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(54) Title: IMMUNE CHECKPOINT INHIBITOR CO-EXPRESSION VECTORS

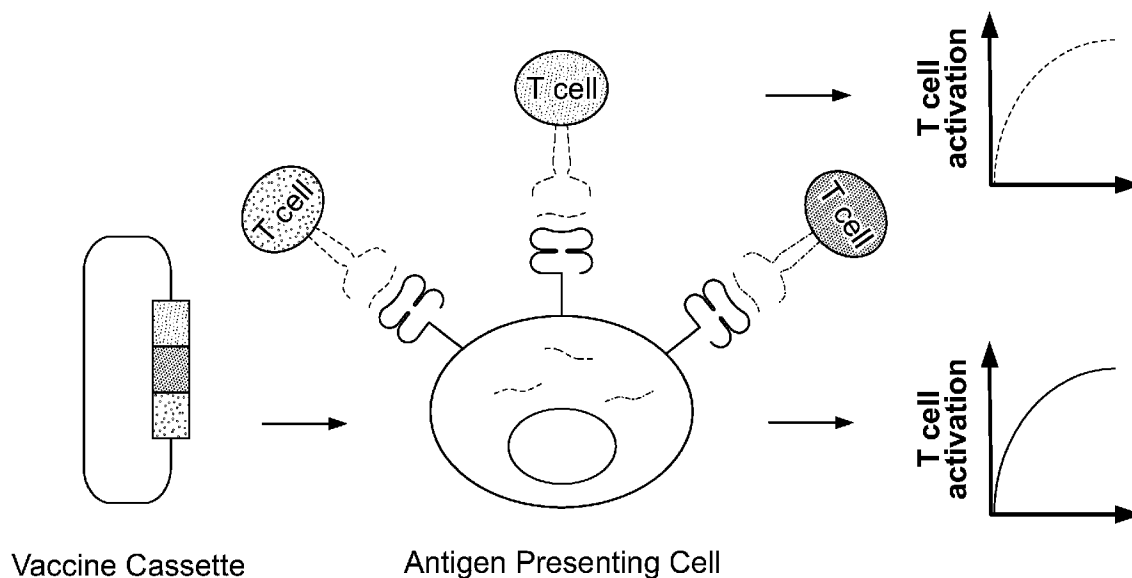


FIG. 1

(57) **Abrégé/Abstract:**

Disclosed herein are vectors that include antigen-encoding nucleic acid sequences and co-express immune modulators. Also disclosed are nucleotides, cells, and methods associated with the vectors including their use as vaccines.

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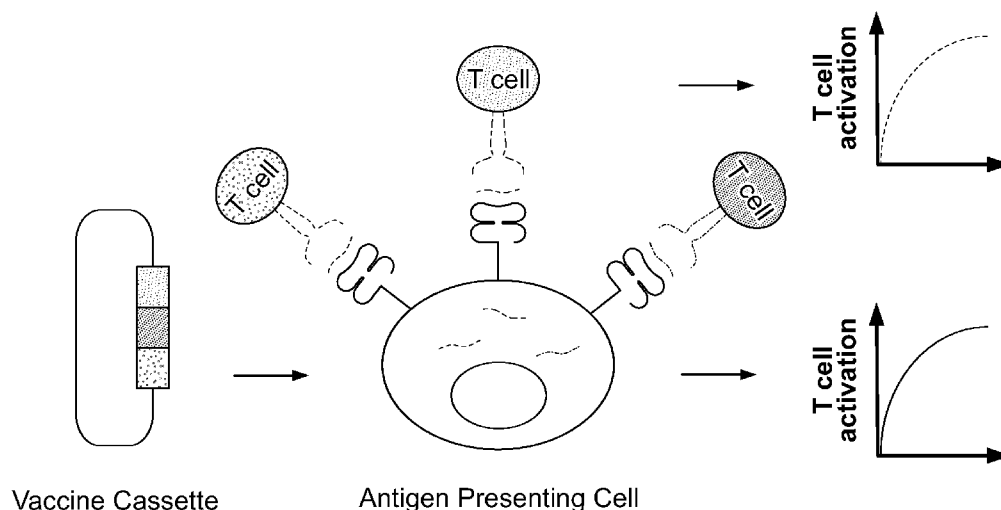


FIG. 1

(57) Abstract: Disclosed herein are vectors that include antigen-encoding nucleic acid sequences and co-express immune modulators. Also disclosed are nucleotides, cells, and methods associated with the vectors including their use as vaccines.

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IMMUNE CHECKPOINT INHIBITOR CO-EXPRESSION VECTORS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/675,624 filed May 23, 2018, which is hereby incorporated in its entirety by reference for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 22, 2019, is named GSO_018_WO_Sequence_Listing.txt and is 422,185 bytes in size.

BACKGROUND

[0003] Therapeutic vaccines based on tumor-specific antigens hold great promise as a next-generation of personalized cancer immunotherapy.¹⁻³ For example, cancers with a high mutational burden, such as non-small cell lung cancer (NSCLC) and melanoma, are particularly attractive targets of such therapy given the relatively greater likelihood of neoantigen generation.^{4,5} Early evidence shows that neoantigen-based vaccination can elicit T-cell responses⁶ and that neoantigen targeted cell-therapy can cause tumor regression under certain circumstances in selected patients.⁷

[0004] In addition to the challenges of current neoantigen prediction methods certain challenges also exist with the available vector systems that can be used for neoantigen delivery in humans, many of which are derived from humans. For example, many humans have pre-existing immunity to human viruses as a result of previous natural exposure, and this immunity can be a major obstacle to the use of recombinant human viruses for neoantigen delivery for cancer treatment.

[0005] The use of immune checkpoint inhibitors has shown great promise in the treatment of cancer. However, improved delivery methods, particularly in the case of DNA or RNA based cancer vaccines, are still needed.

SUMMARY

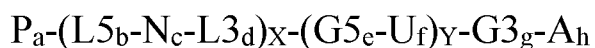
[0006] Disclosed herein is vector system comprising an antigen cassette, the antigen cassette comprising: (1) at least one antigen-encoding nucleic acid sequence associated with a

tumor present within a subject comprising: at least one antigen-encoding nucleic acid sequence, optionally the at least one antigen-encoding nucleic acid sequence comprising an MHC class I antigen-encoding nucleic acid sequence, each comprising: a. an epitope encoding nucleic acid sequence, optionally comprising at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, b. optionally a 5' linker sequence, and c. optionally a 3' linker sequence; (2) at least one promoter sequence operably linked to at least one antigen-encoding nucleic acid sequence, (3) optionally, at least one MHC class II antigen-encoding nucleic acid sequence; (4) optionally, at least one GP GPG linker sequence (SEQ ID NO:56); (5) optionally, at least one polyadenylation sequence; and the vector further comprising, optionally within the cassette, a nucleic acid sequence encoding at least one immune modulator, optionally wherein the nucleic acid sequence encoding the at least one immune modulator is transcribed on: (1) the same transcript as the at least one antigen-encoding nucleic acid sequence with an internal ribosome entry sequence (IRES) sequence separating the sequences encoding the at least one immune modulator and the at least one antigen-encoding nucleic acid sequence, or (2) a different transcript as the at least one antigen-encoding nucleic acid sequence, wherein at least one second promoter sequence is operably linked to the sequences encoding the at least one immune modulator.

[0007] Also disclosed herein is a chimpanzee adenovirus vector comprising: a. a modified ChAdV68 sequence comprising the sequence of SEQ ID NO:1 with an E1 (nt 577 to 3403) deletion and an E3 (nt 27,125-31,825) deletion; b. a CMV promoter sequence; c. an SV40 polyadenylation signal nucleotide sequence; d. a nucleic acid sequence encoding an immune checkpoint inhibitor, and e. an antigen cassette, the antigen cassette comprising: (1) at least one antigen-encoding nucleic acid sequences derived from a tumor present within a subject, the at least one antigen-encoding nucleic acid sequence comprising: at least 10 tumor-specific and subject-specific MHC class I antigen-encoding nucleic acid sequences linearly linked to each other and each comprising: (A) a MHC class I epitope encoding nucleic acid sequence with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, wherein the MHC I epitope encoding nucleic acid sequence encodes a MHC class I epitope 7-15 amino acids in length, (B) a 5' linker sequence, wherein the 5' linker sequence encodes a native N-terminal amino acid sequence of the MHC I epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length, (C) a 3' linker sequence, wherein the 3' linker sequence encodes

a native C-terminal acid sequence of the MHC I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and wherein each of the MHC class I antigen-encoding nucleic acid sequences encodes a polypeptide that is 25 amino acids in length, and wherein each 3' end of each MHC class I antigen-encoding nucleic acid sequence is linked to the 5' end of the following MHC class I antigen-encoding nucleic acid sequence with the exception of the final MHC class I antigen-encoding nucleic acid sequence; and (2) at least two MHC class II antigen-encoding nucleic acid sequences comprising: (A) a PADRE MHC class II sequence (SEQ ID NO:48), (B) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46), (C) a first nucleic acid sequence encoding a GPGPG amino acid linker sequence linking the PADRE MHC class II sequence and the Tetanus toxoid MHC class II sequence, (D) a second nucleic acid sequence encoding a GPGPG amino acid linker sequence linking the 5' end of the at least two MHC class II antigen-encoding nucleic acid sequences to the at least 10 tumor-specific and subject-specific MHC class I neoantigen-encoding nucleic acid sequences, (E) optionally, a third nucleic acid sequence encoding a GPGPG amino acid linker sequence at the 3' end of the at least two MHC class II antigen-encoding nucleic acid sequences; and wherein the antigen cassette is inserted within the E1 deletion and the CMV promoter sequence is operably linked to the antigen cassette, and wherein the nucleic acid sequence encoding the checkpoint inhibitor is transcribed: (1) on the same transcript as the at least one antigen-encoding nucleic acid sequence with an internal ribosome entry sequence (IRES) sequence separating the sequences encoding the checkpoint inhibitor and the at least one antigen-encoding nucleic acid sequence, or (2) on a different transcript as the at least one antigen-encoding nucleic acid sequences, optionally wherein a second CMV promoter sequence is operably linked to the sequences encoding the at least one immune modulator, or optionally wherein the at least one immune modulator is inserted within the E3 deletion.

[0008] In some aspects, an ordered sequence of each element of the vector is described in the formula, from 5' to 3', comprising:



wherein P comprises the at least one promoter sequence operably linked to at least one of the at least one antigen-encoding nucleic acid sequences, where a = 1, N comprises one of the epitope encoding nucleic acid sequence with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by the wild-type nucleic acid sequence, where c = 1, L5 comprises the 5' linker sequence, where b = 0 or 1, L3

comprises the 3' linker sequence, where $d = 0$ or 1 , G5 comprises one of the at least one nucleic acid sequences encoding a GP GPG amino acid linker, where $e = 0$ or 1 , G3 comprises one of the at least one nucleic acid sequences encoding a GP GPG amino acid linker, where $g = 0$ or 1 , U comprises one of the at least one MHC class II antigen-encoding nucleic acid sequence, where $f = 1$, A comprises the at least one polyadenylation sequence, where $h = 0$ or 1 , $X = 2$ to 400 , where for each X the corresponding N_c is an epitope encoding nucleic acid sequence, optionally wherein for each X the corresponding N_c is a distinct MHC class I epitope encoding nucleic acid sequence, and $Y = 0-2$, where for each Y the corresponding U_f is an antigen-encoding nucleic acid sequence, optionally wherein for each Y the corresponding U_f is a distinct MHC class II antigen-encoding nucleic acid sequence. In a particular aspect, $b = 1$, $d = 1$, $e = 1$, $g = 1$, $h = 1$, $X = 10$, $Y = 2$, P is a CMV promoter sequence, each N encodes a MHC class I epitope 7-15 amino acids in length, L5 encodes a native N-terminal amino acid sequence of the MHC I epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length, L3 encodes a native C-terminal amino acid sequence of the MHC I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, U is each of a PADRE class II sequence and a Tetanus toxoid MHC class II sequence, the vector comprises a modified ChAdV68 sequence comprising the sequence of SEQ ID NO:1 with an E1 (nt 577 to 3403) deletion and an E3 (nt 27,125- 31,825) deletion and the neoantigen cassette is inserted within the E1 deletion, and each of the MHC class I antigen-encoding nucleic acid sequences encodes a polypeptide that is 25 amino acids in length.

[0009] In some aspects, at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I on the tumor cell surface. In some aspects, at least 1, 2, or optionally 3 of the antigen-encoding nucleic acid sequences encode polypeptide sequences or portions thereof is presented by MHC class I on the tumor cell surface.

[0010] In some aspects, each antigen-encoding nucleic acid sequence is linked directly to one another. In some aspects, at least one of the at least one antigen-encoding nucleic acid sequences is linked to a distinct antigen-encoding nucleic acid sequence with a nucleic acid sequence encoding a linker.. In some aspects, the linker links two MHC class I sequences or an MHC class I sequence to an MHC class II sequence. In some aspects, the linker is selected from the group consisting of: (1) consecutive glycine residues, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues in length; (2) consecutive alanine residues, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues in length; (3) two arginine residues (RR); (4) alanine, alanine, tyrosine (AAY); (5) a

consensus sequence at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues in length that is processed efficiently by a mammalian proteasome; and (6) one or more native sequences flanking the antigen derived from the cognate protein of origin and that is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 2-20 amino acid residues in length. In some aspects, the linker links two MHC class II sequences or an MHC class II sequence to an MHC class I sequence. In some aspects, the linker comprises the sequence GP GPG.

[0011] In some aspects, at least one of the at least one antigen-encoding nucleic acid sequences is linked, operably or directly, to a separate or contiguous sequence that enhances the expression, stability, cell trafficking, processing and presentation, and/or immunogenicity of the at least one antigen-encoding nucleic acid sequences. In some aspects, the separate or contiguous sequence comprises at least one of: a ubiquitin sequence, a ubiquitin sequence modified to increase proteasome targeting (e.g., the ubiquitin sequence contains a Gly to Ala substitution at position 76), an immunoglobulin signal sequence (e.g., IgK), a major histocompatibility class I sequence, lysosomal-associated membrane protein (LAMP)-1, human dendritic cell lysosomal-associated membrane protein, and a major histocompatibility class II sequence; optionally wherein the ubiquitin sequence modified to increase proteasome targeting is A76.

[0012] In some aspects, at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has increased binding affinity to its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence. In some aspects, at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has increased binding stability to its corresponding MHC allele relative to the translated, corresponding wild-type, parental nucleic acid sequence. In some aspects, at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has an increased likelihood of presentation on its corresponding MHC allele relative to the translated, corresponding wild-type, parental nucleic acid sequence.

[0013] In some aspects, at least one alteration comprises a point mutation, a frameshift mutation, a non-frameshift mutation, a deletion mutation, an insertion mutation, a splice variant, a genomic rearrangement, or a proteasome-generated spliced antigen.

[0014] In some aspects, the tumor is selected from the group consisting of: lung cancer, melanoma, breast cancer, ovarian cancer, prostate cancer, kidney cancer, gastric cancer, colon cancer, testicular cancer, head and neck cancer, pancreatic cancer, brain cancer, B-cell

lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer.

[0015] In some aspects, expression of each of the at least one antigen-encoding nucleic acid sequences is driven by the at least one promoter.

[0016] In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 nucleic acid sequences and wherein at least one of the antigen-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC I on the tumor cell surface. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 nucleic acid sequences and wherein, when administered to the subject and translated, at least one of the antigens are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on the tumor cell surface. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 MHC class I and/or class II antigen-encoding nucleic acid sequences, wherein, when administered to the subject and translated, at least one of the MHC class I or class II antigens are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on the tumor cell surface, and optionally wherein the expression of each of the at least 2-400 MHC class I or class II antigen-encoding nucleic acid sequences is driven by the at least one promoter.

[0017] In some aspects, each MHC class I antigen-encoding nucleic acid sequence encodes a polypeptide sequence between 8 and 35 amino acids in length, optionally 9-17, 9-25, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 amino acids in length.

[0018] In some aspects, at least one MHC class II antigen-encoding nucleic acid sequence is present. In some aspects, at least one MHC class II antigen-encoding nucleic acid sequence is present and comprises at least one MHC class II neoantigen-encoding nucleic acid sequence that comprises at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence. In some aspects, the at least one MHC class II antigen-encoding nucleic acid sequence is 12-20, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 20-40 amino acids in length. In some aspects, the at least one MHC class II antigen-encoding nucleic acid sequence is present and comprises at least one

universal MHC class II antigen-encoding nucleic acid sequence, optionally wherein the at least one universal sequence comprises at least one of Tetanus toxoid and PADRE.

[0019] In some aspects, the at least one promoter sequence is inducible. In some aspects, the at least one promoter sequence is non-inducible. In some aspects, the at least one promoter sequence is a CMV, SV40, EF-1, RSV, PGK, HSA, MCK, or EBV promoter sequence.

[0020] In some aspects, the antigen cassette further comprises at least one poly-adenylation (polyA) sequence operably linked to at least one of the at least one antigen-encoding nucleic acid sequences, optionally wherein the polyA sequence is located 3' of the at least one antigen-encoding nucleic acid sequence. In some aspects, the polyA sequence comprises an SV40 or Bovine Growth Hormone (BGH) polyA sequence. In some aspects, the antigen cassette further comprises at least one of: an intron sequence, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence, an internal ribosome entry sequence (IRES) sequence, or a sequence in the 5' or 3' non-coding region known to enhance the nuclear export, stability, or translation efficiency of mRNA that is operably linked to at least one of the at least one antigen-encoding nucleic acid sequences. In some aspects, the antigen cassette further comprises a reporter gene, including but not limited to, green fluorescent protein (GFP), a GFP variant, secreted alkaline phosphatase, luciferase, or a luciferase variant.

[0021] In some aspects, the at least one immune modulator inhibits an immune checkpoint molecule.

[0022] In some aspects, the immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof. In some aspects, the antibody or antigen-binding fragment thereof is a Fab fragment, a Fab' fragment, a single chain Fv (scFv), a single domain antibody (sdAb) either as single specific or multiple specificities linked together (e.g., camelid antibody domains), or full-length single-chain antibody (e.g., full-length IgG with heavy and light chains linked by a flexible linker). In some aspects, the heavy and light chain sequences of the antibody are a contiguous sequence separated by either a self-cleaving sequence such as 2A, optionally wherein the self-cleaving sequence has a Furin cleavage site sequence 5' of the self-cleaving sequence, or an IRES sequence; or the heavy and light chain sequences of the antibody are linked by a flexible linker such as consecutive glycine residues. In some aspects, the anti-CTLA4 antibody comprises VL CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs:76-78,

respectively, and VH CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs:79-81, respectively. In some aspects, the anti-CTLA4 antibody comprises VL CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs:21-23, respectively, and VH CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs:18-20, respectively.

[0023] In some aspects, the immune modulator is a cytokine. In some aspects, the cytokine is at least one of IL-2, IL-7, IL-12, IL-15, or IL-21 or variants thereof of each.

[0024] In some aspects, the vector is a chimpanzee adenovirus vector. In some aspects, the vector is the chimpanzee adenovirus vector is a ChAdV68 vector. In some aspects, the vector comprises the sequence set forth in SEQ ID NO:1. In some aspects, vector comprises the sequence set forth in SEQ ID NO:1, except that the sequence is fully deleted or functionally deleted in at least one gene selected from the group consisting of the chimpanzee adenovirus E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1, optionally wherein the sequence is fully deleted or functionally deleted in: (1) E1A and E1B; (2) E1A, E1B, and E3; or (3) E1A, E1B, E3, and E4 of the sequence set forth in SEQ ID NO: 1.

[0025] In some aspects, the vector comprises a gene or regulatory sequence obtained from the sequence of SEQ ID NO: 1, optionally wherein the gene is selected from the group consisting of the chimpanzee adenovirus inverted terminal repeat (ITR), E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1.

[0026] In some aspects, the antigen cassette is inserted in the vector at the E1 region, E3 region, and/or any deleted AdV region that allows incorporation of the antigen cassette.

[0027] In some aspects, the vector is generated from one of a first generation, a second generation, or a helper-dependent adenoviral vector.

[0028] In some aspects, the adenovirus vector the vector comprises one or more deletions between base pair number 577 and 3403 or between base pair 456 and 3014, and optionally wherein the vector further comprises one or more deletions between base pair 27,125 and 31,825 or between base pair 27,816 and 31,333 of the sequence set forth in SEQ ID NO:1. In some aspects, the adenovirus vector further comprises one or more deletions between base pair number 3957 and 10346, base pair number 21787 and 23370, and base pair number 33486 and 36193 of the sequence set forth in SEQ ID NO:1.

[0029] In some aspects, the vector comprises +-stranded RNA vector. In some aspects, the +-stranded RNA vector comprises a 5' 7-methylguanosine (m7g) cap. In some aspects, the +-

stranded RNA vectors are produced by *in vitro* transcription. In some aspects, the vectors are self-replicating within a mammalian cell.

[0030] In some aspects, the vector comprises a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) at least one polyadenylation (poly(A)) sequence. In some aspects, the backbone comprises at least one nucleotide sequence of an Aura virus, a Fort Morgan virus, a Venezuelan equine encephalitis virus, a Ross River virus, a Semliki Forest virus, a Sindbis virus, or a Mayaro virus. In some aspects, the backbone comprises at least one nucleotide sequence of a Venezuelan equine encephalitis virus. In some aspects, the backbone comprises at least sequences for nonstructural protein-mediated amplification, a 26S promoter sequence, a poly(A) sequence, a nonstructural protein 1 (nsP1) gene, a nsP2 gene, a nsP3 gene, and a nsP4 gene encoded by the nucleotide sequence of the Aura virus, the Fort Morgan virus, the Venezuelan equine encephalitis virus, the Ross River virus, the Semliki Forest virus, the Sindbis virus, or the Mayaro virus. In some aspects, the backbone comprises at least sequences for nonstructural protein-mediated amplification, a 26S promoter sequence, and a poly(A) sequence encoded by the nucleotide sequence of the Aura virus, the Fort Morgan virus, the Venezuelan equine encephalitis virus, the Ross River virus, the Semliki Forest virus, the Sindbis virus, or the Mayaro virus. In some aspects, sequences for nonstructural protein-mediated amplification are selected from the group consisting of: an alphavirus 5' UTR, a 51-nt CSE, a 24-nt CSE, a 26S subgenomic promoter sequence, a 19-nt CSE, an alphavirus 3' UTR, or combinations thereof.

[0031] In some aspects, the backbone does not encode structural virion proteins capsid, E2 and E1. In some aspects, the neoantigen cassette is inserted in place of the structural virion proteins within the nucleotide sequence of the Aura virus, the Fort Morgan virus, the Venezuelan equine encephalitis virus, the Ross River virus, the Semliki Forest virus, the Sindbis virus, or the Mayaro virus.

[0032] In some aspects, the Venezuelan equine encephalitis virus (VEE) comprises the strain TC-83. In some aspects, the Venezuelan equine encephalitis virus comprises the sequence set forth in SEQ ID NO:3 or SEQ ID NO:5. In some aspects, the Venezuelan equine encephalitis virus comprises the sequence of SEQ ID NO:3 or SEQ ID NO:5 further comprising a deletion between base pair 7544 and 11175. In some aspects, the backbone is the sequence set forth in SEQ ID NO:6 or SEQ ID NO:7. In some aspects, the neoantigen cassette is inserted to replace the deletion between base pair 7544 and 11175 set forth in the sequence of SEQ ID NO:3 or SEQ ID NO:5.

[0033] In some aspects, the insertion of the neoantigen cassette provides for transcription of a polycistronic RNA comprising the nsP1-4 genes and the at least one of antigen-encoding nucleic acid sequences, wherein the nsP1-4 genes and the at least one of antigen-encoding nucleic acid sequences are in separate open reading frames.

[0034] In some aspects, the at least one promoter nucleotide sequence is the native 26S promoter nucleotide sequence encoded by the backbone. In some aspects, the at least one promoter nucleotide sequence is an exogenous RNA promoter. In some aspects, the second promoter nucleotide sequence is a 26S promoter nucleotide sequence. In some aspects, the second promoter nucleotide sequence comprises multiple 26S promoter nucleotide sequences, wherein each 26S promoter nucleotide sequence provides for transcription of one or more of the separate open reading frames.

[0035] In some aspects, the vector is an srRNA vector. In some aspects, the srRNA vector is a Venezuelan equine encephalitis virus srRNA vector.

[0036] In some aspects, the at least one antigen-encoding nucleic acid sequences are selected by performing the steps of: obtaining at least one of exome, transcriptome, or whole genome tumor nucleotide sequencing data from the tumor, wherein the tumor nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens; inputting the peptide sequence of each antigen into a presentation model to generate a set of numerical likelihoods that each of the antigens is presented by one or more of the MHC alleles on the tumor cell surface of the tumor, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens which are used to generate the at least one antigen-encoding nucleic acid sequences.

[0037] In some aspects, each of the epitope encoding nucleic acid sequences are selected by performing the steps of: obtaining at least one of exome, transcriptome, or whole genome tumor nucleotide sequencing data from the tumor, wherein the tumor nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens; inputting the peptide sequence of each antigen into a presentation model to generate a set of numerical likelihoods that each of the antigens is presented by one or more of the MHC alleles on the tumor cell surface of the tumor, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens which are used to generate the at least one antigen-encoding nucleic acid sequences.

[0038] In some aspects, a number of the set of selected antigens is 2-20.

[0039] In some aspects, the presentation model represents dependence between: presence of a pair of a particular one of the MHC alleles and a particular amino acid at a particular position of a peptide sequence; and likelihood of presentation on the tumor cell surface, by the particular one of the MHC alleles of the pair, of such a peptide sequence comprising the particular amino acid at the particular position.

[0040] In some aspects, selecting the set of selected antigen comprises selecting antigens that have an increased likelihood of being presented on the tumor cell surface relative to unselected antigens based on the presentation model. In some aspects, selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of inducing a tumor-specific immune response in the subject relative to unselected antigens based on the presentation model. In some aspects, selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of being presented to naïve T cells by professional antigen presenting cells (APCs) relative to unselected antigens based on the presentation model, optionally wherein the APC is a dendritic cell (DC). In some aspects, selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being subject to inhibition via central or peripheral tolerance relative to unselected antigens based on the presentation model. In some aspects, selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being capable of inducing an autoimmune response to normal tissue in the subject relative to unselected antigens based on the presentation model. In some aspects, exome or transcriptome nucleotide sequencing data is obtained by performing sequencing on the tumor tissue. In some aspects, the sequencing is next generation sequencing (NGS) or any massively parallel sequencing approach.

[0041] In some aspects, the antigen cassette comprises junctional epitope sequences formed by adjacent sequences in the antigen cassette. In some aspects, the at least one or each junctional epitope sequence has an affinity of greater than 500 nM for MHC. In some aspects, each junctional epitope sequence is non-self. In some aspects, the antigen cassette does not encode a non-therapeutic MHC class I or class II epitope nucleic acid sequence comprising a translated, wild-type nucleic acid sequence, wherein the non-therapeutic epitope is predicted to be displayed on an MHC allele of the subject. In some aspects, the non-therapeutic predicted MHC class I or class II epitope sequence is a junctional epitope sequence formed by adjacent sequences in the antigen cassette. In some aspects, the prediction is based on presentation

likelihoods generated by inputting sequences of the non-therapeutic epitopes into a presentation model. In some aspects, an order of the at least one antigen-encoding nucleic acid sequences in the antigen cassette is determined by a series of steps comprising: 1. generating a set of candidate antigen cassette sequences corresponding to different orders of the at least one antigen-encoding nucleic acid sequences; 2. determining, for each candidate antigen cassette sequence, a presentation score based on presentation of non-therapeutic epitopes in the candidate antigen cassette sequence; and 3. selecting a candidate cassette sequence associated with a presentation score below a predetermined threshold as the antigen cassette sequence for a antigen vaccine.

[0042] Also disclosed herein is a pharmaceutical composition comprising a vector disclosed herein (such as a ChAd-based vector disclosed herein) and a pharmaceutically acceptable carrier. In some aspects, the composition further comprises an adjuvant. In some aspects, the composition further comprises an immune modulator. In some aspects, immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof.

[0043] Also disclosed herein is an isolated nucleotide sequence comprising an antigen cassette disclosed herein and at least one promoter disclosed herein. In some aspects, the isolated nucleotide sequence further comprises a ChAd-based gene. In some aspects, the ChAd-based gene is obtained from the sequence of SEQ ID NO: 1, optionally wherein the gene is selected from the group consisting of the chimpanzee adenovirus ITR, E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1, and optionally wherein the nucleotide sequence is cDNA.

[0044] Also disclosed herein is an isolated cell comprising an isolated nucleotide sequence disclosed herein, optionally wherein the cell is a CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.

