recall protein sequence. Exemplary furin cleavage sequence: ARG VAL LYS ARG (SEQ ID NO: 90).

[0148] For functional assays, murine B16 (melanoma/skin), and ID8 (ovarian) tumor cells overexpressing luciferase gene (B16-luc and ID8-luc) were grown in culture. Under normal circumstances, these two murine tumor cell lines B16 and ID8 will not be killed by

murine HPV16 E7-specific CD8+ T cells since these cell lines do not express the HPV16 E7 antigen. Cells were grown in culture and seeded overnight. The next day, cells were then treated with either: (1) 3 different concentrations of HPV16 E7 peptide, RAHYNIVTF (SEQ ID NO: 1) (1 µg/ml, 100 pg/ml, 1 pg/mL), (2) HPV16 E7-conjugated VLP (2.5 µg /mL), (3) an unconjugated (control) VLP (2.5 µg /mL), or (4) were left untreated. The cells were then washed and co-cultured with murine CD8+ HPV16 E7-specific T cells at varying E:T ratios (Effector:Target ratio). The total final volume after co-culture was 200 µL. The cell viability was measured after a certain time of co-culturing by measuring the expression of luciferase, which is used as a surrogate marker for cell viability since these cells over-express luciferase. Reduced luciferase activity indicated more cell death suggesting greater immune redirection and hence greater cytotoxicity.

[0149] Responses to the HPV16 E7-conjugated VLP and HPV16 E7 peptide were time-dependent and concentration-dependent in both cancer cell lines. After 5.5 hrs of co-culturing, both the E7 peptide only (1 µg/mL) and the conjugated VLP (2.5 µg/mL) demonstrated significant levels of T cell mediated cytotoxicity in the B16-luc. (See, FIG. 3A, B16-LUC, and FIG. 3C, ID8). Additional cytotoxicity was seen if co-culturing occurred for 8.5 hours, with cytotoxicity levels of approximately 50% in B16-LUC cells (FIG. 4B) and 38-58% in ID8-LUC cells (FIG. 4D) at E:T ratios of 10. Likewise, FIG. 4 shows similar results achieved over an even longer period of time, 22.5 hrs. Specifically, FIG. 4A and 4B show that cytotoxicity is improved between 8.5 hrs and 22.5 hrs, respectively, for E7-conjugated VLP incubated with B16-LUC cells and murine CD8+ HPV16 E7-specific T cells. Similarly, FIG. 4C and 4D show that cytotoxicity is improved between 8.5 hrs and 22.5 hrs, respectively, for E7-conjugated VLP incubated with ID8 cells and murine CD8+ HPV16 E7-specific T cells.

[0150] These results suggest that both an E7 peptide at higher concentrations (1 µg/mL) and an E7-conjugated conjugated VLP exhibit time dependent tumor cell killing. This further suggests that the E7-conjugated conjugated VLPs undergo furin cleavage and epitope coating of MHC receptors on the tumor cells in order to achieve tumor cell killing.

Example 5

VLP-induced E7 T cell-mediated killing of tumor cells in a concentration-dependent manner

[0151] E7 T cell-mediated killing was also shown to be E7-conjugated VLP concentration dependent. Murine B16 (Melanoma/Skin) and ID8 (ovarian) tumor cells overexpressing luciferase gene (B16-luc and ID8-luc) were seeded. 24 hours later, the cells

were then treated with: (1) an HPV16 E7 peptide RAHYNIVTF (SEQ ID NO: 1) (1 µg/ml, 1 ng/ml); (2) HPV16 E7 conjugated VLP (2.5 µg/ml, 0.025 µg/ml); (3) an unconjugated (control, 2.5 µg/ml) VLP; or (4) were left untreated. The cells were then washed and co-cultured with CD8+ HPV16 E7 specific T cells at varying E:T ratios (Effector:Target ratio). The total final volume after co-culture was 200 µL. The cell viability was measured after a certain time of co-culturing by measuring the expression of luciferase, which is used as a surrogate marker for cell viability since these cells over-express luciferase. Reduced luciferase activity indicated greater immune redirection and hence greater cytotoxicity.

[0152] Responses to the HPV16 E7-conjugated VLP and HPV16 E7 peptide were concentration-dependent and in both cancer cell lines. Both the E7 peptide alone (1 µg/mL) and the E7 conjugated to VLP (2.5 µg/mL) demonstrated significant levels of T cell mediated cytotoxicity in both cell lines. (See, FIG. 3A and 3B, B16-LUC, and FIG. 3C and 3D, ID8). Figure 3 shows particularly that E7 peptide alone at 1 pg/mL exhibit little if any tumor cell killing, regardless of time point, whereas E7 peptide at 100 pg/mL and 1 µg/mL exhibited significant tumor cell cytotoxicity, regardless of time point. Similarly, concentration dependence of cytotoxicity was observed for E7-conjugated VLP in Figure 4. (See, FIG. 4A and 4B, B16-LUC, and FIG. 4C and 4D, ID8). In Figure 4, significant increases in cytotoxicity are observed between 0.025 µg/mL conjugated VLP vs. 2.5 µg/mL conjugated VLP for both cell lines regardless of time point (8.5 hr or 22.5 hr). Thus, Figures 3 and 4 show that cytotoxicity is improved as concentration increases for both E7 peptide and E7-conjugated VLP incubated with B16-LUC cells and murine CD8+ HPV16 E7-specific T cells.

EXAMPLE 6

[0153] Batch consistency of VLP preparations. To ensure that the described process of creating conjugated VLPs that could perform immune redirection was consistent, several separately created batches of E7 peptide-conjugated VLP termed “F1, F2, and F3” were examined. Murine B16 (Melanoma/Skin) and ID8 (ovarian) tumor cells overexpressing luciferase gene (B16-luc and ID8-luc) were seeded. 24 hours later the cells were incubated with: (1) an HPV16 E7 peptide RAHYNIVTF (SEQ ID NO: 1) (1 µg/ml); (2) HPV16 E7-conjugated VLP batch F1, F2, or F3 (2.5 µg/ml); (3) an unconjugated (control, 2.5 µg/ml) VLP; or (4) were left untreated. All batches F1- F3 were consistent and were able to activate E7 T cells, and showed killing of B16-luc and ID8-luc cells after 23 or 23.5 hr time points in two separate experiments respectively. (See, FIG. 5A and 5B, respectively, for B16-luc cells; FIG. 5C and 5D, respectively, for ID8-luc cells).

[0154] To further demonstrate batch consistency in another tumor cell line, MC38 murine colon tumor line, cells were grown in culture per manufacturer suggested conditions. Under normal circumstances, this cell line will not be killed by murine HPV16 E7-specific T cells since the MC38 cell line does not express the murine E7 antigen. MC38 cells were then treated with: (1) an E7 peptide RAHYNIVTF (SEQ ID NO: 1) (1 µg/ml; (2) HPV16 E7-conjugated VLP, from batch F1, F2, or F3 (2.5 µg/ml); (3) an unconjugated (control, 2.5 µg/ml) VLP; or (4) were left untreated. The cells were then washed and co-cultured with CD8+ HPV16 E7-specific T cells at varying E:T ratios (Effector:Target ratio). The total final volume after co-culture was 200 µL. The cell viability was measured after a certain time of co-culturing. In this example, because MC38 does not express luciferase, the cell viability was measured using an establish cell viability assay reagent: CELLTITER-GLO® (PROMEGA, WI, US). This assay provides a luciferase-expressing chemical probe that detects and binds to ATP, a marker of cell viability. Reduced luciferase activity hence indicates more MC38 cell death suggesting greater immune redirection and hence greater cytotoxicity. All batches F1-F3 were consistent in their functional activity and were able to activate E7 T cells, and showed killing of MC38 cells after 23 and 23.5 hr time points in two separate experiments respectively. (See, FIG. 6A and 6B, respectively).

Example 7

***In Vivo* anti-tumor immune redirection in C57BL/6 mice with E7-conjugated VLP**

[0155] Efficacy of HPV16 E7-conjugated VLPs (conjugated-VLPs conjugated to murine MHC-restricted H-2D^b HPV16 E7 peptide) was assessed *in vivo*. A total of 20 C57BL/6 mice were injected intra-peritoneally (I.P.) with ID8-luciferase cells (0.015×10^6 cells/mouse). On Day 6, mice were checked for tumor growth and establishment via luciferase luminescent imaging. (IVIS® Spectrum *in vivo* imaging system, Perkin Elmer, Waltham, MA, US). At day 5, mice are separated into treatment groups and then treated with: (1) buffer only, (2) non-conjugated VLP (100 µg), (3) murine CD8+ HPV16 E7 specific T cells plus E7 peptide conjugated-VLP (n=5, 100 µg), and (4) E7 peptide only (30 µg, n = 5). After 1 hr all mice were injected with approximately 1×10^6 Day 4 post-stimulated CD8+ HPV16 E7 specific T cells (200 µL per mouse). On day 6 and day 7 treatments were administered a second time for each mouse, but no additional stimulated T cells were added. Mice are then monitored and imaged. Efficacy of treatment is monitored by luminescence imaging which acts as a surrogate for tumor growth and metastasis.

[0156] Results are provided in Figure 7 and show that E7 conjugated VLP therapy was able to redirect E7 specific T-cells to the ID8 murine tumor and control tumor formation even after 60 days post-treatment. (FIG. 7A and FIG. 7D). In contrast, controls such as non-conjugated VLP (FIG. 7C) or buffer only (FIG. 7B) failed to show any tumor protection or control. Although some protection was afforded by peptide only (FIG. 7E), it is important to recount that the overall molarity of peptides on E7 conjugated VLP versus peptide alone was less compared to peptide alone. Further, peptide provided incomplete protection, suggesting that the tumor specific targeting of the VLP is important compared to injection of exogenous peptide alone.

Example 8

In Vivo anti-tumor immune redirection in B16.F10 mice with E7-conjugated VLP

[0157] Efficacy of HPV16 E7 VLPs was assessed in another murine tumor model (B16.F10, ATCC, Old Town Manassas, VA, USA). Unlike the ID8 ovarian murine model in which tumors are grown intraperitoneally, the BL16.F10 mouse tumor model is a subcutaneous tumor model. In this experiment, 20 mice were first vaccinated with HPV16 E7 peptide RAHYNIVTF (SEQ ID NO: 1). After 2 weeks, the mice were verified to have an immune response specific to the HPV 16 E7 peptide via HPV16 E7 tetramer (H-2D^b/HPV16 E7 (RAHYNIVTF)(SEQ ID NO: 1) MHC Tetramer staining). Briefly, whole blood (2-3 drops) was collected from the tail vein from the mice and checked for antigen specific CD8⁺ T cell responses by flow cytometry using E7 tetramer (H-2D^b/HPV16 E7 (RAHYNIVTF)(SEQ ID NO: 1) MHC Tetramer, MBL International, Woburn MA, US). Mice were then injected subcutaneously with B16.F10-luciferase cells (0.5×10^6 cells/mouse) at the left flank of the thigh. Tumor growth volume was measured using Vernier calipers and volume was calculated using the formula $(W^2 \times L)/2$, where W (width) is the shorter of the two orthogonal measurements and L (length) is the longer of the two. Mice were monitored every 2 days until palpable tumors (~7 mm in diameter) were observed. Subsequently, mice were divided into various treatment groups, as follows: (1) buffer only (n=5); (2) non-conjugated VLP ("Control" VLP) (n=5); (3) E7-conjugated VLP (n=5); and (4) peptide alone. All treatment groups received 5 µg indicated component per dose. A total of four treatments were administered to the mice, every other day. Mice were then monitored to assess tumor volume. Efficacy of treatment was monitored via tumor volume growth and survival in which the endpoint survival is when tumor growth volume reaches 1500 mm³. Results showed that a delayed effect in tumor

growth was observed in control VLP, E7 conjugated VLP, and E7 peptide groups. (See, FIG. 8A and FIG. 8B). However, eventually all tumors reached the end point size of 1500 mm² (FIG. 8A). All control mice reached their survival endpoint by day 17 (FIG. 8B). Treated mice survived up to day 19 to 22. One mouse from the E7 conjugated VLP-treated group had all tumors regressed, completely, and remained tumor free for over 80 days. (See, FIG. 8B).

[0158] To further test the importance of specific viral memory T-cells and whether their increased presence would lead to an improved therapeutic outcome, the mice were also separated into groups of “high” and “low” T-cell frequencies (see below for explanation), i.e. those mice that had a higher frequency of peripheral HPV16 E7 T cells in their PBMCs, vs. those that did not. Mice were first vaccinated with HPV16 E7 peptide RAHYNIVTF (SEQ ID NO: 1) to generate the anti-viral immune memory response. The vaccinated mice were left alone for approximately 6 weeks to establish immune memory to HPV16 E7. To check for immunogenicity, mice were separated into “high” and “low” T-cell frequencies. Briefly, whole blood (2-3 drops) was collected from the tail vein from the mice and checked for antigen specific CD8⁺ T cell responses by flow cytometry using E7 tetramer (H-2D^b/HPV16 E7 (RAHYNIVTF) (SEQ ID NO: 1) MHC Tetramer, MBL International, Woburn MA, USA). The frequency of HPV16 E7 Tetramer-specific T cell response was between 1-50%. Mice that had a T cell frequency ranging between 1-14% were considered “low” and mice that had a T cell frequency ranging between “15%-50%” were considered “high.” All mice were then injected subcutaneously with B16.F10 melanoma tumor cells (0.5 x 10⁶ cells/mouse) at the left flank of the thigh. Tumor growth volume was measured using Vernier calipers and volume was calculated using the formula (W² x L)/2, where W (width) is the shorter of the two orthogonal measurements, and L (length) is the longer of the two. Tumor volumes were allowed to reach 50 to 100 mm³ before starting treatments with E7 conjugated VLPs or control VLPs (non-conjugated VLP).

