



(51) International Patent Classification:

A61K 35/76 (2015.01) A61K 39/145 (2006.01)
A61K 39/00 (2006.01) A61K 39/193 (2006.01)
A61K 39/12 (2006.01) A61K 39/21 (2006.01)

(21) International Application Number:

PCT/US2018/027093

(22) International Filing Date:

11 April 2018 (11.04.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/487,326 19 April 2017 (19.04.2017) US

(71) Applicant: UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; 112 N. Capitol Street, 6 Gilmore Hall, Iowa City, Iowa 52242-5500 (US).

(72) Inventor: LUBAROFF, David M.; c/o University of Iowa Research Foundation, 112 N. Capitol Street, 6 Gilmore Hall, Iowa City, Iowa 52242-5500 (US).

(74) Agent: SWEENEY, Patricia A. et al.; McKee, Voorhees & Sease, P.L.C., 801 Grand Avenue, Suite 3200, Des Moines, Iowa 50309-2721 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: CANCER VACCINES AND METHODS OF PRODUCING AND USING SAME

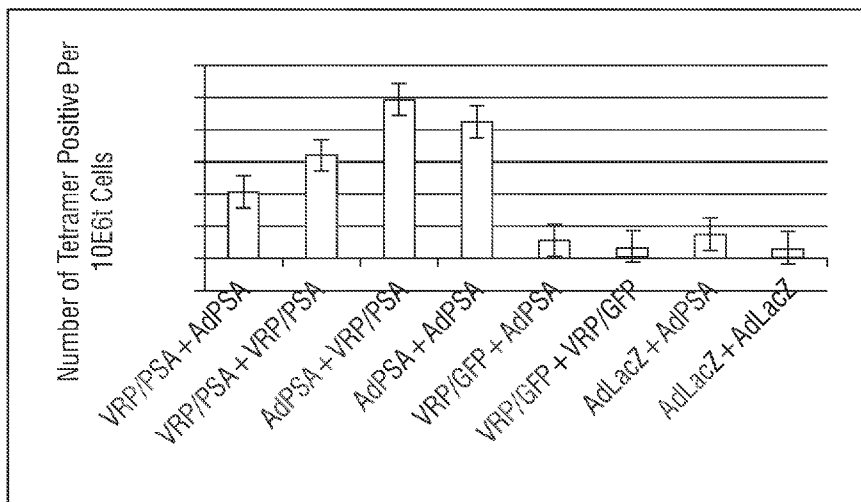


FIG. 1

(57) Abstract: A method of vaccinating a subject is provided, where a cancer protective response is produced. A first vaccine comprises an adenovirus vector comprising at least one nucleic acid molecule that produces a cancer protective response is administered, followed by one or more second vaccines comprising an alphavirus replicon particle comprising RNA comprising or produced from the nucleic acid molecule. In an embodiment the cancer is prostate cancer.



Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

CANCER VACCINES AND METHODS OF PRODUCING AND USING SAME

REFERENCE TO RELATED APPLICATION

This application claims priority to previously filed and co-pending application
5 USSN 62/487,326, filed April 19, 2017, the contents of which are incorporated herein by
reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in
ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said
10 ASCII copy, created on March 29, 2018, is named
LUBAROFF_P12169WO00_SEQ_LISTING_ST25.txt and is 8,014 bytes in size.

BACKGROUND

To date cancer vaccine protocols have met with challenges that have limited
efficacy. Whole cell vaccines, peptide or whole protein vaccines or antigen specific
15 vaccines have been attempted but have unfortunately have shown limited impact on
inhibiting cancer growth or elimination. Prostate cancer is an example. Prostate cancer is
the number one non-skin cancer in males and the second leading cause of cancer deaths,
with an estimated 220,800 men diagnosed in 2015 and 27,540 deaths predicted in the
United States. Globally, the 2012 data (the last published information) reported that there
20 were 1,111,700 new cases and 307,500 deaths. For many prostate cancer patients, primary
therapies of surgery or radiation are not curative and the cancer recurs. Subsequent
therapeutic options are effective in reducing the amount of tumor, but ultimately there is a
breakthrough within an average of 2 years. Additional therapies have been developed, but
they usually only provide an additional 2 to 4 months survival advantage. Eventually
25 patients cease to respond to any treatment, leading to their death. Thus, new and
alternative treatments are required.

Because there is no cure for recurrent prostate and other cancers, although newer
treatments have been shown to extend survival by a modest number of months, the unmet
need is for a therapy or therapies that either significantly extend life or cure the disease.
30 Extension of life must also include a good quality of life. All of the current therapies are
associated with significant adverse events that reduce quality of life. The new treatment(s)

should also carry a reasonable cost to the patient. Current treatment costs are close to \$100,000 per patient.

SUMMARY

5 Provided here are cancer vaccines, which in an embodiment provide for administration to a subject in need thereof. A vaccine comprises a nucleic acid molecule that produces a cancer protective response in a patient. An embodiment provides the vaccine may be delivered by an adenovirus vector, by an alphavirus vector and in a preferred embodiment, both an adenovirus and an alphavirus vector replicon particle vaccine is administered to the patient. In an embodiment the adenovirus vector comprising
10 the antigenic nucleic acid molecule is administered to the patient, followed by administration of an alphavirus vector comprising the antigenic nucleic acid molecule. An embodiment provides the adenovirus vector is administered first, followed by administration of the alphavirus vector replicon particle vaccine in about 14 days.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a graph showing the number of tetramer positive cells produced in response to differing vaccination administration. VRP refers to alphavirus replicon particle; PSA refers to prostate specific antigen; AdPSA refers to adenovirus vector with PSA; AdLacZ refers to a control adenovirus vector carrying the lacZ gene for a non-specific antigen (β -galactosidase).

20 Figures 2A and 2B are graphs showing tumor volume (mm^3) and days post tumor challenges using the AdPSA saline/alphaPSA saline protocol (A) and alphaPSA saline/alphaPSA saline protocol (B).

Figure 3 is a graph showing the percent of tetramer positive cells resulting from differing vaccination administration as indicated.

25 DESCRIPTION

Provided here are cancer vaccines that are safe, highly effective, can be prepared in a short amount of time, usually within 4 to 6 weeks, and provided to patients at a cost far less than currently available cancer treatments. Estimates of costs of the vaccine and with the absence of blood processing, are anticipated to be considerably less than the \$100,000
30 price of the currently available prostate cancer vaccine. An embodiment of the vaccine and process of administration provides for a replication deficient adenovirus vector (Ad) comprising at least one cancer protective nucleic acid molecule that when administered

produces a cancer protective response in the patient. The administration of this vaccine is followed by administration of one or more vaccines comprising a replication deficient alphavirus replicon particle (VRP or RP) comprising the at least one cancer protective nucleic acid molecule. When administering a “prime” vaccine of the Ad vector vaccine
5 followed by one or more “boosting” vaccinations of the VRP vaccine, a synergistic enhancement of cancer protection is provided to the patient. In an embodiment, the nucleic acid molecule is a prostate cancer protective nucleic acid molecule. A further embodiment provides that the prime vaccine is followed by a booster vaccine within about 14 days. Additional booster injections will occur at 14 day intervals. An embodiment provides for
10 one, two, three, four, five, six or more booster vaccinations. Results in preclinical studies demonstrated tumor eradication using three boosters. In still further embodiments, the cancer protective response may be reduction in growth or destruction of cancer tumors and may include elimination of tumors. An embodiment provides for destruction of 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% up to 100% of tumors.

15 The processes and vaccines described here may be used in connection with a cancer in which a nucleic acid molecule can be identified that has a cancer protective effect. The term “cancers,” as used herein, refers to the commonly understood spectrum of diseases including, but not limited to, solid tumors, such as cancers of the breast, respiratory tract, brain, reproductive organs, digestive tract, urinary tract, eye, liver, skin,
20 head and neck, thyroid, parathyroid and their distant metastases, and also includes lymphomas, sarcomas, and leukemias. Examples of breast cancer include, but are not limited to invasive ductal carcinoma, invasive lobular carcinoma, ductal carcinoma in situ, and lobular carcinoma in situ. Examples of cancers of the respiratory tract include but are not limited to small-cell and non-small-cell lung carcinoma, as well as bronchial adenoma and pleuropulmonary blastoma. Examples of brain cancers include but are not limited to
25 brain stem and hypophthalmic glioma, cerebellar and cerebral astrocytoma, medulloblastoma, ependymoma, as well as neuroectodermal and pineal tumor. Tumors of the male reproductive organs include but are not limited to prostate and testicular cancer. Tumors of the female reproductive organs include, but are not limited to endometrial,
30 cervical, ovarian, vaginal, and vulvar cancer, as well as sarcoma of the uterus. Tumors of the digestive tract include, but are not limited to anal, colon, colorectal, esophageal, gallbladder, gastric, pancreatic, rectal, small intestine, and salivary gland cancers. Tumors

of the urinary tract include, but are not limited to bladder, penile, kidney, renal pelvis, ureter, and urethral cancers. Eye cancers include but are not limited to intraocular melanoma and retinoblastoma. Examples of liver cancers include but are not limited to hepatocellular carcinoma (liver cell carcinomas with or without fibrolamellar variant),
5 cholangiocarcinoma (intrahepatic bile duct carcinoma), and mixed hepatocellular cholangiocarcinoma. Skin cancers include, but are not limited to squamous cell carcinoma, Kaposi's sarcoma, malignant melanoma, Merkel cell skin cancer, and non-melanoma skin cancer. Head-and-neck cancers include but are not limited to laryngeal/hypopharyngeal/nasopharyngeal/oropharyngeal cancer, and lip and oral cavity
10 cancer. Lymphomas include, but are not limited to AIDS-related lymphoma, non-Hodgkin's lymphoma, cutaneous T-cell lymphoma, Hodgkin's disease, and lymphoma of the central nervous system. Sarcomas include but are not limited to sarcoma of the soft tissue, fibrosarcoma, osteosarcoma, malignant fibrous histiocytoma, lymphosarcoma, and rhabdomyosarcoma. Leukemias include, but are not limited to acute myeloid leukemia,
15 acute lymphoblastic leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, and hairy cell leukemia. Cancers also specifically include, but are not limited to, chronic myeloid leukemia (CML), acute myeloid leukemia (AML), cutaneous T cell lymphoma (CTCL), cutaneous T cell lymphoma (CTCL), acute T lymphoblast leukemia (ALL), MDR acute T lymphoblast leukemia (MDR ALL), large B-lymphocyte non-
20 Hodgkin's lymphoma, leukemic monocyte lymphoma, epidermal squamous carcinoma, epithelial lung adenocarcinoma, liver hepatocellular carcinoma, colorectal carcinoma, breast adenocarcinoma, brain glioblastoma, prostate adenocarcinoma, gastric carcinoma and other cancerous tissues. These disorders have been characterized in humans, but also exist with a similar etiology in other mammals and can be treated by administering the
25 methods and vaccines here. Examples, without intending to be limiting, of currently identified tumor-associated antigens in non-prostate cancers are Her-2, MUC-1, and CD20.