[0045] Also disclosed herein is a vector comprising an isolated nucleotide sequence disclosed herein.

[0046] Also disclosed herein is a kit comprising a vector disclosed herein and instructions for use.

[0047] Also disclosed herein is a method for treating a subject with cancer, the method comprising administering to the subject a vector disclosed herein or a pharmaceutical

composition disclosed herein. In some aspects, the vector or composition is administered intramuscularly (IM), intradermally (ID), or subcutaneously (SC). In some aspects, the method further comprises administering to the subject an immune modulator, optionally wherein the immune modulator is administered before, concurrently with, or after administration of the vector or pharmaceutical composition. In some aspects, the immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof. In some aspects, the immune modulator is administered intravenously (IV), intramuscularly (IM), intradermally (ID), or subcutaneously (SC). In some aspects, wherein the subcutaneous administration is near the site of the vector or composition administration or in close proximity to one or more vector or composition draining lymph nodes.

[0048] In some aspects, the method further comprises administering to the subject a second vaccine composition. In some aspects, the second vaccine composition is administered prior to the administration of the vector or the pharmaceutical composition of any of the above vectors or compositions. In some aspects, the second vaccine composition is administered subsequent to the administration of the vector or the pharmaceutical composition of any of the above vectors or compositions. In some aspects, the second vaccine composition is the same as the vector or the pharmaceutical composition of any of the above vectors or compositions. In some aspects, the second vaccine composition is different from the vector or the pharmaceutical composition of any of the above vectors or compositions. In some aspects, the second vaccine composition comprises a chimpanzee adenovirus vector. In some aspects, the chimpanzee adenovirus vector is a ChAdV68 vector. In some aspects, the second vaccine composition comprises an srRNA vector. In some aspects, the srRNA vector is a Venezuelan equine encephalitis virus vector. In some aspects, the chimpanzee adenovirus vector or the srRNA vector comprises a nucleic acid sequence encoding at least one immune modulator. In some aspects, the at least one antigen-encoding nucleic acid sequence encoded by the chimpanzee adenovirus vector or the srRNA vector is the same as the at least one antigen-encoding nucleic acid sequences of any of the above vectors. In some aspects, the nucleic acid sequence encoding the at least one immune modulator encoded by the chimpanzee adenovirus vector or the srRNA vector is the same as the the at least one immune modulator of any of the above vectors.

[0049] In some aspects, any of the above compositions further comprise a nanoparticulate delivery vehicle. The nanoparticulate delivery vehicle, in some aspects, may be a lipid nanoparticle (LNP). In some aspects, the LNP comprises ionizable amino lipids. In some aspects, the ionizable amino lipids comprise MC3-like (dilinoleylmethyl- 4-dimethylaminobutyrate) molecules. In some aspects, the nanoparticulate delivery vehicle encapsulates the neoantigen expression system.

[0050] In some aspects, any of the above compositions further comprise a plurality of LNPs, wherein the LNPs comprise: the neoantigen expression system; a cationic lipid; a non-cationic lipid; and a conjugated lipid that inhibits aggregation of the LNPs, wherein at least about 95% of the LNPs in the plurality of LNPs either: have a non-lamellar morphology; or are electron-dense.

[0051] In some aspects, the non-cationic lipid is a mixture of (1) a phospholipid and (2) cholesterol or a cholesterol derivative.

[0052] In some aspects, the conjugated lipid that inhibits aggregation of the LNPs is a polyethyleneglycol (PEG)-lipid conjugate. In some aspects, the PEG-lipid conjugate is selected from the group consisting of: a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG dialkylloxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, and a mixture thereof. In some aspects the PEG-DAA conjugate is a member selected from the group consisting of: a PEG-didecyloxypropyl (C₁₀) conjugate, a PEG-dilauryloxypropyl (C₁₂) conjugate, a PEG-dimyristyloxypropyl (C₁₄) conjugate, a PEG-dipalmitoyloxypropyl (C₁₆) conjugate, a PEG-distearoyloxypropyl (C₁₈) conjugate, and a mixture thereof.

[0053] In some aspects, the neoantigen expression system is fully encapsulated in the LNPs.

[0054] In some aspects, the non-lamellar morphology of the LNPs comprises an inverse hexagonal (H_{II}) or cubic phase structure.

[0055] In some aspects, the cationic lipid comprises from about 10 mol % to about 50 mol % of the total lipid present in the LNPs. In some aspects, the cationic lipid comprises from about 20 mol % to about 50 mol % of the total lipid present in the LNPs. In some aspects, the cationic lipid comprises from about 20 mol % to about 40 mol % of the total lipid present in the LNPs.

[0056] In some aspects, the non-cationic lipid comprises from about 10 mol % to about 60 mol % of the total lipid present in the LNPs. In some aspects, the non-cationic lipid comprises

from about 20 mol % to about 55 mol % of the total lipid present in the LNPs. In some aspects, the non-cationic lipid comprises from about 25 mol % to about 50 mol % of the total lipid present in the LNPs.

[0057] In some aspects, the conjugated lipid comprises from about 0.5 mol % to about 20 mol % of the total lipid present in the LNPs. In some aspects, the conjugated lipid comprises from about 2 mol % to about 20 mol % of the total lipid present in the LNPs. In some aspects, the conjugated lipid comprises from about 1.5 mol % to about 18 mol % of the total lipid present in the LNPs.

[0058] In some aspects, greater than 95% of the LNPs have a non-lamellar morphology. In some aspects, greater than 95% of the LNPs are electron dense.

[0059] In some aspects, any of the above compositions further comprise a plurality of LNPs, wherein the LNPs comprise: a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the LNPs; a conjugated lipid that inhibits aggregation of LNPs comprising from 0.5 mol % to 2 mol % of the total lipid present in the LNPs; and a non-cationic lipid comprising either: a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from 4 mol % to 10 mol % of the total lipid present in the LNPs and the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the LNPs; a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the LNPs and the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the LNPs; or up to 49.5 mol % of the total lipid present in the LNPs and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the LNPs.

[0060] In some aspects, any of the above compositions further comprise a plurality of LNPs, wherein the LNPs comprise: a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the LNPs; a conjugated lipid that inhibits aggregation of LNPs comprising from 0.5 mol % to 2 mol % of the total lipid present in the LNPs; and a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the LNPs.

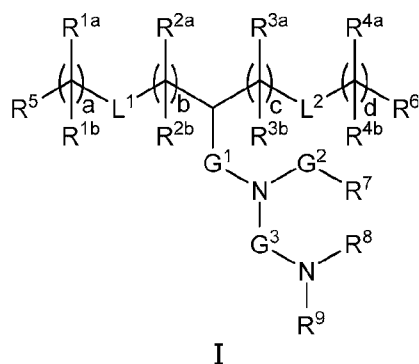
[0061] In some aspects, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

[0062] In some aspects, the conjugated lipid comprises a polyethyleneglycol (PEG)-lipid conjugate. In some aspects, the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-

DAG) conjugate, a PEG-dialkylloxypropyl (PEG-DAA) conjugate, or a mixture thereof. In some aspects, the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearylloxypropyl (PEG-DSA) conjugate, or a mixture thereof. In some aspects, the PEG portion of the conjugate has an average molecular weight of about 2,000 daltons.

[0063] In some aspects, the conjugated lipid comprises from 1 mol % to 2 mol % of the total lipid present in the LNPs.

[0064] In some aspects, the LNP comprises a compound having a structure of Formula I:



or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

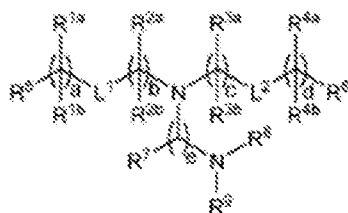
L^1 and L^2 are each independently $-O(C=O)-$, $-(C=O)O-$, $-C(=O)-$, $-O-$, $-S(O)_x-$, $-S-S-$, $-C(=O)S-$, $-SC(=O)-$, $-R^aC(=O)-$, $-C(=O)R^a-$, $-R^aC(=O)R^a-$, $-OC(=O)R^a-$, $-R^aC(=O)O-$ or a direct bond;

G^1 is $Ci-C_2$ alkylene, $-(C=O)-$, $-O(C=O)-$, $-SC(=O)-$, $-R^aC(=O)-$ or a direct bond: $-C(=O)-$, $-(C=O)O-$, $-C(=O)S-$, $-C(=O)R^a-$ or a direct bond; G is $Ci-C_6$ alkylene; R^a is H or C_1-C_{12} alkyl;

R^{1a} and R^{1b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{2a} and R^{2b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{3a} and R^{3b} are, at each occurrence, independently either (a): H or C_1-C_{12} alkyl; or (b) R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{4a} and R^{4b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a

carbon-carbon double bond; R^5 and R^6 are each independently H or methyl; R^7 is C4-C20 alkyl; R^8 and R^9 are each independently C1-C12 alkyl; or R^8 and R^9 , together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring; a, b, c and d are each independently an integer from 1 to 24; and x is 0, 1 or 2.

[0065] In some aspects, the LNP comprises a compound having a structure of Formula II:



II

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

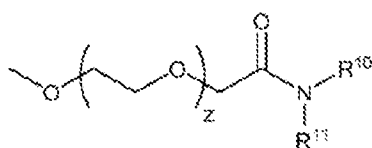
L^1 and L^2 are each independently $-O(C=O)-$, $-(C=O)O-$ or a carbon-carbon double bond; R^{1a} and R^{1b} are, at each occurrence, independently either (a) H or C1-C12 alkyl, or (b) R^{1a} is H or C1-C12 alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{2a} and R^{2b} are, at each occurrence, independently either (a) H or C1-C12 alkyl, or (b) R^{2a} is H or C1-C12 alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{3a} and R^{3b} are, at each occurrence, independently either (a) H or C1-C12 alkyl, or (b) R^{3a} is H or C1-C12 alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{4a} and R^{4b} are, at each occurrence, independently either (a) H or C1-C12 alkyl, or (b) R^{4a} is H or C1-C12 alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^5 and R^6 are each independently methyl or cycloalkyl; R^7 is, at each occurrence, independently H or C1-C12 alkyl; R^8 and R^9 are each independently unsubstituted C1-C12 alkyl; or R^8 and R^9 , together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring comprising one nitrogen atom; a and d are each independently an integer from 0 to 24; b and c are each independently an integer from 1 to 24; and e is 1 or 2, provided that: at least one of R^{1a} , R^{2a} , R^{3a} or R^{4a} is C1-C12 alkyl, or at least one of L^1 or L^2 is $-O(C=O)-$ or $-(C=O)O-$; and R^{1a} and R^{1b} are not isopropyl when a is 6 or n-butyl when a is 8.

[0066] In some aspects, any of the above compositions further comprise one or more excipients comprising a neutral lipid, a steroid, and a polymer conjugated lipid. In some aspects, the neutral lipid comprises at least one of 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). In some aspects, the neutral lipid is DSPC.

[0067] In some aspects, the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1.

[0068] In some aspects, the steroid is cholesterol. In some aspects, the molar ratio of the compound to cholesterol ranges from about 2:1 to 1:1.

[0069] In some aspects, the polymer conjugated lipid is a pegylated lipid. In some aspects, the molar ratio of the compound to the pegylated lipid ranges from about 100:1 to about 25:1. In some aspects, the pegylated lipid is PEG-DAG, a PEG polyethylene (PEG-PE), a PEG-succinoyl-diacylglycerol (PEG-S-DAG), PEG-cer or a PEG dialkoxypolypropylcarbamate. In some aspects, the pegylated lipid has the following structure III:



III

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein: R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and z has a mean value ranging from 30 to 60. In some aspects, R^{10} and R^{11} are each independently straight, saturated alkyl chains having 12 to 16 carbon atoms. In some aspects, the average z is about 45.

start here

[0070] In some aspects, the LNP self-assembles into non-bilayer structures when mixed with polyanionic nucleic acid. In some aspects, the non-bilayer structures have a diameter between 60nm and 120nm. In some aspects, the non-bilayer structures have a diameter of about 70nm, about 80nm, about 90nm, or about 100nm. In some aspects, wherein the nanoparticulate delivery vehicle has a diameter of about 100nm.

[0071] Also disclosed herein is a method of manufacturing a vector disclosed herein, the method comprising: obtaining a plasmid sequence comprising the at least one promoter sequence and the antigen cassette; transfecting the plasmid sequence into one or more host cells; and isolating the vector from the one or more host cells.

[0072] In some aspects, isolating comprises: lysing the host cell to obtain a cell lysate comprising the vector; and purifying the vector from the cell lysate and optionally also from media used to culture the host cell.

[0073] In some aspects, the plasmid sequence is generated using one of the following: DNA recombination or bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells. In some aspects, the one or more host cells are at least one of CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, and AE1-2a cells. In some aspects, purifying the vector from the cell lysate involves one or more of chromatographic separation, centrifugation, virus precipitation, and filtration.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0074] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

[0075] **Figure (FIG.) 1** illustrates development of an *in vitro* T cell activation assay. Schematic of the assay in which the delivery of a vaccine cassette to antigen presenting cells, leads to expression, processing and MHC-restricted presentation of distinct peptide antigens. Reporter T cells engineered with T cell receptors that match the specific peptide-MHC combination become activated resulting in luciferase expression.

[0076] **FIG. 2A** illustrates evaluation of linker sequences in short cassettes and shows five class I MHC restricted epitopes (epitopes 1 through 5) concatenated in the same position relative to each other followed by two universal class II MHC epitopes (MHC-II). Various iterations were generated using different linkers. In some cases the T cell epitopes are directly linked to each other. In others, the T cell epitopes are flanked on one or both sides by its natural sequence. In other iterations, the T cell epitopes are linked by the non-natural sequences AAY, RR, and DPP.

[0077] **FIG. 2B** illustrates evaluation of linker sequences in short cassettes and shows sequence information on the T cell epitopes embedded in the short cassettes.

[0078] **FIG. 3** illustrates evaluation of cellular targeting sequences added to model vaccine cassettes. The targeting cassettes extend the short cassette designs with ubiquitin (Ub), signal peptides (SP) and/or transmembrane (TM) domains, feature next to the five marker human T cell epitopes (epitopes 1 through 5) also two mouse T cell epitopes SIINFELK (SII) and SPSYAYHQF (A5), and use either the non natural linker AAY- or natural linkers flanking the T cell epitopes on both sides (25mer).

[0079] **FIG. 4** illustrates *in vivo* evaluation of linker sequences in short cassettes. A) Experimental design of the *in vivo* evaluation of vaccine cassettes using HLA-A2 transgenic mice.

[0080] **FIG. 5A** illustrates *in vivo* evaluation of the impact of epitope position in long 21-mer cassettes and shows the design of long cassettes entails five marker class I epitopes (epitopes 1 through 5) contained in their 25-mer natural sequence (linker = natural flanking sequences), spaced with additional well-known T cell class I epitopes (epitopes 6 through 21) contained in their 25-mer natural sequence, and two universal class II epitopes (MHC-II0, with only the relative position of the class I epitopes varied.

[0081] **FIG. 5B** illustrates *in vivo* evaluation of the impact of epitope position in long 21-mer cassettes and shows the sequence information on the T cell epitopes used.

[0082] **FIG. 6A** illustrates final cassette design for preclinical IND-enabling studies and shows the design of the final cassettes comprises 20 MHC I epitopes contained in their 25-mer natural sequence (linker = natural flanking sequences), composed of 6 non-human primate (NHP) epitopes, 5 human epitopes, 9 murine epitopes, as well as 2 universal MHC class II epitopes.

[0083] **FIG. 6B** illustrates final cassette design for preclinical IND-enabling studies and shows the sequence information for the T cell epitopes used that are presented on class I MHC of non-human primate, mouse and human origin, as well as sequences of 2 universal MHC class II epitopes PADRE and Tetanus toxoid.

[0084] **FIG. 7A** illustrates ChAdV68.4WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.4WTnt.GFP DNA using the calcium phosphate protocol. Viral replication was observed 10 days after transfection and ChAdV68.4WTnt.GFP viral plaques were visualized using light microscopy (40x magnification).

[0085] **FIG. 7B** illustrates ChAdV68.4WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.4WTnt.GFP DNA using the calcium

phosphate protocol. Viral replication was observed 10 days after transfection and ChAdV68.4WTnt.GFP viral plaques were visualized using fluorescent microscopy at 40x magnification.

[0086] **FIG. 7C** illustrates ChAdV68.4WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.4WTnt.GFP DNA using the calcium phosphate protocol. Viral replication was observed 10 days after transfection and ChAdV68.4WTnt.GFP viral plaques were visualized using fluorescent microscopy at 100x magnification.

[0087] **FIG. 8A** illustrates ChAdV68.5WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.5WTnt.GFP DNA using the lipofectamine protocol. Viral replication (plaques) was observed 10 days after transfection. A lysate was made and used to reinfect a T25 flask of 293A cells. ChAdV68.5WTnt.GFP viral plaques were visualized and photographed 3 days later using light microscopy (40x magnification)

[0088] **FIG. 8B** illustrates ChAdV68.5WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.5WTnt.GFP DNA using the lipofectamine protocol. Viral replication (plaques) was observed 10 days after transfection. A lysate was made and used to reinfect a T25 flask of 293A cells. ChAdV68.5WTnt.GFP viral plaques were visualized and photographed 3 days later using fluorescent microscopy at 40x magnification.

[0089] **FIG. 8C** illustrates ChAdV68.5WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.5WTnt.GFP DNA using the lipofectamine protocol. Viral replication (plaques) was observed 10 days after transfection. A lysate was made and used to reinfect a T25 flask of 293A cells. ChAdV68.5WTnt.GFP viral plaques were visualized and photographed 3 days later using fluorescent microscopy at 100x magnification.

[0090] **FIG. 9** illustrates the viral particle production scheme.

[0091] **FIG. 10** illustrates the alphavirus derived VEE self-replicating RNA (srRNA) vector.

[0092] **FIG. 11** illustrates *in vivo* reporter expression after inoculation of C57BL/6J mice with VEE-Luciferase srRNA. Shown are representative images of luciferase signal following immunization of C57BL/6J mice with VEE-Luciferase srRNA (10 ug per mouse, bilateral intramuscular injection, MC3 encapsulated) at various timepoints.

[0093] **FIG. 12A** illustrates T-cell responses measured 14 days after immunization with VEE srRNA formulated with MC3 LNP in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with 10 ug of VEE-Luciferase srRNA (control), VEE-

UbAAY srRNA (Vax), VEE-Luciferase srRNA and anti-CTLA-4 (aCTLA-4) or VEE-UbAAY srRNA and anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD1 mAb starting at day 7. Each group consisted of 8 mice. Mice were sacrificed and spleens and lymph nodes were collected 14 days after immunization. SIINFEKL-specific T-cell responses were assessed by IFN-gamma ELISPOT and are reported as spot-forming cells (SFC) per 10⁶ splenocytes. Lines represent medians.

[0094] FIG. 12B illustrates T-cell responses measured 14 days after immunization with VEE srRNA formulated with MC3 LNP in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with 10 ug of VEE-Luciferase srRNA (control), VEE-UbAAY srRNA (Vax), VEE-Luciferase srRNA and anti-CTLA-4 (aCTLA-4) or VEE-UbAAY srRNA and anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD1 mAb starting at day 7. Each group consisted of 8 mice. Mice were sacrificed and spleens and lymph nodes were collected 14 days after immunization. SIINFEKL-specific T-cell responses were assessed by MHC I-pentamer staining, reported as pentamer positive cells as a percent of CD8 positive cells. Lines represent medians.

[0095] FIG. 13A illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A third group was treated with the Ad5-GFP prime/VEE-Luciferase srRNA boost in combination with anti-CTLA-4 (aCTLA-4), while the fourth group was treated with the Ad5-UbAAY prime/VEE-UbAAY boost in combination with anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD-1 mAb starting at day 21. T-cell responses were measured by IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 14 days post immunization with adenovirus.

[0096] FIG. 13B illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A third group was treated with the Ad5-GFP prime/VEE-Luciferase srRNA boost in combination with anti-CTLA-4 (aCTLA-4), while the fourth group was treated with the Ad5-UbAAY

prime/VEE-UbAAY boost in combination with anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD-1 mAb starting at day 21. T-cell responses were measured by IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 14 days post immunization with adenovirus and 14 days post boost with srRNA (day 28 after prime).

[0097] FIG. 13C illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A third group was treated with the Ad5-GFP prime/VEE-Luciferase srRNA boost in combination with anti-CTLA-4 (aCTLA-4), while the fourth group was treated with the Ad5-UbAAY prime/VEE-UbAAY boost in combination with anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD-1 mAb starting at day 21. T-cell responses were measured by MHC class I pentamer staining. Mice were sacrificed and spleens and lymph nodes collected at 14 days post immunization with adenovirus.

[0098] FIG. 13D illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A third group was treated with the Ad5-GFP prime/VEE-Luciferase srRNA boost in combination with anti-CTLA-4 (aCTLA-4), while the fourth group was treated with the Ad5-UbAAY prime/VEE-UbAAY boost in combination with anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD-1 mAb starting at day 21. T-cell responses were measured by MHC class I pentamer staining. Mice were sacrificed and spleens and lymph nodes collected at 14 days post immunization with adenovirus and 14 days post boost with srRNA (day 28 after prime).

[0099] FIG. 14A illustrates antigen-specific T-cell responses following heterologous prime/boost in CT26 (Balb/c) tumor bearing mice. Mice were immunized with Ad5-GFP and boosted 15 days after the adenovirus prime with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or primed with Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A separate group

was administered the Ad5-GFP/VEE-Luciferase srRNA prime/boost in combination with anti-PD-1 (aPD1), while a fourth group received the Ad5-UbAAY/VEE-UbAAY srRNA prime/boost in combination with an anti-PD-1 mAb (Vax + aPD1). T-cell responses to the AH1 peptide were measured using IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 12 days post immunization with adenovirus.

[00100] FIG. 14B illustrates antigen-specific T-cell responses following heterologous prime/boost in CT26 (Balb/c) tumor bearing mice. Mice were immunized with Ad5-GFP and boosted 15 days after the adenovirus prime with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or primed with Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A separate group was administered the Ad5-GFP/VEE-Luciferase srRNA prime/boost in combination with anti-PD-1 (aPD1), while a fourth group received the Ad5-UbAAY/VEE-UbAAY srRNA prime/boost in combination with an anti-PD-1 mAb (Vax + aPD1). T-cell responses to the AH1 peptide were measured using IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 12 days post immunization with adenovirus and 6 days post boost with srRNA (day 21 after prime).

[00101] FIG. 15 illustrates ChAdV68 eliciting T-Cell responses to mouse tumor antigens in mice. Mice were immunized with ChAdV68.5WTnt.MAG25mer, and T-cell responses to the MHC class I epitope SIINFEKL (OVA) were measured in C57BL/6J female mice and the MHC class I epitope AH1-A5 measured in Balb/c mice. Mean spot forming cells (SFCs) per 10^6 splenocytes measured in ELISpot assays presented. Error bars represent standard deviation.

[00102] FIG. 16 illustrates cellular immune responses in a CT26 tumor model following a single immunization with either ChAdV6, ChAdV + anti-PD-1, srRNA, srRNA + anti-PD-1, or anti-PD-1 alone. Antigen-specific IFN-gamma production was measured in splenocytes for 6 mice from each group using ELISpot. Results are presented as spot forming cells (SFC) per 10^6 splenocytes. Median for each group indicated by horizontal line. P values determined using the Dunnett's multiple comparison test; *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$. ChAdV = ChAdV68.5WTnt.MAG25mer; srRNA = VEE-MAG25mer srRNA.

[00103] FIG. 17 illustrates CD8 T-Cell responses in a CT26 tumor model following a single immunization with either ChAdV6, ChAdV + anti-PD-1, srRNA, srRNA + anti-PD-1, or anti-PD-1 alone. Antigen-specific IFN-gamma production in CD8 T cells measured using ICS and results presented as antigen-specific CD8 T cells as a percentage of total CD8 T cells. Median

for each group indicated by horizontal line. P values determined using the Dunnett's multiple comparison test; *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$. ChAdV= ChAdV68.5WTnt.MAG25mer; srRNA= VEE-MAG25mer srRNA.

[00104] FIG. 18 illustrates tumor growth in a CT26 tumor model following immunization with a ChAdV/srRNA heterologous prime/boost, a srRNA/ChAdV heterologous prime/boost, or a srRNA/srRNA homologous primer/boost. Also illustrated in a comparison of the prime/boost immunizations with or without administration of anti-PD1 during prime and boost. Tumor volumes measured twice per week and mean tumor volumes presented for the first 21 days of the study. 22-28 mice per group at study initiation. Error bars represent standard error of the mean (SEM). P values determined using the Dunnett's test; *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$. ChAdV= ChAdV68.5WTnt.MAG25mer; srRNA= VEE-MAG25mer srRNA.

[00105] FIG. 19 illustrates survival in a CT26 tumor model following immunization with a ChAdV/srRNA heterologous prime/boost, a srRNA/ChAdV heterologous prime/boost, or a srRNA/srRNA homologous primer/boost. Also illustrated in a comparison of the prime/boost immunizations with or without administration of anti-PD1 during prime and boost. P values determined using the log-rank test; *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$. ChAdV= ChAdV68.5WTnt.MAG25mer; srRNA= VEE-MAG25mer srRNA.

[00106] FIG. 20 illustrates antigen-specific cellular immune responses measured using ELISpot. Antigen-specific IFN-gamma production to six different mamu A01 restricted epitopes was measured in PBMCs for the VEE-MAG25mer srRNA-LNP1(30 μ g) (**FIG. 20A**), VEE-MAG25mer srRNA-LNP1(100 μ g) (**FIG. 20B**), or VEE-MAG25mer srRNA-LNP2(100 μ g) (**FIG. 20C**) homologous prime/boost or the ChAdV68.5WTnt.MAG25mer /VEE-MAG25mer srRNA heterologous prime/boost group (**FIG. 20D**) using ELISpot 1, 2, 3, 4, 5, 6, 8, 9, or 10 weeks after the first boost immunization (6 rhesus macaques per group). Results are presented as mean spot forming cells (SFC) per 10^6 PBMCs for each epitope in a stacked bar graph format. Values for each animal were normalized to the levels at pre-bleed (week 0).

[00107] FIG. 21 shows antigen-specific cellular immune response measured using ELISpot. Antigen-specific IFN-gamma production to six different mamu A01 restricted epitopes was measured in PBMCs after immunization with the ChAdV68.5WTnt.MAG25mer /VEE-MAG25mer srRNA heterologous prime/boost regimen using ELISpot prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks after the initial immunization. Results are presented as mean spot forming cells (SFC) per 10^6 PBMCs for each epitope (6 rhesus macaques per group) in a stacked bar graph format.

[00108] **FIG. 22** shows antigen-specific cellular immune response measured using ELISpot. Antigen-specific IFN-gamma production to six different mamu A01 restricted epitopes was measured in PBMCs after immunization with the VEE-MAG25mer srRNA LNP2 homologous prime/boost regimen using ELISpot prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, or 15 weeks after the initial immunization. Results are presented as mean spot forming cells (SFC) per 10^6 PBMCs for each epitope (6 rhesus macaques per group) in a stacked bar graph format.

[00109] **FIG. 23** shows antigen-specific cellular immune response measured using ELISpot. Antigen-specific IFN-gamma production to six different mamu A01 restricted epitopes was measured in PBMCs after immunization with the VEE-MAG25mer srRNA LNP1 homologous prime/boost regimen using ELISpot prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, or 15 weeks after the initial immunization. Results are presented as mean spot forming cells (SFC) per 10^6 PBMCs for each epitope (6 rhesus macaques per group) in a stacked bar graph format.

[00110] **FIG. 24A and FIG. 24B** show example peptide spectrums generated from Promega's dynamic range standard.

[00111] **FIG. 25** shows the correlation between EDGE score and the probability of detection of candidate shared neoantigen peptides by targeted MS.

[00112] **FIG. 26** illustrates an E1/E3 deleted ChAdV68 viral vector was designed with an expression cassette co-expressing a checkpoint inhibitor introduced into the deleted E1 region.

[00113] **FIG. 27** shows in vitro mouse anti-CTLA4 clone 9D9 antibody expression post infection of 293A cells.

[00114] **FIG. 28A** shows a Western Blot demonstrating human anti-CTLA-4 IgG1 antibody (Ipilimumab) antibody expression in chAd68-MAG-IRES-IPI (IPI-MAG) & chAd68-GFP-IRES-IPI (IPI-GFP) infected cells. HEK293A. Cells were infected at an MOI of 1 and cell pellets and supernatants harvested 48h post infection. The anti-CTLA4 antibody was purified from the supernatant by immunoprecipitation with protein-G beads (ThermoFisher). Pellet and immunoprecipitated supernatant were analyzed by SDS-PAGE electrophoresis and Western blotting using a HRP Donkey anti-human IgG antibody and detected by ECL chemiluminescent substrate (ThermoFisher).