[0159] Results are depicted in Figure 8C and Figure 8D and show that a delayed effect in tumor growth was observed in mice treated with E7 conjugated VLP but not in control groups treated with VLP alone. (See, FIG. 8C). However, eventually all tumors reached the end point of 1500 mm² by day 32 (FIG. 8D). All control mice reached their survival endpoint by day 17. (See, FIG. 8B). Treated mice survived up to day 24 for mice with “low” T-cell frequency, or up to day 32 for mice with “High” T-cell frequency. (FIG. 8D). In the absence of treatment, there was no difference in B16.F10 tumor growth between mice that had higher or lower frequency of E7 specific memory T-cells. (FIG. 8C). In contrast, tumor growth in the treatment groups seemed to suggest that the mice with higher E7 T-frequencies responded

better to E7 conjugated VLP treatment, as the tumor growth was better controlled (FIG. 8D). However, further study of the data revealed no obvious trend when individual mice were scrutinized. This could be due to the “variable” tumor size as treatment was administered once all mice had tumors between 50 to 100 mm³. In general, it was observed that mice who had smaller tumors (closer to 50 mm³) responded better to the conjugated VLP treatment, but this did not necessarily correlate with a higher pre-existing T-cell PBMC frequency. This suggests that the immune redirection effect could be due to other potential E7-specific T cells, i.e. tissue resident memory T-cells. Nonetheless, these data suggest that immune redirection is possible even in a difficult B16.F10 mouse model that is known to be checkpoint refractory.

Example 9

Detection of VLP-peptide loading onto MHC-I receptors on mouse tumor cells *in vitro*

[0160] An assay system was developed to directly detect peptide-loading from conjugated-VLP onto MHC I receptors on tumor cells. This assay involves the use of OVA-conjugated VLPs (“OVA-VLPs”) and an antibody (fluorophore-conjugated anti-OVA (SIINFEKL)/K^b (SEQ ID NO: 2) antibody, MBL International, Woburn, MA, US) that specifically recognizes the OVA (SIINFEKL)/K^b (SEQ ID NO: 2) complex, but not any other peptide/MHC-I complexes.

[0161] To this end, target cells (B16-LUC, ID8-LUC, and MC38) were plated at 0.02 x 10⁶ cells/well on a 96-well plate. The next day, OVA-conjugated VLPs (2.5 µg/ml) were incubated with target tumor cells for 1 hr at 37 °C, followed by extensive washing to remove any unbound materials. Next, fluorophore-conjugated anti-OVA (SIINFEKL)/K^b (SEQ ID NO: 2) antibody was added directly to the cells at a dilution of 1:100 (total volume of 100 µL, i.e. 1 µL) for 30 minutes at 4 °C. Following this incubation, excess antibodies were washed off and the cells were analyzed for the presence of the OVA (SIINFEKL)/K^b (SEQ ID NO: 2) complex by FACS flow cytometry. As expected, the OVA (SIINFEKL)/K^b (SEQ ID NO: 2) complex was detected in three different mouse tumor cells after incubation with OVA-conjugated VLPs. (See, Figure 9).

[0162] FIG. 9A shows FACS data resulting from incubation of ID8-LUC (top panel), B16-LUC (middle panel), and MC38 (lower panel) cells with OVA peptide alone or OVA-conjugated VLP. This indicates that OVA-conjugated VLPs bind to these tumor cells and their peptides were cleaved, followed by binding of the cleaved peptide to the tumor MHC-I molecules. Therefore, the design of the VLPs is verified to perform the intended functions, i.e. the epitope peptide is cleaved by furin and the peptide is bound by MHC I molecules on the

tumor cell surface. These findings should be applicable to other viral peptides, other than OVA. It is also important to note that differential OVA (SIINFEKL)/K^b (SEQ ID NO: 2) complex was detected across the murine tumors and further studies suggest that loading might be dependent on availability of surface MHC Class 1 on tumor surfaces (*not shown*). FIG. 9B shows the same cells, ID8-LUC (top panel), B16-LUC (middle panel), and MC38 (lower panel), stained by binding of an H2-D^b antibody (PE anti-mouse H-2D b antibody, anti-H-2D^b, Biolegend, San Diego, CA, US) specific for MHC-I complex.

Example 10

Redirection of human CD8+ T cell viral immunity against human immortalized tumor cell lines using VLP conjugated to HCMV pp65 antigen

[0163] The ability of the described conjugated VLP system to trigger immune redirection using other antigens or epitopes was further explored by investigating the ability of human cytomegalovirus (HCMV) pp65 antigen-conjugated VLP to redirect immunity. The conjugated VLPs were generated as described above. The rationale to choose HCMV was based on knowledge that HCMV is highly prevalent (infecting 50-90% of the human population) and mostly asymptomatic in healthy individuals. (See, Longmate et al., *Immunogenetics*, 52(3-4):165-73, 2001; Pardieck et al., *F1000Res*, 7, 2018; and van den Berg et al., *Med. Microbiol. Immunol*, 208(3-4):365-373, 2019). Importantly, HCMV establishes a life-long persistent infection that requires long-lived cellular immunity to prevent disease. Hence, it is rational to hypothesize that a complex adaptive cell-mediated anti-viral immunity developed over many years to strongly control a viral infection in an aging person can be repurposed and harnessed to treat cancer.

[0164] **In vitro cytotoxicity assays.** Human target cells, HTC112, human colon cancer, MCF7, human breast cancer or OVCAR 3, human ovarian cancer (ATCC, Manassas, VA, US) were seeded overnight at 0.01 to 0.2 x 10⁶ per well per 100 µL per 96 well plate. The next day (about 20 to 22 hrs later), each cell line was incubated for one hour at 37 °C under the following conditions: (1) CMV peptide at a final concentration of 1 µg / mL, (2) control VLP (no peptide) at a final concentration of 2.5 µg/mL, (3) CMV-conjugated VLP at a final concentration of 2.5 µg/mL, and CMV peptide at a final concentration of 14 pg/mL (equivalent to peptide conjugated to CMV-VLP at 2.5 µg/mL). After 1 hour, the cells were washed vigorously with 200 µL of media for three times to remove non-specific binding. Human patient donor CMV T cells (ASTARTE Biologics, Seattle, WA, US) were added at the E:T (effector cell:target cell) ratio of 10:1 and incubated in a tissue culture incubator for 24 hrs. The total final volume

after co-culture was 200 μ L. The cell viability was measured after a certain time of co-culturing. In this example, because the above human cells does not express luciferase, the cell viability was measured using an establish cell viability assay reagent: CELLTITER-GLO® (PROMEGA, WI, US). This assay provides a luciferase-expressing chemical probe that detects and binds to ATP, a marker of cell viability. Reduced luciferase activity hence indicates more human immortalized cell death suggesting greater immune redirection and hence greater cytotoxicity

[0165] The results are provided in Figure 10. CMV conjugated VLP was effective in killing human healthy donor CMV pp65-specific CD8+ T-cells (Astarte Biologics, Inc., Bothell, WA, US), immortalized HLA.A2 positive human colon cancer cells (HCT116, FIG. 10A), human ovarian cancer cells (OVCAR3, FIG. 10B) and human breast cancer cells (MCF7, FIG. 10C). In both the mouse and human systems, control VLPs, i.e., no peptide conjugation, did not induce killing of the target cells, nor did co-incubation of CD8+ T cells with tumor cells alone. In these series of *in vitro* studies using human cell lines, it was determined that 1 μ g/mL of CMV peptide is equivalent to 1060 nM peptide, whereas the molar equivalent of CMV peptide conjugated to the surface of the VLP in 2.5 μ g/mL of CMV-conjugated VLP was $\sim$ 0.024 nM. Thus, a comparable T-cell redirection killing of tumor cells was triggered by the conjugated VLP despite using almost 44,000 times less than the CMV peptide alone. Importantly, it was determined that this 0.024 nM molar equivalent concentration of CMV (pp65) peptide alone that is conjugated to VLP was 14 pg/mL. When cytotoxicity was tested at 14 pg/mL of the free peptide alone, little to no killing of tumor cells was observed in all three human target cells (Figure 10C). This highlights the potency of the conjugated VLP in enhancing peptide loading compared to exogenous free peptide loading-alone onto tumor cells to facilitate immune redirection.

Example 11

VLP conjugation methods

[0166] Two conjugation approaches were tested as candidates for conjugation of the VLP to the fusion protein: (1) wild type HPV vaccine (WT-VLP); and (2) a conjugated HPV vaccine (RG1 VLP which has 2 additional cysteine residues per single L1 monomer and therefore 720 extra cysteines on the RG1 VLP surface compared to WT VLP). For each test, the VLP was first reduced by reaction with tris(2-carboxyethyl)phosphine (TCEP), for 1 hour at room temperature. This reduction step was required to “release” free cysteines on the VLP surface for the next step - maleimide conjugation of the peptides to the VLP. Protocols followed

for reduction and maleimide conjugation utilizing maleimide molecules directly synthesized upstream of the recall protein were as described above.

[0167] The RG1 VLP L1 protein sequence with the additional RG1 peptide sequence insertion is as follows (SEQ ID NO: 87):

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MSLWLPSEAT VYLPPVPVSK VVSTDEYVAR TNIYYHAGTS RLLAVGHPYF PIKKPNNNKI
LVPKVSGLQY RVFRIHLPDP NKFGFPDTSF YNPDTQRLVW ACVGVEVGRG QPLGVGISGH
PLLNLDDTE NASAYAQLYK TCKQAGTCPP DIIPKVANAG VDNRECISMD YKQTQLCLIG
CKPPIGEHWG KGSPCTNVAV NPGDCPPEL INTVIQDGM VDTGFGAMDF TTLQANKSEV
PLDICTSICK YPDYIKMVSE PYGDSLFFYL RREQMFVRHL FNRAGAVGEN VPDDLVIKGS
GSTANLASSN YFPTPSGSMV TSDAQIFNKP YWLQRAQGHN NGICWGNQLF VTVVDITRST
NMSLCAAIST SETTYKNTNF KEYLRHGEEY DLQFIFQLCK ITLTADVMY IHSMNSTILE
DWNFGLPQPP GGTLEDYRF VTSQAIACQK HTPPAKEDP LKKYTFWEVN LKEKFSADLD
QFPLGRKFL QAGLKAKPKF TLGKRKATPT TSSTSTTAKR KKRKLSR
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[0168] Little to no detectable conjugation occurs if there is no reduction step (see Figures 13, lane 3, and Figure 14, lane 5). The efficiency of conjugation is determined by performing Coomassie staining of the VLP on SDS-PAGE (CRITERION™ gels, 4-20% acrylamide, BioRad, Hercules, CA, US) to determine relative amounts of conjugated and unconjugated VLP. (See, FIG. 11). If no conjugation occurs, there will only be a single band as seen in the TCEP:RG1 VLP 1:1 lane. In contrast, the multiple bands in the TCEP:RG1 ratios from 3:1-10:1 show conjugation occurring and therefore also validating the importance of the reduction step. It is also known that the VLPs are held by disulfide bonds and thus addition of TCEP might harm the VLP, however ratios of TCEP:RG1 of 5:1 and 3:1 show intact conjugated VLPs under TEM (transmission electron microscopy), thereby showing a TCEP amount suitable for RG1 conjugation without affecting particle integrity. (See, FIG. 12)

[0169] The relationship of how TCEP concentration determines the coupling efficiency of the fusion protein to the VLP was further investigated at additional ratios of reducing agent to VLP (up to 74:1). (See, FIG. 13). At the highest ratios of TCEP to RG1 (e.g. 74:1), up to 5 coupling sites were present on a single L1 monomer. In contrast, WT-VLP, no matter what ratio of TCEP was tested, yielded only one coupling site per L1 monomer.

Example 12

Human Conjugated VLP trigger tumor cytotoxicity *in vitro* and *in-vivo*.

[0170] The ability to selectively load tumor cells with a common infectious viral antigenic peptide that can easily be recognized and be eliminated by a pre-existing immune recall response is to be evaluated both *in vitro* and *in vivo* using various murine models. As seen in Examples 1 through 11, using a murine MHC-restricted H-2D^b HPV16 E7 peptide from the HPV (human papillomavirus) type 16 E7 antigen, a HPV16 E7-conjugated conjugated VLP (E7-VLP) was generated and shown to specifically bind to the tumor cells ID8 (ovarian), B16 (melanoma), and MC38 (colon). Cleavage of the E7 peptide from the E7-conjugated VLP leads to specific E7 peptide binding to MHC class I receptors on tumor cells thereby rendering those tumor cells susceptible to antigen-specific CTL-mediated killing, in this case murine CD8+ HPV16 E7 specific T cells. As seen in Example 10, using human cytomegalovirus (HCMV) pp65 antigen-conjugated VLPs, this result has also been extended to human healthy donor CMV pp65-specific CD8+ T-cells.

[0171] Since most human individuals have or might have pre-existing immunity against influenza, HPV16, CMV, EBV, and other childhood vaccines (MMR, chicken pox, HEPB), the same experimental methods may be adopted to conjugate the described VLPs to other immunogenic HLA-A2-restricted peptides. HLA-A2 restricted epitopes will be chosen for these experiments as these are the most prevalent human MHC class I allele in the U.S. Various VLPs were conjugated to an HLA-A2 restricted epitope with a furin cleavage site at its N2-terminus as described above. The specific epitopes are provided in Table 4.