Any nucleic acid molecule that provides a cancer protective response may be used in the processes here. The terms "protecting", "protection", "protective response" as used herein, are intended to mean that the subject morbidity or mortality is improved, and/or the
30 cancer cell or tumor growth or adverse impact on the subject is reduced or eliminated. The nucleic acid molecule may be associated with production of an antigen. In another embodiment the response and may or may not produce antibodies. Where administered

prophylactically, there is a reduction in incidence or growth of the cancer. Such protective response may be observed or measured in a myriad of ways. By way of example without limitation, where referring to prostate cancer, one may measure total PSA values, PSA doubling time, time to progression, reduction or elimination of tumors and/or tumor
5 growth, and overall survival.

In an embodiment of the processes here, the cancer is prostate cancer. The nucleic acid molecule may be selected from any molecule that can provide a protective response, and can include for example, prostate specific antigen (PSA) (See for example Klobeck et al. "Genomic sequence of human prostate specific antigen (PSA)" *Nuc. Acids Research*
10 Vol. 17 No. 10 (1989)EMBL accession No. X14810), prostate stem cell antigen (PSCA; examples include Reiter et al. "Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer *Proc. Natl. Acad. Sci. U.S.A.* 95(4), 1735-1740 (1998); GenBank Ref No. AAC39607.1), prostate acid phosphatase (PAP; examples include Sharief et al. "Human prostatic acid phosphatase: cDNA cloning, gene mapping and
15 protein sequence homology with lysosomal acid phosphatase" *Biochem. Biophys. Res. Commun.* 160 (1), 79 – 86 (1989); GenBank Ref No. AAA60022.1) and prostate specific membrane antigen (PSMA; examples include Israeli et al. "Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen" *Cancer Res.* 53(2), 227-230 (1993); Gen Bank Ref No. AAA60209.1). All or fragments of a molecule that
20 provides a cancer protective response may be used in the processes here.

Further, the vaccine may be monovalent or polyvalent and more than one cancer protective nucleic acid molecule may be provided in the vaccine; either more than one of the selected nucleic acid molecule, or different nucleic acid molecules. The prime and boost vaccine will include at least one nucleic acid molecule that is the same.

25 The methods disclosed include any useful variation of a sequence that provides a cancer protective response. For example, methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm.

Optimal alignment of sequences for comparison can use any means to analyze
30 sequence identity (homology) known in the art, e.g., by the progressive alignment method of termed "PILEUP" (Morrison, (1997) *Mol. Biol. Evol.* 14:428-441, as an example of the use of PILEUP); by the local homology algorithm of Smith & Waterman (*Adv. Appl. Math.*

2: 482 (1981)); by the homology alignment algorithm of Needleman & Wunsch (*J. Mol. Biol.* 48:443-453 (1970)); by the search for similarity method of Pearson (*Proc. Natl. Acad. Sci. USA* 85: 2444 (1988)); by computerized implementations of these algorithms (e.g., GAP, BEST FIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.); ClustalW (CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif., described by, e.g., Higgins(1988), *Gene* 73: 237-244; Corpet (1988), *Nucleic Acids Res.* 16:10881-10890; Huang, *Computer Applications in the Biosciences* 8:155-165 (1992); and Pearson (1994), *Methods in Mol. Biol.* 24:307-331); Pfam (Sonnhammer (1998), *Nucleic Acids Res.* 26:322-325); TreeAlign (Hein (1994), *Methods Mol. Biol.* 25:349-364); MEG-ALIGN, and SAM sequence alignment computer programs; or, by manual visual inspection.

Another example of algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul et al, (1990) *J. Mol. Biol.* 215: 403-410. The BLAST programs (Basic Local Alignment Search Tool) of Altschul, S. F., et al., searches under default parameters for identity to sequences contained in the BLAST "GENEMBL" database. A sequence can be analyzed for identity to all publicly available DNA sequences contained in the GENEMBL database using the BLASTN algorithm under the default parameters.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/; see also Zhang (1997), *Genome Res.* 7:649-656 for the "PowerBLAST" variation. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al (1990), *J. Mol. Biol.* 215: 403-410). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the

sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff (1992), *Proc. Natl. Acad. Sci. USA* 89:10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The term BLAST refers to the BLAST algorithm which
5 performs a statistical analysis of the similarity between two sequences; see, e.g., Karlin (1993), *Proc. Natl. Acad. Sci. USA* 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference
10 sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

In an embodiment, GAP (Global Alignment Program) can be used. GAP uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-453, 1970) to find the
15 alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Default gap creation penalty values and gap extension penalty values in the commonly used Version 10 of the Wisconsin Package® (Accelrys, Inc., San Diego, CA) for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is
20 3. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. A general purpose scoring system is the *BLOSUM62* matrix (Henikoff and Henikoff (1993), *Proteins* 17: 49-61), which is currently the default choice for *BLAST* programs. *BLOSUM62* uses a
25 combination of three matrices to cover all contingencies. Altschul, *J. Mol. Biol.* 36: 290-300 (1993), herein incorporated by reference in its entirety and is the scoring matrix used in Version 10 of the Wisconsin Package® (Accelrys, Inc., San Diego, CA) (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

The following terms are used to describe the sequence relationships between two or
30 more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity" and (d) "percentage of sequence identity."

- (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length promoter sequence, or the complete promoter sequence.
- 5 (b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous
10 nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to accurately reflect the similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.
- (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid
15 sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.
- (d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions
20 or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and
25 multiplying the result by 100 to yield the percentage of sequence identity.

Identity to the sequence of described would mean a polynucleotide sequence having at least 65% sequence identity, more preferably at least 70% sequence identity, more preferably at least 75% sequence identity, more preferably at least 80% identity, more preferably at least 85% 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,
30 97%, 98%, 99% sequence identity.

When referring to hybridization techniques, all or part of a known nucleotide sequence can be used as a probe that selectively hybridizes to other corresponding

nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ³²P, or any other
5 detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the DNA sequences. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed (Sambrook *et al.*, 2001).

For example, the sequences disclosed herein, or one or more portions thereof, may
10 be used as a probe capable of specifically hybridizing to corresponding sequences. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among the sequences to be screened and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such sequences may alternatively be used to amplify corresponding sequences from a chosen
15 plant by PCR. This technique may be used to isolate sequences from a desired plant or as a diagnostic assay to determine the presence of sequences in a plant. Hybridization techniques include hybridization screening of DNA libraries plated as either plaques or colonies (Sambrook *et al.*, (2001) Molecular Cloning--A Laboratory Manual (Third ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY.).

20 Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of
25 the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

30 Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50

nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 50°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 0.1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, *Anal. Biochem.*, 138:267-284 (1984): $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide

solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Part I, Chapter 2 (Elsevier, New York); and Ausubel et al., eds. (1995) Current Protocols in Molecular Biology, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (2001) Molecular Cloning: A Laboratory Manual (3rd ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.) and Haymes et al. (1985) In: Nucleic Acid Hybridization, a Practical Approach, IRL Press, Washington, D.C.

In general, sequences that correspond to the nucleotide sequences described and hybridize to the nucleotide sequence disclosed herein will be at least 50% homologous, 70% homologous, and even 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% homologous or more with the disclosed sequence. That is, the sequence similarity between probe and target may range, sharing at least about 50%, about 70%, and even about 85% or more sequence similarity.

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. The term conservatively modified variants applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are silent variations and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each

silent variation of a nucleic acid which encodes a polypeptide is implicit in each described polypeptide sequence and is within the scope of the products and processes described.

As used herein, the terms nucleic acid or polynucleotide refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-
5 stranded form. As such, the terms include RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence, or the like, and can be single-stranded or double-stranded, as well as a DNA/RNA hybrid. Furthermore, the terms are used herein to include naturally-occurring nucleic acid molecules, which can be isolated from a cell, as well as synthetic molecules, which can be prepared, for example, by
10 methods of chemical synthesis or by enzymatic methods such as by the polymerase chain reaction (PCR). Unless specifically limited, the terms encompass nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly
15 encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al. (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka et al.
20 (1985) *J. Biol. Chem.* 260:2605-2608; Rossolini et al. (1994) *Mol. Cell. Probes* 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

As used herein, a nucleotide segment is referred to as operably linked when it is placed into a functional relationship with another DNA segment. For example, DNA for a
25 signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked it is intended that the coding
30 regions are in the same reading frame. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide to be under

the transcriptional regulation of the regulatory regions. The expression cassette can include one or more enhancers in addition to the promoter. By enhancer is intended a cis-acting sequence that increases the utilization of a promoter. Such enhancers can be native to a gene or from a heterologous gene. Further, it is recognized that some promoters can
5 contain one or more enhancers or enhancer-like elements. An example of one such enhancer is the 35S enhancer, which can be a single enhancer, or duplicated. See for example, McPherson et al, US Patent 5,322,938.

A construct is a package of genetic material inserted into the genome of a cell via various techniques. The term nucleic acid construct refers to a coding sequence or
10 sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a marker gene and/or a reporter gene.

A cassette refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest or produces RNA,
15 and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A vector is a means for the transfer of a nucleic acid into a host cell. A vector may be a replicon to which another DNA segment may be attached so as to bring about the replication of the attached segment. A replicon is any genetic element (e.g., plasmid,
20 phage, cosmid, chromosome, virus) that functions as an autonomous unit of DNA or RNA replication *in vivo*, i.e., capable of replication under its own control. Unless indicated otherwise, the term "vector" includes both viral and nonviral means for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*.

In an example, the nucleic acid molecule may be expressed by a recombinant
25 vector, viral vector, or virus. As described more fully below, in a preferred embodiment the recombinant virus vectors include adenovirus and alphavirus vectors. In another example, Venezuelan equine encephalitis (VEE) vectors such as strains V3526 or TC-83 are employed. The techniques employed to insert such a sequence into the viral vector and make ether alterations in the viral DNA, e.g., to insert linker sequences and the like, are
30 known to one of skill in the art. (See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Third editions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 2001.