[00115] **FIG. 28B** shows a Western Blot demonstrating human anti-CTLA-4 IgG2 antibody (Tremelimumab) antibody expression in (1) chAd68-MAG-IRES-TREME & chAd68-M2.2, a control virus infected cells. HEK293A. Cells were infected at an MOI of 1 and cell pellets and

supernatants harvested 48h post infection. The anti-CTLA4 antibody was purified from the supernatant by immunoprecipitation with protein-G beads (ThermoFisher). Pellet and immunoprecipitated supernatant were analyzed by SDS-PAGE electrophoresis and Western blotting using a HRP Donkey anti-human IgG antibody and detected by ECL chemiluminescent substrate (ThermoFisher).

[00116] FIG. 29 illustrates the general organization of the model epitopes from the various species for large antigen cassettes that had either 30 (L), 40 (XL) or 50 (XXL) epitopes.

[00117] FIG. 30 shows ChAd vectors express long cassettes as indicated by the above Western blot using an anti-class II (PADRE) antibody that recognizes a sequence common to all cassettes. HEK293 cells were infected with chAd68 vectors expressing large cassettes (chAd68-50XXL, chAd68-40XL & chAd68-30L) of variable size. Infections were set up at a MOI of 0.2. Twenty-four hours post infection MG132 a proteasome inhibitor was added to a set of the infected wells (indicated by the plus sign). Another set of virus treated wells were not treated with MG132 (indicated by minus sign). Uninfected HEK293 cells (293F) were used as a negative control. Forty-eight hours post infection cell pellets were harvested and analyzed by SDS/PAGE electrophoresis, and immunoblotting using a rabbit anti-Class II PADRE antibody. A HRP anti-rabbit antibody and ECL chemiluminescent substrate was used for detection.

[00118] FIG. 31 shows CD8⁺ immune responses in chAd68 large cassette immunized mice, detected against AH1 (top) and SIINFEKL (bottom) by ICS. Data is presented as IFN γ ⁺ cells against the model epitope as % of total CD8 cells

[00119] FIG. 32 shows CD8⁺ responses to LD-AH1⁺ (top) and Kb-SIINFEKL⁺ (bottom) Tetramers post chAd68 large cassette vaccination. Data is presented as % of total CD8 cells reactive against the model Tetramer peptide complex. *p<0.05, **p<0.01 by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

[00120] FIG. 33 shows CD8⁺ immune responses in alphavirus large cassette treated mice, detected against AH1 (top) and SIINFEKL (bottom) by ICS. Data is presented as IFN γ ⁺ cells against the model epitope as % of total CD8 cells. *p<0.05, **p<0.01, ***p<0.001 by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

[00121] FIG. 34 illustrates the vaccination strategy used to evaluate immunogenicity of the antigen-cassette containing vectors in rhesus macaques. Triangles indicate chAd68 vaccination (1e12 vp/animal) at weeks 0 & 32. Circles represent alphavirus vaccination at weeks 0, 4, 12, 20, 28 & 32. Squares represent administration of an anti-CTLA4 antibody.

[00122] **FIG. 35** shows a time course of CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG alone (Group 4). Mean SFC/1e6 splenocytes is shown.

[00123] **FIG. 36** shows a time course of CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered IV. (Group 5). Mean SFC/1e6 splenocytes is shown.

[00124] **FIG. 37** shows a time course of CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered SC (Group 6). Mean SFC/1e6 splenocytes is shown.

[00125] **FIG. 38** shows antigen-specific memory responses generated by ChAdV68/samRNA vaccine protocol measured by ELISpot. Results are presented as individual dot plots, with each dot representing a single animal. Pre-immunization baseline (left panel) and memory response at 18 months post-prime (right panel) are shown.

[00126] **FIG. 39** shows memory cell phenotyping of antigen-specific CD8⁺ T-cells by flow cytometry using combinatorial tetramer staining and CD45RA/CCR7 co-staining.

[00127] **FIG. 40** shows the distribution of memory cell types within the sum of the four Mamu-A*01 tetramer⁺ CD8⁺ T-cell populations at study month 18. Memory cells were characterized as follows: CD45RA⁺CCR7⁺=naïve, CD45RA⁺CCR7⁻=effector (Teff), CD45RA⁻CCR7⁺=central memory (Tcm), CD45RA⁻CCR7⁻=effector memory (Tem).

[00128] **FIG. 41** shows antigen-specific T-cell response for immunization with chAd-MAG-CTLA4, chAd-MAG alone, and chAd-MAG with IP delivery of anti-CTLA4 o9D9, at low (1.5e6 IU, left) and high (1.5e7 IU, right) vector doses. Response measured by IFN γ ELISpot and presented as spot-forming cells per 1e6 splenocytes for each mouse (n=8 per group). Bar represents median.

[00129] **FIG. 42** shows antigen-specific T-cell response for immunization with chAd-MAG-CTLA4, chAd-MAG alone, and chAd-MAG with IP delivery of anti-CTLA4 o9D9, at low (1.5e6 IU, left) and high (1.5e7 IU, right) vector doses. Response measured by intracellular staining (ICS) and presented as IFN γ ⁺ cells as a percent of total CD8⁺ cells for each mouse (n=8 per group). Bar represents median.

[00130] **FIG. 43** shows anti-CTLA4 antibody levels in the serum of mice immunized with chAd-MAG-CTLA4 or with chAd-MAG plus IP delivery of anti-CTLA4 o9D9.

Electrochemiluminescence (ECL), mean and standard deviation for each timepoint and group (n=8 mice per group). Black arrows represent timepoints of anti-CTLA4 administration in groups 3 and 4. Mice were immunized with ChAd-MAG and ChAd-MAG-aCTLA4 at Day 0.

Dotted line represents maximum limit of assay. The two groups with systemic (IP) delivery of anti-CTLA4 mAb are both at the maximum limit of the assay at all timepoints measured post-immunization.

[00131] **FIG. 44** shows frequency of CD8+ T cells recognizing the CT26 tumor antigen AH1 in CT26 tumor-bearing mice. P values determined using the one-way ANOVA with Tukey's multiple comparisons test; **P<0.001, *P<0.05. ChAdV=ChAdV68.5WTnt.MAG25mer; aCTLA4=anti-CTLA4 antibody, clone 9D9.

DETAILED DESCRIPTION

I. Definitions

[00132] In general, terms used in the claims and the specification are intended to be construed as having the plain meaning understood by a person of ordinary skill in the art. Certain terms are defined below to provide additional clarity. In case of conflict between the plain meaning and the provided definitions, the provided definitions are to be used.

[00133] As used herein the term “antigen” is a substance that induces an immune response. An antigen can be a neoantigen. An antigen can be a “shared antigen” that is an antigen found among a specific population, *e.g.*, a specific population of cancer patients.

[00134] As used herein the term “neoantigen” is an antigen that has at least one alteration that makes it distinct from the corresponding wild-type antigen, *e.g.*, via mutation in a tumor cell or post-translational modification specific to a tumor cell. A neoantigen can include a polypeptide sequence or a nucleotide sequence. A mutation can include a frameshift or nonframeshift indel, missense or nonsense substitution, splice site alteration, genomic rearrangement or gene fusion, or any genomic or expression alteration giving rise to a neoORF. A mutations can also include a splice variant. Post-translational modifications specific to a tumor cell can include aberrant phosphorylation. Post-translational modifications specific to a tumor cell can also include a proteasome-generated spliced antigen. *See* Liepe et al., A large fraction of HLA class I ligands are proteasome-generated spliced peptides; *Science*. 2016 Oct 21;354(6310):354-358. Such shared neoantigens are useful for inducing an immune response in a subject via administration. The subject can be identified for administration through the use of various diagnostic methods, *e.g.*, patient selection methods described further below.

[00135] As used herein the term “tumor antigen” is a antigen present in a subject's tumor cell or tissue but not in the subject's corresponding normal cell or tissue, or derived from a

polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue.

[00136] As used herein the term “antigen-based vaccine” is a vaccine composition based on one or more antigens, e.g., a plurality of antigens. The vaccines can be nucleotide-based (*e.g.*, virally based, RNA based, or DNA based), protein-based (*e.g.*, peptide based), or a combination thereof.

[00137] As used herein the term “candidate antigen” is a mutation or other aberration giving rise to a sequence that may represent a antigen.

[00138] As used herein the term “coding region” is the portion(s) of a gene that encode protein.

[00139] As used herein the term “coding mutation” is a mutation occurring in a coding region.

[00140] As used herein the term “ORF” means open reading frame.

[00141] As used herein the term “NEO-ORF” is a tumor-specific ORF arising from a mutation or other aberration such as splicing.

[00142] As used herein the term “missense mutation” is a mutation causing a substitution from one amino acid to another.

[00143] As used herein the term “nonsense mutation” is a mutation causing a substitution from an amino acid to a stop codon or causing removal of a canonical start codon.

[00144] As used herein the term “frameshift mutation” is a mutation causing a change in the frame of the protein.

[00145] As used herein the term “indel” is an insertion or deletion of one or more nucleic acids.

[00146] As used herein, the term percent “identity,” in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (*e.g.*, BLASTP and BLASTN or other algorithms available to persons of skill) or by visual inspection. Depending on the application, the percent “identity” can exist over a region of the sequence being compared, *e.g.*, over a functional domain, or, alternatively, exist over the full length of the two sequences to be compared.

[00147] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and

reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Alternatively, sequence similarity or dissimilarity can be established by the combined presence or absence of particular nucleotides, or, for translated sequences, amino acids at selected sequence positions (e.g., sequence motifs).

[00148] Optimal alignment of sequences for comparison can be conducted, e.g., 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 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., *infra*).

[00149] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[00150] As used herein the term “non-stop or read-through” is a mutation causing the removal of the natural stop codon.

[00151] As used herein the term “epitope” is the specific portion of an antigen typically bound by an antibody or T cell receptor.

[00152] As used herein the term “immunogenic” is the ability to elicit an immune response, e.g., via T cells, B cells, or both.

[00153] As used herein the term “HLA binding affinity” “MHC binding affinity” means affinity of binding between a specific antigen and a specific MHC allele.

[00154] As used herein the term “bait” is a nucleic acid probe used to enrich a specific sequence of DNA or RNA from a sample.

[00155] As used herein the term “variant” is a difference between a subject’s nucleic acids and the reference human genome used as a control.

[00156] As used herein the term “variant call” is an algorithmic determination of the presence of a variant, typically from sequencing.

[00157] As used herein the term “polymorphism” is a germline variant, i.e., a variant found in all DNA-bearing cells of an individual.

[00158] As used herein the term “somatic variant” is a variant arising in non-germline cells of an individual.

[00159] As used herein the term “allele” is a version of a gene or a version of a genetic sequence or a version of a protein.

[00160] As used herein the term “HLA type” is the complement of HLA gene alleles.

[00161] As used herein the term “nonsense-mediated decay” or “NMD” is a degradation of an mRNA by a cell due to a premature stop codon.

[00162] As used herein the term “truncal mutation” is a mutation originating early in the development of a tumor and present in a substantial portion of the tumor’s cells.

[00163] As used herein the term “subclonal mutation” is a mutation originating later in the development of a tumor and present in only a subset of the tumor’s cells.

[00164] As used herein the term “exome” is a subset of the genome that codes for proteins. An exome can be the collective exons of a genome.

[00165] As used herein the term “logistic regression” is a regression model for binary data from statistics where the logit of the probability that the dependent variable is equal to one is modeled as a linear function of the dependent variables.

[00166] As used herein the term “neural network” is a machine learning model for classification or regression consisting of multiple layers of linear transformations followed by element-wise nonlinearities typically trained via stochastic gradient descent and back-propagation.

[00167] As used herein the term “proteome” is the set of all proteins expressed and/or translated by a cell, group of cells, or individual.

[00168] As used herein the term “peptidome” is the set of all peptides presented by MHC-I or MHC-II on the cell surface. The peptidome may refer to a property of a cell or a collection of cells (e.g., the tumor peptidome, meaning the union of the peptidomes of all cells that comprise the tumor).

[00169] As used herein the term “ELISPOT” means Enzyme-linked immunosorbent spot assay – which is a common method for monitoring immune responses in humans and animals.

[00170] As used herein the term “dextramers” is a dextran-based peptide-MHC multimers used for antigen-specific T-cell staining in flow cytometry.

[00171] As used herein the term “tolerance or immune tolerance” is a state of immune non-responsiveness to one or more antigens, e.g. self-antigens.

[00172] As used herein the term “central tolerance” is a tolerance affected in the thymus, either by deleting self-reactive T-cell clones or by promoting self-reactive T-cell clones to differentiate into immunosuppressive regulatory T-cells (Tregs).

[00173] As used herein the term “peripheral tolerance” is a tolerance affected in the periphery by downregulating or anergizing self-reactive T-cells that survive central tolerance or promoting these T cells to differentiate into Tregs.

[00174] The term “sample” can include a single cell or multiple cells or fragments of cells or an aliquot of body fluid, taken from a subject, by means including venipuncture, excretion, ejaculation, massage, biopsy, needle aspirate, lavage sample, scraping, surgical incision, or intervention or other means known in the art.

[00175] The term “subject” encompasses a cell, tissue, or organism, human or non-human, whether in vivo, ex vivo, or in vitro, male or female. The term subject is inclusive of mammals including humans.

[00176] The term “mammal” encompasses both humans and non-humans and includes but is not limited to humans, non-human primates, canines, felines, murines, bovines, equines, and porcines.

[00177] The term “clinical factor” refers to a measure of a condition of a subject, e.g., disease activity or severity. “Clinical factor” encompasses all markers of a subject’s health status, including non-sample markers, and/or other characteristics of a subject, such as, without limitation, age and gender. A clinical factor can be a score, a value, or a set of values that can be obtained from evaluation of a sample (or population of samples) from a subject or a subject under a determined condition. A clinical factor can also be predicted by markers and/or other parameters such as gene expression surrogates. Clinical factors can include tumor type, tumor sub-type, and smoking history.

[00178] The term “antigen-encoding nucleic acid sequences derived from a tumor” refers to nucleic acid sequences directly extracted from the tumor, e.g. via RT-PCR; or sequence data obtained by sequencing the tumor and then synthesizing the nucleic acid sequences using the sequencing data, e.g., via various synthetic or PCR-based methods known in the art.

[00179] The term “alphavirus” refers to members of the family *Togaviridae*, and are positive-sense single-stranded RNA viruses. Alphaviruses are typically classified as either Old World, such as Sindbis, Ross River, Mayaro, Chikungunya, and Semliki Forest viruses, or New

World, such as eastern equine encephalitis, Aura, Fort Morgan, or Venezuelan equine encephalitis and its derivative strain TC-83. Alphaviruses are typically self-replicating RNA viruses.

[00180] The term “alphavirus backbone” refers to minimal sequence(s) of an alphavirus that allow for self-replication of the viral genome. Minimal sequences can include conserved sequences for nonstructural protein-mediated amplification, a nonstructural protein 1 (nsP1) gene, a nsP2 gene, a nsP3 gene, a nsP4 gene, and a polyA sequence, as well as sequences for expression of subgenomic viral RNA including a 26S promoter element.

[00181] The term “sequences for nonstructural protein-mediated amplification” includes alphavirus conserved sequence elements (CSE) well known to those in the art. CSEs include, but are not limited to, an alphavirus 5' UTR, a 51-nt CSE, a 24-nt CSE, or other 26S subgenomic promoter sequence, a 19-nt CSE, and an alphavirus 3' UTR.

[00182] The term “RNA polymerase” includes polymerases that catalyze the production of RNA polynucleotides from a DNA template. RNA polymerases include, but are not limited to, bacteriophage derived polymerases including T3, T7, and SP6.

[00183] The term “lipid” includes hydrophobic and/or amphiphilic molecules. Lipids can be cationic, anionic, or neutral. Lipids can be synthetic or naturally derived, and in some instances biodegradable. Lipids can include cholesterol, phospholipids, lipid conjugates including, but not limited to, polyethyleneglycol (PEG) conjugates (PEGylated lipids), waxes, oils, glycerides, fats, and fat-soluble vitamins. Lipids can also include dilinoleylmethyl- 4-dimethylaminobutyrate (MC3) and MC3-like molecules.

[00184] The term “lipid nanoparticle” or “LNP” includes vesicle like structures formed using a lipid containing membrane surrounding an aqueous interior, also referred to as liposomes. Lipid nanoparticles includes lipid-based compositions with a solid lipid core stabilized by a surfactant. The core lipids can be fatty acids, acylglycerols, waxes, and mixtures of these surfactants. Biological membrane lipids such as phospholipids, sphingomyelins, bile salts (sodium taurocholate), and sterols (cholesterol) can be utilized as stabilizers. Lipid nanoparticles can be formed using defined ratios of different lipid molecules, including, but not limited to, defined ratios of one or more cationic, anionic, or neutral lipids. Lipid nanoparticles can encapsulate molecules within an outer-membrane shell and subsequently can be contacted with target cells to deliver the encapsulated molecules to the host cell cytosol. Lipid nanoparticles can be modified or functionalized with non-lipid molecules, including on their surface. Lipid nanoparticles can be single-layered (unilamellar) or multi-layered

(multilamellar). Lipid nanoparticles can be complexed with nucleic acid. Unilamellar lipid nanoparticles can be complexed with nucleic acid, wherein the nucleic acid is in the aqueous interior. Multilamellar lipid nanoparticles can be complexed with nucleic acid, wherein the nucleic acid is in the aqueous interior, or to form or sandwiched between

[00185] Abbreviations: MHC: major histocompatibility complex; HLA: human leukocyte antigen, or the human MHC gene locus; NGS: next-generation sequencing; PPV: positive predictive value; TSNA: tumor-specific neoantigen; FFPE: formalin-fixed, paraffin-embedded; NMD: nonsense-mediated decay; NSCLC: non-small-cell lung cancer; DC: dendritic cell.

[00186] It should be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

[00187] Unless specifically stated or otherwise apparent from context, as used herein the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

[00188] Any terms not directly defined herein shall be understood to have the meanings commonly associated with them as understood within the art of the invention. Certain terms are discussed herein to provide additional guidance to the practitioner in describing the compositions, devices, methods and the like of aspects of the invention, and how to make or use them. It will be appreciated that the same thing may be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein. No significance is to be placed upon whether or not a term is elaborated or discussed herein. Some synonyms or substitutable methods, materials and the like are provided. Recital of one or a few synonyms or equivalents does not exclude use of other synonyms or equivalents, unless it is explicitly stated. Use of examples, including examples of terms, is for illustrative purposes only and does not limit the scope and meaning of the aspects of the invention herein.

[00189] All references, issued patents and patent applications cited within the body of the specification are hereby incorporated by reference in their entirety, for all purposes.

II. Methods of Identifying Antigens

[00190] Methods for identifying shared antigens (*e.g.*, neoantigens) include identifying antigens from a tumor of a subject that are likely to be presented on the cell surface of the tumor or immune cells, including professional antigen presenting cells such as dendritic cells, and/or are likely to be immunogenic. As an example, one such method may comprise the steps of: obtaining at least one of exome, transcriptome or whole genome tumor nucleotide sequencing and/or expression data from the tumor cell of the subject, wherein the tumor nucleotide sequencing and/or expression data is used to obtain data representing peptide sequences of each of a set of antigens (*e.g.*, in the case of neoantigens wherein the peptide sequence of each neoantigen comprises at least one alteration that makes it distinct from the corresponding wild-type peptide sequence or in cases of shared antigens without a mutation where peptides are derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue); inputting the peptide sequence of each antigen into one or more presentation models to generate a set of numerical likelihoods that each of the antigens is presented by one or more MHC alleles on the tumor cell surface of the tumor cell of the subject or cells present in the tumor, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens.

[00191] The presentation model can comprise a statistical regression or a machine learning (*e.g.*, deep learning) model trained on a set of reference data (also referred to as a training data set) comprising a set of corresponding labels, wherein the set of reference data is obtained from each of a plurality of distinct subjects where optionally some subjects can have a tumor, and wherein the set of reference data comprises at least one of: data representing exome nucleotide sequences from tumor tissue, data representing exome nucleotide sequences from normal tissue, data representing transcriptome nucleotide sequences from tumor tissue, data representing proteome sequences from tumor tissue, and data representing MHC peptidome sequences from tumor tissue, and data representing MHC peptidome sequences from normal tissue. The reference data can further comprise mass spectrometry data, sequencing data, RNA sequencing data, expression profiling data, and proteomics data for single-allele cell lines engineered to express a predetermined MHC allele that are subsequently exposed to synthetic protein, normal and tumor human cell lines, and fresh and frozen primary samples, and T cell

assays (e.g., ELISPOT). In certain aspects, the set of reference data includes each form of reference data.

[00192] The presentation model can comprise a set of features derived at least in part from the set of reference data, and wherein the set of features comprises at least one of allele dependent-features and allele-independent features. In certain aspects each feature is included.

[00193] Methods for identifying shared antigens also include generating an output for constructing a personalized cancer vaccine by identifying one or more antigens from one or more tumor cells of a subject that are likely to be presented on a surface of the tumor cells. As an example, one such method may comprise the steps of: obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing and/or expression data from the tumor cells and normal cells of the subject, wherein the nucleotide sequencing and/or expression data is used to obtain data representing peptide sequences of each of a set of antigens identified by comparing the nucleotide sequencing and/or expression data from the tumor cells and the nucleotide sequencing and/or expression data from the normal cells (*e.g.*, in the case of neoantigens wherein the peptide sequence of each neoantigen comprises at least one alteration that makes it distinct from the corresponding wild-type peptide sequence or in cases of shared antigens without a mutation where peptides are derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue), peptide sequence identified from the normal cells of the subject; encoding the peptide sequences of each of the antigens into a corresponding numerical vector, each numerical vector including information regarding a plurality of amino acids that make up the peptide sequence and a set of positions of the amino acids in the peptide sequence; inputting the numerical vectors, using a computer processor, into a deep learning presentation model to generate a set of presentation likelihoods for the set of antigens, each presentation likelihood in the set representing the likelihood that a corresponding antigen is presented by one or more class II MHC alleles on the surface of the tumor cells of the subject, the deep learning presentation model; selecting a subset of the set of antigens based on the set of presentation likelihoods to generate a set of selected antigens; and generating the output for constructing the personalized cancer vaccine based on the set of selected antigens.

[00194] Specific methods for identifying antigens, including neoantigens, are known to those skilled in the art, for example the methods described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00195] A method of treating a subject having a tumor is disclosed herein, comprising performing the steps of any of the antigen identification methods described herein, and further comprising obtaining a tumor vaccine comprising the set of selected antigens, and administering the tumor vaccine to the subject.

[00196] A method disclosed herein can also include identifying one or more T cells that are antigen-specific for at least one of the antigens in the subset. In some embodiments, the identification comprises co-culturing the one or more T cells with one or more of the antigens in the subset under conditions that expand the one or more antigen-specific T cells. In further embodiments, the identification comprises contacting the one or more T cells with a tetramer comprising one or more of the antigens in the subset under conditions that allow binding between the T cell and the tetramer. In even further embodiments, the method disclosed herein can also include identifying one or more T cell receptors (TCR) of the one or more identified T cells. In certain embodiments, identifying the one or more T cell receptors comprises sequencing the T cell receptor sequences of the one or more identified T cells. The method disclosed herein can further comprise genetically engineering a plurality of T cells to express at least one of the one or more identified T cell receptors; culturing the plurality of T cells under conditions that expand the plurality of T cells; and infusing the expanded T cells into the subject. In some embodiments, genetically engineering the plurality of T cells to express at least one of the one or more identified T cell receptors comprises cloning the T cell receptor sequences of the one or more identified T cells into an expression vector; and transfecting each of the plurality of T cells with the expression vector. In some embodiments, the method disclosed herein further comprises culturing the one or more identified T cells under conditions that expand the one or more identified T cells; and infusing the expanded T cells into the subject.

[00197] Also disclosed herein is an isolated T cell that is antigen-specific for at least one selected antigen in the subset.

[00198] Also disclosed herein is a methods for manufacturing a tumor vaccine, comprising the steps of: obtaining at least one of exome, transcriptome or whole genome tumor nucleotide sequencing and/or expression data from the tumor cell of the subject, wherein the tumor nucleotide sequencing and/or expression data is used to obtain data representing peptide sequences of each of a set of antigens (*e.g.*, in the case of neoantigens wherein the peptide sequence of each neoantigen comprises at least one alteration that makes it distinct from the corresponding wild-type peptide sequence or in cases of shared antigens without a mutation

where peptides are derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue); inputting the peptide sequence of each antigen into one or more presentation models to generate a set of numerical likelihoods that each of the antigens is presented by one or more MHC alleles on the tumor cell surface of the tumor cell of the subject, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens; and producing or having produced a tumor vaccine comprising the set of selected antigens.

[00199] Also disclosed herein is a tumor vaccine including a set of selected antigens selected by performing the method comprising the steps of: obtaining at least one of exome, transcriptome or whole genome tumor nucleotide sequencing and/or expression data from the tumor cell of the subject, wherein the tumor nucleotide sequencing and/or expression data is used to obtain data representing peptide sequences of each of a set of antigens, and wherein the peptide sequence of each antigen (*e.g.*, in the case of neoantigens wherein the peptide sequence of each neoantigen comprises at least one alteration that makes it distinct from the corresponding wild-type peptide sequence or in other cases of shared antigens without a mutation where peptides are derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue); inputting the peptide sequence of each antigen into one or more presentation models to generate a set of numerical likelihoods that each of the antigens is presented by one or more MHC alleles on the tumor cell surface of the tumor cell of the subject, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens; and producing or having produced a tumor vaccine comprising the set of selected antigens.

[00200] The tumor vaccine may include one or more of a nucleotide sequence, a polypeptide sequence, RNA, DNA, a cell, a plasmid, or a vector.

[00201] The tumor vaccine may include one or more antigens presented on the tumor cell surface.

[00202] The tumor vaccine may include one or more antigens that is immunogenic in the subject.

- [00203] The tumor vaccine may not include one or more antigens that induce an autoimmune response against normal tissue in the subject.
- [00204] The tumor vaccine may include an adjuvant.
- [00205] The tumor vaccine may include an excipient.
- [00206] A method disclosed herein may also include selecting antigens that have an increased likelihood of being presented on the tumor cell surface relative to unselected antigens based on the presentation model.
- [00207] A method disclosed herein may also include selecting antigens that have an increased likelihood of being capable of inducing a tumor-specific immune response in the subject relative to unselected antigens based on the presentation model.
- [00208] A method disclosed herein may also include selecting antigens that have an increased likelihood of being capable of being presented to naïve T cells by professional antigen presenting cells (APCs) relative to unselected antigens based on the presentation model, optionally wherein the APC is a dendritic cell (DC).
- [00209] A method disclosed herein may also include selecting antigens that have a decreased likelihood of being subject to inhibition via central or peripheral tolerance relative to unselected antigens based on the presentation model.
- [00210] A method disclosed herein may also include selecting antigens that have a decreased likelihood of being capable of inducing an autoimmune response to normal tissue in the subject relative to unselected antigens based on the presentation model.
- [00211] The exome or transcriptome nucleotide sequencing and/or expression data may be obtained by performing sequencing on the tumor tissue.
- [00212] The sequencing may be next generation sequencing (NGS) or any massively parallel sequencing approach.
- [00213] The set of numerical likelihoods may be further identified by at least MHC-allele interacting features comprising at least one of: the predicted affinity with which the MHC allele and the antigen encoded peptide bind; the predicted stability of the antigen encoded peptide-MHC complex; the sequence and length of the antigen encoded peptide; the probability of presentation of antigen encoded peptides with similar sequence in cells from other individuals expressing the particular MHC allele as assessed by mass-spectrometry proteomics or other means; the expression levels of the particular MHC allele in the subject in question (e.g. as measured by RNA-seq or mass spectrometry); the overall neoantigen encoded peptide-sequence-independent probability of presentation by the particular MHC allele in other distinct

subjects who express the particular MHC allele; the overall neoantigen encoded peptide-sequence-independent probability of presentation by MHC alleles in the same family of molecules (e.g., HLA-A, HLA-B, HLA-C, HLA-DQ, HLA-DR, HLA-DP) in other distinct subjects.