Table 4: VLPs Conjugated to an HLA-A2 restricted epitope comprising a furin cleavage site

Virus	Approved Human Vaccine where epitope can be detected (Source)	Epitope	SEQ ID NO
Influenza A	Afluria-Trivalent or Flublok Quadrivalent if aged 18-64, Fluzone High-dose OR Fluad if 65 and older, Flucelvax Quadrivalent for 4-18years old (and older actually)	FMYSDFHFI GILGFVFTL	77
			68
Hepatitis B	Engerix (GSK)	FLPSDFFPSV	41
Measles	MMR-II, (Merck)	KLWESPQEI	36
Chicken Pox	VariVax (Merck)	SLPRSRTPI	4
	VariVax (Merck)	SAPLPSNRV	5
Cytomegalovirus	Natural Infection	NLVPMVATV	3
Human Papilloma virus 16 E7	Natural Infection	YMLDLQPET	88

[0172] Each construct was analyzed by SDS-PAGE as above. (See, FIG. 14). Figure 14A depicts conjugates of WT HPV VLP to the following peptides: MOVA2 (SIINFELK (SEQ ID NO: 2), influenza V1, HepB V1, measles V1, and VZV V1. (fusion proteins are synthesized by Genescript, NJ, US, 80% purity as described above). Likewise, Figure 14B depicts conjugates of RG1 VLPs including MOVA2 (SIINFELK), influenza V1, HepB V1, measles V1, and VZV V1.

[0173] These constructs will be tested *in vitro* for their ability to localize to tumor cells, bind to tumor cells, have their epitopes proteolytically cleaved, and the released epitope bound by the tumor MHC I receptors to trigger a T cell mediated immune response as in the examples above. Cell lines that are positive for HLA-A2 will be used, as above. Such cell lines include, for example, human tumors such as MDA-MB231, HCT116, and PC-3. In addition, a subclone of Murine B16 (Melanoma/Skin), TC-1 (cervical), and ID8 (ovarian) tumor cell subclones were generated that overexpress a luciferase gene and over-express HLA-A2 (B16-AAD, TC1-AAD, and ID8-AAD, respectively). All of these cell lines can be grown in culture. Under normal circumstances, these cell lines will not be killed by the virus-specific CD8+T cells specific for the epitopes listed in the table above since these cell lines do not express or display the respective childhood vaccine or natural infection antigen on their cell surface. Cells

will be then incubated with: (1) a specific virus antigen peptide from Table 4, above at dosages of 1 $\mu\text{g/ml}$, 100 pg/ml , and 1 pg/ml ; (2) a specific virus antigen conjugated VLP (2.5 $\mu\text{g /mL}$); (3) an unconjugated (control) VLP; or (4) untreated (with buffer). The cells are then washed and co-cultured with the respective antigen specific T cells at varying E:T ratios (Effector:Target ratio). The cell viability is to be measured either by CELLTITER-GLO® assay for the human tumors or by measuring the expression of luciferase in the murine tumors. Reduced luciferase activity will indicate greater cytotoxicity, suggesting greater immune redirection.

[0174] To test this *in vivo*, naïve transgenic HLA-A2 or HLA-AAD mice are to be vaccinated with childhood vaccines such as HepB, MMR, and chickenpox (see Table 2, above) to generate the pre-existing cytotoxic T lymphocyte (CTL) viral response. CTL responses are to be evaluated using the HLA-A2 specific tetramers to the respective viral peptides. Once sufficient CTL response is confirmed, the mice are then injected with luciferase expressing HLA-A2 positive tumor lines ID8-AAD (ovarian), B16-AAD (melanoma), or TC-1 AAD (cervical) tumor cells. Tumor growth is monitored daily. Once mice develop palpable tumors with average volume of 10 mm^3 , peptide-conjugated VLP are administered to the tumor-bearing mice at 100 $\mu\text{g/mouse/week}$ and continued for three weeks. The efficacy of anti-tumor immune redirection therapy as described herein using conjugated VLP is measured via tumor volume and survival against the untreated group. In a separate experiment, the same study will be conducted as the above except that the naïve HLA-A2 or HLA-AAD mice will be directly vaccinated with the peptides instead of childhood vaccines.

Example 13

Survival of mice treated with human tumors after exposure to conjugated-VLP

[0175] Immunodeficient mice are to be injected with human tumor cells that overexpress MHC class 1 HLA-A2. Examples include: PC-3, HCT112, and MDA-MB231. At the same time, the mice are injected with human Peripheral Blood Mononuclear Cells (PBMC)(10^6 cells) previously stimulated with a conjugated VLP that is conjugated to a fusion protein comprising one of the following epitopes (Table 5, below):

Table 5: Recall Protein Epitopes from Approved Vaccines

Virus	Approved Human Vaccine where epitope can be detected (Source)	Epitope	SEQ ID NO
Influenza A	Afluria-Trivalent or Flublok Quadrivalent if aged 18-64, Fluzone High-dose OR Fluad if 65 and older, Flucelvax Quadrivalent for 4-18years old (and older actually)	FMYSDFHFI GILGFVFTL	77
			68
Hepatitis B	Energix (GSK)	FLPSDFFPSV	41
Measles	MMR-II, (Merck)	KLWESPQEI	36
Chicken Pox	VariVax (Merck)	SLPRSRTPI	4
	VariVax (Merck)	SAPLPSNRV	5
Cytomegalovirus	Natural Infection	NLVPMVATV	3
Human Papilloma virus 16 E7	Natural Infection	YMLDLQPET	88

[0176] The conjugated-VLP comprising a fusion protein containing a recall protein as described above is then injected into the mouse weekly for three weeks. A second injection of human PBMCs stimulated with the conjugated VLP will then be injected into the mouse at day 14. It is expected that tumor formation will be inhibited compared to controls that were not provided the conjugated VLPs.

Example 14

Conjugated VLP immune redirection properties are dependent on furin cleavage

[0177] Tumor specificity is in part due to the increased presence of furin on tumor cells. In order to confirm the specificity of conjugated VLPs conjugated to a fusion protein containing a furin cleavage site, two experiments will be conducted. In the first experiment, a conjugated VLP will be created without a furin cleavage site. A cytotoxic assay will then be conducted to

demonstrate that without the furin cleavage site, the peptides cannot be loaded onto the tumor cell. Murine B16 (Melanoma/Skin) and ID8 (ovarian) tumor cells overexpressing luciferase gene (B16-luc and ID8-luc) will be grown in culture. Cells will then be treated with: (1) an E7 peptide (1 $\mu\text{g/ml}$, 1 ng/ml); (2) HPV16 E7 (lacking the furin cleavage sequence) conjugated VLP (2.5 $\mu\text{g/ml}$, 0.025 $\mu\text{g/ml}$); or (3) an unconjugated (control, 2.5 $\mu\text{g/ml}$) VLP. The cells are then washed and co-cultured with CD8+ HPV16 E7 specific T cells at varying E:T ratios (Effector:Target ratio). The cell viability is then to be measured by detection of luciferase. Reduced luciferase activity indicates greater immune redirection and hence greater cytotoxicity.

[0178] In a second experiment, FD11, a furin deficient cell line will be engineered to over-express HLA-A2 MHC Class 1 molecules (FD11/AAD). A cytotoxic assay will then be conducted using this cell line to further demonstrate that without furin, the peptides cannot be loaded onto the tumor cell. FD11/AAD tumor cells overexpressing luciferase gene are to be grown in culture. Cells are then treated with: (1) an E7 peptide (1 $\mu\text{g/ml}$, 1 ng/ml); (2) HPV16 E7 conjugated VLP (2.5 $\mu\text{g/ml}$, 0.025 $\mu\text{g/ml}$); or (3) an unconjugated (control, 2.5 $\mu\text{g/ml}$) VLP. The cells are then washed and co-cultured with CD8+ HPV16 E7 specific T cells at varying E:T ratios (Effector:Target ratio). The cell viability is measured by measuring the activity/expression of luciferase. Reduced luciferase activity indicated greater immune redirection and hence greater cytotoxicity

Example 15

Tumor Re-Challenge Experiments

[0179] The ability of mice previously cured of primary tumors by the described conjugated VLP to survive a tumor re-challenge is to be assessed. Mice ($n = 20$) that have survived treatment of a tumor with a conjugated VLP are to be re-challenged with same tumor (1×10^5 live cells) four weeks following disappearance of the last tumor. A group of naïve mice ($n = 20$) that have not been exposed to either a tumor or a conjugated VLP are to be injected with the same tumor as a control. Three treated and three naïve mice are then to be sacrificed pre-VLP therapy, post-VLP, and post re-challenge. Tumors and spleen are then prepared for TCR β high-throughput sequencing by ImmunoSEQ assay (Adaptive Biotechnologies Corp., Seattle, WA). Mice previously cured of primary tumors by conjugated VLPs are expected to exhibit resistance to secondary re-challenge, indicating that the conjugated VLP strategy is able to induce a protective system immune response against tumor

recurrence. Different TCR clonal profiles of mice at various stages, such as pre-VLP therapy, post-VLP, and post re-challenge will be observed.

What is claimed is:

1. A conjugated virus-like particle ("VLP"), comprising:
 - (a) a capsid protein, wherein said capsid protein binds a cancer cell; and
 - (b) a fusion protein, said fusion protein comprising at least: i) a cleavage peptide sequence, wherein the cleavage peptide sequence is cleaved by a protease in a tumor microenvironment; and ii) at least one recall protein sequence, wherein said recall protein sequence encodes a protein or fragment thereof having a sequence that is an epitope capable of being bound by an MHC class I molecule on a tumor cell and thereby recruit a pre-existing T cell in a subject, wherein the cleavage sequence is conjugated to the capsid protein.
2. The VLP of claim 1, wherein the capsid protein is from a *Papillomavirus*.
3. The VLP of claim 2, wherein the *Papillomavirus* comprises an L1 protein.
4. The VLP of claim 1, wherein the epitope is from a pathogen.
5. The VLP of claim 1, wherein the VLP comprises the fusion protein comprises at least one further recall protein, or wherein the VLP comprises at least two fusion proteins each comprising different recall protein sequences.
6. The VLP of claim 5, wherein the at least two recall protein sequences are from different T cell epitopes of the same pathogen.
7. The VLP of claim 5, wherein the at least two recall protein sequences are from different pathogens.
8. The VLP of claim 4, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.
9. The VLP of claim 1, wherein the epitope is or is derived from a human vaccine.
10. The VLP of claim 9, wherein the human vaccine is an early childhood vaccine.

11. The VLP of claim 1, wherein the fusion protein is conjugated to a cysteine, lysine, or arginine of the capsid protein via a disulfide linkage, a maleimide linkage, or an amide linkage.

12. The VLP of claim 1, wherein the capsid protein and the fusion protein are expressed contiguously as a fusion protein.

13. The VLP of claim 9, wherein the vaccine is a vaccine for a pathogen.

14. The VLP of claim 13, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.

15. The VLP of claim 13, wherein the vaccine elicits immunity to a vaccinia virus, varicella zoster virus, a herpes zoster virus, rubella, a hepatitis virus, e.g., hepatitis A virus or hepatitis B virus or hepatitis C virus, an influenza virus type A or type B, a measles virus, a mumps virus, a poliovirus, a variola (smallpox) virus, a rabies virus, dengue virus, Ebola virus, West Nile virus, a yellow fever virus, a zika virus, a cytomegalovirus, or an Epstein–Barr virus.

16. The VLP of claim 15 wherein the vaccine elicits an immunity to a bacteria, and wherein the bacteria is a *Bordetella pertussis*, *Clostridium tetani*, *Chlamydia trachomatis*, diphtheria, *Hemophilus influenza*, *Meningococcus*, e.g., *Meningococcal ACWY*, *Pneumococcus*, *Vibrio cholera*, *Mycobacterium tuberculosis*, *Bacillus Calmette-Guérin* (BCG), typhoid, *Escherichia coli*, *Salmonella*, *Legionella pneumophila*, *Rickettsia*, *Treponema pallidum pallidum*, *Streptococcus* group A or group B, *Streptococcus pneumonia*, *Bacillus anthracis*, *Clostridium botulinum*, or a *Yersinia* bacteria.

17. The VLP of claim 14, where in the parasite is *Entamoeba histolytica*, *Toxoplasma gondii*, *Trichinella*, e.g., *Trichinella spiralis*, *Trichomonas*, e.g., *Trichomonas vaginalis*, *Trypanosoma*, e.g., *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Plasmodium*, e.g., *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

18. The VLP of claim 1, wherein the VLP is capable of eliciting a T cell response at a threshold of at least 2-fold above a baseline of total CD8+ T cells in the subject.

19. The VLP of claim 18, wherein the CD8+ T cells comprise CD69+ cells.

20. The VLP of claim 1, wherein the capsid protein selectively binds a heparin sulfate proteoglycan (HSPG).

21. A method of treating cancer in a subject, which comprises administering to a subject in need thereof a pharmaceutically effective amount of a conjugated virus-like particle (VLP), wherein the conjugated VLP comprises:

- (a) a capsid protein, wherein said capsid protein binds a cancer cell; and
- (b) a fusion protein, said fusion protein comprising at least: i) a cleavage peptide sequence, wherein the cleavage peptide sequence is cleaved by a protease in a tumor microenvironment; and ii) at least one recall protein sequence, wherein said recall protein sequence encodes a protein or fragment thereof having a sequence that is an epitope capable of being bound by an MHC class I molecule on a tumor cell and thereby recruit a pre-existing T cell in a subject, wherein the cleavage sequence is conjugated to the capsid protein.

