CANCER IMMUNOTHERAPY BY DELIVERING CLASS II MHC ANTIGENS USING A VLP-REPLICON

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. §119(e) of Provisional U.S. patent application no. 61/916,394, filed December 16, 2013, the contents of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The invention described herein relates to delivering and transcribing nucleic acid expressing class II major histocompatibility antigens to mammals using replication-defective virus-like particles.

BACKGROUND

[0003] A major challenge in cancer therapy, particularly chemotherapy, is the lack of specificity for target cancer cells as the small molecules are administered systemically and affect all cells with high mitotic index resulting in detrimental side effects. Antibody based therapies display improved specificity for target cells but result in low efficacy. Monoclonal antibodies (mAb) constitute one of the largest classes of reagents or drugs. The current global market for mAbs is more than $36 billion (Ledford, 2020, Nature 468:18-19). Current strategies for antibody based cancer therapy are limited to only a handful of extracellular proteins or receptors (Baker, 2005, Nature Biotech, 23:1065-72; Cohen, et al. 2009, MAbs, 1:56-66), which are accessible and expressed on the outer membranes of cancer cells. Because they are accessible, it was possible to generate antibodies against them. Examples include rituximab (RITUXAN® or MABTHERA®) an anti-CD20 monoclonal antibody (mAb) to treat lymphoma, trastuzumab (HERCEPTIN®) an anti-Her2 mAb to treat some breast cancers and cetuximab (ERBITUX®) an anti-FGF receptor mAb to treat head-neck and colorectal cancers (Adams, 2005, Nature Biotech, 23:1147-57). They are proven to be effective agents for recognizing and destroying cancer cells. However, there are no new accessible cancer markers on cancer cells surface, which limits the future Ab repertoire which can be generated.

[0004] There is an unmet need to exploit a large untapped source of intracellular cancer antigens for cancer therapy. Examples of such intracellular cancer therapeutic targets include: Ras mutants, phosphatases, kinases transcription factors, alpha fetoprotein (AFP), CA15-3,

CA27-29, CA19-9, CA-125, calcitonin, calretinin, carcinoembryonic antigen, chromogranin, cytokeratin, desmin, epithelial membrane protein (EMA), Factor VIII, CD31, FL1, glial fibrillary acidic protein (GFAP), gross cystic disease fluid protein (GCDFP-15), HMB-45, human chorionic gonadotropin (hCG), inhibin, keratin, lymphocyte marker, MART-1 (Melan-A), Myo D1, muscle-specific actin (MSA), neurofilament, neuron-specific enolase (NSE), placental alkaline phosphatase (PLAP), prostate-specific antigen, PTPRC (CD45), S100 protein, smooth muscle actin (SMA), synaptophysin, thyroglobulin, thyroid transcription factor-1, tumor M2-PK, vimentin, cluster of differention 10 (CD10), CD13, CD15, CD20, CD25, CD28, CD30, CD34, CD99, and CD117. Intracellular cancer antigens are not accessible to antibodies and therefore it is more difficult to treat cancer patients with antibodies against these antigens as they may not reach the intracellular location because of large size of the antibodies.

[0005] One approach is to bring those cancer antigens out and display on the tumor cells surface to convert them into induced antigen presenting cells (iAPC) and generate cancer vaccines against the tumor cells, thereby teaching tumor cells to activate cellular machinery to make antibodies against them. It is known that tumor cells synthesize multiple proteins that are potential immunogens and processed peptides from these proteins will activate an antitumor response in the patient. Transfected tumor cells will present those antigens to T(CD4+) helper and activate cytolytic T lymphocytes (CD8+ cells) and generate effector and immune memory T cells effectively generating a cancer vaccine, which is likely to be essential for protection from metastasis-the main cause of cancer death. The attractiveness of this approach for cancer therapy is there is no need for prior knowledge of the cancer antigen(s). Thus, it might work for any type cancer causing protein or antigen. Cancer vaccines are a promising tool for cancer treatment and prevention because of their potential for inducing tumor specific responses in conjunction with minimal toxicity for healthy cells.

[0006] A mouse HLA/CD80 in mouse (autologous) system has been found to prolong survival (Armstrong, et al., 1997, Proc Natl Acad Sci, 94:6886-91; Humphreys, et al., 2004, Cell Mol lmmunol, 1:180-85). This system requires isolating tumor cells, propagating the tumor cells, modifying the cells (*ex vivo*) with a recombinant retrovirus containing these genes, and re-implanting these cells into mice to study immunotherapy. The procedure is cumbersome, lengthy, and susceptible to contamination during ex vivo steps. Further, the reported results are based on genetically and syngeneic, immunologically identical systems. The use of retrovirus creates a chance to retroviral integration in the host genome at a position that could be potentially dangerous.

[0007] There is a need to develop a means of producing a gene therapy that does not have the shortcomings of an *ex vivo* procedure, and which does not require use of a retrovirus that can harm the host.

SUMMARY

[0008] What is described is a novel methodology for inducible immunotherapy of cancer. This immunotherapy is directed to coexpressing class II major histocompatibility (MHC) antigens within the tumor cells of a patient to boost the immune response to the tumor antigens. The transfected cells will act as antigen presenting cells. What was discovered is that the MHC antigens expressed in the transfected tumor cells induces an immune reaction directed against the tumor antigens by acting as antigen presenting molecules. The immune response directed against the tumor cells will then be elaborated by the presence of tumor antigens presented the host antigen presenting cells. What is also described is a virus like particle (VLP) containing RNA coding for MHC class II antigens along with the tumor antigen that can be administered to an animal, which induce host cells of the animal to express these antigens. The VLP mediated RNA delivery system has been previously described in WO 2013148302, hereby incorporated by reference in its entirety, and therein shown to be efficient in expressing proteins in mammalian cells.

[0009] What is described is a method of preventing or treating a disease in a mammalian subject, comprising administering to the subject who is need thereof an effective dosage of a pharmaceutical composition comprising a VLP comprising:

a. an alphavirus replicon comprising a recombinant polynucleotide, wherein the polynucleotide comprises a sequence encoding both subunits of a class II major histocompatibility antigen,
b. a retroviral gag protein, and
c. a fusogenic envelope protein,

wherein the VLP does not contain an alphavirus structural protein gene. The recombinant polypeptide may further comprise a sequence encoding a costimulatory signal protein. The costimulatory signal protein is preferably selected from the group consisting of CD28, CD80, CD86, and CTLA-4. The costimulatory signal protein more preferably comprises CD80.

[0010] The method described herein may be directed to preventing or treating a disease in which the patient has a tumor. The patient may be a cancer patient, preferably a cancer patient is selected from the group of cancer patients having solid tumors derived from breast, cervical, prostate, ovary, renal carcinoma, lung, gastric, pancreas, glioblastoma, and colorectal, most

preferably a breast cancer patient. The recombinant polynucleotide accordingly may further comprise a sequence encoding a tumor-specific antigen, preferably a polypeptide selected from the group consisting of AFP, CA15-3, CA27-29, CA19-9, CA-125, calcitonin, calretinin, carcinoembryonic antigen, chromogranin, cytokeratin, desmin, EMA, Factor VIII, FL1, GFAP, GCDFP-15, HMB-45, hCG, inhibin, keratin, lymphocyte marker, MART-1 (Melan-A), Myo D1, MSA, neurofilament, NSE, PLAP, prostate-specific antigen, S100 protein, SMA, synaptophysin, thyroglobulin, thyroid transcription factor-1, tumor M2-PK, vimentin, CD10, CD13, CD15, CD20, CD25, CD30, CD31, CD34, CD45 (PTPRC), CD99, CD117, and a fragment thereof.