[00214] The set of numerical likelihoods are further identified by at least MHC-allele noninteracting features comprising at least one of: the C- and N-terminal sequences flanking the neoantigen encoded peptide within its source protein sequence; the presence of protease cleavage motifs in the neoantigen encoded peptide, optionally weighted according to the expression of corresponding proteases in the tumor cells (as measured by RNA-seq or mass spectrometry); the turnover rate of the source protein as measured in the appropriate cell type; the length of the source protein, optionally considering the specific splice variants (“isoforms”) most highly expressed in the tumor cells as measured by RNA-seq or proteome mass spectrometry, or as predicted from the annotation of germline or somatic splicing mutations detected in DNA or RNA sequence data; the level of expression of the proteasome, immunoproteasome, thymoproteasome, or other proteases in the tumor cells (which may be measured by RNA-seq, proteome mass spectrometry, or immunohistochemistry); the expression of the source gene of the neoantigen encoded peptide (e.g., as measured by RNA-seq or mass spectrometry); the typical tissue-specific expression of the source gene of the neoantigen encoded peptide during various stages of the cell cycle; a comprehensive catalog of features of the source protein and/or its domains as can be found in e.g. uniProt or PDB <http://www.rcsb.org/pdb/home/home.do>; features describing the properties of the domain of the source protein containing the peptide, for example: secondary or tertiary structure (e.g., alpha helix vs beta sheet); alternative splicing; the probability of presentation of peptides from the source protein of the neoantigen encoded peptide in question in other distinct subjects; the probability that the peptide will not be detected or over-represented by mass spectrometry due to technical biases; the expression of various gene modules/pathways as measured by RNASeq (which need not contain the source protein of the peptide) that are informative about the state of the tumor cells, stroma, or tumor-infiltrating lymphocytes (TILs); the copy number of the source gene of the neoantigen encoded peptide in the tumor cells; the probability that the peptide binds to the TAP or the measured or predicted binding affinity of the peptide to the TAP; the expression level of TAP in the tumor cells (which may be measured by RNA-seq, proteome mass spectrometry, immunohistochemistry); presence or absence of tumor mutations, including, but not limited to: driver mutations in known cancer driver genes such as EGFR,

KRAS, ALK, RET, ROS1, TP53, CDKN2A, CDKN2B, NTRK1, NTRK2, NTRK3, and in genes encoding the proteins involved in the antigen presentation machinery (e.g., B2M, HLA-A, HLA-B, HLA-C, TAP-1, TAP-2, TAPBP, CALR, CNX, ERP57, HLA-DM, HLA-DMA, HLA-DMB, HLA-DO, HLA-DOA, HLA-DOB, HLA-DP, HLA-DPA1, HLA-DPB1, HLA-DQ, HLA-DQA1, HLA-DQA2, HLA-DQB1, HLA-DQB2, HLA-DR, HLA-DRA, HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5 or any of the genes coding for components of the proteasome or immunoproteasome). Peptides whose presentation relies on a component of the antigen-presentation machinery that is subject to loss-of-function mutation in the tumor have reduced probability of presentation; presence or absence of functional germline polymorphisms, including, but not limited to: in genes encoding the proteins involved in the antigen presentation machinery (e.g., B2M, HLA-A, HLA-B, HLA-C, TAP-1, TAP-2, TAPBP, CALR, CNX, ERP57, HLA-DM, HLA-DMA, HLA-DMB, HLA-DO, HLA-DOA, HLA-DOB, HLA-DP, HLA-DPA1, HLA-DPB1, HLA-DQ, HLA-DQA1, HLA-DQA2, HLA-DQB1, HLA-DQB2, HLA-DR, HLA-DRA, HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5 or any of the genes coding for components of the proteasome or immunoproteasome); tumor type (e.g., NSCLC, melanoma); clinical tumor subtype (e.g., squamous lung cancer vs. non-squamous); smoking history; the typical expression of the source gene of the peptide in the relevant tumor type or clinical subtype, optionally stratified by driver mutation.

[00215] The at least one alteration may be a frameshift or nonframeshift indel, missense or nonsense substitution, splice site alteration, genomic rearrangement or gene fusion, or any genomic or expression alteration giving rise to a neoORF.

[00216] The tumor cell may be selected from the group consisting of: lung cancer, melanoma, breast cancer, ovarian cancer, prostate cancer, kidney cancer, gastric cancer, colon cancer, testicular cancer, head and neck cancer, pancreatic cancer, brain cancer, B-cell lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, and T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer.

[00217] A method disclosed herein may also include obtaining a tumor vaccine comprising the set of selected neoantigens or a subset thereof, optionally further comprising administering the tumor vaccine to the subject.

[00218] At least one of neoantigens in the set of selected neoantigens, when in polypeptide form, may include at least one of: a binding affinity with MHC with an IC₅₀ value of less than 1000nM, for MHC Class I polypeptides a length of 8-15, 8, 9, 10, 11, 12, 13, 14, or 15 amino

acids, for MHC Class II polypeptides a length of 6-30, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids, presence of sequence motifs within or near the polypeptide in the parent protein sequence promoting proteasome cleavage, and presence of sequence motifs promoting TAP transport. For MHC Class II, presence of sequence motifs within or near the peptide promoting cleavage by extracellular or lysosomal proteases (e.g., cathepsins) or HLA-DM catalyzed HLA binding.

[00219]

[00220] Disclosed herein is are methods for identifying one or more neoantigens that are likely to be presented on a tumor cell surface of a tumor cell, comprising executing the steps of: receiving mass spectrometry data comprising data associated with a plurality of isolated peptides eluted from major histocompatibility complex (MHC) derived from a plurality of fresh or frozen tumor samples; obtaining a training data set by at least identifying a set of training peptide sequences present in the tumor samples and presented on one or more MHC alleles associated with each training peptide sequence; obtaining a set of training protein sequences based on the training peptide sequences; and training a set of numerical parameters of a presentation model using the training protein sequences and the training peptide sequences, the presentation model providing a plurality of numerical likelihoods that peptide sequences from the tumor cell are presented by one or more MHC alleles on the tumor cell surface.

[00221] The presentation model may represent dependence between: presence of a pair of a particular one of the MHC alleles and a particular amino acid at a particular position of a peptide sequence; and likelihood of presentation on the tumor cell surface, by the particular one of the MHC alleles of the pair, of such a peptide sequence comprising the particular amino acid at the particular position.

[00222] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has an increased likelihood that it is presented on the cell surface of the tumor relative to one or more distinct tumor neoantigens.

[00223] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has an increased likelihood that it is capable of inducing a tumor-specific immune response in the subject relative to one or more distinct tumor neoantigens.

[00224] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has an increased likelihood that it is capable of being presented to naïve T cells by professional antigen presenting cells (APCs)

relative to one or more distinct tumor neoantigens, optionally wherein the APC is a dendritic cell (DC).

[00225] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has a decreased likelihood that it is subject to inhibition via central or peripheral tolerance relative to one or more distinct tumor neoantigens.

[00226] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has a decreased likelihood that it is capable of inducing an autoimmune response to normal tissue in the subject relative to one or more distinct tumor neoantigens.

[00227] A method disclosed herein can also include selecting a subset of neoantigens, wherein the subset of neoantigens is selected because each has a decreased likelihood that it will be differentially post-translationally modified in tumor cells versus APCs, optionally wherein the APC is a dendritic cell (DC).

[00228] The practice of the methods herein will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992).

III. Identification of Tumor Specific Mutations in Neoantigens

[00229] Also disclosed herein are methods for the identification of certain mutations (e.g., the variants or alleles that are present in cancer cells). In particular, these mutations can be present in the genome, transcriptome, proteome, or exome of cancer cells of a subject having cancer but not in normal tissue from the subject. Specific methods for identifying neoantigens, including shared neoantigens, that are specific to tumors are known to those skilled in the art, for example the methods described in more detail in international patent application

publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00230] Genetic mutations in tumors can be considered useful for the immunological targeting of tumors if they lead to changes in the amino acid sequence of a protein exclusively in the tumor. Useful mutations include: (1) non-synonymous mutations leading to different amino acids in the protein; (2) read-through mutations in which a stop codon is modified or deleted, leading to translation of a longer protein with a novel tumor-specific sequence at the C-terminus; (3) splice site mutations that lead to the inclusion of an intron in the mature mRNA and thus a unique tumor-specific protein sequence; (4) chromosomal rearrangements that give rise to a chimeric protein with tumor-specific sequences at the junction of 2 proteins (i.e., gene fusion); (5) frameshift mutations or deletions that lead to a new open reading frame with a novel tumor-specific protein sequence. Mutations can also include one or more of nonframeshift indel, missense or nonsense substitution, splice site alteration, genomic rearrangement or gene fusion, or any genomic or expression alteration giving rise to a neoORF.

[00231] Peptides with mutations or mutated polypeptides arising from for example, splice-site, frameshift, readthrough, or gene fusion mutations in tumor cells can be identified by sequencing DNA, RNA or protein in tumor versus normal cells.

[00232] Also mutations can include previously identified tumor specific mutations. Known tumor mutations can be found at the Catalogue of Somatic Mutations in Cancer (COSMIC) database.

[00233] A variety of methods are available for detecting the presence of a particular mutation or allele in an individual's DNA or RNA. Advancements in this field have provided accurate, easy, and inexpensive large-scale SNP genotyping. For example, several techniques have been described including dynamic allele-specific hybridization (DASH), microplate array diagonal gel electrophoresis (MADGE), pyrosequencing, oligonucleotide-specific ligation, the TaqMan system as well as various DNA "chip" technologies such as the Affymetrix SNP chips. These methods utilize amplification of a target genetic region, typically by PCR. Still other methods, based on the generation of small signal molecules by invasive cleavage followed by mass spectrometry or immobilized padlock probes and rolling-circle amplification. Several of the methods known in the art for detecting specific mutations are summarized below.

[00234] PCR based detection means can include multiplex amplification of a plurality of markers simultaneously. For example, it is well known in the art to select PCR primers to generate PCR products that do not overlap in size and can be analyzed simultaneously.

Alternatively, it is possible to amplify different markers with primers that are differentially labeled and thus can each be differentially detected. Of course, hybridization based detection means allow the differential detection of multiple PCR products in a sample. Other techniques are known in the art to allow multiplex analyses of a plurality of markers.

[00235] Several methods have been developed to facilitate analysis of single nucleotide polymorphisms in genomic DNA or cellular RNA. For example, a single base polymorphism can be detected by using a specialized exonuclease-resistant nucleotide, as disclosed, e.g., in Mundy, C. R. (U.S. Pat. No. 4,656,127). According to the method, a primer complementary to the allelic sequence immediately 3' to the polymorphic site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the polymorphic site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide(s) present in the polymorphic site of the target molecule is complementary to that of the nucleotide derivative used in the reaction. This method has the advantage that it does not require the determination of large amounts of extraneous sequence data.

[00236] A solution-based method can be used for determining the identity of a nucleotide of a polymorphic site. Cohen, D. et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087). As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to allelic sequences immediately 3' to a polymorphic site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the polymorphic site will become incorporated onto the terminus of the primer.

[00237] An alternative method, known as Genetic Bit Analysis or GBA is described by Goelet, P. et al. (PCT Appln. No. 92/15712). The method of Goelet, P. et al. uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the polymorphic site of the target molecule being evaluated. In contrast to the method of Cohen et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. et al. can be a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase.

[00238] Several primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. et al., Nucl. Acids. Res. 17:7779-7784 (1989); Sokolov, B. P., Nucl. Acids Res. 18:3671 (1990); Syvanen, A.-C., et al., Genomics 8:684-692 (1990); Kuppuswamy, M. N. et al., Proc. Natl. Acad. Sci. (U.S.A.) 88:1143-1147 (1991); Prezant, T. R. et al., Hum. Mutat. 1:159-164 (1992); Ugozzoli, L. et al., GATA 9:107-112 (1992); Nyren, P. et al., Anal. Biochem. 208:171-175 (1993)). These methods differ from GBA in that they utilize incorporation of labeled deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide can result in signals that are proportional to the length of the run (Syvanen, A.-C., et al., Amer. J. Hum. Genet. 52:46-59 (1993)).

[00239] A number of initiatives obtain sequence information directly from millions of individual molecules of DNA or RNA in parallel. Real-time single molecule sequencing-by-synthesis technologies rely on the detection of fluorescent nucleotides as they are incorporated into a nascent strand of DNA that is complementary to the template being sequenced. In one method, oligonucleotides 30-50 bases in length are covalently anchored at the 5' end to glass cover slips. These anchored strands perform two functions. First, they act as capture sites for the target template strands if the templates are configured with capture tails complementary to the surface-bound oligonucleotides. They also act as primers for the template directed primer extension that forms the basis of the sequence reading. The capture primers function as a fixed position site for sequence determination using multiple cycles of synthesis, detection, and chemical cleavage of the dye-linker to remove the dye. Each cycle consists of adding the polymerase/labeled nucleotide mixture, rinsing, imaging and cleavage of dye. In an alternative method, polymerase is modified with a fluorescent donor molecule and immobilized on a glass slide, while each nucleotide is color-coded with an acceptor fluorescent moiety attached to a gamma-phosphate. The system detects the interaction between a fluorescently-tagged polymerase and a fluorescently modified nucleotide as the nucleotide becomes incorporated into the de novo chain. Other sequencing-by-synthesis technologies also exist.

[00240] Any suitable sequencing-by-synthesis platform can be used to identify mutations. As described above, four major sequencing-by-synthesis platforms are currently available: the Genome Sequencers from Roche/454 Life Sciences, the 1G Analyzer from Illumina/Solexa, the SOLiD system from Applied BioSystems, and the Heliscope system from Helicos Biosciences. Sequencing-by-synthesis platforms have also been described by Pacific BioSciences and

VisiGen Biotechnologies. In some embodiments, a plurality of nucleic acid molecules being sequenced is bound to a support (e.g., solid support). To immobilize the nucleic acid on a support, a capture sequence/universal priming site can be added at the 3' and/or 5' end of the template. The nucleic acids can be bound to the support by hybridizing the capture sequence to a complementary sequence covalently attached to the support. The capture sequence (also referred to as a universal capture sequence) is a nucleic acid sequence complementary to a sequence attached to a support that may dually serve as a universal primer.

[00241] As an alternative to a capture sequence, a member of a coupling pair (such as, e.g., antibody/antigen, receptor/ligand, or the avidin-biotin pair as described in, e.g., US Patent Application No. 2006/0252077) can be linked to each fragment to be captured on a surface coated with a respective second member of that coupling pair.

[00242] Subsequent to the capture, the sequence can be analyzed, for example, by single molecule detection/sequencing, e.g., as described in the Examples and in U.S. Pat. No. 7,283,337, including template-dependent sequencing-by-synthesis. In sequencing-by-synthesis, the surface-bound molecule is exposed to a plurality of labeled nucleotide triphosphates in the presence of polymerase. The sequence of the template is determined by the order of labeled nucleotides incorporated into the 3' end of the growing chain. This can be done in real time or can be done in a step-and-repeat mode. For real-time analysis, different optical labels to each nucleotide can be incorporated and multiple lasers can be utilized for stimulation of incorporated nucleotides.

[00243] Sequencing can also include other massively parallel sequencing or next generation sequencing (NGS) techniques and platforms. Additional examples of massively parallel sequencing techniques and platforms are the Illumina HiSeq or MiSeq, Thermo PGM or Proton, the Pac Bio RS II or Sequel, Qiagen's Gene Reader, and the Oxford Nanopore MinION. Additional similar current massively parallel sequencing technologies can be used, as well as future generations of these technologies.

[00244] Any cell type or tissue can be utilized to obtain nucleic acid samples for use in methods described herein. For example, a DNA or RNA sample can be obtained from a tumor or a bodily fluid, e.g., blood, obtained by known techniques (e.g. venipuncture) or saliva. Alternatively, nucleic acid tests can be performed on dry samples (e.g. hair or skin). In addition, a sample can be obtained for sequencing from a tumor and another sample can be obtained from normal tissue for sequencing where the normal tissue is of the same tissue type as the tumor. A sample can be obtained for sequencing from a tumor and another sample can

be obtained from normal tissue for sequencing where the normal tissue is of a distinct tissue type relative to the tumor.

[00245] Tumors can include one or more of lung cancer, melanoma, breast cancer, ovarian cancer, prostate cancer, kidney cancer, gastric cancer, colon cancer, testicular cancer, head and neck cancer, pancreatic cancer, brain cancer, B-cell lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, and T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer.

[00246] Alternatively, protein mass spectrometry can be used to identify or validate the presence of mutated peptides bound to MHC proteins on tumor cells. Peptides can be acid-eluted from tumor cells or from HLA molecules that are immunoprecipitated from tumor, and then identified using mass spectrometry.

IV. Antigens

[00247] Antigens can include nucleotides or polypeptides. For example, an antigen can be an RNA sequence that encodes for a polypeptide sequence. Antigens useful in vaccines can therefore include nucleotide sequences or polypeptide sequences.

[00248] Disclosed herein are isolated peptides that comprise tumor specific mutations identified by the methods disclosed herein, peptides that comprise known tumor specific mutations, and mutant polypeptides or fragments thereof identified by methods disclosed herein. Neoantigen peptides can be described in the context of their coding sequence where a neoantigen includes the nucleotide sequence (e.g., DNA or RNA) that codes for the related polypeptide sequence.

[00249] Also disclosed herein are peptides derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue, for example any polypeptide known to or have been found to be aberrantly expressed in a tumor cell or cancerous tissue in comparison to a normal cell or tissue. Suitable polypeptides from which the antigenic peptides can be derived can be found for example in the COSMIC database. COSMIC curates comprehensive information on somatic mutations in human cancer. The peptide contains the tumor specific mutation.

[00250] One or more polypeptides encoded by an antigen nucleotide sequence can comprise at least one of: a binding affinity with MHC with an IC₅₀ value of less than 1000nM, for MHC Class I peptides a length of 8-15, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids, presence of sequence motifs within or near the peptide promoting proteasome cleavage, and presence or

sequence motifs promoting TAP transport. For MHC Class II peptides a length 6-30, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids, presence of sequence motifs within or near the peptide promoting cleavage by extracellular or lysosomal proteases (e.g., cathepsins) or HLA-DM catalyzed HLA binding.

[00251] One or more antigens can be presented on the surface of a tumor.

[00252] One or more antigens can be is immunogenic in a subject having a tumor, e.g., capable of eliciting a T cell response or a B cell response in the subject.

[00253] One or more antigens that induce an autoimmune response in a subject can be excluded from consideration in the context of vaccine generation for a subject having a tumor.

[00254] The size of at least one antigenic peptide molecule can comprise, but is not limited to, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120 or greater amino molecule residues, and any range derivable therein. In specific embodiments the antigenic peptide molecules are equal to or less than 50 amino acids.

[00255] Antigenic peptides and polypeptides can be: for MHC Class I 15 residues or less in length and usually consist of between about 8 and about 11 residues, particularly 9 or 10 residues; for MHC Class II, 6-30 residues, inclusive.

[00256] If desirable, a longer peptide can be designed in several ways. In one case, when presentation likelihoods of peptides on HLA alleles are predicted or known, a longer peptide could consist of either: (1) individual presented peptides with an extensions of 2-5 amino acids toward the N- and C-terminus of each corresponding gene product; (2) a concatenation of some or all of the presented peptides with extended sequences for each. In another case, when sequencing reveals a long (>10 residues) neoepitope sequence present in the tumor (e.g. due to a frameshift, read-through or intron inclusion that leads to a novel peptide sequence), a longer peptide would consist of: (3) the entire stretch of novel tumor-specific amino acids--thus bypassing the need for computational or in vitro test-based selection of the strongest HLA-presented shorter peptide. In both cases, use of a longer peptide allows endogenous processing by patient cells and may lead to more effective antigen presentation and induction of T cell responses.

[00257] Antigenic peptides and polypeptides can be presented on an HLA protein. In some aspects antigenic peptides and polypeptides are presented on an HLA protein with greater affinity than a wild-type peptide. In some aspects, a antigenic peptide or polypeptide can have an IC₅₀ of at least less than 5000 nM, at least less than 1000 nM, at least less than 500 nM, at least less than 250 nM, at least less than 200 nM, at least less than 150 nM, at least less than 100 nM, at least less than 50 nM or less.

[00258] In some aspects, antigenic peptides and polypeptides do not induce an autoimmune response and/or invoke immunological tolerance when administered to a subject.

[00259] Also provided are compositions comprising at least two or more antigenic peptides. In some embodiments the composition contains at least two distinct peptides. At least two distinct peptides can be derived from the same polypeptide. By distinct polypeptides is meant that the peptide vary by length, amino acid sequence, or both. The peptides are derived from any polypeptide known to or have been found to contain a tumor specific mutation or peptides derived from any polypeptide known to or have been found to have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue, for example any polypeptide known to or have been found to be aberrantly expressed in a tumor cell or cancerous tissue in comparison to a normal cell or tissue. Suitable polypeptides from which the antigenic peptides can be derived can be found for example in the COSMIC database or the AACR Genomics Evidence Neoplasia Information Exchange (GENIE) database. COSMIC curates comprehensive information on somatic mutations in human cancer. AACR GENIE aggregates and links clinical-grade cancer genomic data with clinical outcomes from tens of thousands of cancer patients. The peptide contains the tumor specific mutation. In some aspects the tumor specific mutation is a driver mutation for a particular cancer type.

[00260] Antigenic peptides and polypeptides having a desired activity or property can be modified to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, antigenic peptide and polypeptides can be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding, stability or presentation. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu,

Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications can be made using well known peptide synthesis procedures, as described in e.g., Merrifield, *Science* 232:341-347 (1986), Barany & Merrifield, *The Peptides*, Gross & Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart & Young, *Solid Phase Peptide Synthesis*, (Rockford, Ill., Pierce), 2d Ed. (1984).

[00261] Modifications of peptides and polypeptides with various amino acid mimetics or unnatural amino acids can be particularly useful in increasing the stability of the peptide and polypeptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al., *Eur. J. Drug Metab Pharmacokin.* 11:291-302 (1986). Half-life of the peptides can be conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous trichloroacetic acid or ethanol. The cloudy reaction sample is cooled (4 degrees C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

[00262] The peptides and polypeptides can be modified to provide desired attributes other than improved serum half-life. For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. Immunogenic peptides/T helper conjugates can be linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus can be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the peptide can be linked to the T helper peptide without a spacer.

[00263] A antigenic peptide can be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the peptide. The amino terminus of either the

antigenic peptide or the T helper peptide can be acylated. Exemplary T helper peptides include tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389.

[00264] Proteins or peptides can be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteins or peptides from natural sources, or the chemical synthesis of proteins or peptides. The nucleotide and protein, polypeptide and peptide sequences corresponding to various genes have been previously disclosed, and can be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases located at the National Institutes of Health website. The coding regions for known genes can be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

[00265] In a further aspect a antigen includes a nucleic acid (e.g. polynucleotide) that encodes a antigenic peptide or portion thereof. The polynucleotide can be, e.g., DNA, cDNA, PNA, CNA, RNA (e.g., mRNA), either single- and/or double-stranded, or native or stabilized forms of polynucleotides, such as, e.g., polynucleotides with a phosphorothiate backbone, or combinations thereof and it may or may not contain introns. A still further aspect provides an expression vector capable of expressing a polypeptide or portion thereof. Expression vectors for different cell types are well known in the art and can be selected without undue experimentation. Generally, DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, DNA can be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognized by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Guidance can be found e.g. in Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

V. Vaccine Compositions

[00266] Also disclosed herein is an immunogenic composition, e.g., a vaccine composition, capable of raising a specific immune response, e.g., a tumor-specific immune response. Vaccine compositions typically comprise one or a plurality of antigens, e.g., selected using a method described herein. Vaccine compositions can also be referred to as vaccines.

[00267] A vaccine can contain between 1 and 30 peptides, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 different peptides, 6, 7, 8, 9, 10 11, 12, 13, or 14 different peptides, or 12, 13 or 14 different peptides. Peptides can include post-translational modifications. A vaccine can contain between 1 and 100 or more nucleotide sequences, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more different nucleotide sequences, 6, 7, 8, 9, 10 11, 12, 13, or 14 different nucleotide sequences, or 12, 13 or 14 different nucleotide sequences. A vaccine can contain between 1 and 30 antigen sequences, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more different antigen sequences, 6, 7, 8, 9, 10 11, 12, 13, or 14 different antigen sequences, or 12, 13 or 14 different antigen sequences.

[00268] In one embodiment, different peptides and/or polypeptides or nucleotide sequences encoding them are selected so that the peptides and/or polypeptides capable of associating with different MHC molecules, such as different MHC class I molecules and/or different MHC class II molecules. In some aspects, one vaccine composition comprises coding sequence for peptides and/or polypeptides capable of associating with the most frequently occurring MHC class I molecules and/or different MHC class II molecules. Hence, vaccine compositions can comprise different fragments capable of associating with at least 2 preferred, at least 3 preferred, or at least 4 preferred MHC class I molecules and/or different MHC class II molecules.

[00269] The vaccine composition can be capable of raising a specific cytotoxic T-cells response and/or a specific helper T-cell response.

[00270] A vaccine composition can further comprise an adjuvant and/or a carrier. Examples of useful adjuvants and carriers are given herein below. A composition can be associated with a carrier such as e.g. a protein or an antigen-presenting cell such as e.g. a dendritic cell (DC) capable of presenting the peptide to a T-cell.

[00271] Adjuvants are any substance whose admixture into a vaccine composition increases or otherwise modifies the immune response to a antigen. Carriers can be scaffold structures, for

example a polypeptide or a polysaccharide, to which an antigen, is capable of being associated. Optionally, adjuvants are conjugated covalently or non-covalently.

[00272] The ability of an adjuvant to increase an immune response to an antigen is typically manifested by a significant or substantial increase in an immune-mediated reaction, or reduction in disease symptoms. For example, an increase in humoral immunity is typically manifested by a significant increase in the titer of antibodies raised to the antigen, and an increase in T-cell activity is typically manifested in increased cell proliferation, or cellular cytotoxicity, or cytokine secretion. An adjuvant may also alter an immune response, for example, by changing a primarily humoral or Th response into a primarily cellular, or Th response.

[00273] Suitable adjuvants include, but are not limited to 1018 ISS, alum, aluminium salts, Amplivax, AS15, BCG, CP-870,893, CpG7909, CyaA, dSLIM, GM-CSF, IC30, IC31, Imiquimod, ImuFact IMP321, IS Patch, ISS, ISCOMATRIX, JuvImmune, LipoVac, MF59, monophosphoryl lipid A, Montanide IMS 1312, Montanide ISA 206, Montanide ISA 50V, Montanide ISA-51, OK-432, OM-174, OM-197-MP-EC, ONTAK, PepTel vector system, PLG microparticles, resiquimod, SRL172, Virosomes and other Virus-like particles, YF-17D, VEGF trap, R848, beta-glucan, Pam3Cys, Aquila's QS21 stimulon (Aquila Biotech, Worcester, Mass., USA) which is derived from saponin, mycobacterial extracts and synthetic bacterial cell wall mimics, and other proprietary adjuvants such as Ribi's Detox. Quil or Superfos. Adjuvants such as incomplete Freund's or GM-CSF are useful. Several immunological adjuvants (e.g., MF59) specific for dendritic cells and their preparation have been described previously (Dupuis M, et al., Cell Immunol. 1998; 186(1):18-27; Allison A C; Dev Biol Stand. 1998; 92:3-11). Also cytokines can be used. Several cytokines have been directly linked to influencing dendritic cell migration to lymphoid tissues (e.g., TNF-alpha), accelerating the maturation of dendritic cells into efficient antigen-presenting cells for T-lymphocytes (e.g., GM-CSF, IL-1 and IL-4) (U.S. Pat. No. 5,849,589, specifically incorporated herein by reference in its entirety) and acting as immunoadjuvants (e.g., IL-12) (Gabrilovich D I, et al., J Immunother Emphasis Tumor Immunol. 1996 (6):414-418).

[00274] CpG immunostimulatory oligonucleotides have also been reported to enhance the effects of adjuvants in a vaccine setting. Other TLR binding molecules such as RNA binding TLR 7, TLR 8 and/or TLR 9 may also be used.

[00275] Other examples of useful adjuvants include, but are not limited to, chemically modified CpGs (e.g. CpR, Idera), Poly(I:C)(e.g. polyi:CI2U), non-CpG bacterial DNA or RNA

as well as immunoactive small molecules and antibodies such as cyclophosphamide, sunitinib, bevacizumab, celebrex, NCX-4016, sildenafil, tadalafil, vardenafil, sorafenib, XL-999, CP-547632, pazopanib, ZD2171, AZD2171, ipilimumab, tremelimumab, and SC58175, which may act therapeutically and/or as an adjuvant. The amounts and concentrations of adjuvants and additives can readily be determined by the skilled artisan without undue experimentation. Additional adjuvants include colony-stimulating factors, such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, sargramostim).

[00276] A vaccine composition can comprise more than one different adjuvant.

Furthermore, a therapeutic composition can comprise any adjuvant substance including any of the above or combinations thereof. It is also contemplated that a vaccine and an adjuvant can be administered together or separately in any appropriate sequence.