In one embodiment the vaccine is produced using an alphavirus vector (referred to in some instances as RNA particle or RP) technology. RP are produced by introducing into cell in culture a replicon RNA that expresses the foreign gene and two helper RNAs, one that codes for the alphavirus capsid protein and the other that codes for the alphavirus glycoproteins (E2 and E1). These RNAs can be introduced into cells using a number of methods such as lipid transfection or electroporation. After the three RNAs have been introduced into cells the replicon RNA replicates itself *in-cis* and the helper RNAs *in-trans*. The helper RNAs produce the structural proteins which recognize the replicon RNA and package it into RP. RP expressing the foreign gene constitutes the autogenous vaccine.

The methods and variations of same used to produce such replicons are known to one skilled in the art. Illustrative methodology can be found at US patent 6,156,558, incorporated herein by reference in its entirety, and also at US patents 6,521,235; 6,531,135; and US Patents 7,442,381; 6,541,010; 7,045,335; and 5,792,462 all of which are incorporated herein by reference in their entirety.

Alphavirus vectors and alphavirus replicon particles are used in embodiments of the invention. The term "alphavirus" has its conventional meaning in the art and includes the various species of alphaviruses which are members of the *Togaviridae* family. This includes alphaviruses such as Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus (WEE), Sindbis virus, South African Arbovirus No. 86, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylgach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, and Buggy Creek virus. The viral genome is a single-stranded, messenger-sense RNA, modified at the 5'-end with a methylated cap, and at the 3'-end with a variable-length poly (A) tract. Structural subunits containing a single viral protein, C, associate with the RNA genome in an icosahedral nucleocapsid. In the virion, the capsid is surrounded by a lipid envelope covered with a regular array of transmembrane protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2. See Pedersen et al., *J. Virol.* 14:40 (1974). The Sindbis and Semliki Forest viruses are considered the prototypical alphaviruses and have been studied extensively. See

Schlesinger The Togaviridae and Flaviviridae, Plenum Publishing Corp., New York (1986). The VEE virus has also been studied. See U.S. Pat. No. 5,185,440 to Davis et al.

As the above patents illustrate, use of alphavirus replicon particles to produce protective molecules are processes known to one skilled in the art. There are many
5 modifications to the process available, and any process using a replicon subunit or replicon particle methodology can be used with the invention. Thus the system in one embodiment provides for infectious, defective, alphavirus particles, wherein each particle comprises an alphavirus replicon RNA, and wherein the replicon RNA comprises an alphavirus
10 packaging signal, one or more heterologous RNA sequence(s), and a sequence encoding at least one alphavirus structural protein, and wherein the replicon RNA furthermore lacks a sequence encoding at least one alphavirus structural protein; wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture. For example, in US patent 6,531,135, incorporated herein by reference in its entirety is shown in an embodiment an RP system which uses a helper cell
15 for expressing an infectious, replication defective, alphavirus particle in an alphavirus-permissive cell. The helper cell includes (a) a first helper RNA encoding (i) at least one alphavirus structural protein, and (ii) not encoding at least one alphavirus structural protein; and (b) a second helper RNA separate from the first helper RNA, the second helper RNA (i) not encoding at least one alphavirus structural protein encoded by the first
20 helper RNA, and (ii) encoding at least one alphavirus structural protein not encoded by the first helper RNA, such that all of the alphavirus structural proteins assemble together into alphavirus particles in the cell. Preferably, the alphavirus packaging segment is deleted from at least the first helper RNA.

There are many variations that are available to one skilled in the art when preparing
25 such replicons. For example, in another embodiment, the helper cell also includes a replicon RNA, which encodes the alphavirus packaging segment and an inserted heterologous RNA. In the embodiment wherein the helper cell also includes a replicon RNA, the alphavirus packaging segment may be, and preferably is, deleted from both the first helper RNA and the second helper RNA. For example, in the embodiment wherein the
30 helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted heterologous RNA, the first helper RNA includes the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and the second helper RNA includes the alphavirus

capsid protein. The replicon RNA, first helper RNA, and second helper RNA in an embodiment are all on separate molecules and are cotransfected into the host cell.

In an alternative embodiment, the helper cell includes a replicon RNA encoding the alphavirus packaging segment, an inserted heterologous RNA, and the alphavirus capsid protein encoded by the second helper RNA, and the first helper RNA includes the
5 alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein. Thus, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the second helper RNA are on a single molecule. The heterologous RNA comprises a foreign RNA.

The RNA encoding the structural proteins, i.e., the first helper RNA and the second
10 helper RNA, may advantageously include one or more attenuating mutations. In an embodiment, at least one of the first helper RNA and the second helper RNA includes at least one attenuating mutation. The attenuating mutations provide the advantage that in the event of RNA recombination within the cell, the coming together of the structural and non-structural genes will produce a virus of decreased virulence.

In another aspect a method of making infectious, non-living replication defective,
15 alphavirus particles are provided. The method includes transfecting a helper cell as given above with a replication defective replicon RNA, producing the alphavirus particles in the transfected cell, and then collecting the alphavirus particles from the cell. The replicon RNA encodes the alphavirus packaging segment and a heterologous RNA. The transfected
20 cell further includes the first helper RNA and second helper RNA as described above.

In another aspect, a set of RNAs is provided for expressing an infectious, non-living replication defective alphavirus. The set of RNAs comprises, in combination, (a) a replicon RNA encoding a promoter sequence, an inserted heterologous RNA, wherein
25 RNA encoding at least one structural protein of the alphavirus is deleted from the replicon RNA so that the replicon RNA is replication defective, and (b) a first helper RNA separate from the replicon RNA, wherein the first helper RNA encodes in trans, the structural protein which is deleted from the replicon RNA and which may or may not include a promoter sequence. In this embodiment, it is preferred that an RNA segment encoding at least one of the structural proteins is located on an RNA other than the first helper RNA.
30 Thus, for example, the set of RNAs may include a replicon RNA including RNA which encodes the alphavirus packaging sequence, the inserted heterologous RNA, and the alphavirus capsid protein, but both the alphavirus E1 glycoprotein and alphavirus E2

glycoprotein are deleted therefrom; and a first helper RNA includes RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein.

In another embodiment, the set of RNAs also includes a second helper RNA separate from the replicon RNA and the first helper RNA. In this embodiment, the second helper RNA encodes, in trans, at least one structural protein, which is different from the structural protein encoded by the replicon RNA and by the first helper RNA. Thus, for example, the set of RNAs may include a replicon RNA including RNA which encodes the alphavirus packaging sequence, and the inserted heterologous RNA; a first helper RNA including RNA which may encode a promoter sequence and an RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein; and a second helper RNA including RNA which encodes the alphavirus capsid protein, with the replicon RNA, the first helper RNA, and the second helper RNA being in *trans* from each other, on separate molecules.

As another aspect, provided is a pharmaceutical formulation comprising infectious alphavirus particles as described above, in an effective immunogenic amount in a pharmaceutically acceptable carrier. See, for example, the '135 patent at column 2, line 10 – column 11 line 52 which includes examples 1-5.

The phrases "structural protein" or "alphavirus structural protein" as used herein refer to the encoded proteins which are required for production of particles that contain the replicon RNA, and include the capsid protein, E1 glycoprotein, and E2 glycoprotein. As described hereinabove, the structural proteins of the alphavirus are distributed among one or more helper RNAs (i.e., a first helper RNA and a second helper RNA). In addition, one or more structural proteins may be located on the same RNA molecule as the replicon RNA, provided that at least one structural protein is deleted from the replicon RNA such that the replicon and resulting alphavirus particle are replication defective. As used herein, the terms "deleted" or "deletion" mean either total deletion of the specified segment or the deletion of a sufficient portion of the specified segment to render the segment inoperative or nonfunctional, in accordance with standard usage. See, e.g., U.S. Pat. No. 4,650,764 to Temin et al. The term "replication defective" as used herein, means that the replicon RNA cannot produce particles in the host cell in the absence of the helper RNA. That is, no additional particles can be produced in the host cell. The replicon RNA is replication defective inasmuch as the replicon RNA does not include all of the alphavirus structural

proteins required for production of particles because at least one of the required structural proteins has been deleted therefrom.

The helper cell for production of the infectious, replication defective, alphavirus particle comprises a set of RNAs, as described above. The set of RNAs principally include
5 a first helper RNA and a second helper RNA. The first helper RNA includes RNA encoding at least one alphavirus structural protein but does not encode all alphavirus structural proteins. In other words, the first helper RNA does not encode at least one alphavirus structural protein; that is, at least one alphavirus structural protein gene has been deleted from the first helper RNA. In one embodiment, the first helper RNA includes RNA
10 encoding the alphavirus E1 glycoprotein, with the alphavirus capsid protein and the alphavirus E2 glycoprotein being deleted from the first helper RNA. In another embodiment, the first helper RNA includes RNA encoding the alphavirus E2 glycoprotein, with the alphavirus capsid protein and the alphavirus E1 glycoprotein being deleted from the first helper RNA. In a third, preferred embodiment, the first helper RNA includes RNA
15 encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, with the alphavirus capsid protein being deleted from the first helper RNA.

The second helper RNA includes RNA encoding the capsid protein which is different from the structural proteins encoded by the first helper RNA. In the embodiment wherein the first helper RNA includes RNA encoding only the alphavirus E1 glycoprotein, the second
20 helper RNA may include RNA encoding one or both of the alphavirus capsid protein and the alphavirus E2 glycoprotein which are deleted from the first helper RNA. In the embodiment wherein, the first helper RNA includes RNA encoding only the alphavirus E2 glycoprotein, the second helper RNA may include RNA encoding one or both of the alphavirus capsid protein and the alphavirus E1 glycoprotein which are deleted from the
25 first helper RNA. In the embodiment wherein the first helper RNA includes RNA encoding both the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, the second helper RNA may include RNA encoding the alphavirus capsid protein which is deleted from the first helper RNA.

In one embodiment, the packaging segment or "encapsidation sequence" is deleted
30 from at least the first helper RNA. In a preferred embodiment, the packaging segment is deleted from both the first helper RNA and the second helper RNA.