[0011] The method described herein may be directed to preventing or treating an infectious disease. The recombinant polypeptide accordingly may further comprise a sequence of an infectious agent. The infectious agent may be an infectious virus.

[0012] The VLP of the method described herein may comprise an alphavirus replicon derived from Sindbis virus or Venezuelan equine encephalitis virus. The fusogenic envelope protein of the VLP may comprise a glycoprotein, or fragment or derivative thereof. The fusogenic envelope protein may specifically bind to a tumor cell. The VLP accordingly may be capable of binding to a eukaryotic cell. The eukaryotic cell may be a human cell. The binding may be specific to a target cell.

[0013] The method described herein may administer the pharmaceutical composition parenterally. The pharmaceutical composition may be administered by intravenous injection. The pharmaceutical composition may be administered by injection within the tumor.

[0014] In the method described herein the pharmaceutical composition may be administered in combination with a chemotherapeutic drug. The chemotherapeutic drug may be selected from the drug classes consisting of a taxane (paclitaxel or docetaxel), an anthracycline (doxorubicin or epirubicin), cyclophosphamide, capecitabine, tamoxifen, letrozole, carboplatin, gemcitabine, cisplatin, erlotinib, irinotecan, fluorouracil, and oxaliplatin. The pharmaceutical composition may be administered in combination with radiation therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Fig. 1 schematically shows an example of a vector. The DNA sequence of the vector is provided in the Sequence Listing, appended hereto (SEQ ID NO:1).

[0016] Fig. 2 shows the basic concept for producing and using the VLP described herein. The replicon plasmid contains a CMV promoter driving expression of a replicon and a gene of interest. The replicon contains elements NSP1, NSP2, NSP3 and NSP4. The gene of interest contains a nucleic acid encoding the two class II MHC antigen subunits and a disease-

specific antigen. The structural vector contains the Gag from RSV under control of a CMV promoter. The envelope vector contains VSV-G protein under control of a CMV promoter. These are transfected into a producer host cell, which manufactures the RNA containing VLP. The VLP can be used to infect target cells, which produce the proteins encoded by the gene of interest.

[0017] Fig. 3 shows the specific construct of the Examples. This shows the replicon plasmid, VLP-VEE, which contains nucleic acid encoding HLA-DR1 and CD80 protein.

[0018] Fig. 4 shows animal survival after 4T1-Luc2 (breast cancer) cell grafting.

[0019] Fig. 5 shows immunized animal survival after challenge with 4T1-Luc2 cells.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Definitions

[0020] When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

[0021] The term "fusogenic protein" as used herein is meant to refer to a protein that can induce the fusion of the plasma membrane derived envelope of the VLP to the membrane of the recipient cell.

[0022] The terms "express" and "produce" are used synonymously herein, and refer to the biosynthesis of a gene product. These terms encompass the transcription of a gene into RNA. These terms also encompass translation of RNA into one or more polypeptides, and further encompass all naturally occurring post-transcriptional and post-translational modifications. The expression or production of an antibody or antigen-binding fragment thereof may be within the cytoplasm of the cell, or into the extracellular milieu such as the growth medium of a cell culture.

[0023] "Polynucleotide," synonymously referred to as "nucleic acid molecule," "nucleotides" or "nucleic acids," refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons.

"Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short nucleic acid chains, often referred to as oligonucleotides.

[0024] "Replicon" as used herein refers to a polynucleotide having the genetic elements necessary to facilitate replication of its sequence and while also being capable of undergoing translation.

[0025] "Virus-like particle" (VLP), as used herein, refers to a structure resembling a virus particle. In preferred embodiments, a VLP contains at least one fusogenic protein displayed on the surface of the particle. A virus-like particle in accordance with the invention lacks all or part of the replicative components of the viral genome. Typically, a virus-like particle in accordance with the invention does not carry genetic information encoding for the proteins of the virus-like particle.

Vectors

[0026] Alphaviruses belong to the group IV Togaviridae family of viruses. The alphaviruses are small, spherical, enveloped viruses with a genome of a single positive sense strand RNA. The total genome length ranges between 11,000 and 12,000 nucleotides, and has a 5' cap, and 3' poly-A tail. The four non-structural protein genes (NSP genes) are encoded in the 5′ two-thirds of the genome, while the three structural proteins are translated from a subgenomic mRNA colinear with the 3′ one-third of the genome.

[0027] There are two open reading frames (ORFs) in the alphavirus genome, non-structural and structural. The first includes NSP genes and encodes proteins (nsP1–nsP4) necessary for transcription and replication of viral RNA. The second encodes three structural proteins: the core nucleocapsid protein C, and the envelope proteins P62 and E1 that associate as a heterodimer. The viral membrane-anchored surface glycoproteins are responsible for receptor recognition and entry into target cells through membrane fusion.

[0028] The Sindbis virus (SIN) and Venezuelan equine encephalitis virus (VEEV) are alphaviruses whose genome comprises a positive mRNA strand of 11703 nucleotides. SIN infects a variety of vertebrate hosts. The genome of Sindbis virus encodes nonstructural (NS, replicon) and structural proteins (capsid and pH dependent fusogenic envelope) that are directly translated in the cytoplasm of the host cell. The alphaviruses also include Aura virus, Babanki

virus, Barmah Forest virus, Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalitis virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Me Tri virus, Middelburg virus, Mosso das Pedras virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Rio Negro virus, Ross River virus, Salmon pancreas disease virus, Semliki Forest virus (SFV), Southern elephant seal virus, Tonate virus, Trocara virus, Una virus, Western equine encephalitis virus, and Whataroa virus.

[0029] Infection of host cell with an alphavirus results in cytotoxicity culminating with apoptosis, This is mostly due to both: expression of alphavirus genomic RNA in large quantities triggering antiviral state in host cells and direct interaction of alphaviral non-structural proteins (NSP2 of SIN or NC of VEEV) with cellular mRNA synthesis or translational shut-off causing cytophathic effect (CPE) on host cell host cell. A natural SIN variant containing a point mutation in one of the nonstructural proteins, NSP2 (at position 726) demonstrated sustained and noncytopathic growth in infected cells although the viral titer recovered from infected cells was substantially reduced (Frolov, et al., 1999, J Virol, 73:3845-65).

[0030] Alphaviruses are of interest to gene therapy researchers. Ross River virus, SIN, SFV, and VEEV have all been used to develop vectors for gene delivery. Pseudotyped viruses may be formed by combining alphaviral envelopes glycoproteins and retroviral capsids. Alphaviral envelope glycoproteins pseudotyped retroviruses or lentiviruses are able to integrate the genes that they carry into the potential host cells. The pseudotyped alphaviruses are recognized and infected by the alphaviral envelope proteins E2 and E1. Stable integration of viral genes is mediated by retroviral interiors of these vectors.