22. The method of claim 21, wherein the capsid protein is from *Papillomavirus*.

23. The method of claim 22, wherein the *Papillomavirus* comprises an L1 protein.

24. The method of claim 21, wherein the recall protein comprises the epitope of a pathogen.

25. The method of claim 21, wherein the VLP comprises the fusion protein comprises at least one further recall protein, or wherein the VLP comprises at least two fusion proteins each comprising different recall protein sequences.

26. The method of claim 25, wherein the at least two recall protein sequences are from different T cell epitopes of the same pathogen.

27. The method of claim 25, wherein the at least two recall protein sequences are from different pathogens.

28. The method of claim 24, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.

29. The method of claim 21, wherein the epitope is or is derived from a human vaccine.

30. The method of claim 29, wherein the human vaccine is an early childhood vaccine.

31. The method of claim 21, wherein the fusion protein is conjugated to a cysteine, lysine, or arginine of the capsid protein via a disulfide linkage, a maleimide linkage, or an amide linkage.

32. The method of claim 21, wherein the capsid protein and the fusion protein are expressed contiguously as a fusion protein.

33. The method of claim 29, wherein the vaccine is a vaccine for a pathogen.

34. The method of claim 33, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.

35. The method of claim 33, wherein the vaccine elicits immunity to a vaccinia virus, varicella zoster virus, a herpes zoster virus, rubella, a hepatitis virus, e.g., hepatitis A virus or hepatitis B virus or hepatitis C virus, an influenza virus type A or type B, a measles virus, a mumps virus, a poliovirus, a variola (smallpox) virus, a rabies virus, dengue virus, Ebola virus, West Nile virus, a yellow fever virus, a zika virus, a cytomegalovirus, or an Epstein-Barr virus.

36. The method of claim 35 wherein the vaccine elicits an immunity to a bacteria, and wherein the bacteria is a *Bordetella pertussis*, *Clostridium tetani*, *Chlamydia trachomatis*, diphtheria, *Hemophilus influenza*, *Meningococcus*, e.g., *Meningococcal ACWY*,

Pneumococcus, *Vibrio cholera*, *Mycobacterium tuberculosis*, *Bacillus Calmette-Guérin* (BCG), typhoid, *Escherichia coli*, *Salmonella*, *Legionella pneumophila*, *Rickettsia*, *Treponema pallidum pallidum*, *Streptococcus* group A or group B, *Streptococcus pneumonia*, *Bacillus anthracis*, *Clostridium botulinum*, or a *Yersinia* bacteria.

37. The method of claim 34, where in the parasite is *Entamoeba histolytica*, *Toxoplasma gondii*, *Trichinella*, e.g., *Trichinella spiralis*, *Trichomonas*, e.g., *Trichomonas vaginalis*, *Trypanosoma*, e.g., *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Plasmodium*, e.g., *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

38. The method of claim 21, wherein the VLP is capable of eliciting a T cell response at a threshold of at least 2-fold above a baseline of total CD8+ T cells in the subject.

39. The method of claim 38, wherein the CD8+ T cells comprise CD69+ cells.

40. The method of claim 21, wherein the capsid protein selectively binds a heparin sulfate proteoglycan (HSPG).

41. The method of claim 21, which further comprises administering a second dose of the recall protein.

42. The method of claim 41, wherein the second administration of the recall protein is delivered as a conjugated VLP.

43. The method of claim 41, wherein the second administration of the recall protein is delivered as a vaccine.

44. The method of claim 41, wherein the second administration of the recall is as an isolated peptide in an adjuvant.

45. A method of treating a disease or condition in a subject, which comprises administering to the subject a pharmaceutically effective amount of a conjugated virus-like particle (VLP), wherein administering the conjugated VLP comprises:

- i. detecting in the subject the presence of a preexisting immune response;
- ii. preparing the conjugated VLP for administration to the subject, wherein said conjugated VLP comprises:
 - (a) a capsid protein, wherein said capsid protein binds a cancer cell; and
 - (b) a fusion protein, said fusion protein comprising at least: i) a cleavage peptide sequence, wherein the cleavage peptide sequence is cleaved by a protease in a tumor microenvironment; and ii) at least one recall protein sequence,

wherein said recall protein sequence encodes a protein or fragment thereof having a sequence that is an epitope capable of being bound by an MHC class I molecule on a tumor cell and thereby recruit a pre-existing T cell in a subject,

wherein the cleavage sequence is conjugated to the capsid protein, and

wherein the T cell epitope is one that elicits a T cell response based on the preexisting immune response in the subject.

- 46. The method of claim 45, wherein the capsid protein is from *Papillomavirus*.
- 47. The method of claim 46, wherein the *Papillomavirus* comprises an L1 protein.
- 48. The method of claim 45, wherein the recall protein comprises the epitope of a pathogen.
- 49. The method of claim 45, wherein the VLP comprises the fusion protein comprises at least one further recall protein, or wherein the VLP comprises at least two fusion proteins each comprising different recall protein sequences.
- 50. The method of claim 49, wherein the at least two recall protein sequences are from different T cell epitopes of the same pathogen.
- 51. The method of claim 49, wherein the at least two recall protein sequences are from different pathogens.

52. The method of claim 48, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.

53. The method of claim 45, wherein the epitope is or is derived from a human vaccine.

54. The method of claim 53, wherein the human vaccine is an early childhood vaccine.

55. The method of claim 45, wherein the fusion protein is conjugated to a cysteine, lysine, or arginine of the capsid protein via a disulfide linkage, a maleimide linkage, or an amide linkage.

56. The method of claim 45, wherein the capsid protein and the fusion protein are expressed contiguously as a fusion protein.

57. The method of claim 53, wherein the vaccine is a vaccine for a pathogen.

58. The method of claim 57, wherein the pathogen is a virus, a bacterium, a fungus, or a parasite.

59. The method of claim 53, wherein the vaccine elicits immunity to a vaccinia virus, varicella zoster virus, a herpes zoster virus, rubella, a hepatitis virus, e.g., hepatitis A virus or hepatitis B virus or hepatitis C virus, an influenza virus type A or type B, a measles virus, a mumps virus, a poliovirus, a variola (smallpox) virus, a rabies virus, dengue virus, Ebola virus, West Nile virus, a yellow fever virus, a zika virus, a cytomegalovirus, or an Epstein-Barr virus.

60. The method of claim 53, wherein the vaccine elicits an immunity to a bacteria, and wherein the bacteria is a *Bordetella pertussis*, *Clostridium tetani*, *Chlamydia trachomatis*, diphtheria, *Hemophilus influenza*, *Meningococcus*, e.g., *Meningococcal ACWY*, *Pneumococcus*, *Vibrio cholera*, *Mycobacterium tuberculosis*, *Bacillus Calmette-Guérin* (BCG), typhoid, *Escherichia coli*, *Salmonella*, *Legionella pneumophila*, *Rickettsia*, *Treponema*

pallidum pallidum, *Streptococcus* group A or group B, *Streptococcus pneumonia*, *Bacillus anthracis*, *Clostridium botulinum*, or a *Yersinia* bacteria.

61. The method of claim 52, where in the parasite is *Entamoeba histolytica*, *Toxoplasma gondii*, *Trichinella*, e.g., *Trichinella spiralis*, *Trichomonas*, e.g., *Trichomonas vaginalis*, *Trypanosoma*, e.g., *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Plasmodium*, e.g., *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

62. The method of claim 45, wherein the VLP is capable of eliciting a T cell response at a threshold of at least 2-fold above a baseline of total CD8+ T cells in the subject.

63. The method of claim 62, wherein the CD8+ T cells comprise CD69+ cells.

64. The method of claim 45, wherein the capsid protein selectively binds a heparin sulfate proteoglycan (HSPG).

65. The method of claim 45, further comprising a second administration of the recall protein.

66. The method of claim 47, wherein the recall protein administered second is delivered in a conjugated VLP.

67. The method of claim 45, further comprising administering to a patient in need thereof the recall protein selected from step (ii)(b) of claim 1.

68. The method of claim 46, wherein the subject receives a second administration of the recall protein.

69. The method of claim 47, wherein the recall protein administered second is delivered in a conjugated VLP.

70. The method of any one of claims 44 to 47, wherein a boosting vaccine is delivered to the subject.

71. The method of claim 70, wherein the boosting vaccine is delivered at least two weeks following the administration of the conjugated VLP.

72. The method of claim 70, wherein the vaccine is a shingles vaccine, a prevenar vaccine, a HEPLISA V-B vaccine, a MMR-II vaccine, Zostavax, or Energix.

73. The method of claim 72, wherein the vaccine is for shingles.

74. The method of claim 73, wherein the subject is a human patient at least 50 years old.

75. The method of claim 72, wherein the vaccine is Prevenar.

76. The method of claim 75, wherein the subject is a human patient at least 50 years old.

77. The method of claim 72, wherein the vaccine is HEPLISAV-B.

78. The method of claim 77, wherein the subject is a human patient at least 50 years old.

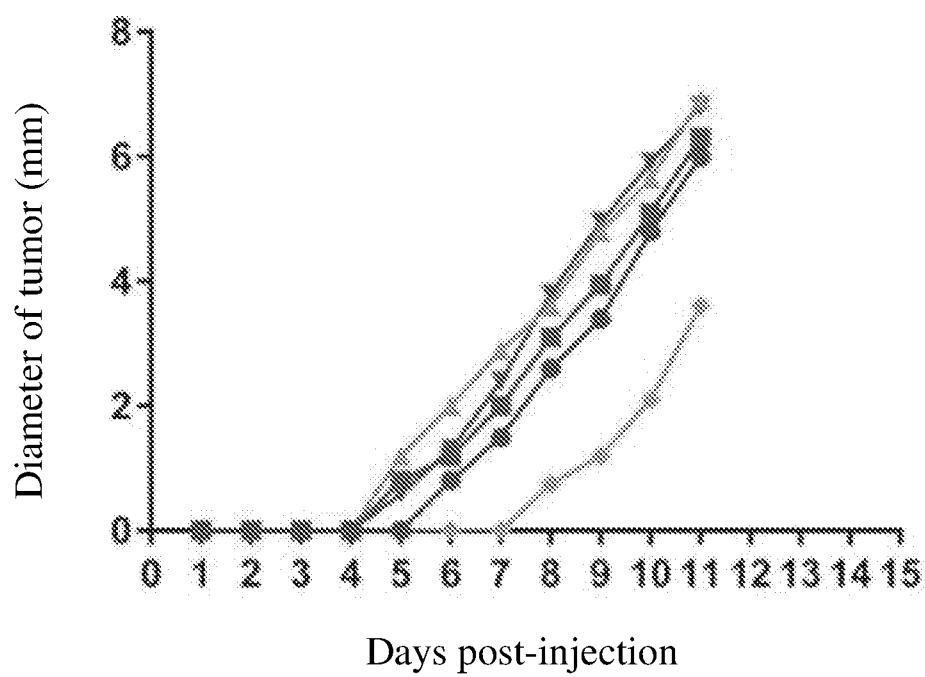
79. The method of any one of claims 45 to 47, wherein the subject was previously treated with a CAR-T, therapeutic vaccine, check point inhibitor, oncolytic virus, neo-antigen vaccine, neo-adjuvant, chemo-therapy, radiation, or surgery, but was previously shown to not have an anti-tumor effect.

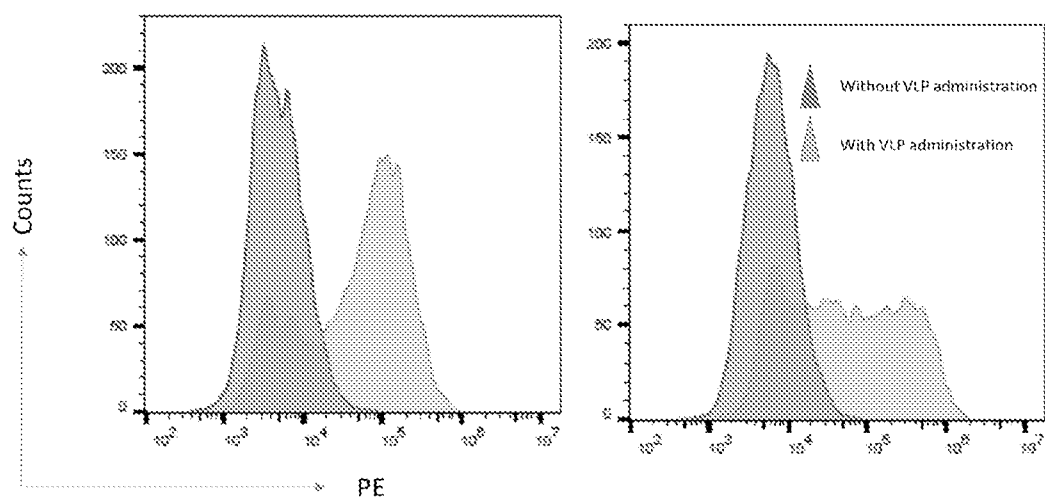
80. A method claim of any one of claims 44 to 52, wherein the recall protein is not a tumor associated antigen.