[00277] A carrier (or excipient) can be present independently of an adjuvant. The function of a carrier can for example be to increase the molecular weight of in particular mutant to increase activity or immunogenicity, to confer stability, to increase the biological activity, or to increase serum half-life. Furthermore, a carrier can aid presenting peptides to T-cells. A carrier can be any suitable carrier known to the person skilled in the art, for example a protein or an antigen presenting cell. A carrier protein could be but is not limited to keyhole limpet hemocyanin, serum proteins such as transferrin, bovine serum albumin, human serum albumin, thyroglobulin or ovalbumin, immunoglobulins, or hormones, such as insulin or palmitic acid. For immunization of humans, the carrier is generally a physiologically acceptable carrier acceptable to humans and safe. However, tetanus toxoid and/or diphtheria toxoid are suitable carriers. Alternatively, the carrier can be dextrans for example sepharose.

[00278] Cytotoxic T-cells (CTLs) recognize an antigen in the form of a peptide bound to an MHC molecule rather than the intact foreign antigen itself. The MHC molecule itself is located at the cell surface of an antigen presenting cell. Thus, an activation of CTLs is possible if a trimeric complex of peptide antigen, MHC molecule, and APC is present. Correspondingly, it may enhance the immune response if not only the peptide is used for activation of CTLs, but if additionally APCs with the respective MHC molecule are added. Therefore, in some embodiments a vaccine composition additionally contains at least one antigen presenting cell.

[00279] Antigens can also be included in viral vector-based vaccine platforms, such as vaccinia, fowlpox, self-replicating alphavirus, marabavirus, adenovirus (*See, e.g.,* Tatsis et al., *Adenoviruses, Molecular Therapy* (2004) 10, 616—629), or lentivirus, including but not limited to second, third or hybrid second/third generation lentivirus and recombinant lentivirus

of any generation designed to target specific cell types or receptors (*See, e.g.,* Hu et al., Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases, *Immunol Rev.* (2011) 239(1): 45-61, Sakuma et al., Lentiviral vectors: basic to translational, *Biochem J.* (2012) 443(3):603-18, Cooper et al., Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter, *Nucl. Acids Res.* (2015) 43 (1): 682-690, Zufferey et al., Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery, *J. Virol.* (1998) 72 (12): 9873-9880). Dependent on the packaging capacity of the above mentioned viral vector-based vaccine platforms, this approach can deliver one or more nucleotide sequences that encode one or more neoantigen peptides. The sequences may be flanked by non-mutated sequences, may be separated by linkers or may be preceded with one or more sequences targeting a subcellular compartment (*See, e.g.,* Gros et al., Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients, *Nat Med.* (2016) 22 (4):433-8, Stronen et al., Targeting of cancer neoantigens with donor-derived T cell receptor repertoires, *Science.* (2016) 352 (6291):1337-41, Lu et al., Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions, *Clin Cancer Res.* (2014) 20(13):3401-10). Upon introduction into a host, infected cells express the antigens, and thereby elicit a host immune (e.g., CTL) response against the peptide(s). Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Pat. No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)). A wide variety of other vaccine vectors useful for therapeutic administration or immunization of antigens, e.g., *Salmonella typhi* vectors, and the like will be apparent to those skilled in the art from the description herein.

V.A. Antigen Cassette

[00280] The methods employed for the selection of one or more antigens, the cloning and construction of a “cassette” and its insertion into a viral vector are within the skill in the art given the teachings provided herein. By “antigen cassette” is meant the combination of a selected antigen or plurality of antigens and the other regulatory elements necessary to transcribe the antigen(s) and express the transcribed product. A antigen or plurality of antigens can be operatively linked to regulatory components in a manner which permits transcription. Such components include conventional regulatory elements that can drive expression of the antigen(s) in a cell transfected with the viral vector. Thus the antigen cassette can also contain a

selected promoter which is linked to the antigen(s) and located, with other, optional regulatory elements, within the selected viral sequences of the recombinant vector.

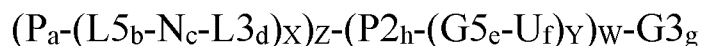
[00281] Useful promoters can be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of antigen(s) to be expressed. For example, a desirable promoter is that of the cytomegalovirus immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken cytoplasmic beta-actin promoter [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. Other suitable or desirable promoters can be selected by one of skill in the art.

[00282] The antigen cassette can also include nucleic acid sequences heterologous to the viral vector sequences including sequences providing signals for efficient polyadenylation of the transcript (poly(A), poly-A or pA) and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally can be inserted in the cassette following the antigen-based sequences and before the viral vector sequences. A common intron sequence can also be derived from SV-40, and is referred to as the SV-40 T intron sequence. A antigen cassette can also contain such an intron, located between the promoter/enhancer sequence and the antigen(s). Selection of these and other common vector elements are conventional [see, e.g., Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989) and references cited therein] and many such sequences are available from commercial and industrial sources as well as from Genbank.

[00283] A antigen cassette can have one or more antigens. For example, a given cassette can include 1-10, 1-20, 1-30, 10-20, 15-25, 15-20, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more antigens. Antigens can be linked directly to one another. Antigens can also be linked to one another with linkers. Antigens can be in any orientation relative to one another including N to C or C to N.

[00284] As above stated, the antigen cassette can be located in the site of any selected deletion in the viral vector, such as the site of the E1 gene region deletion or E3 gene region deletion, among others which may be selected.

[00285] The antigen cassette can be described using the following formula to describe the ordered sequence of each element, from 5' to 3':



[00286] wherein P and P2 comprise promoter nucleotide sequences, N comprises an MHC class I epitope encoding nucleic acid sequence, L5 comprises a 5' linker sequence, L3 comprises a 3' linker sequence, G5 comprises a nucleic acid sequences encoding an amino acid linker, G3 comprises one of the at least one nucleic acid sequences encoding an amino acid linker, U comprises an MHC class II antigen-encoding nucleic acid sequence, where for each X the corresponding N_c is a epitope encoding nucleic acid sequence, where for each Y the corresponding U_f is an antigen-encoding nucleic acid sequence. The composition and ordered sequence can be further defined by selecting the number of elements present, for example where a = 0 or 1, where b = 0 or 1, where c = 1, where d = 0 or 1, where e = 0 or 1, where f = 1, where g = 0 or 1, where h = 0 or 1, X = 1 to 400, Y = 0, 1, 2, 3, 4 or 5, Z = 1 to 400, and W = 0, 1, 2, 3, 4 or 5.

[00287] In one example, elements present include where a = 0, b = 1, d = 1, e = 1, g = 1, h = 0, X = 10, Y = 2, Z = 1, and W = 1, describing where no additional promoter is present (*i.e.* only the promoter nucleotide sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone) is present), 20 MHC class I epitope are present, a 5' linker is present for each N, a 3' linker is present for each N, 2 MHC class II epitopes are present, a linker is present linking the two MHC class II epitopes, a linker is present linking the 5' end of the two MHC class II epitopes to the 3' linker of the final MHC class I epitope, and a linker is present linking the 3' end of the two MHC class II epitopes to the to a vector backbone (e.g., a viral backbone such as an alphavirus backbone). Examples of linking the 3' end of the antigen cassette to a vector backbone (e.g., a viral backbone such as an alphavirus backbone) include linking directly to the 3' UTR elements provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone), such as a 3' 19-nt CSE. Examples of linking the 5' end of the antigen cassette to a vector backbone (e.g., a viral backbone such as an alphavirus backbone) include linking directly to a 26S promoter sequence, an alphavirus 5' UTR, a 51-nt CSE, or a 24-nt CSE.

[00288] Other examples include: where a = 1 describing where a promoter other than the promoter nucleotide sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone) is present; where a = 1 and Z is greater than 1 where multiple promoters other than the promoter nucleotide sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone) are present each driving expression of 1 or more

distinct MHC class I epitope encoding nucleic acid sequences; where $h = 1$ describing where a separate promoter is present to drive expression of the MHC class II antigen-encoding nucleic acid sequences; and where $g = 0$ describing the MHC class II antigen-encoding nucleic acid sequence, if present, is directly linked to a vector backbone (e.g., a viral backbone such as an alphavirus backbone).

[00289] Other examples include where each MHC class I epitope that is present can have a 5' linker, a 3' linker, neither, or both. In examples where more than one MHC class I epitope is present in the same antigen cassette, some MHC class I epitopes may have both a 5' linker and a 3' linker, while other MHC class I epitopes may have either a 5' linker, a 3' linker, or neither. In other examples where more than one MHC class I epitope is present in the same antigen cassette, some MHC class I epitopes may have either a 5' linker or a 3' linker, while other MHC class I epitopes may have either a 5' linker, a 3' linker, or neither.

[00290] In examples where more than one MHC class II epitope is present in the same antigen cassette, some MHC class II epitopes may have both a 5' linker and a 3' linker, while other MHC class II epitopes may have either a 5' linker, a 3' linker, or neither. In other examples where more than one MHC class II epitope is present in the same antigen cassette, some MHC class II epitopes may have either a 5' linker or a 3' linker, while other MHC class II epitopes may have either a 5' linker, a 3' linker, or neither.

[00291] The promoter nucleotide sequences P and/or P2 can be the same as a promoter nucleotide sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone). For example, the promoter sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone), P_n and P₂, can each comprise a 26S subgenomic promoter. The promoter nucleotide sequences P and/or P2 can be different from the promoter nucleotide sequence provided by a vector backbone (e.g., a viral backbone such as an alphavirus backbone), as well as can be different from each other.

[00292] The 5' linker L5 can be a native sequence or a non-natural sequence. Non-natural sequence include, but are not limited to, AAY, RR, and DPP. The 3' linker L3 can also be a native sequence or a non-natural sequence. Additionally, L5 and L3 can both be native sequences, both be non-natural sequences, or one can be native and the other non-natural. For each X, the amino acid linkers can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93,

94,95, 96, 97, 98, 99, 100 or more amino acids in length. For each X, the amino acid linkers can be also be at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, or at least 30 amino acids in length.

[00293] The amino acid linker G5, for each Y, can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94,95, 96, 97, 98, 99, 100 or more amino acids in length. For each Y, the amino acid linkers can be also be at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, or at least 30 amino acids in length.

[00294] The amino acid linker G3 can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94,95, 96, 97, 98, 99, 100 or more amino acids in length. G3 can be also be at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, or at least 30 amino acids in length.

[00295] For each X, each N can encodes a MHC class I epitope 7-15 amino acids in length. For each X, each N can also encodes a MHC class I epitope 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids in length. For each X, each N can also encodes a MHC class I epitope at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, or at least 30 amino acids in length.

V.B. Immune Checkpoints

[00296] Vectors described herein, such as C68 vectors described herein or alphavirus vectors described herein, can comprise a nucleic acid which encodes at least one antigen and the same or a separate vector can comprise a nucleic acid which encodes at least one immune modulator (e.g., an antibody such as an scFv) which binds to and blocks the activity of an immune checkpoint molecule. Vectors can comprise a antigen cassette and one or more nucleic acid molecules encoding a checkpoint inhibitor.

[00297] Illustrative immune checkpoint molecules that can be targeted for blocking or inhibition include, but are not limited to, CTLA-4, 4-1BB (CD137), 4-1BBL (CD137L), PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, TIM3, B7H3, B7H4, VISTA, KIR, 2B4 (belongs to the CD2 family of molecules and is expressed on all NK, $\gamma\delta$, and memory CD8+ ($\alpha\beta$) T cells), CD160 (also referred to as BY55), and CGEN-15049. Immune checkpoint inhibitors include antibodies, or antigen binding fragments thereof, or other binding proteins, that bind to and block or inhibit the activity of one or more of CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, TIM3, B7H3, B7H4, VISTA, KIR, 2B4, CD160, and CGEN-15049. Illustrative immune checkpoint inhibitors include Tremelimumab (CTLA-4 blocking antibody), anti-OX40, PD-L1 monoclonal Antibody (Anti-B7-H1; MEDI4736), ipilimumab, MK-3475 (PD-1 blocker), Nivolumab (anti-PD1 antibody), CT-011 (anti-PD1 antibody), BY55 monoclonal antibody, AMP224 (anti-PDL1 antibody), BMS-936559 (anti-PDL1 antibody), MPLDL3280A (anti-PDL1 antibody), MSB0010718C (anti-PDL1 antibody) and Yervoy/ipilimumab (anti-CTLA-4 checkpoint inhibitor). Antibody-encoding sequences can be engineered into vectors such as C68 using ordinary skill in the art. An exemplary method is described in Fang et al., Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol.* 2005 May;23(5):584-90. Epub 2005 Apr 17; herein incorporated by reference for all purposes.

[00298] Immune modulators (e.g., checkpoint inhibitor antibodies such as anti-CTLA4 antibodies or anti-PD1 antibodies) encoded in the same vector system as the antigen encoding cassette can also be encoded such that the nucleic acid sequence encoding the immune modulator is transcribed as part of the same transcript as the antigen-encoding nucleic acid sequence(s). Additional elements can be incorporated into the nucleic acid sequence cassette that allow for translation of both the antigens and the immune modulator. For example, an internal ribosome entry sequence (IRES) sequence can be used to separate sequences encoding

antigens and immune modulator(s), allowing separate translation of the antigens and the immune modulator(s). In another example, a sequence encoding a self-cleaving 2A peptide can be incorporated between antigens and immune modulator(s), allowing translation of both the antigens and the immune modulator(s) as part of the same protein, followed by cleavage of the 2A peptide; resulting in separate proteins for the antigens and the immune modulator(s). These examples are not meant to be limiting, and it is also understood that multiple elements can be combined to facilitate co-expression of both antigens and immune modulator(s), such as use of both an IRES sequence and a 2A peptide encoding sequence. Additionally, a Furin cleavage site encoding sequence can be incorporated 5' of the 2A peptide encoding sequence. The Furin cleavage site allows for removal of the 2A peptide residues following self-cleavage.

[00299] In examples where antigens and immune modulator(s) are encoded on the same transcript, the order of the antigens and the immune modulator can be in any order. For example, in the case of using an IRES sequence to separate the antigens and the immune modulator, the order, from 5' to 3', can either be in an antigen-IRES-immune modulator orientation, or in a immune modulator-IRES-antigen orientation.

[00300] In addition, immune modulators encoded in the same vector system as the antigen encoding cassette can also be encoded such that the nucleic acid sequence encoding the immune modulator is transcribed on a different transcript from the antigen-encoding nucleic acid sequence(s). For example, separate promoters can be incorporated to independently drive transcription of the immune modulator and the antigen-encoding nucleic acid sequence(s). The separate promoters can be the same or different promoters, and each can be an inducible or constitutive promoter. Exemplary promoter sequences include, but are not limited to, CMV, SV40, EF-1, RSV, PGK, MCK, HSA, and EBV promoter sequences. In another example, the antigen encoding cassette and the nucleic acid sequence encoding the immune modulator can be inserted into different regions, including deleted regions, of the same viral vector such that each are independently transcribed. In one example, a vector is designed with an expression cassette introduced into the deleted E1 region and the immune checkpoint inhibitor is introduced into the deleted E3 region in an E1/E3 deleted ChAdV68 viral vector.

V.C. Additional Considerations for Vaccine Design and Manufacture

V.C.1. Determination of a Set of Peptides that Cover All Tumor

Subclones

[00301] Truncal peptides, meaning those presented by all or most tumor subclones, can be prioritized for inclusion into the vaccine.⁵³ Optionally, if there are no truncal peptides predicted to be presented and immunogenic with high probability, or if the number of truncal peptides predicted to be presented and immunogenic with high probability is small enough that additional non-truncal peptides can be included in the vaccine, then further peptides can be prioritized by estimating the number and identity of tumor subclones and choosing peptides so as to maximize the number of tumor subclones covered by the vaccine.⁵⁴

V.C.2. Antigen Prioritization

[00302] After all of the above antigen filters are applied, more candidate antigens may still be available for vaccine inclusion than the vaccine technology can support. Additionally, uncertainty about various aspects of the antigen analysis may remain and tradeoffs may exist between different properties of candidate vaccine antigens. Thus, in place of predetermined filters at each step of the selection process, an integrated multi-dimensional model can be considered that places candidate antigens in a space with at least the following axes and optimizes selection using an integrative approach.

1. Risk of auto-immunity or tolerance (risk of germline) (lower risk of auto-immunity is typically preferred)
2. Probability of sequencing artifact (lower probability of artifact is typically preferred)
3. Probability of immunogenicity (higher probability of immunogenicity is typically preferred)
4. Probability of presentation (higher probability of presentation is typically preferred)
5. Gene expression (higher expression is typically preferred)
6. Coverage of HLA genes (larger number of HLA molecules involved in the presentation of a set of antigens may lower the probability that a tumor will escape immune attack via downregulation or mutation of HLA molecules)
7. Coverage of HLA classes (covering both HLA-I and HLA-II may increase the probability of therapeutic response and decrease the probability of tumor escape)

[00303] Additionally, optionally, antigens can be deprioritized (e.g., excluded) from the vaccination if they are predicted to be presented by HLA alleles lost or inactivated in either all or part of the patient's tumor. HLA allele loss can occur by either somatic mutation, loss of heterozygosity, or homozygous deletion of the locus. Methods for detection of HLA allele somatic mutation are well known in the art, e.g. (Shukla et al., 2015). Methods for detection of somatic LOH and homozygous deletion (including for HLA locus) are likewise well described. (Carter et al., 2012; McGranahan et al., 2017; Van Loo et al., 2010). Antigens can also be

deprioritized if mass-spectrometry data indicates a predicted antigen is not presented by a predicted HLA allele.

V.D. Alphavirus

V.D.1. Alphavirus Biology

[00304] Alphaviruses are members of the family *Togaviridae*, and are positive-sense single stranded RNA viruses. Members are typically classified as either Old World, such as Sindbis, Ross River, Mayaro, Chikungunya, and Semliki Forest viruses, or New World, such as eastern equine encephalitis, Aura, Fort Morgan, or Venezuelan equine encephalitis virus and its derivative strain TC-83 (Strauss Microbial Review 1994). A natural alphavirus genome is typically around 12kb in length, the first two-thirds of which contain genes encoding non-structural proteins (nsPs) that form RNA replication complexes for self-replication of the viral genome, and the last third of which contains a subgenomic expression cassette encoding structural proteins for virion production (Frolov RNA 2001).

[00305] A model lifecycle of an alphavirus involves several distinct steps (Strauss Microbial Review 1994, Jose Future Microbiol 2009). Following virus attachment to a host cell, the virion fuses with membranes within endocytic compartments resulting in the eventual release of genomic RNA into the cytosol. The genomic RNA, which is in a plus-strand orientation and comprises a 5' methylguanylate cap and 3' polyA tail, is translated to produce non-structural proteins nsP1-4 that form the replication complex. Early in infection, the plus-strand is then replicated by the complex into a minus-strand template. In the current model, the replication complex is further processed as infection progresses, with the resulting processed complex switching to transcription of the minus-strand into both full-length positive-strand genomic RNA, as well as the 26S subgenomic positive-strand RNA containing the structural genes. Several conserved sequence elements (CSEs) of alphavirus have been identified to potentially play a role in the various RNA replication steps including; a complement of the 5' UTR in the replication of plus-strand RNAs from a minus-strand template, a 51-nt CSE in the replication of minus-strand synthesis from the genomic template, a 24-nt CSE in the junction region between the nsPs and the 26S RNA in the transcription of the subgenomic RNA from the minus-strand, and a 3' 19-nt CSE in minus-strand synthesis from the plus-strand template.

[00306] Following the replication of the various RNA species, virus particles are then typically assembled in the natural lifecycle of the virus. The 26S RNA is translated and the

resulting proteins further processed to produce the structural proteins including capsid protein, glycoproteins E1 and E2, and two small polypeptides E3 and 6K (Strauss 1994). Encapsulation of viral RNA occurs, with capsid proteins normally specific for only genomic RNA being packaged, followed by virion assembly and budding at the membrane surface.

V.D.2. Alphavirus as a delivery vector

[00307] Alphaviruses (including alphavirus sequences, features, and other elements) can be used to generate alphavirus-based delivery vectors (also be referred to as alphavirus vectors, alphavirus viral vectors, alphavirus vaccine vectors, self-replicating RNA (srRNA) vectors, or self-amplifying RNA (samRNA) vectors). Alphaviruses have previously been engineered for use as expression vector systems (Pushko 1997, Rheme 2004). Alphaviruses offer several advantages, particularly in a vaccine setting where heterologous antigen expression can be desired. Due to its ability to self-replicate in the host cytosol, alphavirus vectors are generally able to produce high copy numbers of the expression cassette within a cell resulting in a high level of heterologous antigen production. Additionally, the vectors are generally transient, resulting in improved biosafety as well as reduced induction of immunological tolerance to the vector. The public, in general, also lacks pre-existing immunity to alphavirus vectors as compared to other standard viral vectors, such as human adenovirus. Alphavirus based vectors also generally result in cytotoxic responses to infected cells. Cytotoxicity, to a certain degree, can be important in a vaccine setting to properly illicit an immune response to the heterologous antigen expressed. However, the degree of desired cytotoxicity can be a balancing act, and thus several attenuated alphaviruses have been developed, including the TC-83 strain of VEE. Thus, an example of a antigen expression vector described herein can utilize an alphavirus backbone that allows for a high level of antigen expression, elicits a robust immune response to antigen, does not elicit an immune response to the vector itself, and can be used in a safe manner.

Furthermore, the antigen expression cassette can be designed to elicit different levels of an immune response through optimization of which alphavirus sequences the vector uses, including, but not limited to, sequences derived from VEE or its attenuated derivative TC-83.

[00308] Several expression vector design strategies have been engineered using alphavirus sequences (Pushko 1997). In one strategy, a alphavirus vector design includes inserting a second copy of the 26S promoter sequence elements downstream of the structural protein genes, followed by a heterologous gene (Frolov 1993). Thus, in addition to the natural non-structural and structural proteins, an additional subgenomic RNA is produced that expresses the

heterologous protein. In this system, all the elements for production of infectious virions are present and, therefore, repeated rounds of infection of the expression vector in non-infected cells can occur.

[00309] Another expression vector design makes use of helper virus systems (Pushko 1997). In this strategy, the structural proteins are replaced by a heterologous gene. Thus, following self-replication of viral RNA mediated by still intact non-structural genes, the 26S subgenomic RNA provides for expression of the heterologous protein. Traditionally, additional vectors that expresses the structural proteins are then supplied *in trans*, such as by co-transfection of a cell line, to produce infectious virus. A system is described in detail in USPN 8,093,021, which is herein incorporated by reference in its entirety, for all purposes. The helper vector system provides the benefit of limiting the possibility of forming infectious particles and, therefore, improves biosafety. In addition, the helper vector system reduces the total vector length, potentially improving the replication and expression efficiency. Thus, an example of a antigen expression vector described herein can utilize an alphavirus backbone wherein the structural proteins are replaced by a antigen cassette, the resulting vector both reducing biosafety concerns, while at the same time promoting efficient expression due to the reduction in overall expression vector size.

V.D.3. Alphavirus production *in vitro*

[00310] Alphavirus delivery vectors are generally positive-sense RNA polynucleotides. A convenient technique well-known in the art for RNA production is *in vitro* transcription IVT. In this technique, a DNA template of the desired vector is first produced by techniques well-known to those in the art, including standard molecular biology techniques such as cloning, restriction digestion, ligation, gene synthesis, and polymerase chain reaction (PCR). The DNA template contains a RNA polymerase promoter at the 5' end of the sequence desired to be transcribed into RNA. Promoters include, but are not limited to, bacteriophage polymerase promoters such as T3, T7, or SP6. The DNA template is then incubated with the appropriate RNA polymerase enzyme, buffer agents, and nucleotides (NTPs). The resulting RNA polynucleotide can optionally be further modified including, but limited to, addition of a 5' cap structure such as 7-methylguanosine or a related structure, and optionally modifying the 3' end to include a polyadenylate (polyA) tail. The RNA can then be purified using techniques well-known in the field, such as phenol-chloroform extraction.

V.D.4. Delivery via lipid nanoparticle

[00311] An important aspect to consider in vaccine vector design is immunity against the vector itself (Riley 2017). This may be in the form of preexisting immunity to the vector itself, such as with certain human adenovirus systems, or in the form of developing immunity to the vector following administration of the vaccine. The latter is an important consideration if multiple administrations of the same vaccine are performed, such as separate priming and boosting doses, or if the same vaccine vector system is to be used to deliver different antigen cassettes.

[00312] In the case of alphavirus vectors, the standard delivery method is the previously discussed helper virus system that provides capsid, E1, and E2 proteins *in trans* to produce infectious viral particles. However, it is important to note that the E1 and E2 proteins are often major targets of neutralizing antibodies (Strauss 1994). Thus, the efficacy of using alphavirus vectors to deliver antigens of interest to target cells may be reduced if infectious particles are targeted by neutralizing antibodies.

[00313] An alternative to viral particle mediated gene delivery is the use of nanomaterials to deliver expression vectors (Riley 2017). Nanomaterial vehicles, importantly, can be made of non-immunogenic materials and generally avoid eliciting immunity to the delivery vector itself. These materials can include, but are not limited to, lipids, inorganic nanomaterials, and other polymeric materials. Lipids can be cationic, anionic, or neutral. The materials can be synthetic or naturally derived, and in some instances biodegradable. Lipids can include fats, cholesterol, phospholipids, lipid conjugates including, but not limited to, polyethyleneglycol (PEG) conjugates (PEGylated lipids), waxes, oils, glycerides, and fat soluble vitamins.

[00314] Lipid nanoparticles (LNPs) are an attractive delivery system due to the amphiphilic nature of lipids enabling formation of membranes and vesicle like structures (Riley 2017). In general, these vesicles deliver the expression vector by absorbing into the membrane of target cells and releasing nucleic acid into the cytosol. In addition, LNPs can be further modified or functionalized to facilitate targeting of specific cell types. Another consideration in LNP design is the balance between targeting efficiency and cytotoxicity. Lipid compositions generally include defined mixtures of cationic, neutral, anionic, and amphipathic lipids. In some instances, specific lipids are included to prevent LNP aggregation, prevent lipid oxidation, or provide functional chemical groups that facilitate attachment of additional moieties. Lipid composition can influence overall LNP size and stability. In an example, the lipid composition comprises dilinoleylmethyl- 4-dimethylaminobutyrate (MC3) or MC3-like molecules. MC3

and MC3-like lipid compositions can be formulated to include one or more other lipids, such as a PEG or PEG-conjugated lipid, a sterol, or neutral lipids.

[00315] Nucleic-acid vectors, such as expression vectors, exposed directly to serum can have several undesirable consequences, including degradation of the nucleic acid by serum nucleases or off-target stimulation of the immune system by the free nucleic acids. Therefore, encapsulation of the alphavirus vector can be used to avoid degradation, while also avoiding potential off-target effects. In certain examples, an alphavirus vector is fully encapsulated within the delivery vehicle, such as within the aqueous interior of an LNP. Encapsulation of the alphavirus vector within an LNP can be carried out by techniques well-known to those skilled in the art, such as microfluidic mixing and droplet generation carried out on a microfluidic droplet generating device. Such devices include, but are not limited to, standard T-junction devices or flow-focusing devices. In an example, the desired lipid formulation, such as MC3 or MC3-like containing compositions, is provided to the droplet generating device in parallel with the alphavirus delivery vector and other desired agents, such that the delivery vector and desired agents are fully encapsulated within the interior of the MC3 or MC3-like based LNP. In an example, the droplet generating device can control the size range and size distribution of the LNPs produced. For example, the LNP can have a size ranging from 1 to 1000 nanometers in diameter, e.g., 1, 10, 50, 100, 500, or 1000 nanometers. Following droplet generation, the delivery vehicles encapsulating the expression vectors can be further treated or modified to prepare them for administration.

V.E. Chimpanzee adenovirus (ChAd)

V.E.1. Viral delivery with chimpanzee adenovirus

[00316] Vaccine compositions for delivery of one or more antigens (e.g., via a antigen cassette and including one or more neoantigens) can be created by providing adenovirus nucleotide sequences of chimpanzee origin, a variety of novel vectors, and cell lines expressing chimpanzee adenovirus genes. A nucleotide sequence of a chimpanzee C68 adenovirus (also referred to herein as ChAdV68) can be used in a vaccine composition for antigen delivery (*See* SEQ ID NO: 1). Use of C68 adenovirus derived vectors is described in further detail in USPN 6,083,716, which is herein incorporated by reference in its entirety, for all purposes.

[00317] In a further aspect, provided herein is a recombinant adenovirus comprising the DNA sequence of a chimpanzee adenovirus such as C68 and a antigen cassette operatively linked to regulatory sequences directing its expression. The recombinant virus is capable of

infecting a mammalian, preferably a human, cell and capable of expressing the antigen cassette product in the cell. In this vector, the native chimpanzee E1 gene, and/or E3 gene, and/or E4 gene can be deleted. A antigen cassette can be inserted into any of these sites of gene deletion. The antigen cassette can include a antigen against which a primed immune response is desired.