[0031] There are limitations to the use of alphaviruses in the field of gene therapy due to their lack of specificity of targeting. However, through the introduction of variable antibody domains in a non-conserved loop in the structure of E2, specific populations of cells have been targeted. Furthermore, the use of whole alphaviruses for gene therapy is of limited efficacy both because several internal alphaviral proteins are involved in the induction of apoptosis upon infection and also because the alphaviral capsid mediates only the transient introduction of mRNA into host cells. Neither of these limitations extends to alphaviral envelope pseudotypes of retroviruses or lentiviruses. Various alphavirus-based expression vectors for transgene expression in target cells have been described (Xiong, et al., 1989, Science, 243:1188-91; and Bredenbeek, et al., 1993, J Virol, 67:6439-46). For safety considerations these expression systems usually comprise two plasmids. One plasmid contains the coding sequence of the viral replicon (i.e., non-structural proteins) and an internal promoter and transgene coding region, while the second plasmid encodes the viral structural genes. These plasmids are used to generate

mRNA in vitro, which is then electroporated into host cells to generate one-round infectious virus particles. These viral particles are then used to infect target cells for transgene expression. These particles raise safety concerns, however, because recombination between the sequence elements encoding the non-structural and the structural viral elements can yield replication-competent alphavirus particles having the ability to mediate a significant cytopathic effect in vivo.

[0032] A possible solution to this problem is to use unrelated VLPs to deliver alphavirus replicons to the cytoplasm of mammalian cells where they can replicate autonomously and express genes of interest without any nuclear involvement. These VLPs can be produced using three vectors. The first vector comprises the coding sequence for the alphavirus replicon under the control of a mammalian promoter (e.g., CMV), a retroviral-specific RNA packaging signal, and a gene or polynucleotide of interest. The gene may express a protein with therapeutic or research applications, or a shRNA or other regulatory nucleic acid. The second vector comprises retroviral Gag. The third vector would provide the suitable envelope glycoprotein for infection of target cells.

[0033] Upon co-transfection into an appropriate packing cell line, RNA molecules transcribed from the cellular promoter present in the first vector will be packaged into VLPs produced from the second vector. These VLPs can deliver the alphavirus-based replicon to a target cell based on the envelope glycoprotein present in the VLPs. Once inside the cell, the host translational machinery will translate the introduced alphavirus RNA and produce alphavirus replication proteins, which will in turn amplify the RNA and express the gene or polynucleotide of interest. Mutant replicons such as the one described above can greatly prolong the duration of expression with minimal impact on the host cell. Moreover, DNA encoding genes for alphavirus structural elements will be absent in the target cell, so the safety of the proposed system is greatly enhanced.

[0034] Described herein are compositions relating to VLPs and methods for making and using the described VLPs. The described compositions include VLPs, and vectors and cells used to produce the described VLPs. The related methods described herein relate to methods of producing the VLPs, methods of transducing cells using the VLPs, and methods of producing a protein or polynucleotide of interest in a target cell using the VLPs described herein. Also described are alphavirus-based replicons that allow for expression of proteins or polynucleotides of interest in a target cell without the risk of viral infection.

[0035] Described herein are vectors for use in producing VLPs carrying an alphavirus-based replicon that does not encode alphavirus structural proteins. To produce VLPS of this sort,

several components may be produced by transfecting or nucleofecting one or more vectors encoding these components into a cell line for in vitro production. In some embodiments, these components are encoded by separate vectors to reduce the likelihood that the resulting VLP will be replication competent. For example, a multi-plasmid system may be used where one plasmid encodes the genetic material, such as an RNA polynucleotide encoding Sindbis virus or VEEV nonstructural proteins, to be packaged by the VLP; another encodes the structural proteins of the VLP, such as gag; and another plasmid encodes a fusion protein, such as VSV-G, to facilitate fusion of the VLP to the membrane of a target cell.

[0036] The vectors encoding the genetic material to be packaged by a host cell can take a variety of forms, such as selectable or inducible plasmids, but generally have some common characteristics. For example, vectors encoding an RNA alphavirus-based replicon described herein may include a promoter sequence, a retroviral packaging signal sequence, translation initiation sequences, nonstructural alphavirus proteins, a cloning site for inserting a gene or polynucleotide of interest, an inserted gene or polynucleotide of interest, a 3' untranslated region, and a poly-adenosine tail segment.

[0037] In some embodiments the described vectors include a promoter element that allows for segments of the vector to be transcribed by a host cell. In some embodiments the vector sequence may be transcribed into RNA to be packaged into a VLP. In most embodiments of the described vectors, the promoter sequence will be located upstream of all of the translatable elements included within the vector (see for example, Fig. 1 illustrating the location of the cytomegalovirus promoter "pCMV"). In some embodiments described herein the promoter sequence will be derived from a virus, such as CMV, or simian virus 40 (SV40). Numerous other promoter sequences are well known to those skilled in the art and their use with the vectors described herein would be apparent based on the description provided.

[0038] In some embodiments the described vectors encoding the genetic material to be packaged by a host cell can include a polynucleotide sequence that encodes a retroviral packaging signal sequence (also known as a psi (Ψ) element) to allow one or two copies of the RNA sequence transcribed from the vector to be packaged into a VLP particle formed in a host cell. Most, if not all, retroviruses have a packaging sequence of this nature, thus these sequences, and their incorporation into the described vectors, will be readily apparent to those skilled in the art. In some embodiments the vectors described herein include a polynucleotide sequence that encodes a retroviral packaging sequence derived from Rous sarcoma virus, Moloney murine leukemia virus, simian immunodeficiency virus (SIV), HIV, human T-lymphotropic virus, and the like. In a particular embodiment, the retroviral packaging sequence is derived from Rous

sarcoma virus. Alternatively, the retroviral packaging sequence is derived from murine leukemia virus.

[0039] Another aspect of the vectors encoding the genetic material to be packaged by a host cell described herein are translation initiation sequences, which allow the RNA sequence encoded by the vector to be translated in a host cell. In some embodiments the described translation initiation sequences may be capable of allowing for expression of alphavirus-based nonstructural proteins, which can replicate the RNA sequence carried by the described VLPs once it is delivered to the host cell. In some embodiments, the described translation initiation sequences may be capable of allowing for expression of a gene of interest. In some embodiments the translation initiation sequence may allow for the gene of interest to be translated by host cell translation complexes. In some embodiments the translation initiation sequences described herein may be derived from an alphavirus, such as Sindbis virus or VEEV. In other embodiments the translation initiation sequences may be derived from other genes, such as virus genes known to have translation initiation sequences capable of initiating translation of an RNA sequence by mammalian translation complexes. Alternatively, the translation initiation sequences may be derived from other genes, such as the native translation initiation sequence of the gene of interest inserted into the described alphavirus replicon. In some embodiments the translation initiation sequences described herein may be located at more than one location in the packaged RNA molecule, and thus may be encoded one or more times by the described vectors. For example, it may be desirable to translate the described Sindbis or VEEV nonstructural proteins separately from a gene of interest encoded by the package RNA molecule. In such an instance, both the polynucleotide(s) encoding the nonstructural proteins and the polynucleotide encoding the protein of interest will have separate translation initiation sequences located 5' of their position in the vector and packaged RNA. Based on this description, those skilled in the art will understand that a variety of translation initiation sequences capable of promoting the translation of RNA in a mammalian cell may be incorporated to the described VLP-packaged RNAs described herein.