81. A method of claim 45, wherein the method inhibits the growth, progression, or metastasis of a tumor.

82. A method of claim 81, wherein the tumor is a small lung cell cancer, hepatocellular carcinoma, liver cancer, hepatocellular carcinoma, melanoma, metastatic melanoma, adrenal cancer, anal cancer, aplastic anemia, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, cancer of unknown primary origin, Castleman disease, cervical cancer, colon/rectum cancer, endometrial cancer, esophagus cancer, Ewing family of tumors, eye cancer, gallbladder cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (gist), gestational trophoblastic disease, Hodgkin disease, Kaposi sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, leukemia, liver cancer, lung cancer, lymphoma, malignant mesothelioma, multiple myeloma, myelodysplastic syndrome, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, penile cancer, pituitary tumors, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma, skin cancer, stomach cancer, testicular cancer, thymus cancer, thyroid cancer, uterine sarcoma, vaginal cancer, vulvar cancer, Waldenstrom Macroglobulinemia, Wilms Tumor, non-Hodgkin lymphoma, Hodgkin lymphoma, Burkitt's lymphoma, lymphoblastic lymphomas, mantle cell lymphoma (MCL), multiple myeloma (MM), small lymphocytic lymphoma (SLL), splenic marginal zone lymphoma, marginal zone lymphoma (extra-nodal or nodal), mixed cell type diffuse aggressive lymphomas of adults, large cell type diffuse aggressive lymphomas of adults, large cell immunoblastic diffuse aggressive lymphomas of adults, small non-cleaved cell diffuse aggressive lymphomas of adults, or follicular lymphoma, head and neck cancer, endometrial or uterine carcinoma, non-small cell lung cancer, osteosarcoma, glioblastoma, or metastatic cancer.



**FIGURE 2A**

**FIGURE 2B**

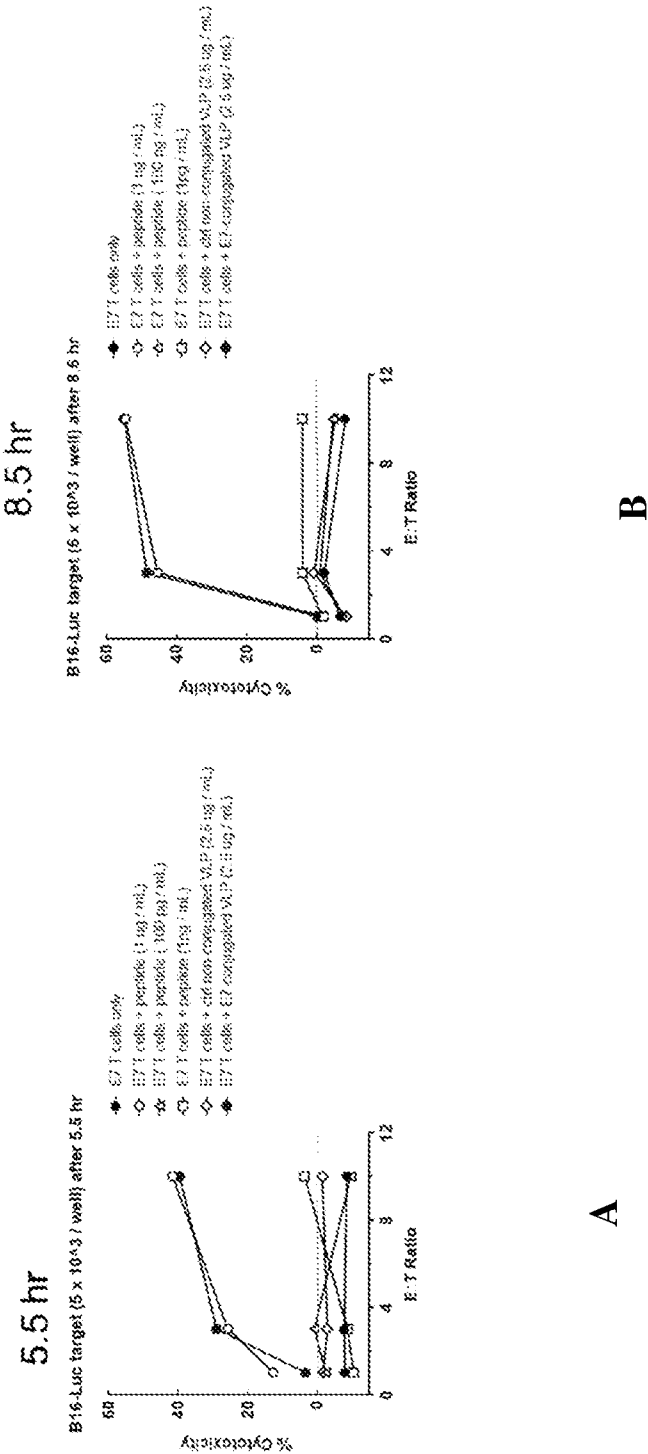
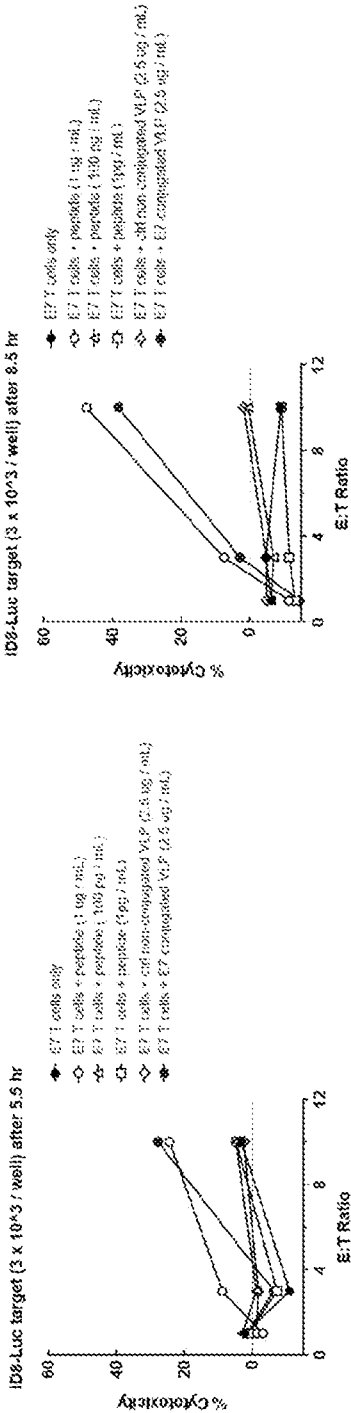


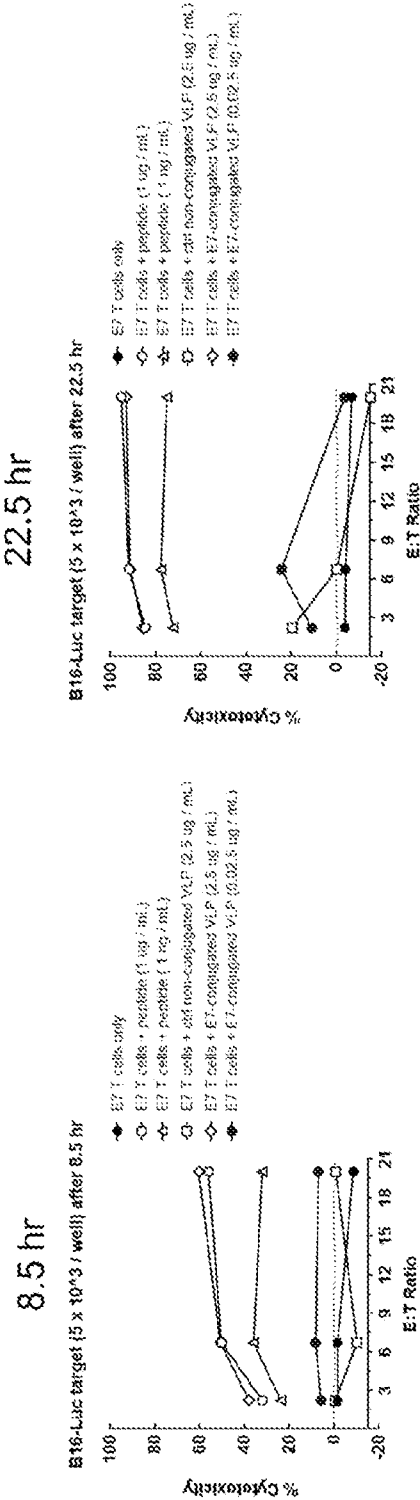
FIGURE 3



D

C

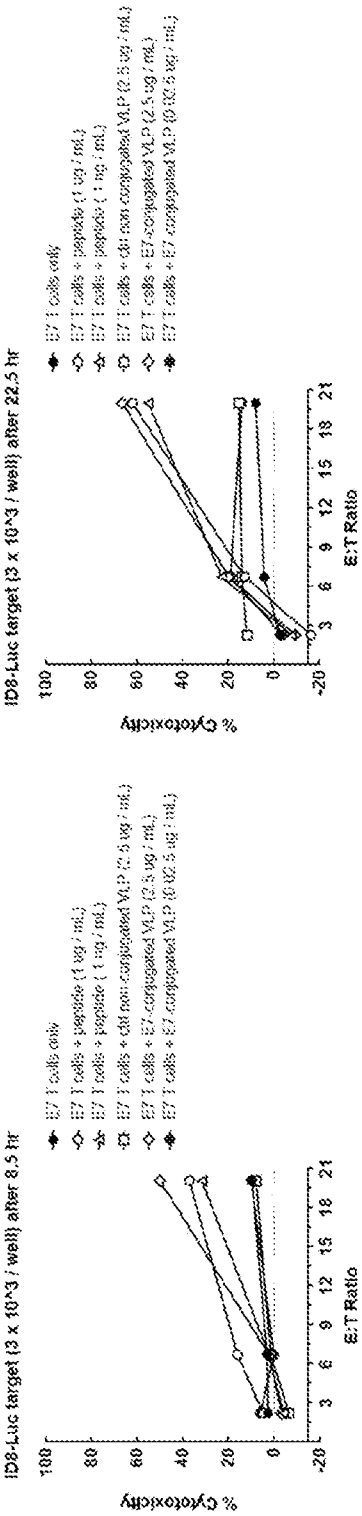
FIGURE 3



B

A

FIGURE 4



C

D

FIGURE 4

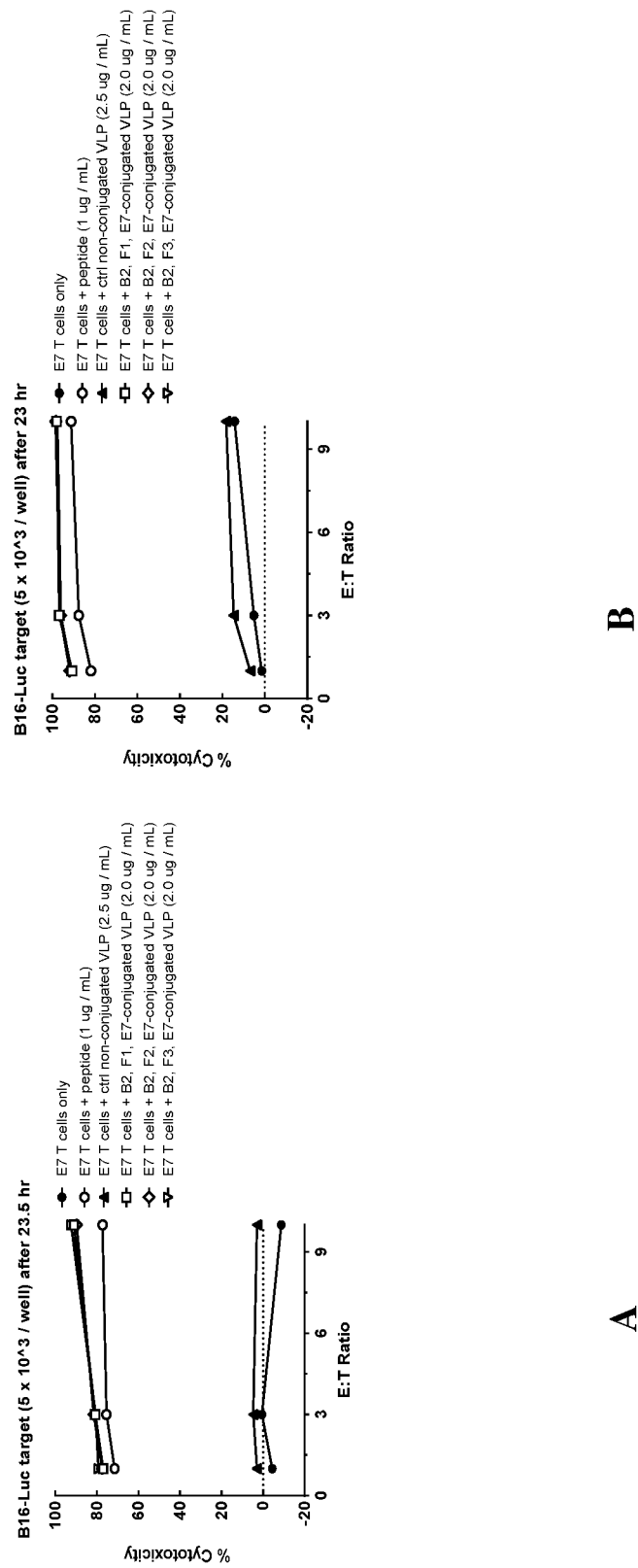
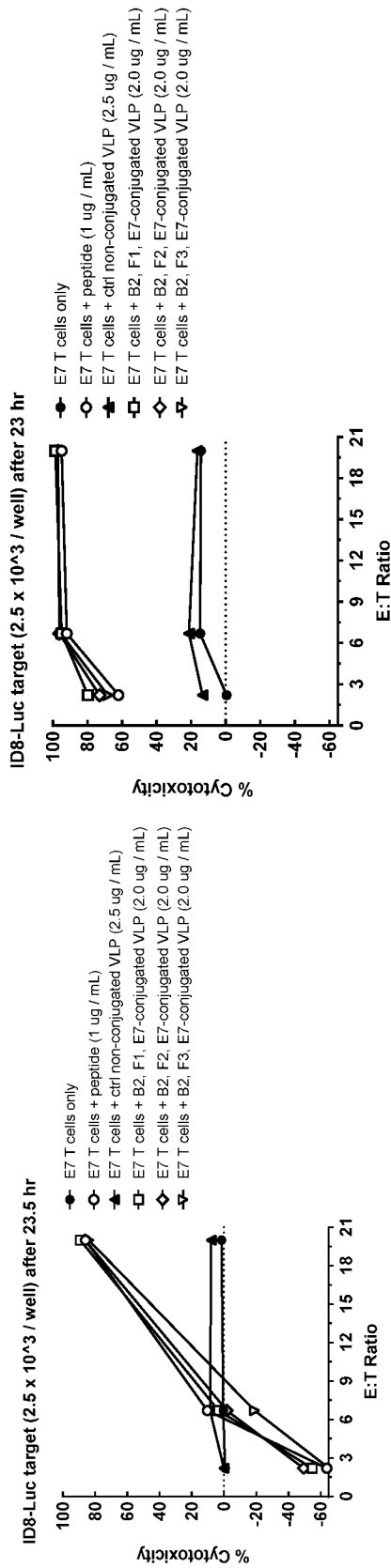