In an embodiment wherein the packaging segment is deleted from both the first helper RNA and the second helper RNA, preferably the helper cell contains a replicon RNA in addition to the first helper RNA and the second helper RNA. The replicon RNA encodes the packaging segment and an inserted heterologous RNA. The inserted
5 heterologous RNA may be RNA encoding a protein or a peptide. The inserted heterologous RNA may encode a protein or a peptide which is desirously expressed by the host, alphavirus-permissive cell, and includes the promoter and regulatory segments necessary for the expression of that protein or peptide in that cell.

For example, in one embodiment, the helper cell includes a set of RNAs which
10 include (a) a replicon RNA including RNA encoding an alphavirus packaging sequence and an inserted heterologous RNA, (b) a first helper RNA including RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and (c) a second helper RNA including RNA encoding the alphavirus capsid protein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into
15 alphavirus particles in the host cell.

In an alternate embodiment, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the second helper RNA are on a single molecule together, such that a first molecule, i.e., the first helper RNA, including RNA encoding at least one but not all of the required alphavirus structural proteins, and a second
20 molecule, i.e., the replicon RNA and second helper RNA, including RNA encoding the packaging segment, the inserted heterologous DNA and the capsid protein. Thus, the capsid protein is encoded by the second helper RNA, but the second helper RNA is located on the second-molecule together with the replicon RNA. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs including (a) a
25 replicon RNA including RNA encoding an alphavirus packaging sequence, an inserted heterologous RNA, and an alphavirus capsid protein, and (b) a first helper RNA including RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles in the host cell.

30 In one embodiment, the RNA encoding the alphavirus structural proteins, i.e., the capsid, E1 glycoprotein and E2 glycoprotein, contains at least one attenuating mutation. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a

nucleotide mutation or an amino acid coded for in view of such a mutation which result in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with standard terminology in the art. See, e.g., B. Davis, et al., *Microbiology* 132 (3d ed. 1980), whether the mutation be a substitution mutation or an in-frame deletion mutation. The phrase "attenuating mutation" excludes mutations which would be lethal to the virus. Thus, according to this embodiment, at least one of the first helper RNA and the second helper RNA includes at least one attenuating mutation. In a more preferred embodiment, at least one of the first helper RNA and the second helper RNA includes at least two, or multiple, attenuating mutations. The multiple attenuating mutations may be positioned in either the first helper RNA or in the second helper RNA, or they may be distributed randomly with one or more attenuating mutations being positioned in the first helper RNA and one or more attenuating mutations positioned in the second helper RNA. Appropriate attenuating mutations will be dependent upon the alphavirus used. For example, when the alphavirus is VEE, suitable attenuating mutations may be selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating mutation, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating mutation, preferably isoleucine or leucine as E1 amino acid 81; and codons at E1 amino acid 253 which specify an attenuating mutation, preferably serine or threonine as E1 amino acid 253.

In an alternate embodiment, wherein the alphavirus is the South African Arbovirus No. 86 (S.A.AR86), suitable attenuating mutations may be selected from the group consisting of codons at nsP1 amino acid position 538 which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid position 376 which specify an attenuating amino acid, preferably alanine as E2 amino acid 376; codons at E2 amino acid position 372 which specify an attenuating amino acid, preferably leucine as E2 amino acid

372; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid 96; and codons at nsP2 amino acid position 372 which specify an attenuating amino acid, preferably valine as nsP2 amino acid 372. Suitable attenuating mutations useful in embodiments wherein other alphaviruses are employed are known to those skilled in the art. Attenuating mutations may be introduced into the RNA by performing site-directed mutagenesis on the cDNA which encodes the RNA, in accordance with known procedures. See, Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488 (1985). Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures.

A synthetic plasmid vector is used called pVEK. The pVEK plasmid vectors incorporate derivatives of the nonstructural genes from the attenuated Venezuelan equine encephalitis VEE virus vaccine, TC-83. (See, for example US Patent 9,079,943, incorporated herein by reference in its entirety.) The pVEK plasmid vectors have been modified for optimal donor gene expression (Kamrud, K. I., Custer, M., Dudek, J. M., Owens, G., Alterson, K. D., Lee, J. S., Groebner, J. L. & Smith, J. F. (2007). Alphavirus replicon approach to promoterless analysis of IRES elements. *Virology*. 360, 376-87. Epub 2006 Dec 6.). The plasmids are linearized and RNA is transcribed from the plasmid DNA with T7 Express enzyme in the presence of Cap analog (Promega, Madison, WI) and purified. The purified RNA is electroporated into Vero cells (derived from Master Cells) for translation into autogenous recombinant proteins. Neither the pVEK plasmid vector nor the transcribed RNA contain the structural, capsid, or glycoprotein genes for TC-83.

The nonstructural genes aid in the expression of the autogenous recombinant proteins by forming a complex which transcribes additional autogenous recombinant gene RNA. Nsp1 serves as the capping enzyme and is believed to play a major role in the binding and assembly of the complex. Nsp2 is an RNA binding protein that has NTPase activity and likely functions as a RNA helicase to unwind duplex RNA. Nsp2 also functions as a protease that is required for post-translational processing of the nonstructural polyproteins. Nsp3 is a phosphoprotein whose role has not been determined. Nsp4 has been identified as the RNA polymerase. Rayner, et al. (2002). Alphavirus vectors and vaccination. *Rev Med Virol* 12, 279-96.

The pVEK vectors control protein expression at the level of translation by incorporating internal ribosome entry site (IRES) elements associated with each donor gene (Kamrud, et al. (2007). Alphavirus replicon approach to promoterless analysis of IRES elements. *Virology*. 360, 376-87. Epub 2006 Dec 6.). The sequence differs from the
5 TC-83 genomic sequence by four mutations and the absence of all TC-83 structural genes. In addition, a kanamycin resistance Open Reading Frame has been inserted into the plasmid backbone as a selective marker to amplify E. coli that contain the replicon plasmid DNA. A multiple cloning site has also been inserted in place of the structural protein genes. The resulting plasmid, pVEK, is replicated in bacteria using the COLE1 origin of
10 replication and contains a 5' untranslated region, TC-83 nonstructural protein sequences, a 26S promoter, a multiple cloning site and a 3' untranslated region, all placed downstream of a T7 polymerase promoter for *in vitro* RNA transcription.

As with alphavirus vectors, an adenoviral vector is a replication deficient virus vector that can express a nucleic acid molecule. They have been used to express a variety
15 of nucleic acid molecules. Examples of adenovirus vectors and their production are discussed, for example, where used to produce a vaccine expressing a sequence of the Dengue virus, see US Patent No. 8,920,813; or a human CFTR protein, see US Patent No. 6,136,594, each of which are incorporated herein by reference in their entirety. There are many variations on specific components and processes of producing a replication deficient
20 adenovirus vector, and the following is provided by way of example and not limitation.

Adenovirus from various origins, subtypes, or mixture of subtypes can be used as the source of the viral genome for the adenoviral vector. Non-human adenovirus (e.g., simian, avian, canine, ovine, or bovine adenoviruses) can be used to generate the adenoviral vector. For example, the adenoviral vector can be based on a simian adenovirus,
25 including both new world and old world monkeys (see, e.g., Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses (2005)). The phylogeny of adenoviruses that infect primates is disclosed in, e.g., Roy et al., *PLoS Pathog.*, 5(7): e100050. doi:10.1371/journal.ppat.1000503 (2009). For instance, a simian adenovirus can be of serotype 1, 3, 6, 7, 11, 16, 18, 19, 20, 27, 33, 38, 39, 48, 49, or 50, or any other
30 simian adenoviral serotype. Other non-human adenoviruses which can be used in the invention include non-human primate adenoviruses that are genetically and/or phenotypically similar to group C human adenoviruses. A human adenovirus can be used

as the source of the viral genome for the adenoviral vector. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, and 31), subgroup B (e.g., serotypes 3, 7, 11, 14, 16, 21, 34, 35, and 50), subgroup C (e.g., serotypes 1, 2, 5, and 6), subgroup D (e.g., serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, and 42-48), subgroup E (e.g., serotype 4), subgroup F (e.g., serotypes 40 and 41), an unclassified serogroup (e.g., serotypes 49 and 51), or any other adenoviral serotype. Adenoviral serotypes 1 through 51 (i.e., Ad1 through Ad51) are available from the American Type Culture Collection (ATCC, Manassas, Va.). In an embodiment, the adenoviral vector is of human subgroup C, especially serotype 2 or even more desirably serotype 5. However, non-group C adenoviruses can be used to prepare adenoviral gene transfer vectors for delivery of gene products to host cells. Examples of adenoviruses used in the construction of non-group C adenoviral gene transfer vectors include Ad12 (group A), Ad7 and Ad35 (group B), Ad30 and Ad36 (group D), Ad4 (group E), and Ad41 (group F). Non-group C adenoviral vectors, methods of producing non-group C adenoviral vectors, and methods of using non-group C adenoviral vectors are disclosed in, for example, U.S. Pat. Nos. 5,801,030, 5,837,511, and 5,849,561, and International Patent Application Publications WO 1997/012986 and WO 1998/053087.

The '813 patent describes variations on preparation of adenovirus vectors, such as where the adenoviral vector can comprise portions of an adenoviral genome of two or more (e.g., a mixture of) subtypes, in addition to containing a nucleic acid sequence encoding the chimeric hexon protein as described herein, and thereby be a "chimeric" adenoviral vector. A chimeric adenoviral vector can comprise an adenoviral genome that is derived from two or more (e.g., 2, 3, 4, etc.) different adenovirus serotypes. In one version, a chimeric adenoviral vector can comprise approximately different or equal amounts of the genome of each of the two or more different adenovirus serotypes.

In an embodiment, the adenoviral vector is replication-deficient, such that the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of one or more regions of the adenoviral genome for propagation (e.g., to form adenoviral vector particles). A replication-deficient adenoviral vector is an adenoviral vector that requires complementation of one or more gene functions or regions of the adenoviral genome that are required for replication, as a result of, for example, a deficiency in one or more replication-essential gene function or regions, such

that the adenoviral vector does not replicate in typical host cells, especially those in a human to be infected by the adenoviral vector.