[0040] The vectors encoding genetic material to be packaged by a host cell may also include polynucleotides that encode nonstructural alphavirus proteins, such as nonstructural proteins from SIN or VEEV. For example, in some embodiments the described vectors may include polynucleotides that encode one or more Sindbis virus nonstructural proteins. In some embodiments the described vectors may include polynucleotides that encode one or more VEEV nonstructural proteins. In some embodiments described vectors may include polynucleotides that encode the SIN or VEEV nonstructural protein NSP1. In some embodiments described vectors

may include polynucleotides that encode the Sindbis virus or VEEV nonstructural protein NSP2. In some embodiments described vectors may include polynucleotides that encode the SIN or VEEV nonstructural protein NSP3. In some embodiments described vectors may include polynucleotides that encode the Sindbis virus or VEEV nonstructural protein NSP4. In some embodiments described vectors may include polynucleotides that encode the SIN or VEEV nonstructural proteins NSP1, NSP2, NSP3, and NSP4. In some embodiments the polynucleotide of the described vector that encodes the alphavirus nonstructural proteins will be derived from the corresponding genomic sequence of an alphavirus genome, such as that of Sindbis virus or VEEV. In some embodiments, the polynucleotides encoding the alphavirus nonstructural proteins are void of any polynucleotides that encode the alphavirus structural proteins, regardless of whether the structural proteins are from the same or a different alphavirus than the nonstructural protein sequences present.

[0041] The vector described herein for incorporating genetic material to be packaged by a host cell may also contain a polynucleotide of interest that may be expressed in a host cell transduced by a VLP carrying the genetic material encoded by the vector. In some embodiments the described vectors may encode an RNA polynucleotide sequence to be packaged into a VLP, which can then be delivered to a host cell by VLP-mediated transduction of the cell. Once the RNA polynucleotide sequence has been delivered to the target cell a polynucleotide of interest encoded by the RNA may provide for expression of a protein of interest. Accordingly, the vectors described herein are designed to encode a RNA for packaging into a VLP that can express a gene of interest once inside a target cell. Therefore, in some embodiments the described vectors will include a polynucleotide sequence of interest. In some embodiments of the described vector, the polynucleotide sequence of interest may encode a protein of interest. For example, the polynucleotide sequence of interest may encode green fluorescent protein (GFP) in some embodiments and serve a detectable marker of viral transduction of a target cell. In another embodiment, the polynucleotide sequence of interest may encode a functional version of a protein endogenous to the target cell. In another embodiment, the polynucleotide sequence of interest may encode a functional version of a protein endogenous to the target subject. In another embodiment, the polynucleotide sequence of interest may encode a protein that is foreign to the target cell. In another embodiment, the polynucleotide sequence of interest may encode a protein that is foreign to the target subject. In some embodiments the polynucleotide sequence of interest may encode a protein capable of having a therapeutic effect on a target cell. In some embodiments the polynucleotide sequence of interest may encode a protein capable of having a therapeutic effect on a target subject. In an alternative embodiment the polynucleotide sequence

of interest may server as an interfering RNA molecule and function to regulate endogenous gene expression in a host cell. For example, in some embodiments the polynucleotide sequence of interest may comprise a sequence that provides for the formation of an RNA hairpin loop to initiate RNA interference. In addition, the polynucleotide of interest could be a positive or negative sense strand of RNA that can be transcribed by the RNA-dependent RNA polymerase complex formed by the alphavirus nonstructural proteins encoded by the packaged RNA molecule. Since this RNA-dependent RNA polymerase can transcribe RNA in the positive-sense and negative-sense directions, an interfering RNA sequence, such as a miRNA or shRNA, may be inserted into the packaged RNA replicon and can be designed to encode an interfering polynucleotide in either direction. Those of skill in the art will appreciate the therapeutic characteristic of this aspect of the described transduction system, as it can allow for expression of selected proteins in a subject. In accordance with this aspect of the described vector, a cloning site having one or more restriction endonuclease sites may also be included in the vector, to facilitate insertion of a polynucleotide sequence of interest.

[0042] Another vector useful in the production of the VLPs described herein is a vector that encodes a virus structural protein. One such class of proteins is the retroviral group-specific antigen (gag) protein. The gag protein is the core structural protein of retroviruses and, in some instances, is capable of forming enveloped virus cores when expressed in eukaryotic cells. This property makes gag proteins particularly useful in the production of VLPs, because they can form the basic structural aspect of the particle and allow for packaging of RNA associated with a retroviral packaging signal sequence. Accordingly, described herein are vectors that include a polynucleotide that encodes a retroviral gag protein. In some embodiments, the described vectors include a polynucleotide that encodes a retroviral gag protein and a promoter polynucleotide sequence that allows for the gag gene sequence to be transcribed into mRNA by host cell RNA polymerase. In one embodiment, the promoter polynucleotide sequence is derived from a virus, such as SV40 or CMV. In some embodiments, the vector will further include a polynucleotide that encodes a protein of interest. Those skilled in the relevant art will understand that a polynucleotide sequence of a gag protein from any retrovirus may be used to produce the vectors and VLPs described herein. In some embodiments the polynucleotide sequence encoding the gag protein may be derived from Rous sarcoma virus. In some embodiments the polynucleotide sequence encoding the gag protein may be derived from murine leukemia virus. In some embodiments the polynucleotide sequence encoding the gag protein may be derived from SIV. In some embodiments the polynucleotide sequence encoding the gag protein may be derived from human T-lymphotropic virus.

[0043] Another vector useful in the production of the VLPs described herein is a vector that encodes a protein to mediate fusion between the VLP envelope and a host cell. A class of proteins suitable for this purpose is viral fusion proteins, which facilitate virus infection of cells by allowing an enveloped virus to fuse its membrane with that of a host cell. Many of viral fusion proteins also have known, or suspected, cellular receptor proteins that may allow for targeting of selected cell types, or in cases of more ubiquitous receptors, such as sialic acid for influenza virus, more generalized targeting may be desired. In some instances, viral fusion proteins work in conjunction with viral attachment proteins, ligands for cellular receptor, a receptor for a cell ligand, or accessory proteins, thus proteins of this sort may also be encoded by the described vectors, in addition to, or also by, the vector encoding a viral fusion protein. Alternatively, in some embodiments a viral fusion protein from one virus may be encoded by the described vector along with a viral attachment protein of another virus, a ligand of a cellular receptor, a receptor of a cell ligand, or an accessory protein to facilitate, or direct, targeting of a VLP to a desired cell type. In some embodiments the viral fusion protein, viral attachment protein, ligand of a cellular receptor, receptor of a cell ligand, or accessory protein will be a type-I membrane protein, which will allow the extracellular domain of the protein to be oriented extracellularly when present on the cell surface. This will also allow the fusion protein to be correctly oriented following budding of a VLP from a packaging cell. Expression of such proteins in a cell will typically result in the cell surface being coated with the proteins, so that budding of a VLP from any part of the cell membrane will provide the VLP with some amount of the protein(s) on its surface. In some embodiments, the described vectors include a polynucleotide that encodes a viral fusion protein and a promoter polynucleotide sequence that allows for the fusion protein gene sequence to be translated into mRNA by host cell RNA polymerase. In one embodiment, the promoter polynucleotide sequence is derived from a virus, such as SV40 or CMV. In some embodiments, the described vectors include a polynucleotide that encodes a viral attachment protein and a promoter polynucleotide sequence that allows for the attachment protein gene sequence to be translated into mRNA by host cell RNA polymerase. In one embodiment, the promoter polynucleotide sequence is derived from a virus, such as SV40 or CMV. In some embodiments the vectors described herein include a polynucleotide that encodes a vesicular stomatitis virus G protein. In some embodiments the vectors described herein include a polynucleotide that encodes the influenza hemaglutinin protein. In some embodiments the vectors described herein include a polynucleotide that encodes the influenza neuraminidase protein. In some embodiments the vectors described herein include a polynucleotide that encodes the respiratory syncytial virus fusion protein. In some embodiments the vectors described herein

include a polynucleotide that encodes the rotavirus VP7 protein. Other such fusion proteins will be apparent to those skilled in the art based on desired tropism or cell target of the associated virus.