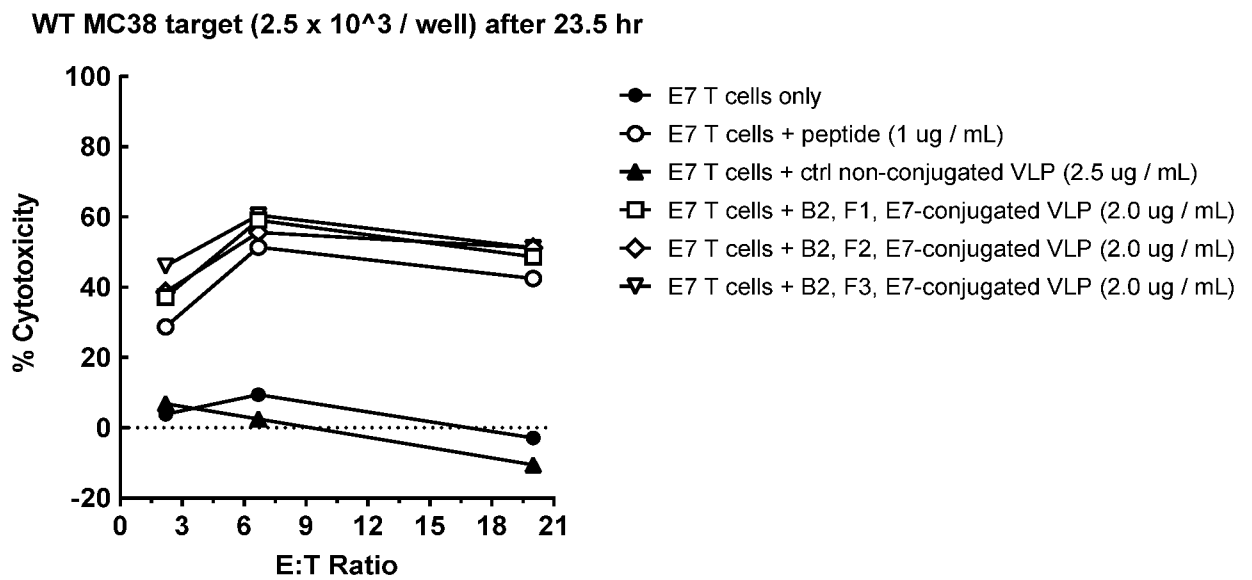
FIGURE 5

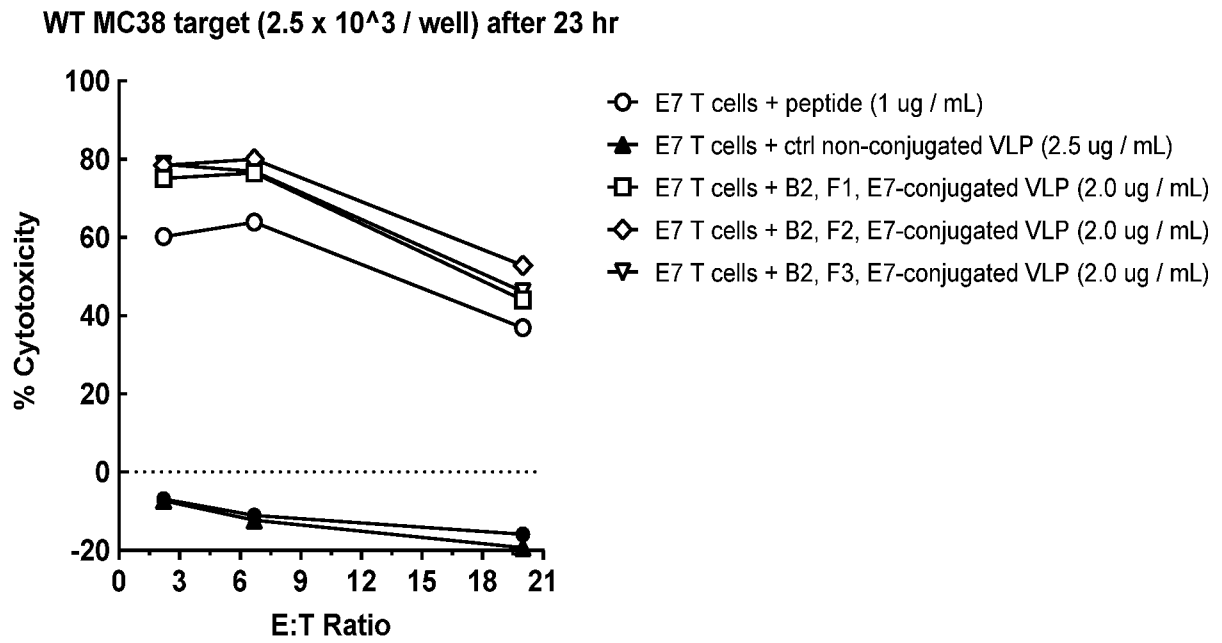


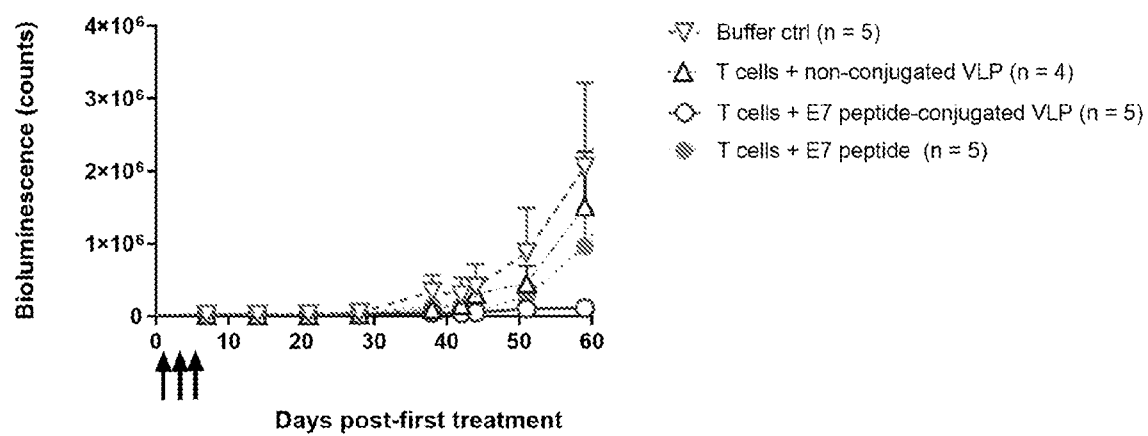
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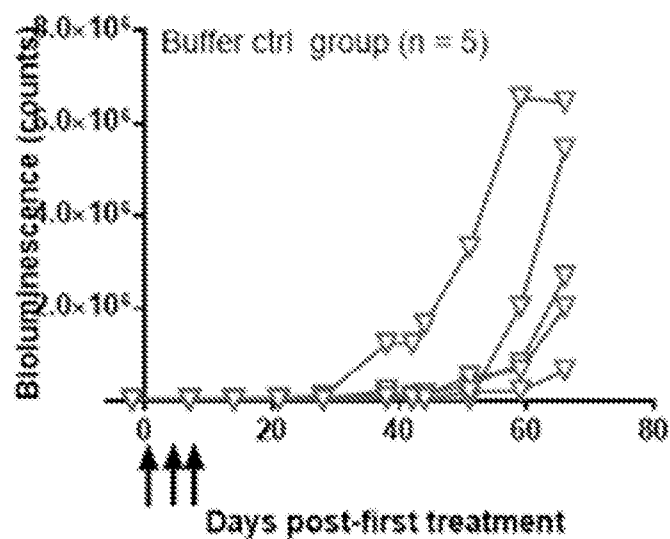
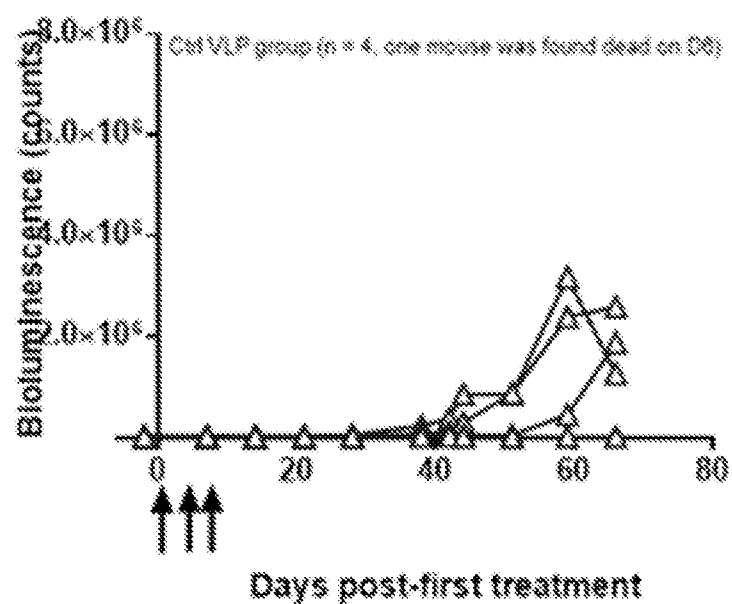
D

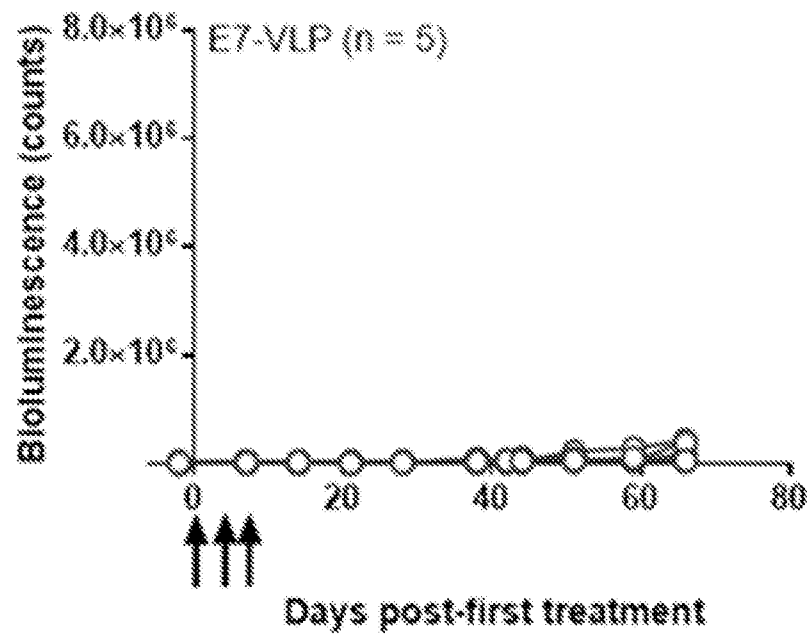
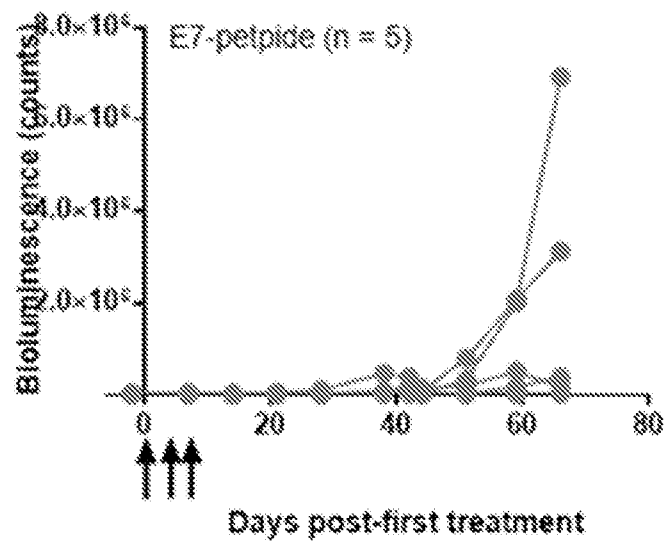
FIGURE 5