[00318] In another aspect, provided herein is a mammalian cell infected with a chimpanzee adenovirus such as C68.

[00319] In still a further aspect, a novel mammalian cell line is provided which expresses a chimpanzee adenovirus gene (e.g., from C68) or functional fragment thereof.

[00320] In still a further aspect, provided herein is a method for delivering a antigen cassette into a mammalian cell comprising the step of introducing into the cell an effective amount of a chimpanzee adenovirus, such as C68, that has been engineered to express the antigen cassette.

[00321] Still another aspect provides a method for eliciting an immune response in a mammalian host to treat cancer. The method can comprise the step of administering to the host an effective amount of a recombinant chimpanzee adenovirus, such as C68, comprising a antigen cassette that encodes one or more antigens from the tumor against which the immune response is targeted.

[00322] Also disclosed is a non-simian mammalian cell that expresses a chimpanzee adenovirus gene obtained from the sequence of SEQ ID NO: 1. The gene can be selected from the group consisting of the adenovirus E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4 and L5 of SEQ ID NO: 1.

[00323] Also disclosed is a nucleic acid molecule comprising a chimpanzee adenovirus DNA sequence comprising a gene obtained from the sequence of SEQ ID NO: 1. The gene can be selected from the group consisting of said chimpanzee adenovirus E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4 and L5 genes of SEQ ID NO: 1. In some aspects the nucleic acid molecule comprises SEQ ID NO: 1. In some aspects the nucleic acid molecule comprises the sequence of SEQ ID NO: 1, lacking at least one gene selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4 and L5 genes of SEQ ID NO: 1.

[00324] Also disclosed is a vector comprising a chimpanzee adenovirus DNA sequence obtained from SEQ ID NO: 1 and a antigen cassette operatively linked to one or more regulatory sequences which direct expression of the cassette in a heterologous host cell, optionally wherein the chimpanzee adenovirus DNA sequence comprises at least the *cis*-

elements necessary for replication and virion encapsidation, the *cis*-elements flanking the antigen cassette and regulatory sequences. In some aspects, the chimpanzee adenovirus DNA sequence comprises a gene selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4 and L5 gene sequences of SEQ ID NO: 1. In some aspects the vector can lack the E1A and/or E1B gene.

[00325] Also disclosed herein is a host cell transfected with a vector disclosed herein such as a C68 vector engineered to expression a antigen cassette. Also disclosed herein is a human cell that expresses a selected gene introduced therein through introduction of a vector disclosed herein into the cell.

[00326] Also disclosed herein is a method for delivering a antigen cassette to a mammalian cell comprising introducing into said cell an effective amount of a vector disclosed herein such as a C68 vector engineered to expression the antigen cassette.

[00327] Also disclosed herein is a method for producing a antigen comprising introducing a vector disclosed herein into a mammalian cell, culturing the cell under suitable conditions and producing the antigen.

V.E.2. E1-Expressing Complementation Cell Lines

[00328] To generate recombinant chimpanzee adenoviruses (Ad) deleted in any of the genes described herein, the function of the deleted gene region, if essential to the replication and infectivity of the virus, can be supplied to the recombinant virus by a helper virus or cell line, i.e., a complementation or packaging cell line. For example, to generate a replication-defective chimpanzee adenovirus vector, a cell line can be used which expresses the E1 gene products of the human or chimpanzee adenovirus; such a cell line can include HEK293 or variants thereof. The protocol for the generation of the cell lines expressing the chimpanzee E1 gene products (Examples 3 and 4 of USPN 6,083,716) can be followed to generate a cell line which expresses any selected chimpanzee adenovirus gene.

[00329] An AAV augmentation assay can be used to identify a chimpanzee adenovirus E1-expressing cell line. This assay is useful to identify E1 function in cell lines made by using the E1 genes of other uncharacterized adenoviruses, e.g., from other species. That assay is described in Example 4B of USPN 6,083,716.

[00330] A selected chimpanzee adenovirus gene, e.g., E1, can be under the transcriptional control of a promoter for expression in a selected parent cell line. Inducible or constitutive promoters can be employed for this purpose. Among inducible promoters are included the sheep metallothioneine promoter, inducible by zinc, or the mouse mammary tumor virus (MMTV) promoter, inducible by a glucocorticoid, particularly, dexamethasone. Other inducible promoters, such as those identified in International patent application WO95/13392, incorporated by reference herein can also be used in the production of packaging cell lines. Constitutive promoters in control of the expression of the chimpanzee adenovirus gene can be employed also.

[00331] A parent cell can be selected for the generation of a novel cell line expressing any desired C68 gene. Without limitation, such a parent cell line can be HeLa [ATCC Accession No. CCL 2], A549 [ATCC Accession No. CCL 185], KB [CCL 17], Detroit [e.g., Detroit 510, CCL 72] and WI-38 [CCL 75] cells. Other suitable parent cell lines can be obtained from other sources. Parent cell lines can include CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a.

[00332] An E1-expressing cell line can be useful in the generation of recombinant chimpanzee adenovirus E1 deleted vectors. Cell lines constructed using essentially the same procedures that express one or more other chimpanzee adenoviral gene products are useful in

the generation of recombinant chimpanzee adenovirus vectors deleted in the genes that encode those products. Further, cell lines which express other human Ad E1 gene products are also useful in generating chimpanzee recombinant Ads.

V.E.3. Recombinant Viral Particles as Vectors

[00333] The compositions disclosed herein can comprise viral vectors, that deliver at least one antigen to cells. Such vectors comprise a chimpanzee adenovirus DNA sequence such as C68 and a antigen cassette operatively linked to regulatory sequences which direct expression of the cassette. The C68 vector is capable of expressing the cassette in an infected mammalian cell. The C68 vector can be functionally deleted in one or more viral genes. A antigen cassette comprises at least one antigen under the control of one or more regulatory sequences such as a promoter. Optional helper viruses and/or packaging cell lines can supply to the chimpanzee viral vector any necessary products of deleted adenoviral genes.

[00334] The term "functionally deleted" means that a sufficient amount of the gene region is removed or otherwise altered, e.g., by mutation or modification, so that the gene region is no longer capable of producing one or more functional products of gene expression. Mutations or modifications that can result in functional deletions include, but are not limited to, nonsense mutations such as introduction of premature stop codons and removal of canonical and non-canonical start codons, mutations that alter mRNA splicing or other transcriptional processing, or combinations thereof. If desired, the entire gene region can be removed.

[00335] Modifications of the nucleic acid sequences forming the vectors disclosed herein, including sequence deletions, insertions, and other mutations may be generated using standard molecular biological techniques and are within the scope of this invention.

V.E.4. Construction of The Viral Plasmid Vector

[00336] The chimpanzee adenovirus C68 vectors useful in this invention include recombinant, defective adenoviruses, that is, chimpanzee adenovirus sequences functionally deleted in the E1a or E1b genes, and optionally bearing other mutations, e.g., temperature-sensitive mutations or deletions in other genes. It is anticipated that these chimpanzee sequences are also useful in forming hybrid vectors from other adenovirus and/or adeno-associated virus sequences. Homologous adenovirus vectors prepared from human adenoviruses are described in the published literature [see, for example, Kozarsky I and II, cited above. and references cited therein, U.S. Pat. No. 5,240,846].

[00337] In the construction of useful chimpanzee adenovirus C68 vectors for delivery of a antigen cassette to a human (or other mammalian) cell, a range of adenovirus nucleic acid sequences can be employed in the vectors. A vector comprising minimal chimpanzee C68 adenovirus sequences can be used in conjunction with a helper virus to produce an infectious recombinant virus particle. The helper virus provides essential gene products required for viral infectivity and propagation of the minimal chimpanzee adenoviral vector. When only one or more selected deletions of chimpanzee adenovirus genes are made in an otherwise functional viral vector, the deleted gene products can be supplied in the viral vector production process by propagating the virus in a selected packaging cell line that provides the deleted gene functions in trans.

V.E.5. Recombinant Minimal Adenovirus

[00338] A minimal chimpanzee Ad C68 virus is a viral particle containing just the adenovirus cis-elements necessary for replication and virion encapsidation. That is, the vector contains the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of the adenoviruses (which function as origins of replication) and the native 5' packaging/enhancer domains (that contain sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter). See, for example, the techniques described for preparation of a "minimal" human Ad vector in International Patent Application WO96/13597 and incorporated herein by reference.

V.E.6. Other Defective Adenoviruses

[00339] Recombinant, replication-deficient adenoviruses can also contain more than the minimal chimpanzee adenovirus sequences. These other Ad vectors can be characterized by deletions of various portions of gene regions of the virus, and infectious virus particles formed by the optional use of helper viruses and/or packaging cell lines.

[00340] As one example, suitable vectors may be formed by deleting all or a sufficient portion of the C68 adenoviral immediate early gene E1a and delayed early gene E1b, so as to eliminate their normal biological functions. Replication-defective E1-deleted viruses are capable of replicating and producing infectious virus when grown on a chimpanzee adenovirus-transformed, complementation cell line containing functional adenovirus E1a and E1b genes which provide the corresponding gene products in trans. Based on the homologies to known adenovirus sequences, it is anticipated that, as is true for the human recombinant E1-deleted adenoviruses of the art, the resulting recombinant chimpanzee adenovirus is capable of

infecting many cell types and can express antigen(s), but cannot replicate in most cells that do not carry the chimpanzee E1 region DNA unless the cell is infected at a very high multiplicity of infection.

[00341] As another example, all or a portion of the C68 adenovirus delayed early gene E3 can be eliminated from the chimpanzee adenovirus sequence which forms a part of the recombinant virus.

[00342] Chimpanzee adenovirus C68 vectors can also be constructed having a deletion of the E4 gene. Still another vector can contain a deletion in the delayed early gene E2a.

[00343] Deletions can also be made in any of the late genes L1 through L5 of the chimpanzee C68 adenovirus genome. Similarly, deletions in the intermediate genes IX and IVa2 can be useful for some purposes. Other deletions may be made in the other structural or non-structural adenovirus genes.

[00344] The above discussed deletions can be used individually, i.e., an adenovirus sequence can contain deletions of E1 only. Alternatively, deletions of entire genes or portions thereof effective to destroy or reduce their biological activity can be used in any combination. For example, in one exemplary vector, the adenovirus C68 sequence can have deletions of the E1 genes and the E4 gene, or of the E1, E2a and E3 genes, or of the E1 and E3 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on. As discussed above, such deletions can be used in combination with other mutations, such as temperature-sensitive mutations, to achieve a desired result.

[00345] The cassette comprising antigen(s) be inserted optionally into any deleted region of the chimpanzee C68 Ad virus. Alternatively, the cassette can be inserted into an existing gene region to disrupt the function of that region, if desired.

V.E.7. Helper Viruses

[00346] Depending upon the chimpanzee adenovirus gene content of the viral vectors employed to carry the antigen cassette, a helper adenovirus or non-replicating virus fragment can be used to provide sufficient chimpanzee adenovirus gene sequences to produce an infective recombinant viral particle containing the cassette.

[00347] Useful helper viruses contain selected adenovirus gene sequences not present in the adenovirus vector construct and/or not expressed by the packaging cell line in which the vector is transfected. A helper virus can be replication-defective and contain a variety of adenovirus

genes in addition to the sequences described above. The helper virus can be used in combination with the E1-expressing cell lines described herein.

[00348] For C68, the "helper" virus can be a fragment formed by clipping the C terminal end of the C68 genome with SspI, which removes about 1300 bp from the left end of the virus. This clipped virus is then co-transfected into an E1-expressing cell line with the plasmid DNA, thereby forming the recombinant virus by homologous recombination with the C68 sequences in the plasmid.

[00349] Helper viruses can also be formed into poly-cation conjugates as described in Wu et al, J. Biol. Chem., 264:16985-16987 (1989); K. J. Fisher and J. M. Wilson, Biochem. J., 299:49 (Apr. 1, 1994). Helper virus can optionally contain a reporter gene. A number of such reporter genes are known to the art. The presence of a reporter gene on the helper virus which is different from the antigen cassette on the adenovirus vector allows both the Ad vector and the helper virus to be independently monitored. This second reporter is used to enable separation between the resulting recombinant virus and the helper virus upon purification.

V.E.8. Assembly of Viral Particle and Infection of a Cell Line

[00350] Assembly of the selected DNA sequences of the adenovirus, the antigen cassette, and other vector elements into various intermediate plasmids and shuttle vectors, and the use of the plasmids and vectors to produce a recombinant viral particle can all be achieved using conventional techniques. Such techniques include conventional cloning techniques of cDNA, in vitro recombination techniques (e.g., Gibson assembly), use of overlapping oligonucleotide sequences of the adenovirus genomes, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO₄ precipitation techniques or liposome-mediated transfection methods such as lipofectamine. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

[00351] For example, following the construction and assembly of the desired antigen cassette-containing viral vector, the vector can be transfected in vitro in the presence of a helper virus into the packaging cell line. Homologous recombination occurs between the helper and the vector sequences, which permits the adenovirus-antigen sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant viral vector particles.

[00352] The resulting recombinant chimpanzee C68 adenoviruses are useful in transferring a antigen cassette to a selected cell. In in vivo experiments with the recombinant virus grown in the packaging cell lines, the E1-deleted recombinant chimpanzee adenovirus demonstrates utility in transferring a cassette to a non-chimpanzee, preferably a human, cell.

V.E.9. Use of the Recombinant Virus Vectors

[00353] The resulting recombinant chimpanzee C68 adenovirus containing the antigen cassette (produced by cooperation of the adenovirus vector and helper virus or adenoviral vector and packaging cell line, as described above) thus provides an efficient gene transfer vehicle which can deliver antigen(s) to a subject in vivo or ex vivo.

[00354] The above-described recombinant vectors are administered to humans according to published methods for gene therapy. A chimpanzee viral vector bearing a antigen cassette can be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

[00355] The chimpanzee adenoviral vectors are administered in sufficient amounts to transduce the human cells and to provide sufficient levels of antigen transfer and expression to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the liver, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

[00356] Dosages of the viral vector will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. The dosage will be adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed. The levels of expression of antigen(s) can be monitored to determine the frequency of dosage administration.

[00357] Recombinant, replication defective adenoviruses can be administered in a "pharmaceutically effective amount", that is, an amount of recombinant adenovirus that is effective in a route of administration to transfect the desired cells and provide sufficient levels

of expression of the selected gene to provide a vaccinal benefit, i.e., some measurable level of protective immunity. C68 vectors comprising a antigen cassette can be co-administered with adjuvant. Adjuvant can be separate from the vector (e.g., alum) or encoded within the vector, in particular if the adjuvant is a protein. Adjuvants are well known in the art.

[00358] Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, intranasal, intramuscular, intratracheal, subcutaneous, intradermal, rectal, oral and other parental routes of administration. Routes of administration may be combined, if desired, or adjusted depending upon the immunogen or the disease. For example, in prophylaxis of rabies, the subcutaneous, intratracheal and intranasal routes are preferred. The route of administration primarily will depend on the nature of the disease being treated.

[00359] The levels of immunity to antigen(s) can be monitored to determine the need, if any, for boosters. Following an assessment of antibody titers in the serum, for example, optional booster immunizations may be desired

VI. Therapeutic and Manufacturing Methods

[00360] Also provided is a method of inducing a tumor specific immune response in a subject, vaccinating against a tumor, treating and or alleviating a symptom of cancer in a subject by administering to the subject one or more antigens such as a plurality of antigens identified using methods disclosed herein.

[00361] In some aspects, a subject has been diagnosed with cancer or is at risk of developing cancer. A subject can be a human, dog, cat, horse or any animal in which a tumor specific immune response is desired. A tumor can be any solid tumor such as breast, ovarian, prostate, lung, kidney, gastric, colon, testicular, head and neck, pancreas, brain, melanoma, and other tumors of tissue organs and hematological tumors, such as lymphomas and leukemias, including acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, T cell lymphocytic leukemia, and B cell lymphomas.

[00362] A antigen can be administered in an amount sufficient to induce a CTL response.

[00363] A antigen can be administered alone or in combination with other therapeutic agents. The therapeutic agent is for example, a chemotherapeutic agent, radiation, or immunotherapy. Any suitable therapeutic treatment for a particular cancer can be administered.

[00364] In addition, a subject can be further administered an anti-immunosuppressive/immunostimulatory agent such as a checkpoint inhibitor. For example, the subject can be further administered an anti-CTLA antibody or anti-PD-1 or anti-PD-L1.

Blockade of CTLA-4 or PD-L1 by antibodies can enhance the immune response to cancerous cells in the patient. In particular, CTLA-4 blockade has been shown effective when following a vaccination protocol.

[00365] The optimum amount of each antigen to be included in a vaccine composition and the optimum dosing regimen can be determined. For example, a antigen or its variant can be prepared for intravenous (i.v.) injection, sub-cutaneous (s.c.) injection, intradermal (i.d.) injection, intraperitoneal (i.p.) injection, intramuscular (i.m.) injection. Methods of injection include s.c., i.d., i.p., i.m., and i.v. Methods of DNA or RNA injection include i.d., i.m., s.c., i.p. and i.v. Other methods of administration of the vaccine composition are known to those skilled in the art.

[00366] A vaccine can be compiled so that the selection, number and/or amount of antigens present in the composition is/are tissue, cancer, and/or patient-specific. For instance, the exact selection of peptides can be guided by expression patterns of the parent proteins in a given tissue or guided by mutation status of a patient. The selection can be dependent on the specific type of cancer, the status of the disease, earlier treatment regimens, the immune status of the patient, and, of course, the HLA-haplotype of the patient. Furthermore, a vaccine can contain individualized components, according to personal needs of the particular patient. Examples include varying the selection of antigens according to the expression of the antigen in the particular patient or adjustments for secondary treatments following a first round or scheme of treatment.

[00367] A patient can be identified for administration of an antigen vaccine through the use of various diagnostic methods, e.g., patient selection methods described further below. Patient selection can involve identifying mutations in, or expression patterns of, one or more genes. In some cases, patient selection involves identifying the haplotype of the patient. The various patient selection methods can be performed in parallel, *e.g.*, a sequencing diagnostic can identify both the mutations and the haplotype of a patient. The various patient selection methods can be performed sequentially, *e.g.*, one diagnostic test identifies the mutations and separate diagnostic test identifies the haplotype of a patient, and where each test can be the same (*e.g.*, both high-throughput sequencing) or different (*e.g.*, one high-throughput sequencing and the other Sanger sequencing) diagnostic methods.

[00368] For a composition to be used as a vaccine for cancer, antigens with similar normal self-peptides that are expressed in high amounts in normal tissues can be avoided or be present in low amounts in a composition described herein. On the other hand, if it is known that the

tumor of a patient expresses high amounts of a certain antigen, the respective pharmaceutical composition for treatment of this cancer can be present in high amounts and/or more than one antigen specific for this particular antigen or pathway of this antigen can be included.

[00369] Compositions comprising an antigen can be administered to an individual already suffering from cancer. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the tumor antigen and to cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician. It should be kept in mind that compositions can generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations, especially when the cancer has metastasized. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of an antigen, it is possible and can be felt desirable by the treating physician to administer substantial excesses of these compositions.

[00370] For therapeutic use, administration can begin at the detection or surgical removal of tumors. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter.

[00371] The pharmaceutical compositions (e.g., vaccine compositions) for therapeutic treatment are intended for parenteral, topical, nasal, oral or local administration. A pharmaceutical composition can be administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. The compositions can be administered at the site of surgical excision to induce a local immune response to the tumor. Disclosed herein are compositions for parenteral administration which comprise a solution of the antigen and vaccine compositions are dissolved or suspended in an acceptable carrier, e.g., an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions can be sterilized by conventional, well known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. 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, triethanolamine oleate, etc.

[00372] Antigens can also be administered via liposomes, which target them to a particular cells tissue, such as lymphoid tissue. Liposomes are also useful in increasing half-life. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the antigen to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired antigen can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic compositions. Liposomes can be formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9; 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,501,728, 4,837,028, and 5,019,369.

[00373] For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension can be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

[00374] For therapeutic or immunization purposes, nucleic acids encoding a peptide and optionally one or more of the peptides described herein can also be administered to the patient. A number of methods are conveniently used to deliver the nucleic acids to the patient. For instance, the nucleic acid can be delivered directly, as "naked DNA". This approach is described, for instance, in Wolff et al., *Science* 247: 1465-1468 (1990) as well as U.S. Pat. Nos. 5,580,859 and 5,589,466. The nucleic acids can also be administered using ballistic delivery as described, for instance, in U.S. Pat. No. 5,204,253. Particles comprised solely of DNA can be administered. Alternatively, DNA can be adhered to particles, such as gold particles. Approaches for delivering nucleic acid sequences can include viral vectors, mRNA vectors, and DNA vectors with or without electroporation.

[00375] The nucleic acids can also be delivered complexed to cationic compounds, such as cationic lipids. Lipid-mediated gene delivery methods are described, for instance, in 9618372WOAWO 96/18372; 9324640WOAWO 93/24640; Mannino & Gould-Fogerite, *BioTechniques* 6(7): 682-691 (1988); U.S. Pat. No. 5,279,833 Rose U.S. Pat. No. 5,279,833; 9106309WOAWO 91/06309; and Felgner et al., *Proc. Natl. Acad. Sci. USA* 84: 7413-7414 (1987).

[00376] Antigens can also be included in viral vector-based vaccine platforms, such as vaccinia, fowlpox, self-replicating alphavirus, marabavirus, adenovirus (*See, e.g.,* Tatsis et al., *Adenoviruses, Molecular Therapy* (2004) 10, 616—629), or lentivirus, including but not limited to second, third or hybrid second/third generation lentivirus and recombinant lentivirus of any generation designed to target specific cell types or receptors (*See, e.g.,* Hu et al., *Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases, Immunol Rev.* (2011) 239(1): 45-61, Sakuma et al., *Lentiviral vectors: basic to translational, Biochem J.* (2012) 443(3):603-18, Cooper et al., *Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter, Nucl. Acids Res.* (2015) 43 (1): 682-690, Zufferey et al., *Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery, J. Virol.* (1998) 72 (12): 9873-9880). Dependent on the packaging capacity of the above mentioned viral vector-based vaccine platforms, this approach can deliver one or more nucleotide sequences that encode one or more antigen peptides. The sequences may be flanked by non-mutated sequences, may be separated by linkers or may be preceded with one or more sequences targeting a subcellular compartment (*See, e.g.,* Gros et al., *Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients, Nat Med.* (2016) 22 (4):433-8, Stronen et al., *Targeting of cancer neoantigens with donor-derived T cell receptor repertoires, Science.* (2016) 352 (6291):1337-41, Lu et al., *Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions, Clin Cancer Res.* (2014) 20(13):3401-10). Upon introduction into a host, infected cells express the antigens, and thereby elicit a host immune (e.g., CTL) response against the peptide(s). Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Pat. No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)). A wide variety of other vaccine vectors useful for therapeutic administration or immunization of antigens, e.g., *Salmonella typhi* vectors, and the like will be apparent to those skilled in the art from the description herein.

[00377] A means of administering nucleic acids uses minigene constructs encoding one or multiple epitopes. To create a DNA sequence encoding the selected CTL epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes are reverse translated. A human codon usage table is used to guide the codon choice for each amino acid. These epitope-encoding DNA sequences are directly adjoined, creating a continuous polypeptide sequence. To optimize expression and/or immunogenicity, additional elements can be incorporated into the minigene design. Examples of amino acid sequence that could be reverse translated and included in the minigene sequence include: helper T lymphocyte, epitopes, a leader (signal) sequence, and an endoplasmic reticulum retention signal. In addition, MHC presentation of CTL epitopes can be improved by including synthetic (e.g. poly-alanine) or naturally-occurring flanking sequences adjacent to the CTL epitopes. The minigene sequence is converted to DNA by assembling oligonucleotides that encode the plus and minus strands of the minigene. Overlapping oligonucleotides (30-100 bases long) are synthesized, phosphorylated, purified and annealed under appropriate conditions using well known techniques. The ends of the oligonucleotides are joined using T4 DNA ligase. This synthetic minigene, encoding the CTL epitope polypeptide, can then be cloned into a desired expression vector.

[00378] Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). A variety of methods have been described, and new techniques can become available. As noted above, nucleic acids are conveniently formulated with cationic lipids. In addition, glycolipids, fusogenic liposomes, peptides and compounds referred to collectively as protective, interactive, non-condensing (PINC) could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

[00379] Also disclosed is a method of manufacturing a tumor vaccine, comprising performing the steps of a method disclosed herein; and producing a tumor vaccine comprising a plurality of antigens or a subset of the plurality of antigens.

[00380] Antigens disclosed herein can be manufactured using methods known in the art. For example, a method of producing an antigen or a vector (e.g., a vector including at least one sequence encoding one or more antigens) disclosed herein can include culturing a host cell under conditions suitable for expressing the antigen or vector wherein the host cell comprises at least one polynucleotide encoding the antigen or vector, and purifying the antigen or vector.

Standard purification methods include chromatographic techniques, electrophoretic, immunological, precipitation, dialysis, filtration, concentration, and chromatofocusing techniques.

[00381] Host cells can include a Chinese Hamster Ovary (CHO) cell, NS0 cell, yeast, or a HEK293 cell. Host cells can be transformed with one or more polynucleotides comprising at least one nucleic acid sequence that encodes a antigen or vector disclosed herein, optionally wherein the isolated polynucleotide further comprises a promoter sequence operably linked to the at least one nucleic acid sequence that encodes the antigen or vector. In certain embodiments the isolated polynucleotide can be cDNA.

VII. Antigen Use and Administration

[00382] A vaccination protocol can be used to dose a subject with one or more antigens. A priming vaccine and a boosting vaccine can be used to dose the subject. The priming vaccine can be based on C68 (e.g., the sequences shown in SEQ ID NO:1 or 2) or srRNA (e.g., the sequences shown in SEQ ID NO:3 or 4) and the boosting vaccine can be based on C68 (e.g., the sequences shown in SEQ ID NO:1 or 2) or srRNA (e.g., the sequences shown in SEQ ID NO:3 or 4). Each vector typically includes a cassette that includes antigens. Cassettes can include about 20 antigens, separated by spacers such as the natural sequence that normally surrounds each antigen or other non-natural spacer sequences such as AAY. Cassettes can also include MHCII antigens such a tetanus toxoid antigen and PADRE antigen, which can be considered universal class II antigens. Cassettes can also include a targeting sequence such as a ubiquitin targeting sequence. In addition, each vaccine dose can be administered to the subject in conjunction with (e.g., concurrently, before, or after) a checkpoint inhibitor (CPI). CPI's can include those that inhibit CTLA4, PD1, and/or PDL1 such as antibodies or antigen-binding portions thereof. Such antibodies can include tremelimumab or durvalumab.

[00383] A priming vaccine can be injected (e.g., intramuscularly) in a subject. Bilateral injections per dose can be used. For example, one or more injections of ChAdV68 (C68) can be used (e.g., total dose 1×10^{12} viral particles); one or more injections of self-replicating RNA (srRNA) at low vaccine dose selected from the range 0.001 to 1 ug RNA, in particular 0.1 or 1 ug can be used; or one or more injections of srRNA at high vaccine dose selected from the range 1 to 100 ug RNA, in particular 10 or 100 ug can be used.

[00384] A vaccine boost (boosting vaccine) can be injected (e.g., intramuscularly) after prime vaccination. A boosting vaccine can be administered about every 1, 2, 3, 4, 5, 6, 7, 8, 9,

or 10 weeks, e.g., every 4 weeks and/or 8 weeks after the prime. Bilateral injections per dose can be used. For example, one or more injections of ChAdV68 (C68) can be used (e.g., total dose 1×10^{12} viral particles); one or more injections of self-replicating RNA (srRNA) at low vaccine dose selected from the range 0.001 to 1 ug RNA, in particular 0.1 or 1 ug can be used; or one or more injections of srRNA at high vaccine dose selected from the range 1 to 100 ug RNA, in particular 10 or 100 ug can be used.

[00385] Anti-CTLA-4 (e.g., tremelimumab) can also be administered to the subject. For example, anti-CTLA4 can be administered subcutaneously near the site of the intramuscular vaccine injection (ChAdV68 prime or srRNA low doses) to ensure drainage into the same lymph node. Tremelimumab is a selective human IgG2 mAb inhibitor of CTLA-4. Target Anti-CTLA-4 (tremelimumab) subcutaneous dose is typically 70-75 mg (in particular 75 mg) with a dose range of, e.g., 1-100 mg or 5-420 mg.

[00386] In certain instances an anti-PD-L1 antibody can be used such as durvalumab (MEDI 4736). Durvalumab is a selective, high affinity human IgG1 mAb that blocks PD-L1 binding to PD-1 and CD80. Durvalumab is generally administered at 20 mg/kg i.v. every 4 weeks.

[00387] Immune monitoring can be performed before, during, and/or after vaccine administration. Such monitoring can inform safety and efficacy, among other parameters.