Cells expressing the described vectors

[0044] Provided herein are cells comprising the vectors described to produce VLPs. These cells may be used to produce the VLPs described herein by transcribing or expressing the polynucleotides of the vectors. For instance, a mammalian cell transfected with a vector having a polynucleotide sequence encoding an alphavirus RNA construct having a gene or polynucleotide of interest and a packaging signal, a vector encoding a retroviral gag protein, and a vector encoding a viral fusion protein could produce a VLP having the expressed viral fusion protein on its surface with one or two copies of the encoded alphavirus RNA construct housed inside the VLP. Furthermore, because none of these vectors encode alphavirus structural proteins the possibility of creating an infectious virus is substantially reduced compared to systems that do include alphavirus structural proteins.

[0045] The described cells may be any eukaryotic cell capable of transcribing, and where necessary (such as in the case of the gag and fusion proteins), translating the polynucleotides of the described vectors. The cells will likely be mammalian cells in many embodiments. For example, rodent cells, such as murine, hamster (CHO or BHK-21), or rat cells could be used to express the described vectors; canine cells, such as Madin Darby canine kidney cells, could be used to express the described vectors; primate cells, such as vero cells, could be used to express the described vectors; and human cells, such as HEK293T cells (human kidney), Hep-2 cells (human airway), Caco-2 (intestine), HeLa (epithelium), and other such cell lines known in the art, could be used to express the described vectors. In some embodiments the described cells can be transfected and selected, using standard transfection and selection methods known in the art, to stably comprise one or more of the described vectors.

[0046] In some embodiments the cell lines described herein will contain a vector comprising a polynucleotide sequence encoding an alphavirus replicon wherein the alphavirus replicon encodes a protein of interest, a vector comprising a polynucleotide sequence encoding a gag protein, and a vector comprising a polynucleotide sequence encoding a heterologous fusogenic envelope protein, wherein neither the vectors nor the cell contain a gene encoding an alphavirus structural protein. In some embodiments the alphavirus replicon may be derived from Sindbis virus or VEEV. In some embodiments the alphavirus replicon may have polynucleotide sequences that encode Sindbis virus or VEEV nonstructural proteins NSP1, NSP2, NSP3, NSP4,

and a retroviral packaging signal. In some embodiments the retroviral packaging signal may be derived from either Rous sarcoma virus or murine leukemia virus. In some embodiments the polynucleotide sequence encoding the gag protein is derived from Rous sarcoma virus. In some embodiments the polynucleotide sequence encoding the heterologous fusogenic envelope protein encodes VSV-G.

Virus-like particles

[0047] VLPs produced using the vectors and cells are also described herein. The VLPs described herein will have four general characteristics: they will comprise one or two RNA molecules encoding an alphavirus replicon, and optionally a protein of interest; they will have a viral core comprising a retroviral gag protein, or, in some embodiments, a gag fusion protein; they will have a surface protein to facilitate fusion with a cell, and they will not contain a polynucleotide that encodes an alphavirus structural protein.

[0048] The VLPs described herein will be useful in transducing cells in order to express a protein of interest therein. Accordingly, the described VLPs may incorporate one or two alphavirus-based RNA polynucleotides capable of encoding a protein of interest. To facilitate translation of the RNA sequence some embodiments of the described packaged RNA may include translation initiation sequences as described herein. In some embodiments the RNA sequence incorporated into the VLP will include a retroviral packaging sequence that will facilitate inclusion of the RNA into a forming VLP. In some embodiments the retroviral packaging sequence is derived from Rous sarcoma virus, Moloney murine leukemia virus, SIV, HIV, human T-lymphotropic virus, and the like. In a particular embodiment, the retroviral packaging sequence is derived from Rous sarcoma virus. Alternatively, the retroviral packaging sequence may be derived from murine leukemia virus. In addition, the RNA sequences included in the VLP may be capable of encoding nonstructural alphavirus proteins. For example, in some embodiments the packaged RNA may encode one or more Sindbis virus or VEEV nonstructural proteins. In some embodiments the packaged RNA may encode the Sindbis virus or VEEV nonstructural protein NSP1. In some embodiments the packaged RNA may encode the Sindbis virus or VEEV nonstructural protein NSP2. In some embodiments the packaged RNA may encode the Sindbis virus or VEEV nonstructural protein NSP3. In some embodiments the packaged RNA may encode the Sindbis virus or VEEV nonstructural protein NSP4. In some embodiments the packaged RNA may encode the Sindbis virus or VEEV nonstructural proteins NSP1, NSP2, NSP3, and NSP4. The packaged RNA may also include the polynucleotide sequence of a protein of interest. For example, the polynucleotide sequence of interest may

encode GFP in some embodiments and serve a detectable marker of viral transduction of a target cell. In another embodiment, the polynucleotide sequence of interest may encode a functional version of a protein endogenous to the target cell. In another embodiment, the polynucleotide sequence of interest may encode a functional version of a protein endogenous to the target subject. In another embodiment, the polynucleotide sequence of interest may encode a protein that is foreign to the target cell. In another embodiment, the polynucleotide sequence of interest may encode a protein that is foreign to the target subject. In some embodiments the polynucleotide sequence of interest may encode a protein capable of having a therapeutic effect on a target cell. In some embodiments the polynucleotide sequence of interest may encode a protein capable of having a therapeutic effect on a target subject. Those of skill in the art will appreciate the therapeutic characteristic of this aspect of the described VLPs, as they can allow for expression of selected proteins in a cell or subject.

[0049] The VLPs described herein may also comprise a viral gag protein to provide a viral core structure to the particle. The gag protein is the core structural protein of retroviruses and, in some instances, is capable of forming enveloped virus cores when expressed in eukaryotic cells. This property makes gag proteins particularly useful in the production of VLPs, because they can form the basic structural aspect of the particle and allow for packaging of RNA associated with a retroviral packaging signal sequence. Those skilled in the relevant art will understand that a gag protein from any retrovirus may be used to produce the vectors and VLPs described herein. In some embodiments the gag protein may be derived from Rous sarcoma virus. In some embodiments the gag protein may be derived from murine leukemia virus. In alternative embodiments the gag protein may be derived from SIV, HIV, human T-lymphotropic virus, or similar retrovirus.