**FIGURE 6A**

**FIGURE 6B**

**FIGURE 7A**

**FIGURE 7B****FIGURE 7C**

**FIGURE 7D****FIGURE 7E**

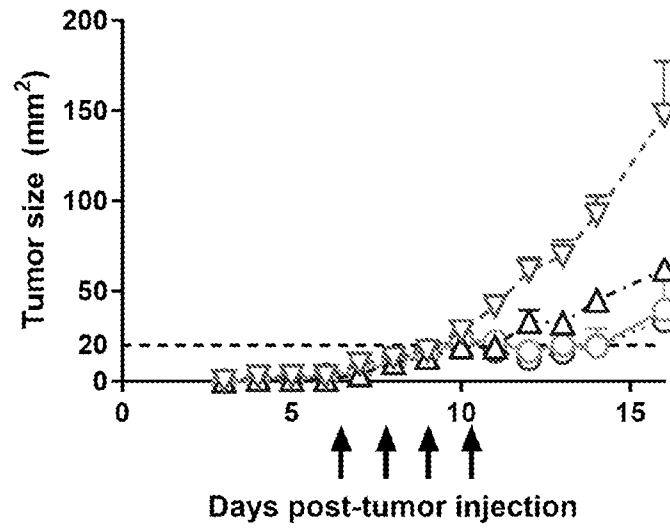


FIGURE 8A

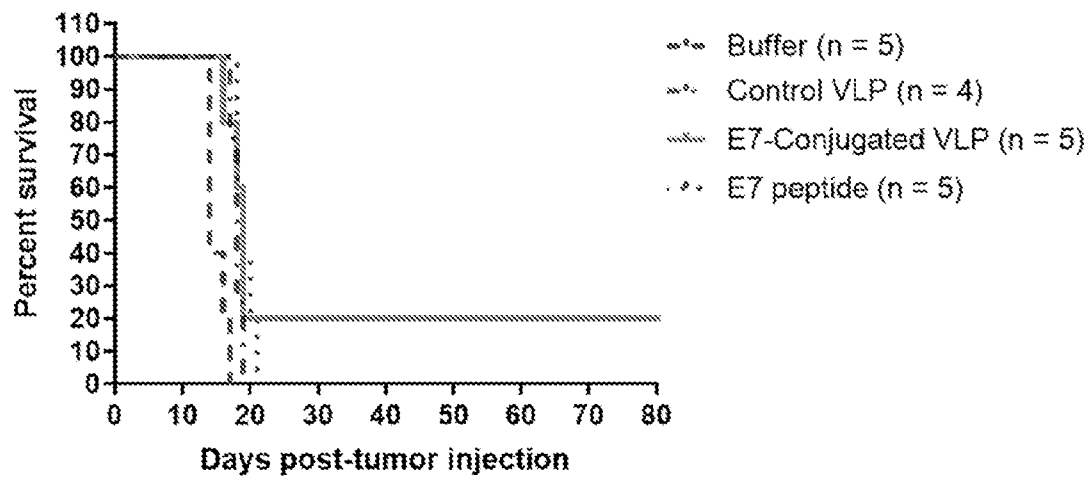


FIGURE 8B

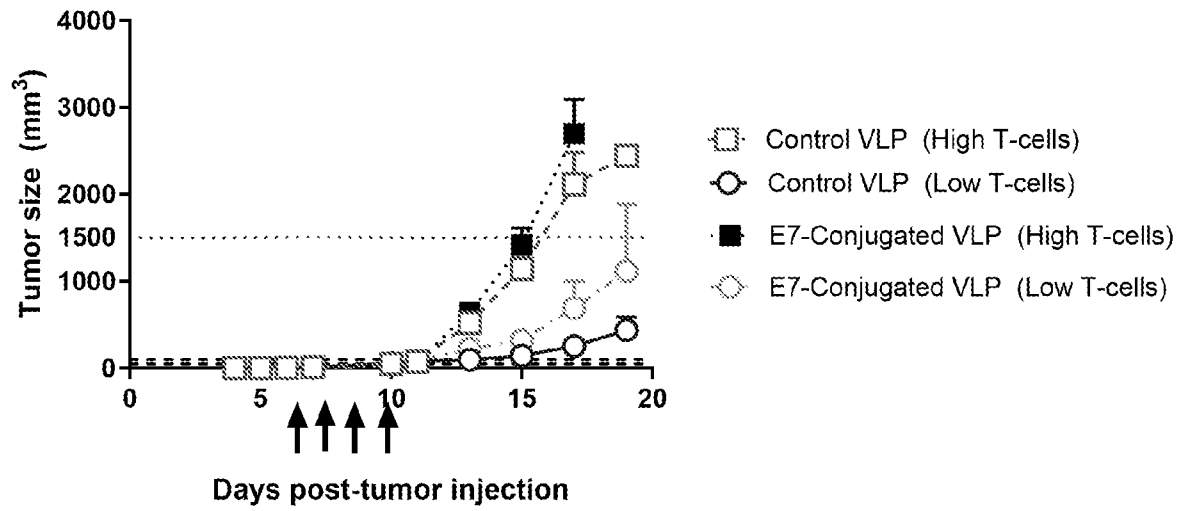


FIGURE 8C

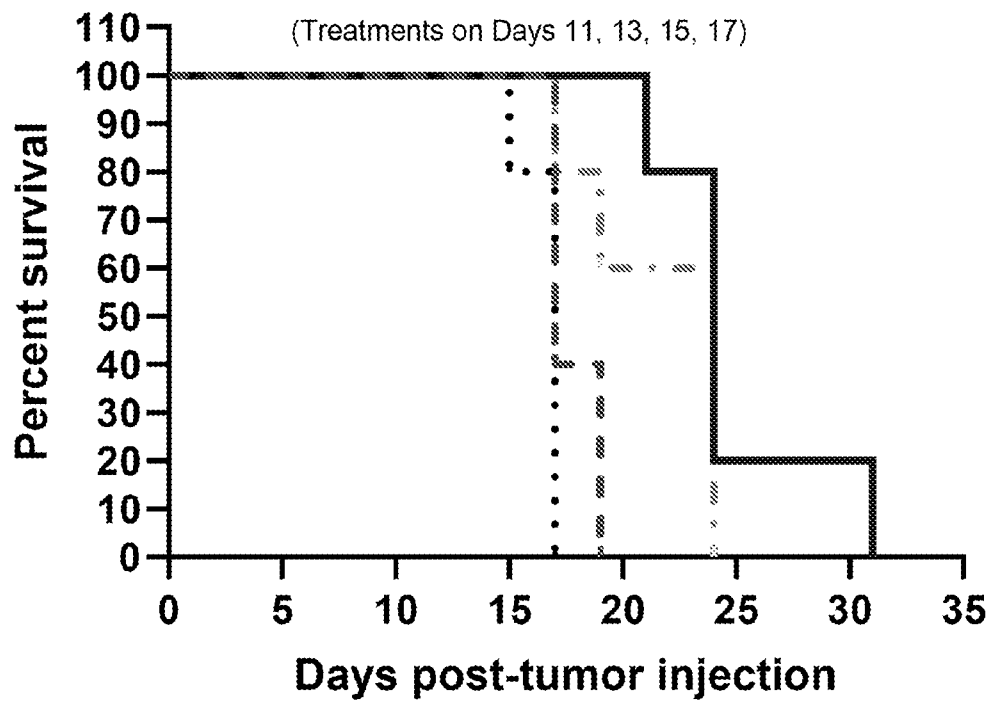
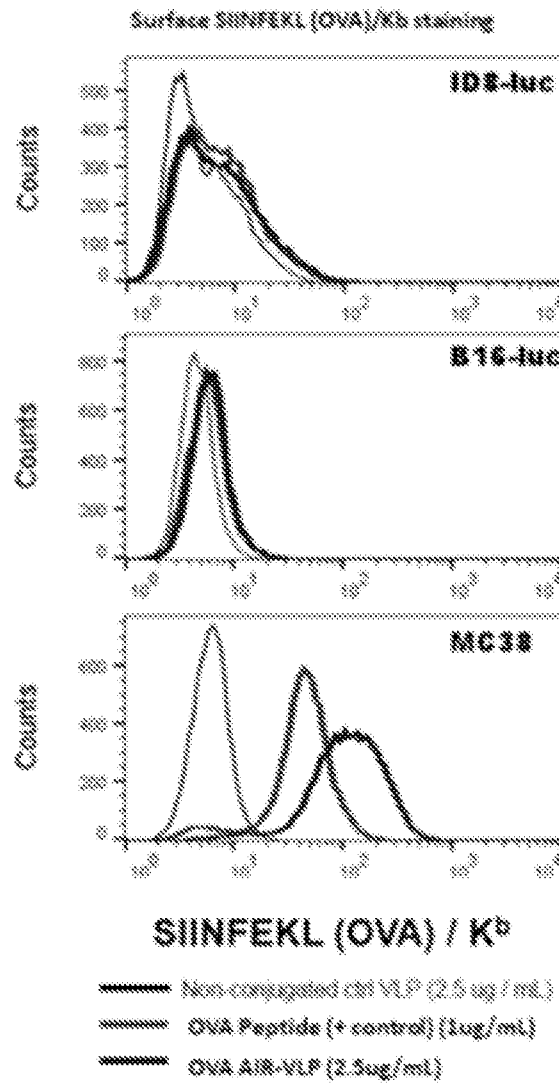
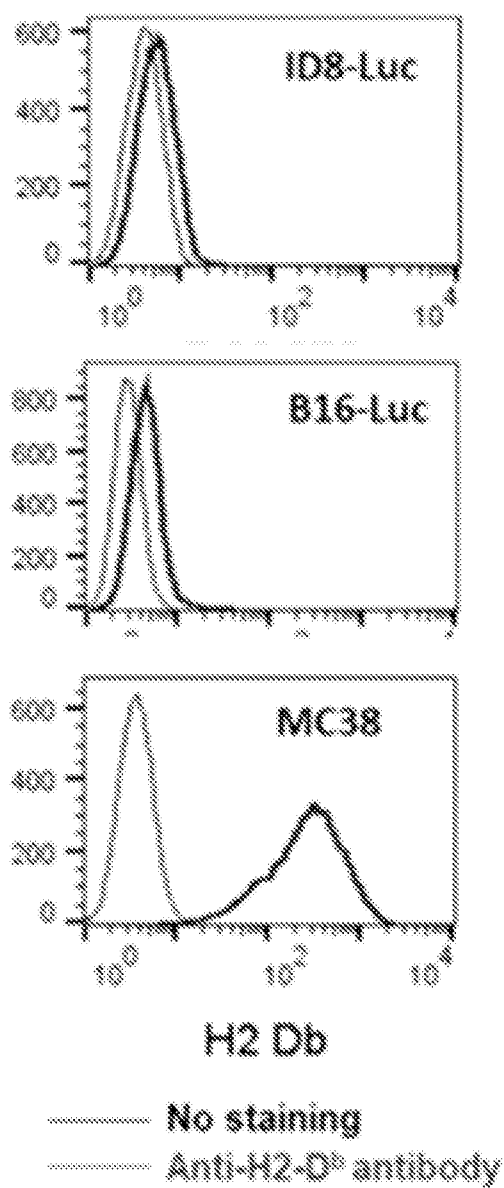