[00388] To perform immune monitoring, PBMCs are commonly used. PBMCs can be isolated before prime vaccination, and after prime vaccination (e.g. 4 weeks and 8 weeks). PBMCs can be harvested just prior to boost vaccinations and after each boost vaccination (e.g. 4 weeks and 8 weeks).

[00389] T cell responses can be assessed as part of an immune monitoring protocol. T cell responses can be measured using one or more methods known in the art such as ELISpot, intracellular cytokine staining, cytokine secretion and cell surface capture, T cell proliferation, MHC multimer staining, or by cytotoxicity assay. T cell responses to epitopes encoded in vaccines can be monitored from PBMCs by measuring induction of cytokines, such as IFN-gamma, using an ELISpot assay. Specific CD4 or CD8 T cell responses to epitopes encoded in vaccines can be monitored from PBMCs by measuring induction of cytokines captured intracellularly or extracellularly, such as IFN-gamma, using flow cytometry. Specific CD4 or CD8 T cell responses to epitopes encoded in the vaccines can be monitored from PBMCs by measuring T cell populations expressing T cell receptors specific for epitope/MHC class I complexes using MHC multimer staining. Specific CD4 or CD8 T cell responses to epitopes encoded in the vaccines can be monitored from PBMCs by measuring the ex vivo expansion of

T cell populations following 3H-thymidine, bromodeoxyuridine and carboxyfluoresceine-diacetate– succinimidylester (CFSE) incorporation. The antigen recognition capacity and lytic activity of PBMC-derived T cells that are specific for epitopes encoded in vaccines can be assessed functionally by chromium release assay or alternative colorimetric cytotoxicity assays.

VIII. Antigen Identification

VIII.A. Antigen Candidate Identification

[00390] Research methods for NGS analysis of tumor and normal exome and transcriptomes have been described and applied in the antigen identification space.^{6,14,15} Certain optimizations for greater sensitivity and specificity for antigen identification in the clinical setting can be considered. These optimizations can be grouped into two areas, those related to laboratory processes and those related to the NGS data analysis. Examples of optimizations are known to those skilled in the art, for example the methods described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00391]

VIII.B. Isolation and Detection of HLA Peptides

[00392] Isolation of HLA-peptide molecules was performed using classic immunoprecipitation (IP) methods after lysis and solubilization of the tissue sample (55-58). A clarified lysate was used for HLA specific IP.

[00393] Immunoprecipitation was performed using antibodies coupled to beads where the antibody is specific for HLA molecules. For a pan-Class I HLA immunoprecipitation, a pan-Class I CR antibody is used, for Class II HLA – DR, an HLA-DR antibody is used. Antibody is covalently attached to NHS-sepharose beads during overnight incubation. After covalent attachment, the beads were washed and aliquoted for IP. (59, 60) Immunoprecipitations can also be performed with antibodies that are not covalently attached to beads. Typically this is done using sepharose or magnetic beads coated with Protein A and/or Protein G to hold the antibody to the column. Some antibodies that can be used to selectively enrich MHC/peptide complex are listed below.

Antibody Name	Specificity
W6/32	Class I HLA-A, B, C
L243	Class II – HLA-DR
Tu36	Class II – HLA-DR
LN3	Class II – HLA-DR
Tu39	Class II – HLA-DR, DP, DQ

[00394] The clarified tissue lysate is added to the antibody beads for the immunoprecipitation. After immunoprecipitation, the beads are removed from the lysate and the lysate stored for additional experiments, including additional IPs. The IP beads are washed to remove non-specific binding and the HLA/peptide complex is eluted from the beads using standard techniques. The protein components are removed from the peptides using a molecular weight spin column or C18 fractionation. The resultant peptides are taken to dryness by SpeedVac evaporation and in some instances are stored at -20C prior to MS analysis.

[00395] Dried peptides are reconstituted in an HPLC buffer suitable for reverse phase chromatography and loaded onto a C-18 microcapillary HPLC column for gradient elution in a Fusion Lumos mass spectrometer (Thermo). MS1 spectra of peptide mass/charge (m/z) were collected in the Orbitrap detector at high resolution followed by MS2 low resolution scans collected in the ion trap detector after HCD fragmentation of the selected ion. Additionally, MS2 spectra can be obtained using either CID or ETD fragmentation methods or any combination of the three techniques to attain greater amino acid coverage of the peptide. MS2 spectra can also be measured with high resolution mass accuracy in the Orbitrap detector.

[00396] MS2 spectra from each analysis are searched against a protein database using Comet (61, 62) and the peptide identification are scored using Percolator (63-65). Additional sequencing is performed using PEAKS studio (Bioinformatics Solutions Inc.) and other search engines or sequencing methods can be used including spectral matching and *de novo* sequencing (97).

VIII.B.1. MS limit of detection studies in support of comprehensive HLA peptide sequencing.

[00397] Using the peptide YVYVADVAAK it was determined what the limits of detection are using different amounts of peptide loaded onto the LC column. The amounts of peptide tested were 1 pmol, 100fmol, 10 fmol, 1 fmol, and 100amol. (Table 1) The results are shown in Fig. 24A and 24B. These results indicate that the lowest limit of detection (LoD) is in the attomol range (10^{-18}), that the dynamic range spans five orders of magnitude, and that the

signal to noise appears sufficient for sequencing at low femtomol ranges (10^{-15}). Mass spectrometry can be used in conjunction with prediction algorithms described herein to validate HLA presentation. For example, mass spectrometry can be used to validate epitope candidates generated by EDGE prediction model (a deep learning model trained on HLA presented peptides sequenced by MS/MS, as described in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856). An example of the correlation between EDGE score and the probability of detection of candidate shared neoantigen peptides by targeted MS is shown in Fig. 25.

Table 1

Peptide m/z	Loaded on Column	Copies/Cell in 1e9cells
566.830	1 pmol	600
562.823	100 fmol	60
559.816	10 fmol	6
556.810	1 fmol	0.6
553.802	100 amol	0.06

IX. Presentation Model

[00398] Presentation models can be used to identify likelihoods of peptide presentation in patients. Various presentation models are known to those skilled in the art, for example the presentation models described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, WO/2018/208856, WO2016187508, and US patent application US20110293637, each herein incorporated by reference, in their entirety, for all purposes.

X. Training Module

[00399] Training modules can be used to construct one or more presentation models based on training data sets that generate likelihoods of whether peptide sequences will be presented by MHC alleles associated with the peptide sequences. Various training modules are known to those skilled in the art, for example the presentation models described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes. A

training module can construct a presentation model to predict presentation likelihoods of peptides on a per-allele basis. A training module can also construct a presentation model to predict presentation likelihoods of peptides in a multiple-allele setting where two or more MHC alleles are present.

XI. Prediction Module

[00400] A prediction module can be used to receive sequence data and select candidate antigens in the sequence data using a presentation model. Specifically, the sequence data may be DNA sequences, RNA sequences, and/or protein sequences extracted from tumor tissue cells of patients. A prediction module may identify candidate neoantigens that are mutated peptide sequences by comparing sequence data extracted from normal tissue cells of a patient with the sequence data extracted from tumor tissue cells of the patient to identify portions containing one or more mutations. A prediction module may identify candidate antigens that have altered expression in a tumor cell or cancerous tissue in comparison to a normal cell or tissue by comparing sequence data extracted from normal tissue cells of a patient with the sequence data extracted from tumor tissue cells of the patient to identify improperly expressed candidate antigens.

[00401] A presentation module can apply one or more presentation model to processed peptide sequences to estimate presentation likelihoods of the peptide sequences. Specifically, the prediction module may select one or more candidate antigen peptide sequences that are likely to be presented on tumor HLA molecules by applying presentation models to the candidate antigens. In one implementation, the presentation module selects candidate antigen sequences that have estimated presentation likelihoods above a predetermined threshold. In another implementation, the presentation model selects the N candidate antigen sequences that have the highest estimated presentation likelihoods (where N is generally the maximum number of epitopes that can be delivered in a vaccine). A vaccine including the selected candidate antigens for a given patient can be injected into the patient to induce immune responses.

XI.B.Cassette Design Module

XI.B.1 Overview

[00402] A cassette design module can be used to generate a vaccine cassette sequence based on selected candidate peptides for injection into a patient. Various cassette design modules are known to those skilled in the art, for example the cassette design modules

described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00403] A set of therapeutic epitopes may be generated based on the selected peptides determined by a prediction module associated with presentation likelihoods above a predetermined threshold, where the presentation likelihoods are determined by the presentation models. However it is appreciated that in other embodiments, the set of therapeutic epitopes may be generated based on any one or more of a number of methods (alone or in combination), for example, based on binding affinity or predicted binding affinity to HLA class I or class II alleles of the patient, binding stability or predicted binding stability to HLA class I or class II alleles of the patient, random sampling, and the like.

[00404] Therapeutic epitopes may correspond to selected peptides themselves. Therapeutic epitopes may also include C- and/or N-terminal flanking sequences in addition to the selected peptides. N- and C-terminal flanking sequences can be the native N- and C-terminal flanking sequences of the therapeutic vaccine epitope in the context of its source protein. Therapeutic epitopes can represent a fixed-length epitope. Therapeutic epitopes can represent a variable-length epitope, in which the length of the epitope can be varied depending on, for example, the length of the C- or N-flanking sequence. For example, the C-terminal flanking sequence and the N-terminal flanking sequence can each have varying lengths of 2-5 residues, resulting in 16 possible choices for the epitope.

[00405] A cassette design module can also generate cassette sequences by taking into account presentation of junction epitopes that span the junction between a pair of therapeutic epitopes in the cassette. Junction epitopes are novel non-self but irrelevant epitope sequences that arise in the cassette due to the process of concatenating therapeutic epitopes and linker sequences in the cassette. The novel sequences of junction epitopes are different from the therapeutic epitopes of the cassette themselves.

[00406] A cassette design module can generate a cassette sequence that reduces the likelihood that junction epitopes are presented in the patient. Specifically, when the cassette is injected into the patient, junction epitopes have the potential to be presented by HLA class I or HLA class II alleles of the patient, and stimulate a CD8 or CD4 T-cell response, respectively. Such reactions are often times undesirable because T-cells reactive to the junction epitopes have no therapeutic benefit, and may diminish the immune response to the selected therapeutic epitopes in the cassette by antigenic competition.⁷⁶

[00407] A cassette design module can iterate through one or more candidate cassettes, and determine a cassette sequence for which a presentation score of junction epitopes associated with that cassette sequence is below a numerical threshold. The junction epitope presentation score is a quantity associated with presentation likelihoods of the junction epitopes in the cassette, and a higher value of the junction epitope presentation score indicates a higher likelihood that junction epitopes of the cassette will be presented by HLA class I or HLA class II or both.

[00408] In one embodiment, a cassette design module may determine a cassette sequence associated with the lowest junction epitope presentation score among the candidate cassette sequences.

[00409] A cassette design module may iterate through one or more candidate cassette sequences, determine the junction epitope presentation score for the candidate cassettes, and identify an optimal cassette sequence associated with a junction epitope presentation score below the threshold.

[00410] A cassette design module may further check the one or more candidate cassette sequences to identify if any of the junction epitopes in the candidate cassette sequences are self-epitopes for a given patient for whom the vaccine is being designed. To accomplish this, the cassette design module checks the junction epitopes against a known database such as BLAST. In one embodiment, the cassette design module may be configured to design cassettes that avoid junction self-epitopes.

[00411] A cassette design module can perform a brute force approach and iterate through all or most possible candidate cassette sequences to select the sequence with the smallest junction epitope presentation score. However, the number of such candidate cassettes can be prohibitively large as the capacity of the vaccine increases. For example, for a vaccine capacity of 20 epitopes, the cassette design module has to iterate through $\sim 10^{18}$ possible candidate cassettes to determine the cassette with the lowest junction epitope presentation score. This determination may be computationally burdensome (in terms of computational processing resources required), and sometimes intractable, for the cassette design module to complete within a reasonable amount of time to generate the vaccine for the patient. Moreover, accounting for the possible junction epitopes for each candidate cassette can be even more burdensome. Thus, a cassette design module may select a cassette sequence based on ways of iterating through a number of candidate cassette sequences that are significantly smaller than the number of candidate cassette sequences for the brute force approach.

[00412] A cassette design module can generate a subset of randomly or at least pseudo-randomly generated candidate cassettes, and selects the candidate cassette associated with a junction epitope presentation score below a predetermined threshold as the cassette sequence. Additionally, the cassette design module may select the candidate cassette from the subset with the lowest junction epitope presentation score as the cassette sequence. For example, the cassette design module may generate a subset of ~1 million candidate cassettes for a set of 20 selected epitopes, and select the candidate cassette with the smallest junction epitope presentation score. Although generating a subset of random cassette sequences and selecting a cassette sequence with a low junction epitope presentation score out of the subset may be sub-optimal relative to the brute force approach, it requires significantly less computational resources thereby making its implementation technically feasible. Further, performing the brute force method as opposed to this more efficient technique may only result in a minor or even negligible improvement in junction epitope presentation score, thus making it not worthwhile from a resource allocation perspective. A cassette design module can determine an improved cassette configuration by formulating the epitope sequence for the cassette as an asymmetric traveling salesman problem (TSP). Given a list of nodes and distances between each pair of nodes, the TSP determines a sequence of nodes associated with the shortest total distance to visit each node exactly once and return to the original node. For example, given cities A, B, and C with known distances between each other, the solution of the TSP generates a closed sequence of cities, for which the total distance traveled to visit each city exactly once is the smallest among possible routes. The asymmetric version of the TSP determines the optimal sequence of nodes when the distance between a pair of nodes are asymmetric. For example, the “distance” for traveling from node A to node B may be different from the “distance” for traveling from node B to node A. By solving for an improved optimal cassette using an asymmetric TSP, the cassette design module can find a cassette sequence that results in a reduced presentation score across the junctions between epitopes of the cassette. The solution of the asymmetric TSP indicates a sequence of therapeutic epitopes that correspond to the order in which the epitopes should be concatenated in a cassette to minimize the junction epitope presentation score across the junctions of the cassette. A cassette sequence determined through this approach can result in a sequence with significantly less presentation of junction epitopes while potentially requiring significantly less computational resources than the random sampling approach, especially when the number of generated candidate cassette sequences is large. Illustrative

examples of different computational approaches and comparisons for optimizing cassette design are described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

XIII. Example Computer

[00413] A computer can be used for any of the computational methods described herein. One skilled in the art will recognize a computer can have different architectures. Examples of computers are known to those skilled in the art, for example the computers described in more detail in international patent application publications WO/2017/106638, WO/2018/195357, and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00414]

XIV. Antigen Delivery Vector Example

[00415] Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

[00416] The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992).

XIV.A. Neoantigen Cassette Design

[00417] Through vaccination, multiple class I MHC restricted tumor-specific neoantigens (TSNAs) that stimulate the corresponding cellular immune response(s) can be delivered. In one example, a vaccine cassette was engineered to encode multiple epitopes as a single gene

product where the epitopes were either embedded within their natural, surrounding peptide sequence or spaced by non-natural linker sequences. Several design parameters were identified that could potentially impact antigen processing and presentation and therefore the magnitude and breadth of the TSNA specific CD8 T cell responses. In the present example, several model cassettes were designed and constructed to evaluate: (1) whether robust T cell responses could be generated to multiple epitopes incorporated in a single expression cassette; (2) what makes an optimal linker placed between the TSNA within the expression cassette- that leads to optimal processing and presentation of all epitopes; (3) if the relative position of the epitopes within the cassette impact T cell responses; (4) whether the number of epitopes within a cassette influences the magnitude or quality of the T cell responses to individual epitopes; (5) if the addition of cellular targeting sequences improves T cell responses.

[00418] Two readouts were developed to evaluate antigen presentation and T cell responses specific for marker epitopes within the model cassettes: (1) an *in vitro* cell-based screen which allowed assessment of antigen presentation as gauged by the activation of specially engineered reporter T cells (Aarnoudse et al., 2002; Nagai et al., 2012); and (2) an *in vivo* assay that used HLA-A2 transgenic mice (Vitiello et al., 1991) to assess post-vaccination immunogenicity of cassette-derived epitopes of human origin by their corresponding epitope-specific T cell responses (Cornet et al., 2006; Depla et al., 2008; Ishioka et al., 1999).

XIV.B. Antigen Cassette Design Evaluation

XIV.B.1. Methods and Materials

TCR and cassette design and cloning

[00419] The selected TCRs recognize peptides NLVPMVATV (PDB# 5D2N), CLGGLLTMV (PDB#3REV), GILGFVFTL (PDB#1OGA) LLFGYPVYV (PDB#1AO7) when presented by A*0201. Transfer vectors were constructed that contain 2A peptide-linked TCR subunits (beta followed by alpha), the EMCV IRES, and 2A-linked CD8 subunits (beta followed by alpha and by the puromycin resistance gene). Open reading frame sequences were codon-optimized and synthesized by GeneArt.

Cell line generation for in vitro epitope processing and presentation studies

[00420] Peptides were purchased from ProImmune or Genscript diluted to 10mg/mL with 10mM tris(2-carboxyethyl)phosphine (TCEP) in water/DMSO (2:8, v/v). Cell culture medium and supplements, unless otherwise noted, were from Gibco. Heat inactivated fetal bovine serum (FBS_{hi}) was from Seradigm. QUANTI-Luc Substrate, Zeocin, and Puromycin were from InvivoGen. Jurkat-Lucia NFAT Cells (InvivoGen) were maintained in RPMI 1640 supplemented with 10% FBS_{hi}, Sodium Pyruvate, and 100µg/mL Zeocin. Once transduced, these cells additionally received 0.3 µg/mL Puromycin. T2 cells (ATCC CRL-1992) were cultured in Iscove's Medium (IMDM) plus 20% FBS_{hi}. U-87 MG (ATCC HTB-14) cells were maintained in MEM Eagles Medium supplemented with 10% FBS_{hi}.

[00421] Jurkat-Lucia NFAT cells contain an NFAT-inducible Lucia reporter construct. The Lucia gene, when activated by the engagement of the T cell receptor (TCR), causes secretion of a coelenterazine-utilizing luciferase into the culture medium. This luciferase can be measured using the QUANTI-Luc luciferase detection reagent. Jurkat-Lucia cells were transduced with lentivirus to express antigen-specific TCRs. The HIV-derived lentivirus transfer vector was obtained from GeneCopoeia, and lentivirus support plasmids expressing VSV-G (pCMV-VsvG), Rev (pRSV-Rev) and Gag-pol (pCgpV) were obtained from Cell Design Labs.

[00422] Lentivirus was prepared by transfection of 50-80% confluent T75 flasks of HEK293 cells with Lipofectamine 2000 (Thermo Fisher), using 40 µl of lipofectamine and 20 µg of the DNA mixture (4:2:1:1 by weight of the transfer plasmid:pCgpV:pRSV-Rev:pCMV-VsvG). 8-10 mL of the virus-containing media were concentrated using the Lenti-X system (Clontech), and the virus resuspended in 100-200 µl of fresh medium. This volume was used to overlay an equal volume of Jurkat-Lucia cells (5x10⁴-1x10⁶ cells were used in different experiments).

Following culture in 0.3 µg/ml puromycin-containing medium, cells were sorted to obtain clonality. These Jurkat-Lucia TCR clones were tested for activity and selectivity using peptide loaded T2 cells.

In vitro epitope processing and presentation assay

[00423] T2 cells are routinely used to examine antigen recognition by TCRs. T2 cells lack a peptide transporter for antigen processing (TAP deficient) and cannot load endogenous peptides in the endoplasmic reticulum for presentation on the MHC. However, the T2 cells can easily be loaded with exogenous peptides. The five marker peptides (NLVPMVATV, CLGGLLTMV, GLCTLVAML, LLFGYPVYV, GILGFVFTL) and two irrelevant peptides (WLSLLVPFV, FLLTRICT) were loaded onto T2 cells. Briefly, T2 cells were counted and diluted to 1x10⁶ cells/mL with IMDM plus 1% FBS_{hi}. Peptides were added to result in 10 µg peptide/1x10⁶ cells. Cells were then incubated at 37°C for 90 minutes. Cells were washed twice with IMDM plus 20% FBS_{hi}, diluted to 5x10⁵ cells/mL and 100 µL plated into a 96-well Costar tissue culture plate. Jurkat-Lucia TCR clones were counted and diluted to 5x10⁵ cells/mL in RPMI 1640 plus 10% FBS_{hi} and 100 µL added to the T2 cells. Plates were incubated overnight at 37°C, 5% CO₂. Plates were then centrifuged at 400g for 3 minutes and 20 µL supernatant removed to a white flat bottom Greiner plate. QUANTI-Luc substrate was prepared according to instructions and 50 µL/well added. Luciferase expression was read on a Molecular Devices SpectraMax iE3x.

[00424] To test marker epitope presentation by the adenoviral cassettes, U-87 MG cells were used as surrogate antigen presenting cells (APCs) and were transduced with the adenoviral vectors. U-87 MG cells were harvested and plated in culture media as 5x10⁵ cells/100 µl in a 96-well Costar tissue culture plate. Plates were incubated for approximately 2 hours at 37°C. Adenoviral cassettes were diluted with MEM plus 10% FBS_{hi} to an MOI of 100, 50, 10, 5, 1 and 0 and added to the U-87 MG cells as 5µl/well. Plates were again incubated for approximately 2 hours at 37°C. Jurkat-Lucia TCR clones were counted and diluted to 5x10⁵ cells/mL in RPMI plus 10% FBS_{hi} and added to the U-87 MG cells as 100 µL/well. Plates were then incubated for approximately 24 hours at 37°C, 5% CO₂. Plates were centrifuged at 400g for 3 minutes and 20 µL supernatant removed to a white flat bottom Greiner plate. QUANTI-Luc substrate was prepared according to instructions and 50 µL/well added. Luciferase expression was read on a Molecular Devices SpectraMax iE3x.

Mouse strains for immunogenicity studies

[00425] Transgenic HLA-A2.1 (HLA-A2 Tg) mice were obtained from Taconic Labs, Inc. These mice carry a transgene consisting of a chimeric class I molecule comprised of the human HLA-A2.1 leader, $\alpha 1$, and $\alpha 2$ domains and the murine H2-Kb $\alpha 3$, transmembrane, and cytoplasmic domains (Vitiello et al., 1991). Mice used for these studies were the first generation offspring (F1) of wild type BALB/cAnNTac females and homozygous HLA-A2.1 Tg males on the C57Bl/6 background.

Adenovirus vector (Ad5v) immunizations

[00426] HLA-A2 Tg mice were immunized with 1×10^{10} to 1×10^6 viral particles of adenoviral vectors via bilateral intramuscular injection into the tibialis anterior. Immune responses were measured at 12 days post-immunization.

Lymphocyte isolation

[00427] Lymphocytes were isolated from freshly harvested spleens and lymph nodes of immunized mice. Tissues were dissociated in RPMI containing 10% fetal bovine serum with penicillin and streptomycin (complete RPMI) using the GentleMACS tissue dissociator according to the manufacturer's instructions.

Ex vivo enzyme-linked immunospot (ELISPOT) analysis

[00428] ELISPOT analysis was performed according to ELISPOT harmonization guidelines (Janetzki et al., 2015) with the mouse IFN γ ELISpotPLUS kit (MABTECH). 1×10^5 splenocytes were incubated with 10 μ M of the indicated peptides for 16 hours in 96-well IFN γ antibody coated plates. Spots were developed using alkaline phosphatase. The reaction was timed for 10 minutes and was quenched by running the plate under tap water. Spots were counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation $> 50\%$ were recorded as "too numerous to count". Samples with deviation of replicate wells $> 10\%$ were excluded from analysis. Spot counts were then corrected for well confluency using the formula: spot count + 2 x (spot count x %confluence / [100% - %confluence]). Negative background was corrected by subtraction of spot counts in the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count were set to the highest observed corrected value, rounded up to the nearest hundred.

Ex vivo intracellular cytokine staining (ICS) and flow cytometry analysis

[00429] Freshly isolated lymphocytes at a density of $2-5 \times 10^6$ cells/mL were incubated with 10 μ M of the indicated peptides for 2 hours. After two hours, brefeldin A was added to a concentration of 5 μ g/ml and cells were incubated with stimulant for an additional 4 hours. Following stimulation, viable cells were labeled with fixable viability dye eFluor780 according to manufacturer's protocol and stained with anti-CD8 APC (clone 53-6.7, BioLegend) at 1:400

dilution. Anti-IFN γ PE (clone XMG1.2, BioLegend) was used at 1:100 for intracellular staining. Samples were collected on an Attune NxT Flow Cytometer (Thermo Scientific). Flow cytometry data was plotted and analyzed using FlowJo. To assess degree of antigen-specific response, both the percent IFN γ ⁺ of CD8⁺ cells and the total IFN γ ⁺ cell number/ 1×10^6 live cells were calculated in response to each peptide stimulant.

XIV.B.2. *In Vitro* Evaluation of Antigen Cassette Designs

[00430] As an example of antigen cassette design evaluation, an *in vitro* cell-based assay was developed to assess whether selected human epitopes within model vaccine cassettes were being expressed, processed, and presented by antigen-presenting cells (Fig. 1). Upon recognition, Jurkat-Lucia reporter T cells that were engineered to express one of five TCRs specific for well-characterized peptide-HLA combinations become activated and translocate the nuclear factor of activated T cells (NFAT) into the nucleus which leads to transcriptional activation of a luciferase reporter gene. Antigenic stimulation of the individual reporter CD8 T cell lines was quantified by bioluminescence.

[00431] Individual Jurkat-Lucia reporter lines were modified by lentiviral transduction with an expression construct that includes an antigen-specific TCR beta and TCR alpha chain separated by a P2A ribosomal skip sequence to ensure equimolar amounts of translated product (Banu et al., 2014). The addition of a second CD8 beta-P2A-CD8 alpha element to the lentiviral construct provided expression of the CD8 co-receptor, which the parent reporter cell line lacks, as CD8 on the cell surface is crucial for the binding affinity to target pMHC molecules and enhances signaling through engagement of its cytoplasmic tail (Lyons et al., 2006; Yachi et al., 2006).

[00432] After lentiviral transduction, the Jurkat-Lucia reporters were expanded under puromycin selection, subjected to single cell fluorescence assisted cell sorting (FACS), and the monoclonal populations tested for luciferase expression. This yielded stably transduced reporter cell lines for specific peptide antigens 1, 2, 4, and 5 with functional cell responses. (Table 2).

Table 2: Development of an *in vitro* T cell activation assay. Peptide-specific T cell recognition as measured by induction of luciferase indicates effective processing and presentation of the vaccine cassette antigens.

	Short Cassette Design
Epitope	AAY
1	24.5 ± 0.5
2	11.3 ± 0.4
3*	n/a
4	26.1 ± 3.1
5	46.3 ± 1.9

* Reporter T cell for epitope 3 not yet generated

[00433] In another example, a series of short cassettes, all marker epitopes were incorporated in the same position (Fig. 2A) and only the linkers separating the HLA-A*0201 restricted epitopes (Fig. 2B) were varied. Reporter T cells were individually mixed with U-87 antigen-presenting cells (APCs) that were infected with adenoviral constructs expressing these short cassettes, and luciferase expression was measured relative to uninfected controls. All four antigens in the model cassettes were recognized by matching reporter T cells, demonstrating efficient processing and presentation of multiple antigens. The magnitude of T cell responses follow largely similar trends for the natural and AAY-linkers. The antigens released from the RR-linker based cassette show lower luciferase inductions (Table 3). The DPP-linker, designed to disrupt antigen processing, produced a vaccine cassette that led to low epitope presentation (Table 3).

Table 3: Evaluation of linker sequences in short cassettes. Luciferase induction in the *in vitro* T cell activation assay indicated that, apart from the DPP-based cassette, all linkers facilitated efficient release of the cassette antigens. T cell epitope only (no linker) = 9AA, natural linker one side = 17AA, natural linker both sides = 25AA, non-natural linkers = AAY, RR, DPP

Epitope	Short Cassette Designs					
	9AA	17AA	25AA	AAY	RR	DPP
1	33.6 ± 0.9	42.8 ± 2.1	42.3 ± 2.3	24.5 ± 0.5	21.7 ± 0.9	0.9 ± 0.1
2	12.0 ± 0.9	10.3 ± 0.6	14.6 ± 0.4	11.3 ± 0.4	8.5 ± 0.3	1.1 ± 0.2
3*	n/a	n/a	n/a	n/a	n/a	n/a
4	26.6 ± 2.5	16.1 ± 0.6	16.6 ± 0.8	26.1 ± 3.1	12.5 ± 0.8	1.3 ± 0.2
5	29.7 ± 0.6	21.2 ± 0.7	24.3 ± 1.4	46.3 ± 1.9	19.7 ± 0.4	1.3 ± 0.1

* Reporter T cell for epitope 3 not yet generated

[00434] In another example, an additional series of short cassettes were constructed that, besides human and mouse epitopes, contained targeting sequences such as ubiquitin (Ub), MHC and Ig-kappa signal peptides (SP), and/or MHC transmembrane (TM) motifs positioned on either the N- or C-terminus of the cassette. (Fig. 3). When delivered to U-87 APCs by adenoviral vector, the reporter T cells again demonstrated efficient processing and presentation of multiple cassette-derived antigens. However, the magnitude of T cell responses were not substantially impacted by the various targeting features (Table 4).