A deficiency in a gene function or genomic region, can be a disruption (e.g., deletion) of sufficient genetic material of the adenoviral genome to obliterate or impair the function of the gene (e.g., such that the function of the gene product is reduced by at least about 2-fold, 5-fold, 10-fold, 20-fold, 30-fold, or 50-fold) whose nucleic acid sequence was disrupted (e.g., deleted) in whole or in part. Deletion of an entire gene region often is not required for disruption of a replication-essential gene function. However, for the purpose of providing sufficient space in the adenoviral genome for one or more transgenes, removal of a majority of one or more gene regions may be desirable. While deletion of genetic material is preferred, mutation of genetic material by addition or substitution also is appropriate for disrupting gene function. Replication-essential gene functions are those gene functions that are required for adenovirus replication (e.g., propagation) and are encoded by, for example, the adenoviral early regions (e.g., the E1, E2, and E4 regions), late regions (e.g., the L1, L2, L3, L4, and L5 regions), genes involved in viral packaging (e.g., the IVa2 gene), and virus-associated RNAs (e.g., VA-RNA-1 and/or VA-RNA-2).

The replication-deficient adenoviral vector can be modified in any suitable manner to cause the deficiencies in the one or more replication-essential gene functions in one or more regions of the adenoviral genome for propagation. The complementation of the deficiencies in the one or more replication-essential gene functions of one or more regions of the adenoviral genome can refer to the use of exogenous means to provide the deficient replication-essential gene functions. Such complementation can be effected in any suitable manner, for example, by using complementing cells and/or exogenous DNA (e.g., helper adenovirus) encoding the disrupted replication-essential gene functions.

The adenoviral vector can be deficient in one or more replication-essential gene functions of only the early regions (i.e., E1-E4 regions) of the adenoviral genome, only the late regions (i.e., L1-L5 regions) of the adenoviral genome, both the early and late regions of the adenoviral genome, or all adenoviral genes (i.e., a high capacity adenovector (HC-Ad). See Morsy et al., Proc. Natl. Acad. Sci. USA, 95: 965-976 (1998); Chen et al., Proc. Natl. Acad. Sci. USA, 94: 1645-1650 (1997); and Kochanek et al., Hum. Gene Ther., 10: 2451-2459 (1999). Examples of replication-deficient adenoviral vectors are disclosed in U.S. Pat. Nos. 5,837,511; 5,851,806; 5,994,106; 6,127,175; 6,482,616; and 7,195,896, and

International Patent Application Publications WO 1994/028152, WO 1995/002697, WO 1995/016772, WO 1995/034671, WO 1996/022378, WO 1997/012986, WO 1997/021826, and WO 2003/022311.

The early regions of the adenoviral genome include the E1, E2, E3, and E4 regions. 5 The E1 region comprises the E1A and E1B subregions, and one or more deficiencies in replication-essential gene functions in the E1 region can include one or more deficiencies in replication-essential gene functions in either or both of the E1A and E1B subregions, thereby requiring complementation of the E1A subregion and/or the E1B subregion of the adenoviral genome for the adenoviral vector to propagate (e.g., to form adenoviral vector 10 particles). The E2 region comprises the E2A and E2B subregions, and one or more deficiencies in replication-essential gene functions in the E2 region can include one or more deficiencies in replication-essential gene functions in either or both of the E2A and E2B subregions, thereby requiring complementation of the E2A subregion and/or the E2B subregion of the adenoviral genome for the adenoviral vector to propagate (e.g., to form 15 adenoviral vector particles).

The E3 region does not include any replication-essential gene functions, such that a deletion of the E3 region in part or in whole does not require complementation of any gene functions in the E3 region for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). In the context of the invention, the E3 region is defined as the region that 20 initiates with the open reading frame of the 12.5K protein from the E3 region of human adenovirus 5 (NCBI reference sequence AP_000218) and ends with the open reading frame that encodes the 14.7K protein from the E3 region of human adenovirus 5 (NCBI reference sequence AP_000224.1). The E3 region may be deleted in whole or in part or retained in whole or in part. The size of the deletion may be tailored so as to retain an adenoviral 25 vector whose genome closely matches the optimum genome packaging size. A larger deletion will accommodate the insertion of larger heterologous nucleic acid sequences in the adenoviral genome.

The E4 region comprises multiple open reading frames (ORFs). An adenoviral vector with a deletion of all of the open reading frames of the E4 region except ORF6, and 30 in some cases ORF3, does not require complementation of any gene functions in the E4 region for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). Conversely, an adenoviral vector with a disruption or deletion of ORF6, and in some cases

ORF3, of the E4 region (e.g., with a deficiency in a replication-essential gene function based in ORF6 and/or ORF3 of the E4 region), with or without a disruption or deletion of any of the other open reading frames of the E4 region or the native E4 promoter, polyadenylation sequence, and/or the right-side inverted terminal repeat (ITR), requires
5 complementation of the E4 region (specifically, of ORF6 and/or ORF3 of the E4 region) for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). The late regions of the adenoviral genome include the L1, L2, L3, L4, and L5 regions. The adenoviral vector also can have a mutation in the major late promoter (MLP), as discussed in International Patent Application Publication WO 2000/000628, which can render the
10 adenoviral vector replication-deficient if desired.

The one or more regions of the adenoviral genome that contain one or more deficiencies in replication-essential gene functions desirably are one or more early regions of the adenoviral genome, i.e., the E1, E2, and/or E4 regions, optionally with the deletion in part or in whole of the E3 region.

15 The replication-deficient adenoviral vector also can have one or more mutations as compared to the wild-type adenovirus (e.g., one or more deletions, insertions, and/or substitutions) in the adenoviral genome that do not inhibit viral replication in host cells. Thus, in addition to one or more deficiencies in replication-essential gene functions, the adenoviral vector can be deficient in other respects that are not replication-essential. For
20 example, the adenoviral vector can have a partial or entire deletion of the adenoviral early region known as the E3 region, which is not essential for propagation of the adenoviral genome.

In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E1 region or the E4 region of the adenoviral genome, for
25 propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of the E1A subregion and/or the E1B region of the adenoviral genome (denoted an E1-deficient adenoviral vector) or the E4 region of the adenoviral genome (denoted an E4-deficient adenoviral vector) for propagation (e.g., to form adenoviral vector particles). The
30 adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral

genome (denoted an E1/E3-deficient adenoviral vector). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E4 region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral genome (denoted an E3/E4-
5 deficient adenoviral vector).

In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E2 region, preferably the E2A subregion, of the adenoviral genome, for propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential
10 gene function of the E2A subregion of the adenoviral genome (denoted an E2A-deficient adenoviral vector) for propagation (e.g., to form adenoviral vector particles). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E2A region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral
15 genome (denoted an E2A/E3-deficient adenoviral vector).

In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E1 and E4 regions of the adenoviral genome for propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of both the E1
20 and E4 regions of the adenoviral genome (denoted an E1/E4-deficient adenoviral vector) for propagation (e.g., to form adenoviral vector particles). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region of the adenoviral genome, at least one replication-essential gene function of the E4 region of the adenoviral genome, and at least
25 one gene function of the nonessential E3 region of the adenoviral genome (denoted an E1/E3/E4-deficient adenoviral vector). The adenoviral vector in an embodiment requires, at most, complementation of the E1 region of the adenoviral genome for propagation and does not require complementation of any other deficiency of the adenoviral genome for propagation. The adenoviral vector can in another embodiment require, at most,
30 complementation of the E1 and E4 regions of the adenoviral genome for propagation and does not require complementation of any other deficiency of the adenoviral genome for propagation.

The adenoviral vector, when deficient in multiple replication-essential gene functions of the adenoviral genome (e.g., an E1/E4-deficient adenoviral vector), can include a spacer sequence to provide viral growth in a complementing cell line similar to that achieved by adenoviral vectors deficient in a single replication-essential gene function (e.g., an E1-deficient adenoviral vector). The spacer sequence can contain any nucleotide sequence or sequences which are of a desired length, such as sequences at least about 15 base pairs (e.g., between about 15 nucleotides and about 12,000 nucleotides), preferably about 100 nucleotides to about 10,000 nucleotides, more preferably about 500 nucleotides to about 8,000 nucleotides, even more preferably about 1,500 nucleotides to about 6,000 nucleotides, and most preferably about 2,000 to about 3,000 nucleotides in length, or a range defined by any two of the foregoing values. The spacer sequence can be coding or non-coding and native or non-native with respect to the adenoviral genome but does not restore the replication-essential function to the deficient region. The spacer also can contain an expression cassette. More preferably, the spacer comprises a polyadenylation sequence and/or a gene that is non-native with respect to the adenoviral vector. The use of a spacer in an adenoviral vector is further described in, for example, U.S. Pat. No. 5,851,806 and International Patent Application Publication WO 1997/021826.

By removing all or part of the adenoviral genome, for example, the E1, E3, and E4 regions of the adenoviral genome, the resulting adenoviral vector is able to accept inserts of heterologous nucleic acid sequences while retaining the ability to be packaged into adenoviral capsids. A heterologous nucleic acid sequence can be inserted at any position in the adenoviral genome so long as insertion in the position allows for the formation of the adenoviral vector particle. The heterologous nucleic acid sequence preferably is positioned in the E1 region, the E3 region, or the E4 region of the adenoviral genome.

The replication-deficient adenoviral vector can be produced in complementing cell lines that provide gene functions not present in the replication-deficient adenoviral vector, but required for viral propagation, at appropriate levels in order to generate high titers of viral vector stock. Such complementing cell lines are known and include, but are not limited to, 293 cells (described in, e.g., Graham et al., *J. Gen. Virol.*, 36: 59-72 (1977)), PER.C6 cells (described in, e.g., International Patent Application Publication WO 1997/000326, and U.S. Pat. Nos. 5,994,128 and 6,033,908), and 293-ORF6 cells (described in, e.g., International Patent Application Publication WO 1995/034671 and Brough et al., J.

Virology, 71: 9206-9213 (1997)). Other suitable complementing cell lines to produce the replication-deficient adenoviral vector of the invention include complementing cells that have been generated to propagate adenoviral vectors encoding transgenes whose expression inhibits viral growth in host cells (see, e.g., U.S. Patent Application Publication 5 2008/0233650). Additional suitable complementing cells are described in, for example, U.S. Pat. Nos. 6,677,156 and 6,682,929, and International Patent Application Publication WO 2003/020879. In some instances, the cellular genome need not comprise nucleic acid sequences, the gene products of which complement for all of the deficiencies of a replication-deficient adenoviral vector. One or more replication-essential gene functions 10 lacking in a replication-deficient adenoviral vector can be supplied by a helper virus, e.g., an adenoviral vector that supplies in trans one or more essential gene functions required for replication of the replication-deficient adenoviral vector. Alternatively, the inventive adenoviral vector can comprise a non-native replication-essential gene that complements for the one or more replication-essential gene functions lacking in the inventive replication- 15 deficient adenoviral vector. For example, an E1/E4-deficient adenoviral vector can be engineered to contain a nucleic acid sequence encoding E4 ORF 6 that is obtained or derived from a different adenovirus (e.g., an adenovirus of a different serotype than the inventive adenoviral vector, or an adenovirus of a different species than the inventive adenoviral vector).