[0050] Another component of the VLPs described herein is a protein to mediate fusion between the VLP envelope and a host cell. A class of proteins suitable for this purpose is viral fusion proteins, which facilitate virus infection of cells by allowing an enveloped virus to fuse its membrane with that of a host cell. Many of viral fusion proteins also have known, or suspected, cellular receptor proteins that may allow for targeting of selected cell types, or in cases of more ubiquitous receptors, such as sialic acid for influenza virus, more generalized targeting may be achieved. In some instances, viral fusion proteins may work in conjunction with viral attachment proteins, ligands of cellular receptors, receptors of cellular ligands, or accessory proteins, thus proteins of this sort may also be present on the VLP surface in addition to a viral fusion protein. Alternatively, in some embodiments the described VLPs may have a viral fusion protein from one virus and a viral attachment protein of another virus, a ligand of a cellular receptor, a

receptor of a cellular ligand, or an accessory protein to facilitate, or direct, targeting of a VLP to a desired cell type. Similarly, the described VLPs may be produced to have more than one fusion protein in the VLP envelope, as this may facilitate fusion to a select variety of cell types. In some embodiments the VLP surface protein(s) will be a type-I membrane protein, which will allow the extracellular domain of the protein to be oriented extracellularly when present on the cell surface. This will also allow the fusion protein to be correctly oriented following budding of a VLP from a packaging cell. Expression of such proteins in a cell will typically result in the cell surface being coated with the proteins, so that budding of a VLP from any part of the cell membrane will provide the VLP with some amount of the fusion protein on its surface. In some embodiments the VLPs described herein include a VSV-G protein to mediate cell fusion. In some embodiments the VLPs described herein include an influenza hemaglutinin protein to mediate cell fusion. In some embodiments the VLPs described herein include an influenza neuraminidase protein to facilitate cell fusion. In some embodiments the VLPs described herein include respiratory syncytial virus fusion protein. In some embodiments the VLPs described herein include the rotavirus VP7 protein. Other such fusion proteins will be apparent to those skilled in the art based on desired tropism or cell target of the associated virus.

[0051] The VLPs described herein may comprise an alphavirus replicon, wherein the alphavirus replicon includes a polynucleotide of interest or encodes a protein of interest, retroviral gag protein, and heterologous fusogenic envelope protein; wherein the VLP does not contain an alphavirus structural protein gene. In some embodiments the alphavirus replicon of the VLP is derived from Sindbis virus or VEEV. For example, the VLPs described herein may have an alphavirus replicon encoding Sindbis virus or VEEV nonstructural proteins NSP1, NSP2, NSP3, and NSP4. In some embodiments the retroviral packaging signal associated with the packaged RNA in the described VLPs is derived from either Rous sarcoma virus or murine leukemia virus. Based on this description, those skilled in the art will readily understand that the described VLPs may be modified to incorporate aspects of viruses that may facilitate VLP stability, RNA packaging, or cell entry. Such modifications should be understood to be within the scope of the disclosures provided herein.

One embodiment consists of a VLP comprising: an alphavirus replicon comprising a recombinant polynucleotide, wherein the polynucleotide comprises a sequence encoding both subunits of a class II major histocompatibility antigen; a retroviral gag protein, and a fusogenic envelope protein, in which the VLP does not contain an alphavirus structural protein gene.

The recombinant polypeptide of the VLP may further comprise a sequence encoding a costimulatory signal protein. The costimulatory signal protein is preferably selected from the

group consisting of CD28, CD80, CD86, and CTLA-4. The costimulatory signal protein most preferably comprises CD80.

Methods of producing the described VLPs

[0052] The VLPs described herein may be produced in a variety of ways, as will be apparent to those skilled in the art based on the provided disclosure. The commonality to these various methods is the expression of the described vectors in a cell capable of expressing the necessary proteins (gag and a fusion protein) and producing the alphavirus-based RNA replicon. Accordingly, a method of producing a VLP described herein may include co-transforming, transfecting, or nucleofecting a eukaryotic cell with a vector comprising a polynucleotide sequence encoding an alphavirus replicon, wherein the alphavirus replicon includes a polynucleotide of interest or encodes a protein of interest; a vector comprising a polynucleotide sequence encoding a retroviral gag protein; and a vector comprising a polynucleotide sequence encoding a heterologous fusogenic envelope protein; and culturing the co-transformed cell under conditions suitable to cause each vector to produce its encoded product, thereby producing a virus-like particle. In some embodiments the polynucleotide sequence encoding the alphavirus replicon may be derived from Sindbis virus or VEEV. In some embodiments the alphavirus replicon may have polynucleotide sequences that encode Sindbis virus or VEEV nonstructural proteins NSP1, NSP2, NSP3, NSP4, and a retroviral packaging signal. In some embodiments the retroviral packaging signal may be derived from either Rous sarcoma virus or murine leukemia virus. In some embodiments the polynucleotide sequence encoding the gag protein is derived from Rous sarcoma virus. In some embodiments the polynucleotide sequence encoding the heterologous fusogenic envelope protein encodes VSV-G.

Compositions and methods of administration

[0053] Described herein are compositions comprising at least one described VLP and a pharmaceutically acceptable carrier. Such compositions are useful, for example, for administration to subjects in need of expression of an exogenous protein or increased expression of a protein normally found in those of the same species as the subject. The compositions may be formulated as any of various preparations that are known and suitable in the art, including those described and exemplified herein. In some embodiments, the compositions are aqueous formulations. Aqueous solutions may be prepared by admixing the VLPs in water or suitable physiologic buffer, and optionally adding suitable colorants, flavors, preservatives, stabilizing and thickening agents and the like as desired. Aqueous suspensions may also be made by

dispersing the VLPs in water or physiologic buffer with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

[0054] The compositions may be formulated for injection into a subject. For injection, the compositions described may be formulated in aqueous solutions such as water or in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution may contain one or more formulatory agents such as suspending, stabilizing or dispersing agents. Injection formulations may also be prepared as solid form preparations which are intended to be converted, shortly before use, to liquid form preparations suitable for injection, for example, by constitution with a suitable vehicle, such as sterile water, saline solution, or alcohol, before use. The method described herein may administer the pharmaceutical composition parenterally. The pharmaceutical composition may be administered by intravenous injection. The pharmaceutical composition may be administered by injection within a tissue, preferably a tumor or a cancer.

[0055] The compositions may be formulated for aerosolized delivery to a subject. For aerosol delivery, the compositions described may be formulated in aqueous solutions such as water or in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution may contain one or more formulatory agents such as suspending, stabilizing or dispersing agents.

[0056] The compositions may be formulated in sustained release vehicles or depot preparations. Such long-acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compositions may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well-known examples of delivery vehicles suitable for use as carriers for hydrophobic drugs.

Modes of therapy

[0057] The method described herein may be directed to preventing or treating a disease in which the patient has a tumor. The patient may be a cancer patient, preferably a cancer patient is selected from the group of cancer patients having solid tumors derived from breast, cervical, prostate, ovary, renal carcinoma, lung, gastric, pancreas, glioblastoma, and colorectal, most preferably a breast cancer patient. The recombinant polynucleotide accordingly may further comprise a sequence encoding a tumor-specific antigen, preferably a polypeptide selected from

the group consisting of alpha fetoprotein (AFP), CA15-3, CA27-29, CA19-9, CA-125, calcitonin, calretinin, carcinoembryonic antigen, chromogranin, cytokeratin, desmin, epithelial membrane protein (EMA), Factor VIII, FL1, glial fibrillary acidic protein (GFAP), gross cystic disease fluid protein (GCDFP-15), HMB-45, human chorionic gonadotropin (hCG), inhibin, keratin, lymphocyte marker, MART-1 (Melan-A), Myo D1, muscle-specific actin (MSA), neurofilament, neuron-specific enolase (NSE), placental alkaline phosphatase (PLAP), prostate-specific antigen, S100 protein, smooth muscle actin (SMA), synaptophysin, thyroglobulin, thyroid transcription factor-1, tumor M2-PK, vimentin, cluster of differention 10 (CD10), CD13, CD15, CD20, CD25, CD30, CD31, CD34, CD45 (PTPRC), CD99, CD117, and a fragment thereof.