FIGURE 8D

**FIGURE 9A**

**FIGURE 9B**

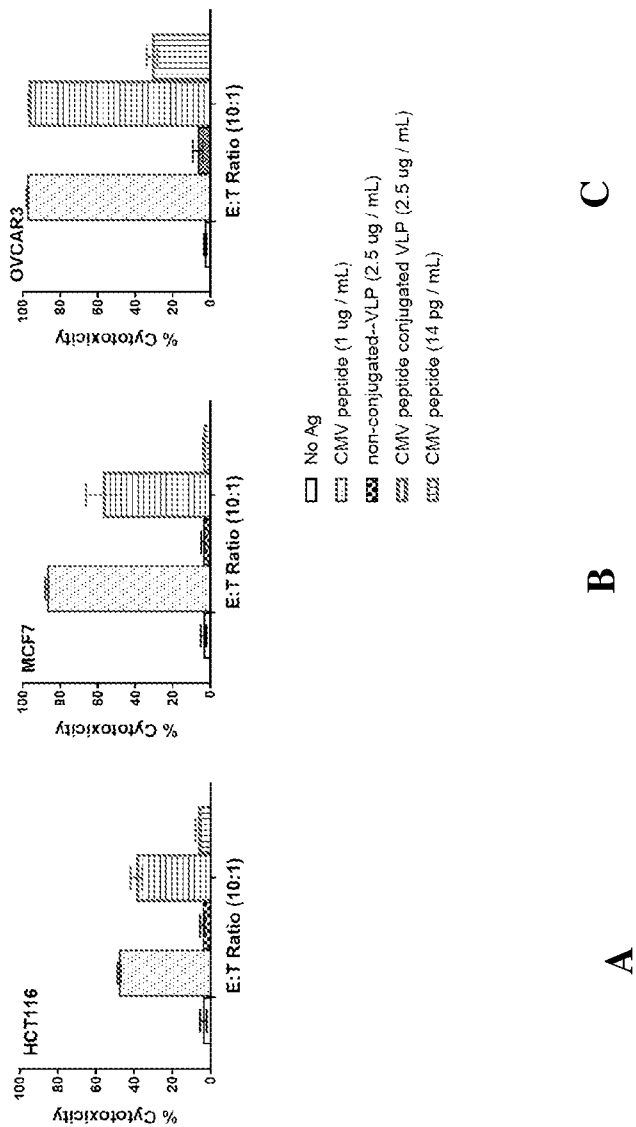


FIGURE 10

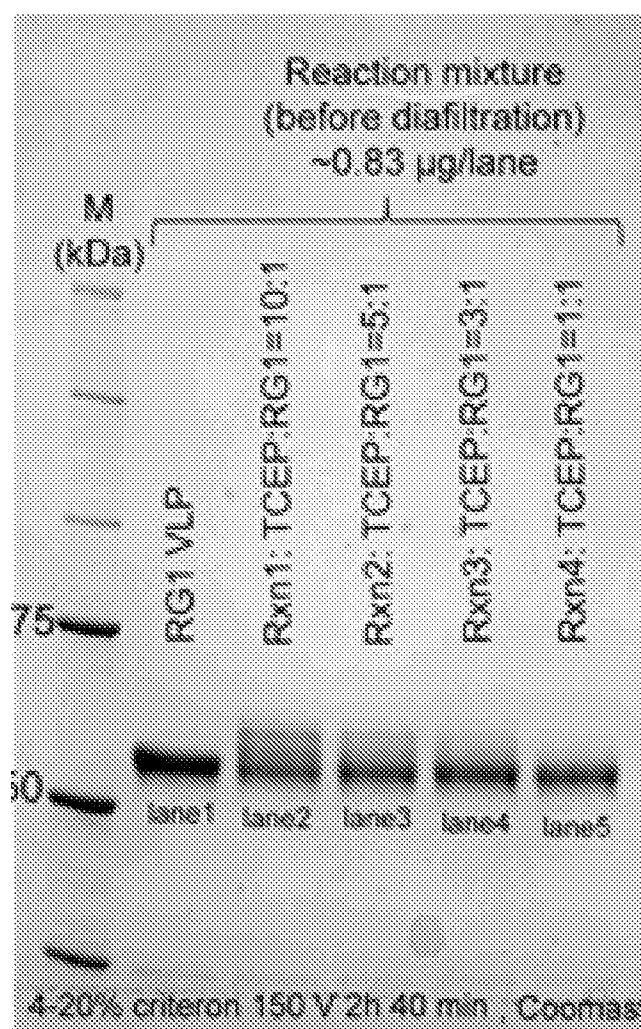
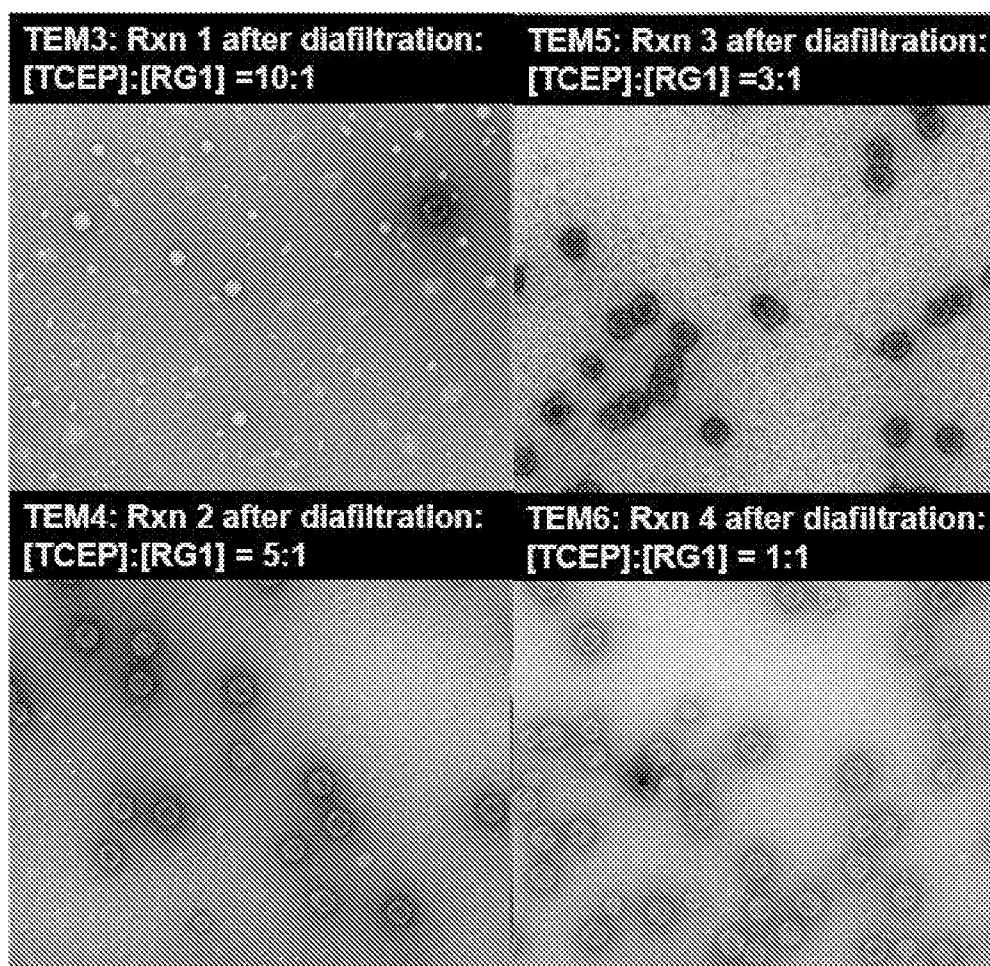
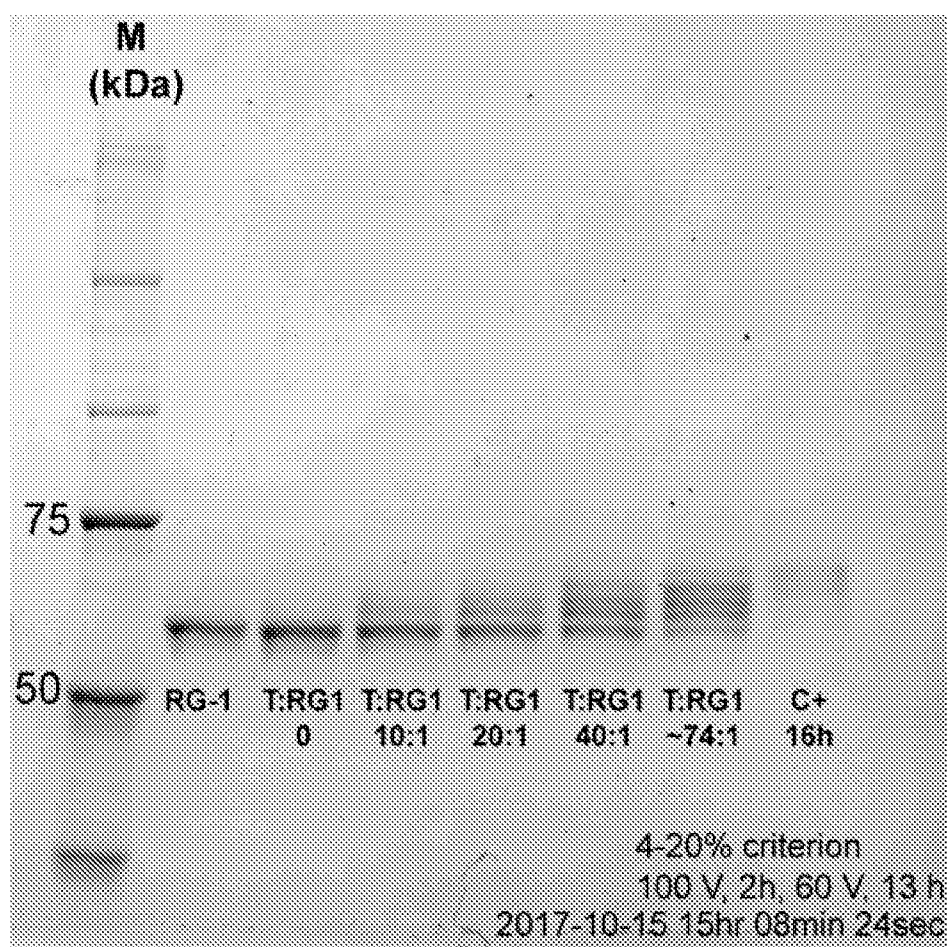
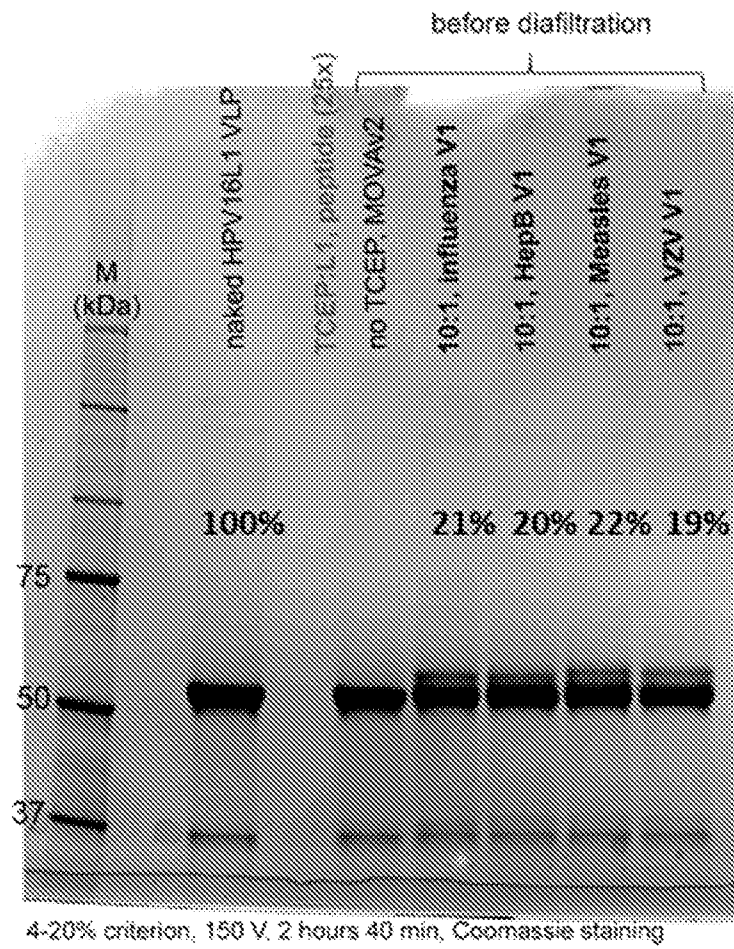


FIGURE 11

**FIGURE 12**

**FIGURE 13**

**FIGURE 14A**

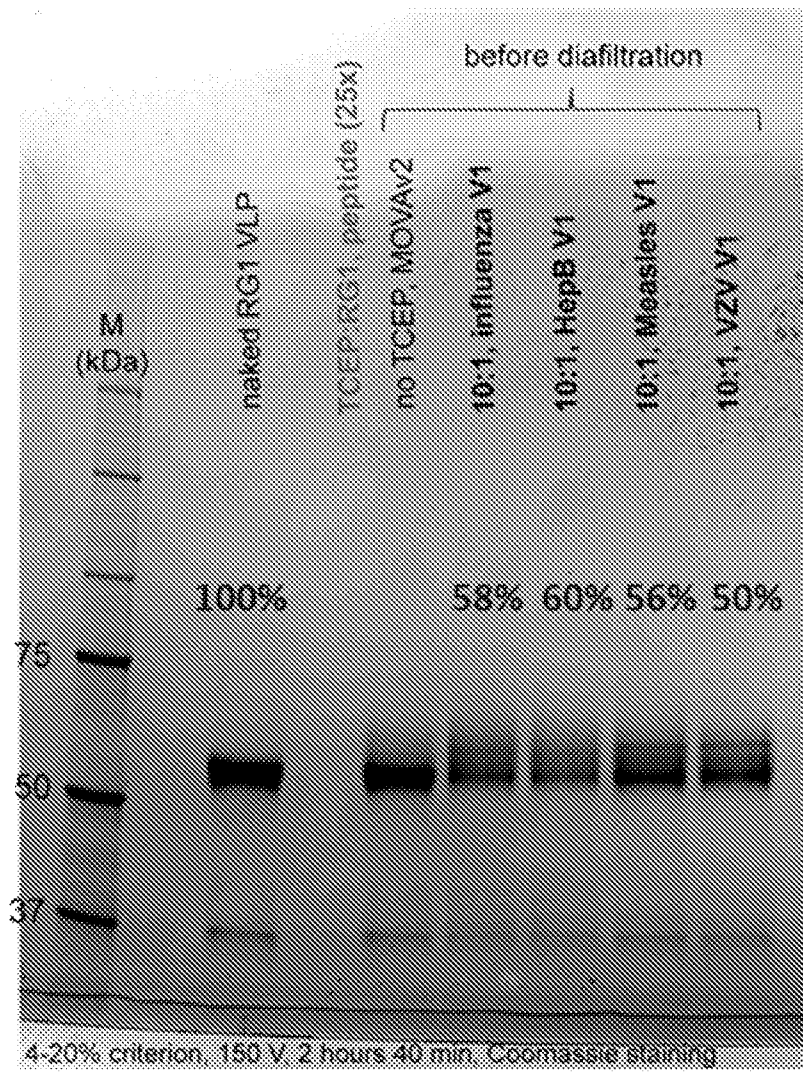


FIGURE 14B