20 As can be seen, there are a variety of means of producing and variations to adenovirus and alphavirus vectors and their use available to one skilled in the art.

The vaccine can be administered as described herein to an animal. The method of the present invention is useful in animals including, but not limited to, humans, canine (e.g., dogs), feline (e.g., cats); equine (e.g., horses), bovine (e.g., cattle), and other animals 25 which can develop cancer.

The vaccine may be administered prior to tumor development, upon diagnosis of cancer, where there is a recurrence of cancer after treatment of the cancer, and where other therapies have been employed. The methods and vaccines here are particularly useful with treating prostate cancer and is in an embodiment particularly useful where there has been 30 prior treatment, such as surgery or radiation, but the cancer has recurred.

As used herein, the term "vaccine" as used herein refers to a pharmaceutical composition comprising at least one molecule, nucleic acid or polypeptide or fragment

thereof that induces a protective response in an animal and possibly, but not necessarily, one or more additional components that enhance the activity of the active component. A vaccine may additionally comprise further components typical to pharmaceutical compositions. A vaccine may comprise one or simultaneously more than one of the
5 elements described above.

The vaccine composition may be introduced into an animal, with a physiologically acceptable vehicle and/or adjuvant. Useful vehicles are well known in the art, and include, e.g., water, buffered water, saline, glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized
10 preparation being rehydrated prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate,
15 triethanolamine oleate, and the like. In an embodiment, the molecule is combined with a binder that assists in associating the molecule with feed, which is particularly useful for oral administration. Such a water resistant binding substance can be any substance having such properties. Examples include, without limitation, agarose or other sugar compounds, albumin, alginate or any similar composition. In an embodiment the immunization
20 composition includes an adjuvant to further enhance immune response. Any convenient adjuvant may be used, and many are well known to one skilled in the art. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to
25 cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses. Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Examples, without meant to be limiting, include, *E. coli*, lipopolysaccharides, aluminum hydroxide and aluminum phosphate (alum), saponins
30 complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as

well as lipid A, and liposomes. Desirable characteristics of ideal adjuvants may include: (1) lack of toxicity; (2) ability to stimulate a long-lasting immune response; (3) simplicity of manufacture and stability in long-term storage; (4) ability to elicit both cell mediated immunity (CMI) and humoral immune response (HIR) to antigens administered by various routes; (5) synergy with other adjuvants; (6) capability of selectively interacting with populations of antigen presenting cells (APC); (7) ability to specifically elicit appropriate T-cell helper 1 (TH 1) or TH 2 cell-specific immune responses; and (8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens. An adjuvant used need not possess all these characteristics.

10 In another embodiment, the nucleic acid molecule be administered with other protective or desirable compounds which may be administered sequentially or progressively or alternately administered simultaneously in an admixture.

In referring to administration of the vaccine, the vaccine may be "administered" in any suitable manner, including but not limited to, parenterally, by injection subcutaneously or intramuscularly, into an organ or cavity of the subject, reverse gavage (rectally), and oral. The vaccine can be administered by any means which includes, but is not limited to, syringes, nebulizers, misters, needleless injection devices, or microprojectile bombardment gene guns (Biolistic bombardment), via a liposome delivery system, naked delivery system, electroporation, viruses, vectors, viral vectors, or an ingestible delivery system wherein the protective molecules are consumed, for example, in feed or water or in any other suitable manner.

As used herein, "effective amount" refers to an amount, which is effective in reducing, eliminating, treating, preventing or controlling the symptoms of the cancer. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual to mount a protective response. Under normal circumstances the vaccine volume injected varies from 0.1 ml to 0.15 ml. We used 0.125 ml. in our Phase I and Phase II clinical trials of the adenovirus vaccine.

The following is provided by way of exemplification and is not intended to limit the scope of the invention. All references referred to herein are incorporated herein by reference.

EXAMPLES

Example 1

Preclinical studies by the inventors demonstrated the ability of a replication-deficient adenovirus, transformed with the gene for human prostate specific antigen (PSA) to induce strong anti-PSA and hence, anti-prostate tumor immune responses [Elzey, B.D.,
5 et al., Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors. *Int J Cancer*, 2001. 94(6): p. 842-9.]. Immunized mice were able to destroy PSA-secreting mouse prostate cancer cells and the immune destruction was a function of antigen-specific CD8+ T cells. *Id.* Following a large
10 number of additional experiments Phase I and Phase II clinical trials were carried out in prostate cancer patients. The former trial demonstrated the safety of the vaccine and also provided preliminary data indicating the induction of immune response in patients as well improved clinical parameters [Lubaroff, D.M., et al., Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic
15 results. *Clin Cancer Res*, 2009. 15(23): p. 7375-80.]. In a further Phase II trial it was confirmed that the majority of patients had both immunologic and clinical benefits following immunization with the adenovirus/PSA (AdPSA) vaccine. Lubaroff et al., An ongoing phase II trial of an adenovirus vaccine for prostate cancer. *Amer Assoc Cancer Res Annual Meeting*, 2012.; Lubaroff, D.M., Prostate cancer vaccines in clinical trials. *Expert
20 Rev Vaccines*, 2012. 11(7): p. 857-68. However, the results were not absolute. Not all of the patients responded to the vaccine and those that did respond did not always have a sustained response.

Example 2**Preparation of vectors**

25 Adenovirus vectors and alphavirus vectors comprising the human prostate specific antigen (PSA) were produced. The PSA nucleic acid molecule used here is that found at Lundwall “Characterization of the gene for prostate-specific antigen, a human glandular kallikrein.” *Biochem Biophys Res Comm* 1989;161:1151-9 EMBL Accession No. X14810 (SEQ ID NO: 1). In the experiments here, the alphavirus vector employed was
30 TC-83. The PSA gene was cloned into the *AscI/PacI* sites of the pVEK (TC-83) replicon vector (Hooper et al., 2009, “Molecular Smallpox Vaccine Delivered by Alphavirus Replicons Elicits Protective Immunity in Mice and Non-Human Primates” *Vaccine* 13(13))

and an optimized construct was selected. (Kamrud et al. Alphavirus replicon approach to promoterless analysis of IRES elements. *Virology*. 2007 Apr 10;360(2):376-87. Epub 2006 Dec 6. PubMed PMID: 17156813; PubMed Central PMCID: PMC1885372). The adenovirus/PSA vaccine was produced as follows: The PSA cDNA provided by Donald
5 Tindall, Mayo Clinic, Rochester, MN, was placed 3' to the CMV promoter in a shuttle vector containing Ad5 DNA. The sequence inserted was the pre-pro form of PSA described by Lundwall. The cDNA encodes for 262 amino acids with a predicted molecular weight of 28.8 kDa. The shuttle vector and E1a-E1b deletion mutant Ad5 DNA were transfected into HEK 293 cells, and recombination between the DNA species was
10 allowed to occur. The amplification and purification of Ad/PSA was performed by the University of Iowa Gene Transfer Vector Core.

The alphavirus platform approach is designed to produce a vaccine containing the prostate specific antigen (*PSA*) gene. Briefly, the *PSA* gene is cloned into DNA plasmids using the same full-length DNA incorporated into the adenovirus for the AdPSA
15 vaccine. The plasmids are transcribed, producing RNA. The RNA is purified and electroporated into Vero cells. Once in the cells, the RNA is translated resulting in Viral Replicon Particles (VRP), encapsulating the replicon *PSA* RNA, are then harvested for formulation into VRP/PSA vaccine.

Preparation of mice

20 Male Balb/c mice, at least 8 weeks old are obtained from The Jackson Laboratories, Bar Harbor, Maine. The mice are injected subcutaneously with the E6 clone of the mouse prostate cancer cell line RM11/PSA 24 hours prior to the initiation of the heterologous prime-boost vaccine protocol. All rules and regulations of animal care are followed to insure humane care of mice throughout the studies. Tumor growth is monitored twice
25 weekly and measurements made using a vernier caliper.

Preparation of vaccines

The AdPSA and VRP/PSA vaccines are obtained from the manufacturer and stocks stored at -80 C in small volume cryotubes. For the preparation of treatment vaccines, the stock is diluted in phosphate-buffered saline (PBS) to obtain the desired dose for injection.
30 For the AdPSA the concentration is 1×10^9 pfu/ml so that an injection dose of 0.1 ml contains 1×10^8 pfu. For the VRP/PSA the concentration is 5×10^9 particles/ml in order to deliver 5×10^8 particles in 0.1 ml.

Vaccination regimes*Prime/boost vaccinations*

The following prime/boost protocol was used on mice, wherein adeno vector prostate specific antigen vaccine (AdPSA) or alphavirus vector PSA antigen vaccine (alphaPSA or VRP/PSA) was administered. The E6 tumor cells were injected subcutaneously on day 0 and the first vaccination was administered at day 1, the second vaccination at day 14.

Table 1

Prime injection	Booster injection
AdPSA	alphaPSA
alphaPSA	AdPSA
alphaPSA	alphaPSA
AdPSA	AdPSA

Control mice received vaccines carrying the DNA or RNA for indifferent antigen and did not generate anti-PSA immune responses.

The results, shown in Figures 1-3, demonstrated the superiority of using a priming injection of AdPSA with a booster injection of alphaPSA. The reverse combination or homologous combinations were not as effective in inducing anti-PSA immune responses.

Production of anti-PSA tetramer positive CD8⁺ T cells following heterologous prime-boost vaccination.

Mice were vaccinated with 1×10^8 AdPSA, 5×10^8 VRP/PSA, or controls of AdLacZ or VRP/GFP. Fourteen (14) days later the mice received booster vaccinations of either homologous or heterologous vaccines or controls. Tetramer assays were run 7 days after the boost. The strongest responses were produced by an AdPSA prime followed by a VRP/PSA boost.