[0058] The method described herein may administer the pharmaceutical composition parenterally. The pharmaceutical composition may be administered by intravenous injection. The pharmaceutical composition may be administered by injection within the tumor.

[0059] In the method described herein the pharmaceutical composition may be administered in combination with a chemotherapeutic drug. The chemotherapeutic drug may be selected from the drug classes consisting of a taxane (paclitaxel or docetaxel), an anthracycline (doxorubicin or epirubicin), cyclophosphamide, capecitabine, tamoxifen, letrozole, carboplatin, gemcitabine, cisplatin, erlotinib, irinotecan, fluorouracil, and oxaliplatin. The pharmaceutical composition may be administered in combination with radiation therapy.

[0060] In the method described herein, the pharmaceutical composition induces tumor-specific CD8+ cytolytic T cells. The method further is directed to a pharmaceutical composition comprising the VLP comprising a class II MHC antigen that induces tumor-specific immunity.

[0061] The method described herein may be directed to preventing or treating an infectious disease. The recombinant polypeptide accordingly may further comprise a sequence of an infectious agent. The infectious agent may be an infectious virus.

[0062] The following examples are provided for illustrative purposes and are meant to enhance, not limit, the preceding disclosure.

EXAMPLE 1 – Production of an Alphavirus-based Gene Expression System

[0063] An alphavirus gene expression system was designed to allow for VLP-mediated delivery an exogenous gene of interest (GOI) or protein of interest (POI) to a target cell with low risk of causing cytopathic viral infection. The expression system was designed using three vectors, which can be expressed in a packaging cell line to produce a transducing VLP. One vector codes for the alphavirus-based expression construct, another vector codes for a retroviral

gag protein to facilitate VLP formation, and a third vector codes for a fusion protein to mediate VLP fusion to the host cell. In addition, the system was constructed to work without the need for alphavirus structural proteins being present.

[0064] To accomplish this, an alphavirus-based DNA plasmid was produced having a cytomegalovirus promoter (CMV); followed by a retroviral packaging signal of respective retroviral packaging protein GAG; followed by a Sindbis or VEE virus genes encoding nonstructural proteins NSP1, NSP2, NSP3, and NSP4; and finally, one or more subgenomic promoter (SGP; a promoter for virus-encoded RNA-dependent RNA polymerase, resulting in the formation of mRNA) to drive expression of a of a gene of interest (GOI), consisting of a recombinant polynucleotide, and inserted into a multiple cloning site; a 3' untranslated region (URT); and a polyA tail (Fig. 1).

[0065] Another plasmid was constructed to encode a retroviral gag protein and a second, optional protein of interest (POI). A third plasmid was constructed to provide expression of a VSV-G viral fusion protein.

[0066] Once constructed the plasmids were tested for the ability to produce VLPs carrying a Sindbis virus replicon having a gene of interest. For these experiments, GFP was used as the gene of interest in order to facilitate detection of delivery and intracellular expression of the gene. To produce VLPs, each of the three plasmids described above were transfected into baby hamster kidney (BHK-21) cells using a standard nucleofection procedure with an Amaxa system according to manufacturer instructions (Lonza).

[0067] Briefly, the BHK-21 cells at $3x10^6$ were re-suspended in 100 µl nucleofection solution L (Amaxa) and transferred to tube containing 4.5 µg of plasmid coding for GAG, 3 µg plasmid coding for VSV-G glycoprotein and 100 nanograms of plasmid coding for Sindbis alphavirus replicon or 2.5 micrograms for VEE replicon (in total volume of 10 µl). The mixture of cells and plasmids was transferred to nucleofection cuvette and nucleofected using Amaxa nucleofector II apparatus using settings for BHK-21. The nucleofected cells were re-suspended in 500 µl of completed culture medium and transferred to tissue culture plate containing culture medium solution and incubated at 37°C for 72-96 hours or for 72 hours at 32°C. After this time supernatants consisting of VLPs and encapsidated alphavirus replicon was clarified by centrifugation at 3000 RPM/10 min at 4°C, filtered by 0.45 um filter and exposed to 10 units of DNAse I (turbo^{TM}-DNAse: Ambion) for 30 min at room temperature. Processed VLPs were stored at 4°C or frozen on dry ice and transferred to -80°C. As a negative control (fusion-defective VLPs), BHK-21 cells were also nucleofected with only the pCMV-Sin Rep-POI-2 or VEEV-Rep-POI and pGAG-POI-1 plasmids, but not the pEnvelope plasmid encoding VSV-G.

Following transfection, the cells were incubated for 48–72 hours in tissue culture medium under normal growth conditions to allow for plasmid-driven production of VLPs. Once the transfected cells were finished incubating, the tissue culture supernatant, which should contain any produced VLPs, was collected. The collected cell supernatants were then added to cultured BHK-21 cells to determine if the cells could be successfully transduced with GFP. Cell supernatants collected from BHK-21 cells transfected with all three plasmids resulted in robust GFP expression when exposed to untransfected BHK-21 cells. Conversely, no GFP expression was observed for untransfected BHK-21 cells incubated with cell supernatants collected from BHK-21 cells transfected with only the pCMV-Sin Rep-POI-2 and pGAG-POI-1 plasmids. Similar experiments were also conducted using human embryonic kidney (HEK293T) cells to demonstrate that the constructed VLPs could transduce human cells. Furthermore, the constructed VLPs can also be stored at 4 °C for at least 30 days without losing the ability to transduce cells.

[0068] Experiments were also conducted to assess the ability of VEEV-based alpha virus replicon to express protein in cells. For these studies BHK-21 cells were transduced with VLPs having a *Gaussia* luciferase gene inserted into a VEEV replicon. Following transduction, cell supernatants monitored for expression of luciferase protein. High amounts of luciferase were detected in the supernatants of cells transduced with the VEEV replicon having the *Gaussia* luciferase gene, relative to control VEEV replicons without an exogenous gene, or with a gene encoding GFP. Additionally, expression of the luciferase protein increased rapidly after transduction. Similar results were also observed in the context of delivering functional cre recombinase (red cells) to cells engineered to express GFP in the absence of cre recombinase.

[0069] Cells were transduced in parallel with either Sindbis-based VLPs encoding GFP or VEEV-based VLPs encoding GFP. Both alphavirus-based VLPs caused robust GFP expression, while the cells transduced with VEEV-based VLPs were observed to have the higher expression levels.

EXAMPLE 2 – Expression of Multiple Proteins in Target Cells Transduced with VEE VLPs

[0070] The basic concept of RNA delivery using VLP is shown in Fig. 2. In this experiment, a breast cancer model was used. A double promoter vector of the replicon was developed for expressing HLA-DR1 and CD80 (Fig. 3). A VLP encoding these two antigens was used to infect 4T1-Luc2 breast cancer cells. Using anti-HLA-DR and CD80 antibodies, the

results showed that both proteins are indeed express in this cell. HLA-DR1 and CD80 are expressed in 4T1-Luc2 cells transfected with a VLP with the VLP-VEE replicon of Fig. 2. HLA-DR1 expressing cells were identified by a FITC-labeled, anti-HLA-DR antibody. CD80 expressing cells were identified by a PE-labeled, anti-CD80 antibody.

[0071] To assess whether an alphavirus replicon can express two separate proteins in the same cell, experiments were performed using a VEE replicon having HLA-DR1 under the control of one subgenomic promoter and CD80 under a another subgenomic promoter. Following production of VLPs having the described VEE replicon, cells were transduced and examined for expression of both proteins. As shown in Fig. 4, transduced cells were able to express both proteins (immunolabeled HLA-DR1 is shown in green (FITC) and CD80 shown in red (phycoerythrin).

EXAMPLE 3 – Expression of Multiple Proteins in Target Cells Transduced with VEE VLPs

[0072] To test the VLP in a mouse animal system. 4T1-Luc2 cells, highly aggressive and metastatic breast cancer cells, were implanted into the mammary fat pad of ten mice. Tumors were visible within a few days and were apparent and palpable (5-7 mm) within a week. Three of the mice were injected intratumorally with a VLP expressing an inert protein (GFP), two mice were injected with a VLP expressing human HLA-DR1/CD80 (xenogeneic), and five mice remained non-injected as a control.

[0073] After one week, tumors were surgically removed from all mice and tumor re-growth and metastasis were followed over several weeks. The results (Fig. 5) showed that 4 out of 5 non-injected mice (untreated controls) died within 4-6 weeks due to cancer regrowth and metastasis in lung, breast and liver. All three VLP/GFP (non-specific protein controls) injected mice were also died within 4-6 weeks due to cancer regrowth and metastasis in lung, breast and liver. However, both VLP/HLA-DR1/CD80 treated mice remained alive for more than 25 weeks with no sign of tumor development.

[0074] In order to show that mice indeed developed immunity against the tumor cells, the mice that survived the previous experiment were challenged again with 4T1-Luc2 cells. Four new controls mice were also included. In the challenge experiment, all mice were intravenously injected with 10,000 4T1-Luc2 cells and tumor growth and progression were monitored. If the mice developed immunity, the mice that survived will not develop a tumor and no metastasis will be found. If there is no immunity, the mice will succumb to metastatic cancer. Four control animals succumbed to metastatic cancer within two weeks of intravenous injection of 4T1-Luc2 cells, whereas the animals treated with therapeutic VLPs survived after more than 4 weeks of challenge (Fig. 5). This is indicative that the HLA/CD80 treated animals have acquired immunity against tumor cells.

[0075] The various features and processes described above may be used independently of one another, or may be combined in various ways. All possible combinations and subcombinations are intended to fall within the scope of this disclosure. In addition, certain method or process blocks may be omitted in some implementations. The methods and processes described herein are also not limited to any particular sequence, and the blocks or states relating thereto can be performed in other sequences that are appropriate. For example, described blocks or states may be performed in an order other than that specifically disclosed, or multiple blocks or states may be combined in a single block or state. The example blocks or states may be

performed in serial, in parallel, or in some other manner. Blocks or states may be added to or removed from the disclosed example embodiments. The example systems and components described herein may be configured differently than described. For example, elements may be added to, removed from, or rearranged compared to the disclosed example embodiments.

[0076] Conditional language used herein, such as, among others, "can," "could," "might," "may," "e.g.," and the like, unless specifically stated otherwise, or otherwise understood within the context as used, is generally intended to convey that certain embodiments include, while other embodiments do not include, certain features, elements, and/or steps. Thus, such conditional language is not generally intended to imply that features, elements and/or steps are in any way required for one or more embodiments or that one or more embodiments necessarily include logic for deciding, with or without author input or prompting, whether these features, elements and/or steps are included or are to be performed in any particular embodiment. The terms "comprising," "including," "having," and the like are synonymous and are used inclusively, in an open-ended fashion, and do not exclude additional elements, features, acts, operations, and so forth. Also, the term "or" is used in its inclusive sense (and not in its exclusive sense) so that when used, for example, to connect a list of elements, the term "or" means one, some, or all of the elements in the list.

[0077] While certain example embodiments have been described, these embodiments have been presented by way of example only, and are not intended to limit the scope of the inventions disclosed herein. Thus, nothing in the foregoing description is intended to imply that any particular feature, characteristic, step, module, or block is necessary or indispensable. Indeed, the novel methods and systems described herein may be embodied in a variety of other forms; furthermore, various omissions, substitutions and changes in the form of the methods and systems described herein may be made without departing from the spirit of the inventions disclosed herein. The accompanying claims and their equivalents are intended to cover such forms or modifications as would fall within the scope and spirit of certain of the inventions disclosed herein.

Table 1: SEQ ID NO: 1

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WHAT IS CLAIMED :

1. Use of an effective dosage of a pharmaceutical composition comprising

   an alphavirus replicon comprising a recombinant polynucleotide, wherein the polynucleotide comprises a sequence encoding both subunits of a human class II major histocompatibility complex protein and a sequence encoding an antigen expressed in a tumor cell;

   a retroviral gag protein, and

   a fusogenic envelope protein, wherein the VLP does not contain an alphavirus structural protein gene

   in the manufacture of a medicament for treating a tumor in a mammalian subject.

2. The use of claim 1, wherein the class II major histocompatibility complex protein (class II MHC protein) encoded by the VLP differs from the class II MHC protein of the mammalian subject.

3. The use of any of claim 1 or claim 2, wherein the pharmaceutical composition induces tumor-specific immunity in the mammalian subject.

4. The use of any one of claims 1-3, wherein the mammalian subject is a cancer patient selected from the group of cancer patients having solid tumors derived from breast, cervical, prostate, ovary, renal carcinoma, lung, gastric, pancreas, glioblastoma, and colorectal.

5. The use of any one of claims 1-4, wherein the antigen expressed in a tumor cell comprises a polypeptide selected from the group consisting of alpha fetoprotein (AFP), CA15-3, CA27-29, CA19-9, CA-125, calcitonin, calretinin, carcinoembryonic antigen, chromogranin, cytokeratin, desmin, epithelial membrane protein (EMA), Factor VIII, FL1, glial fibrillary acidic protein (GFAP), gross cystic disease fluid protein (GCDFP-15), HMB-45, human chorionic gonadotropin (hCG), inhibin, keratin, lymphocyte marker, MART-1 (Melan-A), Myo D1, muscle-specific actin (MSA), neurofilament, neuron-specific enolase (NSE), placental alkaline phosphatase (PLAP), prostate-specific antigen, S100 protein, smooth

muscle actin (SMA), synaptophysin, thyroglobulin, thyroid transcription factor-1, tumor M2-PK, vimentin, cluster of differentiation10 (CD10), CD13, CD15, CD20, CD25, CD30, CD31, CD34, CD45 (PTPRC), CD80, CD99, CD117, and a fragment thereof.

6. The use of any one of claims 1-5, wherein the antigen expressed in a tumor cell and the class II major histocompatibility complex protein are coexpressed in a tumor cell in the mammalian subject.

7. The use of any one of claims 1-6, wherein the VLP causes antigen-presentation to cells of the immune system of the mammalian subject.

8. The use of claim 1, wherein the pharmaceutical composition is to be used in combination with a chemotherapeutic drug.

9. The use of claim 8, wherein the combination therapy further comprises use of a chemotherapeutic drug selected from the drug classes consisting of a taxane (paclitaxel or docetaxel), an anthracycline (doxorubicin or epirubicin), cyclophosphamide, capecitabine, tamoxifen, letrozole, carboplatin, gemcitabine, cisplatin, erlotinib, irinotecan, fluorouracil, and oxaliplatin.

10. The use of claim 1, wherein the pharmaceutical composition is to be used in combination with radiation.

11. The use of any one of claims 1-4, wherein the antigen expressed in a tumor cell is CD80 and the class II MHC protein is HLA-DR1.