Complete destruction of established tumors

Mice were injected subcutaneously with the E6 clone of RM11/PSA mouse prostate tumor cells, followed 24 hours later by a prime vaccination with AdPSA or VRP/PSA vaccine. All of the mice were boosted 14 days later with VRP/PSA vaccine as homologous or heterologous immunization. Tumor growth was monitored twice per week. The results (Figure 2A and 2B) demonstrate that, although the homologous

vaccination of VRP/PSA (alphaPSA in figure) + VRP/PSA was effective in destroying tumors in 7/10 mice (70%), the heterologous vaccination of AdPSA + VRP/PSA resulted in the destruction of 10/10 (100%) mice. (In the graphs of Figures 2A and 2B, the numbers 1 – 10 represent separate mice.)

5 **Example 4**

Additional cancer protective nucleic acid molecule vaccines will be tested. These will be selected from, prostate stem cell antigen (PSCA), prostatic acid phosphatase (PAP), and prostate specific membrane antigen (PSMA) in addition to prostate specific antigen (PSA) These studies will identify the best method to induce the strongest antigen-specific
10 immune responses and the destruction of mouse prostate tumor cells that express one or both antigens.

Example 5

Monovalent vaccines will contain RNA for a single antigen whereas a bivalent vaccine will contain the RNA for two different antigens, and trivalent vaccines will contain
15 the RNA for three different antigens. Initial experiments will compare the efficacy of monovalent PSA and PSCA vaccines, bivalent PSA+PSCA vaccine, and a mixture of monovalent PSA and PSCA vaccines. Mice will be vaccinated with VRP/PSA, VRP/PSCA, VRP/PSA+PSCA, or a mixture of VRP/PSA and VRP/PSCA vaccines. We will perform dose escalation studies using 1×10^7 , 5×10^7 , 1×10^8 , and 5×10^8 ffu with three
20 vaccinations 14 days apart. Appropriate controls will be included. Seven days after the last vaccination blood will be analyzed for anti-PSA and anti-PSCA T cells and the mice injected with RM11/PSA and RM11/PSCA on opposite flanks. Tumor measurements and survival analysis will be performed as previously described.

Example 6

25 This experiment will use PAP and PSMA using the same protocols described above for PSA and PSCA. In addition to monovalent and bivalent studies trivalent vaccines in various combinations will be investigated.

Example 7

Immune responses to foreign antigens involve a large number of cells, receptors,
30 ligands, cytokines, etc. which can have both positive and negative effects. The goal of cancer immunotherapy is to produce positive responses that result in the destruction of

tumor antigen- expressing antigens. However, there are a number of factors that have a negative effect on the immune responses. These negative regulatory elements have been the target of investigators in attempts to induce the strongest and most effective anti-tumor immunity. Currently the most vigorously studied elements are the programmed cell death 1 (PD1) receptor and its ligand (PD- L1). The receptor is involved in down regulation of the immune system and referred to as an immune checkpoint that guards against autoimmunity. See for example Balar AV¹, Weber JS² PD-1 and PD-L1 antibodies in cancer: current status and future directions. *Cancer Immunol Immunother.* 2017 Feb 17. 1954-6. [Epub ahead of print]. Antibodies to PD1 and PD-L1 have shown great promise as monotherapies for several cancers, but not all cancer patients respond. It now appears that the best responses are in patients that have some level of anti-tumor immunity. Our goal in these studies, both preclinical and clinical, is to combine the best vaccine immunotherapy with anti-PD1 or anti-PD-L1 antibody therapy. We predict that the establishment of anti-prostate tumor antigen(s) immune response will result in a high percentage of patients that will respond to checkpoint inhibitor therapies.

We will combine checkpoint inhibition therapy with the vaccine or vaccines that prove most efficacious by themselves in both preclinical and clinical studies. These may be monovalent VRP vaccines, bivalent VRP vaccines, trivalent VRP vaccines, or AdPSA-VRP/PSA prime- boost. Preclinical studies will be followed by clinical trials.

20 **Example 8**

The above experiment was repeated and Figure 3 shows further verification of the prime:boost strategy outlined. AdPSA prime vaccination was followed by VRP/PSA booster vaccination, inducing the highest number of anti-PSA T cells as shown in column 1.

What is claimed is:

1. A method of producing an increased cancer protective response in a subject having cancer, the method comprising,
 - 5 a) administering a first vaccine comprising an adenovirus vector comprising at least one nucleic acid molecule that produces a cancer protective response,
 - b) administering one or more second vaccines comprising an alphavirus replicon particle (VRP) comprising RNA comprising or produced from said at least nucleic acid molecule, wherein administration of said first and at least one second vaccines produces a cancer protective response in said subject, and
 - 10 c) producing a cancer protective response that is increased compared to administering a first vaccine comprising an alphavirus replicon particle and a second vaccine comprising an alphavirus replicon particle.
2. The method of claim 1, wherein said first and second vaccine comprise at least two nucleic acid molecules that produce a cancer protective response.
- 15 3. The method of claim 1, wherein said cancer is prostate cancer.
4. The method of claim 1 wherein said nucleic acid molecule is selected from prostate specific antigen (PSA), prostate stem cell antigen (PSCA), prostate acid phosphatase (PAP) or prostate specific membrane antigen (PSMA).
5. The method of claim 3, wherein said protective response comprises reducing growth of
20 prostate tumors.
6. The method of claim 3, wherein said protective response comprises elimination of prostate cancer tumors.
7. The method of claim 1, wherein at least three of said second vaccines is administered.
8. A method for treating a cancer patient, the method comprising,
 - 25 a) administering a first vaccine comprising an adenovirus vector comprising at least one nucleic acid molecule that produces a cancer protective response, and
 - b) administering one or more second vaccines comprising an alphavirus RNA replicon particle comprising RNA comprising or produced from said at least one nucleic acid molecule, wherein administration of said first and at least one second
30 vaccines produces a cancer protective response in said subject.

9. The method of claim 8, wherein said first and second vaccine comprise at least two nucleic acid molecules that produce a cancer protective response.
10. The method of claim 8, wherein said cancer is prostate cancer.
11. The method of claim 8 wherein said nucleic acid molecule is selected from prostate specific antigen (PSA), prostate stem cell antigen (PSCA), prostate acid phosphatase (PAP)
5 or prostate specific membrane antigen (PSMA).
12. The method of claim 10, wherein said protective response comprises reducing growth of prostate tumors.
13. The method of claim 10, wherein said protective response comprises elimination of
10 prostate cancer tumors.
14. The method of claim 10, wherein at least three of said second vaccines is administered
15. A method of vaccination of a subject having cancer, said method comprising,
a) administering a first vaccine comprising an adenovirus vector comprising at least one nucleic acid molecule that produces a cancer protective response, and
15 b) administering one or more second vaccines comprising an alphavirus replicon particle comprising RNA comprising or produced from said at least one nucleic acid molecule, wherein administration of said first and at least one second vaccines produces a cancer protective response in said subject.
16. The method of claim 15, wherein said cancer is prostate cancer.
- 20 17. The method of claim 15 wherein said nucleic acid molecule is selected from prostate specific antigen (PSA), prostate stem cell antigen (PSCA), prostate acid phosphatase (PAP) or prostate specific membrane antigen (PSMA).
18. The method of claim 16, wherein said protective response comprises reducing growth of prostate tumors.
- 25 19. The method of claim 16, wherein said protective response comprises elimination of prostate cancer tumors.
20. The method of claim 15, wherein at least three of said second vaccines is administered.

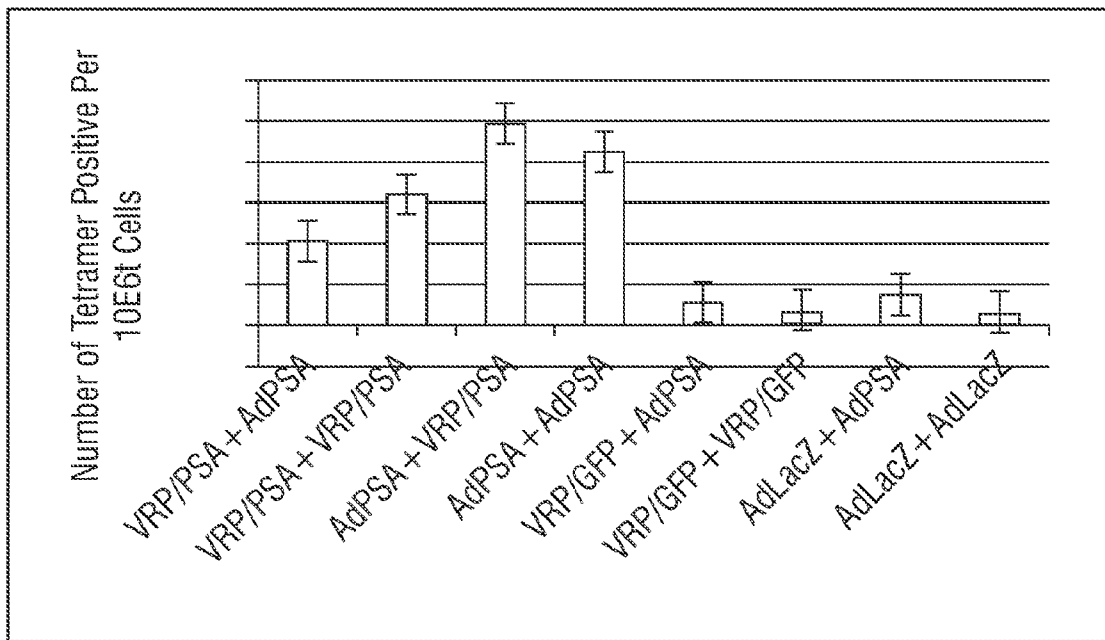


FIG. 1

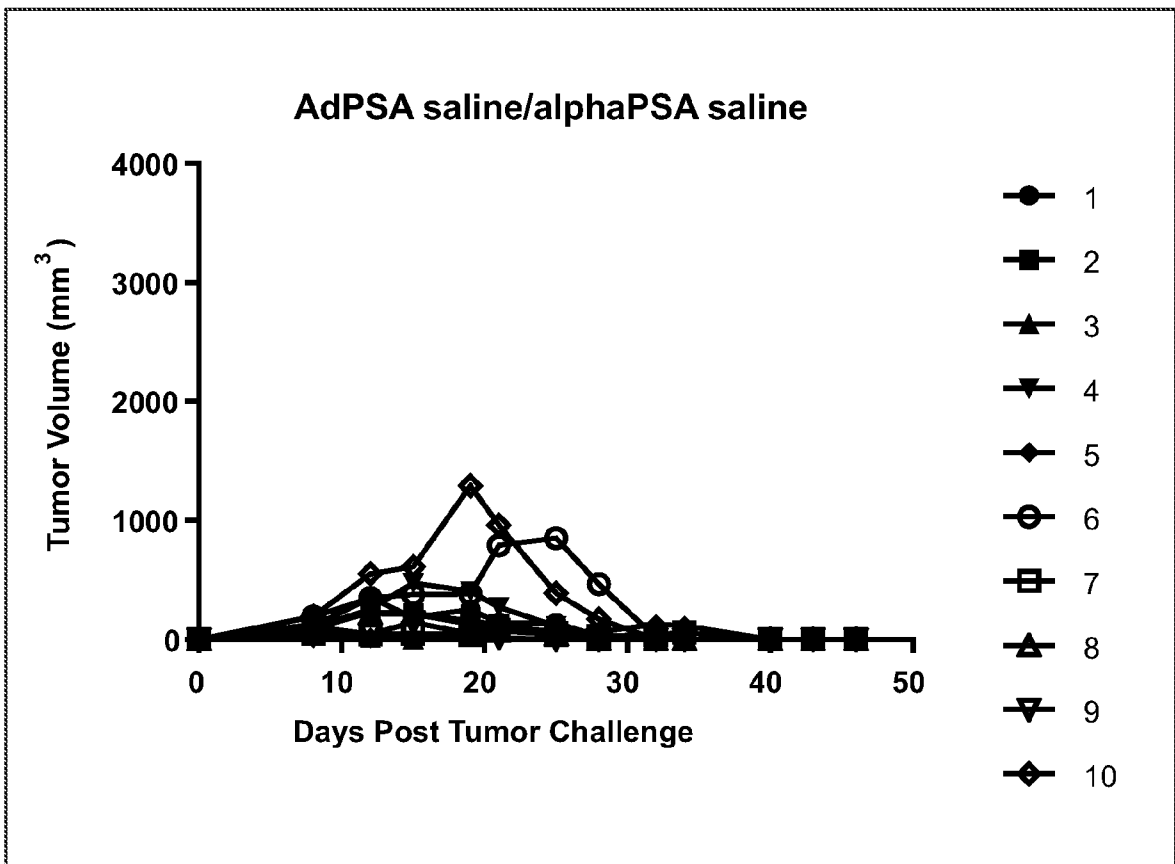


FIG. 2A

3/4

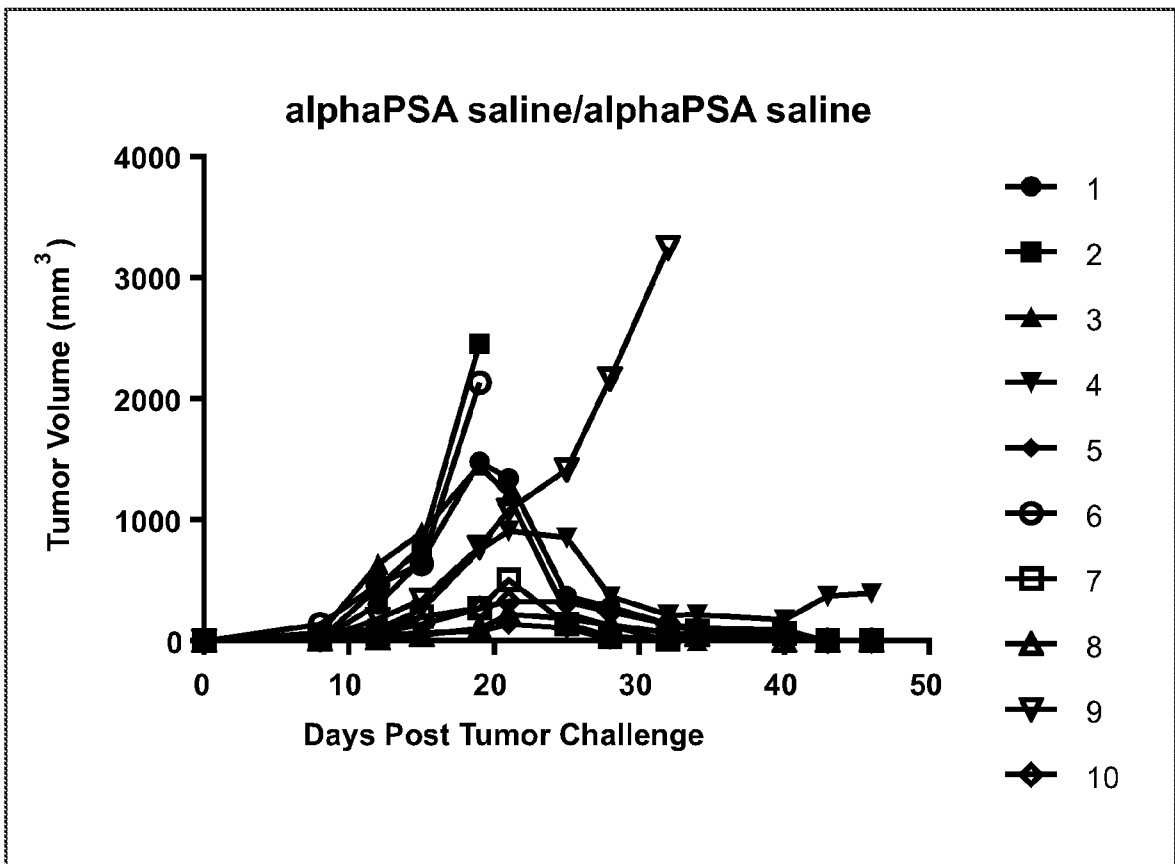


FIG. 2B

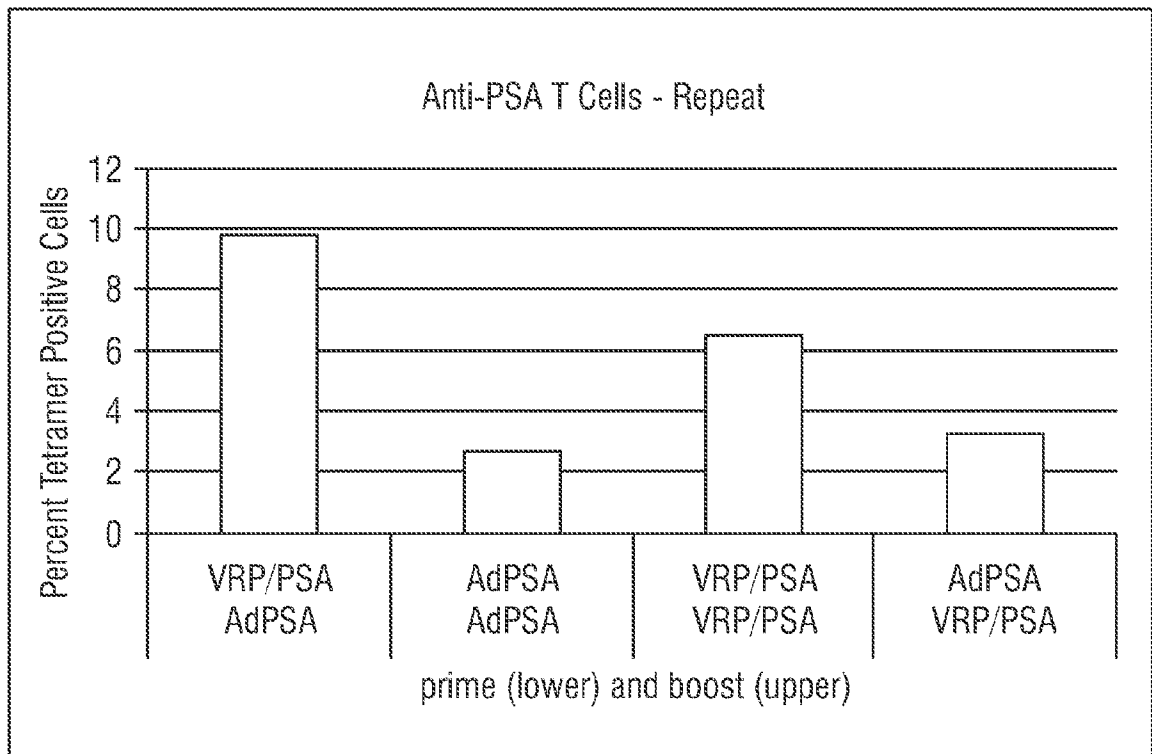


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/027093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/76; A61K 39/00; A61K 39/12; A61K 39/145; A61K 39/193; A61K 39/21 (2018.01)
 CPC - A61K 38/208; A61K 39/0011; A61K 39/12; A61K 39/145; A61K 39/21; A61K 39/39; A61K 48/00;
 A61K 2039/5252; A61K 2039/5256; A61K 2039/5258; A61K 2039/53; A61K 2039/54; A61K
 2039/541; A61K 2039/543; A61K 2039/545; A61K 2039/55538; A61K 2039/5555; A61K
 2039/55566; A61K 2039/57; A61K 2039/575; A61K 2039/70; C07K 14/005; C12N 7/00; C12N
 15/86; C12N 2710/16634; C12N 2740/16134; C12N 2740/16222; C12N 2740/16234 (2018.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/196.11; 424/199.1; 424/208.1; 424/281.1; 435/91.1; 435/320.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 1069908 B1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 10 February 2010 (10.02.2010) entire document	1-20
Y	US 2005/0208020 A1 (DOOLAN et al) 22 September 2005 (22.09.2005) entire document	1-20
Y	US 2016/0375115 A1 (PFIZER INC.) 29 December 2016 (29.12.2016) entire document	4, 5, 11, 12, 17, 18
A	LUBAROFF et al. "Phase I Clinical Trial of an Adenovirus/PSA Vaccine for Prostate Cancer: Safety and Immunologic Results," Clinical Cancer Research, 17 November 2009 (17.11.2009), Vol. 15, No. 23, Pgs. 7375-7380. entire document	1-20
A	US 2005/0266550 A1 (RAYNER et al) 01 December 2005 (01.12.2005) entire document	1-20
A	US 2012/0213813 A1 (SMITH et al) 23 August 2012 (23.08.2012) entire document	1-20
A	SWEENEY et al. "Oncolytic Adenovirus-Mediated Therapy for Prostate Cancer," Oncolytic Virotherapy, 14 July 2016 (14.07.2016), Vol. 5, Pgs. 45-57. entire document	1-20

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 June 2018

Date of mailing of the international search report

03 JUL 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774