Table 4: Evaluation of cellular targeting sequences added to model vaccine cassettes. Employing the *in vitro* T cell activation assay demonstrated that the four HLA-A*0201 restricted marker epitopes are liberated efficiently from the model cassettes and targeting sequences did not substantially improve T cell recognition and activation.

Epitope	Short Cassette Designs									
	A	B	C	D	E	F	G	H	I	J
1	32.5 ± 1.5	31.8 ± 0.8	29.1 ± 1.2	29.1 ± 1.1	28.4 ± 0.7	20.4 ± 0.5	35.0 ± 1.3	30.3 ± 2.0	22.5 ± 0.9	38.1 ± 1.6
2	6.1 ± 0.2	6.3 ± 0.2	7.6 ± 0.4	7.0 ± 0.5	5.9 ± 0.2	3.7 ± 0.2	7.6 ± 0.4	5.4 ± 0.3	6.2 ± 0.4	6.4 ± 0.3
3*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	12.3 ± 1.1	14.1 ± 0.7	12.2 ± 0.8	13.7 ± 1.0	11.7 ± 0.8	10.6 ± 0.4	11.0 ± 0.6	7.6 ± 0.6	16.1 ± 0.5	8.7 ± 0.5
5	44.4 ± 2.8	53.6 ± 1.6	49.9 ± 3.3	50.5 ± 2.8	41.7 ± 2.8	36.1 ± 1.1	46.5 ± 2.1	31.4 ± 0.6	75.4 ± 1.6	35.7 ± 2.2

* Reporter T cell for epitope 3 not yet generated

XIV.B.3. *In Vivo* Evaluation of Antigen Cassette Designs

[00435] As another example of antigen cassette design evaluation, vaccine cassettes were designed to contain 5 well-characterized human class I MHC epitopes known to stimulate CD8 T cells in an HLA-A*02:01 restricted fashion (Fig. 2A, 3, 5A). For the evaluation of their in vivo immunogenicity, vaccine cassettes containing these marker epitopes were incorporated in adenoviral vectors and used to infect HLA-A2 transgenic mice (Fig. 4). This mouse model carries a transgene consisting partly of human HLA-A*0201 and mouse H2-Kb thus encoding a chimeric class I MHC molecule consisting of the human HLA-A2.1 leader, $\alpha 1$ and $\alpha 2$ domains ligated to the murine $\alpha 3$, transmembrane and cytoplasmic H2-Kb domain (Vitiello et al., 1991). The chimeric molecule allows HLA-A*02:01-restricted antigen presentation whilst maintaining the species-matched interaction of the CD8 co-receptor with the $\alpha 3$ domain on the MHC.

[00436] For the short cassettes, all marker epitopes generated a T cell response, as determined by IFN-gamma ELISPOT, that was approximately 10-50x stronger of what has been commonly reported (Cornet et al., 2006; Depla et al., 2008; Ishioka et al., 1999). Of all the linkers evaluated, the concatamer of 25mer sequences, each containing a minimal epitope flanked by their natural amino acids sequences, generated the largest and broadest T cell response (Table 5). Intracellular cytokine staining (ICS) and flow cytometry analysis revealed that the antigen-specific T cell responses are derived from CD8 T cells.

Table 5: In vivo evaluation of linker sequences in short cassettes. ELISPOT data indicated that HLA-A2 transgenic mice, 17 days post-infection with 1e11 adenovirus viral particles, generated a T cell response to all class I MHC restricted epitopes in the cassette.

Epitope	Short Cassette Designs					
	9AA	17AA	25AA	AAV	RR	DPP
1	2020+/-583	2505+/-1281	6844+/-956	1489+/-762	1675+/-690	1781+/-774
2	4472+/-755	3792+/-1319	7629+/-996	3851+/-1748	4726+/-1715	5868+/-1427
3	5830+/-315	3629+/-862	7253+/-491	4813+/-1761	6779+/-1033	7328+/-1700
4	5536+/-375	2446+/-955	2961+/-1487	4230+/-1759	6518+/-909	7222+/-1824
5	8800+/-0	7943+/-821	8423+/-442	8312+/-696	8800+/-0	1836+/-328

[00437] In another example, a series of long vaccine cassettes was constructed and incorporated in adenoviral vectors that, next to the original 5 marker epitopes, contained an additional 16 HLA-A*02:01, A*03:01 and B*44:05 epitopes with known CD8 T cell reactivity (Fig. 5A, B). The size of these long cassettes closely mimicked the final clinical cassette design, and only the position of the epitopes relative to each other was varied. The CD8 T cell responses were comparable in magnitude and breadth for both long and short vaccine cassettes,

demonstrating that (a) the addition of more epitopes did not substantially impact the magnitude of immune response to the original set of epitopes, and (b) the position of an epitope in a cassette did not substantially influence the ensuing T cell response to it (Table 6).

Table 6: In vivo evaluation of the impact of epitope position in long cassettes. ELISPOT data indicated that HLA-A2 transgenic mice, 17 days post-infection with 5e10 adenovirus viral particles, generated a T cell response comparable in magnitude for both long and short vaccine cassettes.

Epitope	Long Cassette Designs		
	Standard	Scrambled	Short
1	863+/-1080	804+/-1113	1871+/-2859
2	6425+/-1594	28+/-62	5390+/-1357
3*	23+/-30	36+/-18	0+/-48
4	2224+/-1074	2727+/-644	2637+/-1673
5	7952+/-297	8100+/-0	8100+/-0

* Suspected technical error caused an absence of a T cell response.

XIV.B.4. Antigen Cassette Design for Immunogenicity and

Toxicology Studies

[00438] In summary, the findings of the model cassette evaluations (Fig. 2-5, Tables 2-6) demonstrated that, for model vaccine cassettes, robust immunogenicity was achieved when a “string of beads” approach was employed that encodes around 20 epitopes in the context of an adenovirus-based vector. The epitopes were assembled by concatenating 25mer sequences, each embedding a minimal CD8 T cell epitope (e.g. 9 amino acid residues) that were flanked on both sides by its natural, surrounding peptide sequence (e.g. 8 amino acid residues on each side). As used herein, a “natural” or “native” flanking sequence refers to the N- and/or C-terminal flanking sequence of a given epitope in the naturally occurring context of that epitope within its source protein. For example, the HCMV pp65 MHC I epitope NLVPMVATV is flanked on its 5’ end by the native 5’ sequence WQAGILAR and on its 3’ end by the native 3’ sequence QGQNLKYQ, thus generating the WQAGILARNLVPMVATVQGQNLKYQ 25mer peptide found within the HCMV pp65 source protein. The natural or native sequence can also refer to a nucleotide sequence that encodes an epitope flanked by native flanking sequence(s). Each 25mer sequence is directly connected to the following 25mer sequence. In instances where the minimal CD8 T cell epitope is greater than or less than 9 amino acids, the flanking peptide length can be adjusted such that the total length is still a 25mer peptide sequence. For example, a 10 amino acid CD8 T cell epitope can be flanked by an 8 amino acid sequence and a 7 amino acid. The concatamer was followed by two universal class II MHC epitopes that were included to stimulate CD4 T helper cells and improve overall *in vivo* immunogenicity of

the vaccine cassette antigens. (Alexander et al., 1994; Panina-Bordignon et al., 1989) The class II epitopes were linked to the final class I epitope by a GP GPG amino acid linker (SEQ ID NO:56). The two class II epitopes were also linked to each other by a GP GPG amino acid linker, as well as flanked on the C-terminus by a GP GPG amino acid linker. Neither the position nor the number of epitopes appeared to substantially impact T cell recognition or response. Targeting sequences also did not appear to substantially impact the immunogenicity of cassette-derived antigens.

[00439] As a further example, based on the in vitro and in vivo data obtained with model cassettes (Fig. 2-5, Tables 2-6), a cassette design was generated that alternates well-characterized T cell epitopes known to be immunogenic in nonhuman primates (NHPs), mice and humans. The 20 epitopes, all embedded in their natural 25mer sequences, are followed by the two universal class II MHC epitopes that were present in all model cassettes evaluated (Fig. 6). This cassette design was used to study immunogenicity as well as pharmacology and toxicology studies in multiple species.

XIV.B.5. Antigen Cassette Design and Evaluation for 30, 40, and 50 Antigens

[00440] Large antigen cassettes were designed that had either 30 (L), 40 (XL) or 50 (XXL) epitopes, each 25 amino acids in length. The epitopes were a mix of human, NHP and mouse epitopes to model disease antigens including tumor antigens. FIG. 29 illustrates the general organization of the epitopes from the various species. The model antigens used are described in Tables 37, 38 and 39 for human, primate, and mouse model epitopes, respectively. Each of Tables 37, 38 and 39 described the epitope position, name, minimal epitope description, and MHC class.

[00441] These cassettes were cloned into the chAd68 and srRNA vaccine vectors as described to evaluate the efficacy of longer multiple-epitope cassettes. FIG. 30 shows that each of the large antigen cassettes were expressed from a ChAdV vector as indicated by at least one major band of the expected size by Western blot.

[00442] Mice were immunized as described to evaluate the efficacy of the large cassettes. T cell responses were analyzed by ICS and tetramer staining following immunization with a chAd68 vector (FIG. 31/Table 40 and FIG. 32/Table 41, respectively) and by ICS following immunization with a srRNA vector (FIG. 33/Table 42) for epitopes AH1 (top panels) and SINNF EKL (bottom panels). Immunizations using chAd68 and srRNA vaccine vectors

expressing either 30 (L), 40 (XL) or 50 (XXL) epitopes induced CD8⁺ immune responses to model disease epitopes.

Table 37 - Human epitopes in large cassettes

Epitope position in each cassette		Name	Minimal epitope	25mer	MHC	Restriction	Strain	Species
L	XL							
3	3	3 5.Influenza M	GLGFVFTL	PLSLPTKGLGFVFTLTPSERGL	Class I	A*02:01	Human	Human
6	6	6 4.HTLV-1 Tax	LLFGYPVYV	HFPGFGQSLLFGYPVYVFGDCVQGD	Class I	A*02:01	Human	Human
9	9	9 3.EBV BMFL1	GLCTLVAML	RMQAIQNAGLCTLVAMLEETIFWLQ	Class I	A*02:01	Human	Human
12	12	12 1.HCMV pp65	NLVPNVATV	WQAGILARNLVPNVATVQGNLIKYQ	Class I	A*02:01	Human	Human
15	15	15 2.EBV LMP2A	CLGGLTMV	RTYGPVFMCLGGLTMVAGAVWLTV	Class I	A*02:01	Human	Human
18	18	18 CT83	NTDNNLAVY	SSSGLNSNTDNNLAVYDLSRDILN	Class I	A*01:01	Human	Human
21	21	21 MAGEA6	EVDPIGHVY	LVFGIELMEVDPIGHVYFATCIGL	Class I	B*35:01	Human	Human
25	25	25 CT83	LLASSILCA	MNFYLLASSILCALIVFWKRRFQ	Class I	A*02:01	Human	Human
28	28	28 FOXE1	ALFPGAVPAA	AAAAAALFPGAVPAARPPYPGAV	Class I	A*02:01	Human	Human
31	31	32 CT83	VVDLSRDIL	SNTDNNLAVYDLSRDILNFPHSIA	Class I	A*24:02	Human	Human
35	35	36 MAGE3/6	ASSLPPTMNV	DPPQSPQGASSLPPTMNVPLWSQSY	Class I	A*01:01	Human	Human
38	38	40 Influenza HA	PKVVKONTLKLAT	ITYGACPKVVKONTLKLATGMRNV	Class II	DRB1*0101 / Human	Human	Human
40	40	44 CMV pp65	LPLKMLNIPSNVH	SIYVVALPLKMLNIPSNVHHYPSA	Class II	DRB1*0101	Human	Human
47	47	47 EBV EBNA3A	PEQWMFQGAPPSQGT	EGPWVPEQWMFQGAPPSQGTDVVQH	Class II	DRB1*0102	Human	Human
50	50	50 CMV pp65	EHPFTSQYRIQGL	RGPDQYSEHPFTSQYRIQGLEVRH	Class II	DRB1*1101	Human	Human

Table 38 – NHP epitopes in large cassettes

Epitope position in each cassette		Name	Minimal epitope	25mer	MHC	Restriction	Strain	Species
L								
	XL	XXL						
1	1	1	1 Gag CM9	CTPYDINQM	MFQALSEGCTPYDINQMNLVIGDHQ	Class I	Mamu-A*01 Rhesus	NHP
4	4	4	4 Tat TL8	TTPEANL	SCSEADATTPESANLGEILSLQY	Class I	Mamu-A*01 Rhesus	NHP
7	7	7	7 Env CL9	CAPPGYALL	WDAIRFRYCAPPGYALLRCNDTYS	Class I	Mamu-A*01 Rhesus	NHP
10	10	10	10 Pol SV9	SGPKTNIV	AFILMALTDSPKTNIVDSQYVMGI	Class I	Mamu-A*01 Rhesus	NHP
13	13	13	13 Gag LW9	LSPTLNAW	GNVWVHTPLSPRTLNAWWKAVEEK	Class I	Mamu-A*01 Rhesus	NHP
16	16	16	16 Env TL9	TVPWPNASL	AFRQVCHTTPWPNASLTPKWNNET	Class I	Mamu-A*01 Rhesus	NHP
19	19	19	19 Ag85B	PNGTSHWEWGAQLN	VFNFPNGTSHWEWGAQLNAMKGD	Class II	Mamu-DR*V Rhesus	NHP
19	19	19	23 HIV-1 Env	YKRVKKEPLGV	NWRSELYKRVKKEPLGVAPTAK	Class II	Mamu-DR*W Rhesus	NHP
23	23	23	26 Gag TE15	TEEAKQVQRHLVE	EKVHTEEAKQVQRHLVVEGTTE	Class II	Mamu-DRB* Rhesus	NHP
27	27	27	30 CFP-10 36-48	AGSLQGWRGAAG	DQVESTAGSLQGWRGAAGTAQAA	Class II	Mafa-DRB1* Cyno	NHP
29	29	29	34 CFP-10 71-86	EISTNIRQAGVQYSRA	QELDEISTNIRQAGVQYSRADEEQ	Class II	Mafa-DRB1* Cyno	NHP
33	33	33	38 Env 338-346	RPKQAWCWF	FHSQPINERPKQAWGFGGSWKEAI	Class I	Mafa-A1*06: Cyno	NHP
37	37	37	42 Nef 103-111	RPKVPLRTM	DDDEEDDLVGVSVRKVPLRTMS	Class I	Mafa-A1*06: Cyno	NHP
48	48	48	45 Gag 386-394	GPRKPKICW	PFAAAQQRGPRKPKICWNGKEGHS	Class I	Mafa-A1*06: Cyno	NHP
			48 Nef LT9	LNWADKKET	RRLTARGLNWDKKETRTPKAKA	Class I	Mafa-B*104: Cyno	NHP

Table 39 - Mouse epitopes in large cassettes

Epitope position in each cassette		Name	Minimal epitope	25mer	MHC	Restriction	Strain	Species
L								
XL	XXL							
2	2	2 OVA257	SINFEKL	VSGLEQLSINFEKLTWTSNNVM	Class I	H2-Kb	B6	Mouse
		5 B16-EGP	EGPRNQDWL	ALAVGALEGRNQDWLGVPRLVT	Class I	H2-Db	B6	Mouse
		8 B16-TRP1 455-463	TAPDNLGYM	VTNTEMFVTAPDNLGYWVEVQWPGQ	Class I	H2-Db	B6	Mouse
5	5	11 Trp2180-188	SYVDFEVL	TOPQIANGSVDFEVLHYSYVDT	Class I	H2-Kb	B6	Mouse
	8	14 CT26 AH1-A5	SPSYAVHQF	LWPRVTYHSPSYAVHQFERAKYKR	Class I	H2-Ld	Balb/C	Mouse
	11	17 CT26 AH1-39	MNKYAYHML	LWPRVTYHMKYAYAHMLERRAKYKR	Class I	H2-Ld	Balb/C	Mouse
	14	20 MC38 Dpagt1	SIVFNLL	GOSLVISASIVFNLLIEGDYRDD	Class I	H2-Kb	B6	Mouse
	17	22 MC38 Adpgk	ASMTNMELM	GIPVHELASMTNMELMSSIVHQV	Class I	H2-Db	B6	Mouse
	20	24 MC38 Reps1	AQLANDVYL	RVLELFRAAQLANDVLIQIMELGA	Class I	H2-Db	B6	Mouse
8	11	27 P815 P1A 35-44	LPYLGWLVF	HRYSLEELPYLGWLVFVAVTTSFL	Class I	H2-Ld	DBA/2	Mouse
	14	29 P815 P1E	GYCGLRGTGV	YLSKNPDGCGLRGTGVSQPMALK	Class I	H2-Kd	DBA/2	Mouse
	22	31 Panc02 Mesothelir	LSIFKHL	NEIPTYEQLSIFKHLDKTYPOGY	Class I	H2-Kb	B6	Mouse
11	24	33 Panc02 Mesothelir	LIWIPALL	SRASLGPGLVLIWIPALLPALRLS	Class I	H2-Kb	B6	Mouse
17	26	35 ID8 Fra 161-169	SSGHNECPV	NWHKGWNWSSGHNECPVGASCHFT	Class I	H2-Kb	B6	Mouse
20	28	37 ID8 Mesothelin 40	GOKMNAQAI	KTLLKYSKGOKMNAQAIALVACYLR	Class I	H2-Db	B6	Mouse
23	30	39 OVA-II	ISQAVHAHAINEAGR	ESLIKISQAVHAHAINEAGREVVG	Class II	I-Ab, I-Ad	B6	Mouse
26	32	41 ESAT-6	MTEQQWNFAGIEAASAIQ	MTEQQWNFAGIEAASAIQGNVTSI	Class II	I-Ab	B6	Mouse
29	34	43 TT p30	FNNFTVSFWLRVPKVSASHL	DMFNNFTVSFWLRVPKVSASHLEQY	Class II	I-Ad	Balb/C	Mouse
	36	46 HEL	DGSTDYGILQINSRW	TNRNTDGYGILQINSRWWCNDG	Class II	I-Ak	CBA	Mouse
	39	49 MOG	MEVGWYRSPFSRVVHL	YRNTGMEVGWYRSPFSRVVHL	Class II	I-Ab	B6	Mouse

Table 40: Average IFN γ + cells in response to AH1 and SIINFEKL peptides in ChAd large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL	5.308	0.660	n/a	8
30	SIINFEKL	4.119	1.019	0.978	8
40	SIINFEKL	6.324	0.954	0.986	8
50	SIINFEKL	8.169	1.469	0.751	8
20	AH1	6.405	2.664	n/a	8
30	AH1	4.373	1.442	0.093	8
40	AH1	4.126	1.135	0.050	8
50	AH1	4.216	0.808	0.063	8

Table 41: Average tetramer+ cells for AH1 and SIINFEKL antigens in ChAd large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL	10.314	2.384	n/a	8
30	SIINFEKL	4.551	2.370	0.003	8
40	SIINFEKL	5.186	3.254	0.009	8
50	SIINFEKL	14.113	3.660	0.072	8
20	AH1	6.864	2.207	n/a	8
30	AH1	4.713	0.922	0.036	8
40	AH1	5.393	1.452	0.223	8
50	AH1	5.860	1.041	0.543	8

Table 42: Average IFN γ + cells in response to AH1 and SIINFEKL peptides in SAM large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL	1.843	0.422	n/a	8
30	SIINFEKL	2.112	0.522	0.879	7
40	SIINFEKL	1.754	0.978	0.995	7
50	SIINFEKL	1.409	0.766	0.606	8
20	AH1	3.050	0.909	n/a	8
30	AH1	0.618	0.427	1.91E-05	7
40	AH1	1.286	0.284	0.001	7
50	AH1	1.309	1.149	0.001	8

XV. ChAd Antigen Cassette Delivery Vector

XV.A. ChAd Antigen Cassette Delivery Vector Construction

[00443] In one example, Chimpanzee adenovirus (ChAd) was engineered to be a delivery vector for antigen cassettes. In a further example, a full-length ChAdV68 vector was synthesized based on AC_000011.1 (sequence 2 from Patent US 6083716) with E1 (nt 457 to 3014) and E3 (nt 27,816- 31,332) sequences deleted. Reporter genes under the control of the CMV promoter/enhancer were inserted in place of the deleted E1 sequences. Transfection of this clone into HEK293 cells did not yield infectious virus. To confirm the sequence of the wild-type C68 virus, isolate VR-594 was obtained from the ATCC, passaged, and then independently sequenced (SEQ ID NO:10). When comparing the AC_000011.1 sequence to the ATCC VR-594 sequence (SEQ ID NO:10) of wild-type ChAdV68 virus, 6 nucleotide differences were identified. In one example, a modified ChAdV68 vector was generated based on AC_000011.1, with the corresponding ATCC VR-594 nucleotides substituted at five positions (ChAdV68.5WTnt SEQ ID NO:1).

[00444] In another example, a modified ChAdV68 vector was generated based on AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,816- 31,332) sequences deleted and the corresponding ATCC VR-594 nucleotides substituted at four positions. A GFP reporter (ChAdV68.4WTnt.GFP; SEQ ID NO:11) or model neoantigen cassette (ChAdV68.4WTnt.MAG25mer; SEQ ID NO:12) under the control of the CMV promoter/enhancer was inserted in place of deleted E1 sequences.

[00445] In another example, a modified ChAdV68 vector was generated based on AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,125- 31,825) sequences deleted and the corresponding ATCC VR-594 nucleotides substituted at five positions. A GFP reporter (ChAdV68.5WTnt.GFP; SEQ ID NO:13) or model neoantigen cassette (ChAdV68.5WTnt.MAG25mer; SEQ ID NO:2) under the control of the CMV promoter/enhancer was inserted in place of deleted E1 sequences

[00446] Relevant vectors are described below:

- Full-Length ChAdVC68 sequence “ChAdV68.5WTnt” (SEQ ID NO:1); AC_000011.1 sequence with corresponding ATCC VR-594 nucleotides substituted at five positions.
- ATCC VR-594 C68 (SEQ ID NO:10); Independently sequenced; Full-Length C68

- ChAdV68.4WTnt.GFP (SEQ ID NO:11); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,816- 31,332) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at four positions; GFP reporter under the control of the CMV promoter/enhancer inserted in place of deleted E1
- ChAdV68.4WTnt.MAG25mer (SEQ ID NO:12); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,816- 31,332) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at four positions; model neoantigen cassette under the control of the CMV promoter/enhancer inserted in place of deleted E1
- ChAdV68.5WTnt.GFP (SEQ ID NO:13); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,125- 31,825) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at five positions; GFP reporter under the control of the CMV promoter/enhancer inserted in place of deleted E1

XV.B. ChAd Antigen Cassette Delivery Vector Testing

XV.B.1. ChAd Vector Evaluation Methods and Materials

Transfection of HEK293A cells using lipofectamine

[00447] DNA for the ChAdV68 constructs (ChAdV68.4WTnt.GFP, ChAdV68.5WTnt.GFP, ChAdV68.4WTnt.MAG25mer and ChAdV68.5WTnt.MAG25mer) was prepared and transfected into HEK293A cells using the following protocol.

[00448] 10 ug of plasmid DNA was digested with PacI to liberate the viral genome. DNA was then purified using GeneJet DNA cleanup Micro columns (Thermo Fisher) according to manufacturer's instructions for long DNA fragments, and eluted in 20 ul of pre-heated water; columns were left at 37 degrees for 0.5-1 hours before the elution step.

[00449] HEK293A cells were introduced into 6-well plates at a cell density of 10^6 cells/well 14-18 hours prior to transfection. Cells were overlaid with 1 ml of fresh medium (DMEM-10% hiFBS with pen/strep and glutamate) per well. 1-2 ug of purified DNA was used per well in a transfection with twice the ul volume (2-4 ul) of Lipofectamine2000, according to the manufacturer's protocol. 0.5 ml of OPTI-MEM medium containing the transfection mix was added to the 1 ml of normal growth medium in each well, and left on cells overnight.

[00450] Transfected cell cultures were incubated at 37°C for at least 5-7 days. If viral plaques were not visible by day 7 post-transfection, cells were split 1:4 or 1:6, and incubated at 37°C to monitor for plaque development. Alternatively, transfected cells were harvested and

subjected to 3 cycles of freezing and thawing and the cell lysates were used to infect HEK293A cells and the cells were incubated until virus plaques were observed.

Transfection of ChAdV68 vectors into HEK293A cells using calcium phosphate and generation of the tertiary viral stock

[00451] DNA for the ChAdV68 constructs (ChAdV68.4WTnt.GFP, ChAdV68.5WTnt.GFP, ChAdV68.4WTnt.MAG25mer, ChAdV68.5WTnt.MAG25mer) was prepared and transfected into HEK293A cells using the following protocol.

[00452] HEK293A cells were seeded one day prior to the transfection at 10^6 cells/ well of a 6 well plate in 5% BS/DMEM/ 1XP/S, 1XGlutamax. Two wells are needed per transfection. Two to four hours prior to transfection the media was changed to fresh media. The ChAdV68.4WTnt.GFP plasmid was linearized with PacI. The linearized DNA was then phenol chloroform extracted and precipitated using one tenth volume of 3M Sodium acetate pH 5.3 and two volumes of 100% ethanol. The precipitated DNA was pelleted by centrifugation at 12,000xg for 5 min before washing 1X with 70% ethanol. The pellet was air dried and re-suspended in 50 μ L of sterile water. The DNA concentration was determined using a NanoDrop™ (ThermoFisher) and the volume adjusted to 5 μ g of DNA/50 μ L.

[00453] 169 μ L of sterile water was added to a microfuge tube. 5 μ L of 2M CaCl_2 was then added to the water and mixed gently by pipetting. 50 μ L of DNA was added dropwise to the CaCl_2 water solution. Twenty six μ L of 2M CaCl_2 was then added and mixed gently by pipetting twice with a micro-pipettor. This final solution should consist of 5 μ g of DNA in 250 μ L of 0.25M CaCl_2 . A second tube was then prepared containing 250 μ L of 2XHBS (Hepes buffered solution). Using a 2 mL sterile pipette attached to a Pipet-Aid air was slowly bubbled through the 2XHBS solution. At the same time the DNA solution in the 0.25M CaCl_2 solution was added in a dropwise fashion. Bubbling was continued for approximately 5 seconds after addition of the final DNA droplet. The solution was then incubated at room temperature for up to 20 minutes before adding to 293A cells. 250 μ L of the DNA/Calcium phosphate solution was added dropwise to a monolayer of 293A cells that had been seeded one day prior at 10^6 cells per well of a 6 well plate. The cells were returned to the incubator and incubated overnight. The media was changed 24h later. After 72h the cells were split 1:6 into a 6 well plate. The monolayers were monitored daily by light microscopy for evidence of cytopathic effect (CPE). 7-10 days post transfection viral plaques were observed and the monolayer harvested by pipetting the media in the wells to lift the cells. The harvested cells and media

were transferred to a 50 mL centrifuge tube followed by three rounds of freeze thawing (at -80 °C and 37 °C). The subsequent lysate, called the primary virus stock was clarified by centrifugation at full speed on a bench top centrifuge (4300Xg) and a proportion of the lysate (10-50%) used to infect 293A cells in a T25 flask. The infected cells were incubated for 48h before harvesting cells and media at complete CPE. The cells were once again harvested, freeze thawed and clarified before using this secondary viral stock to infect a T150 flask seeded at 1.5×10^7 cells per flask. Once complete CPE was achieved at 72h the media and cells were harvested and treated as with earlier viral stocks to generate a tertiary stock.

Production in 293F cells

[00454] ChAdV68 virus production was performed in 293F cells grown in 293 FreeStyle™ (ThermoFisher) media in an incubator at 8% CO₂. On the day of infection cells were diluted to 10^6 cells per mL, with 98% viability and 400 mL were used per production run in 1L Shake flasks (Corning). 4 mL of the tertiary viral stock with a target MOI of >3.3 was used per infection. The cells were incubated for 48-72h until the viability was <70% as measured by Trypan blue. The infected cells were then harvested by centrifugation, full speed bench top centrifuge and washed in 1XPBS, re-centrifuged and then re-suspended in 20 mL of 10mM Tris pH7.4. The cell pellet was lysed by freeze thawing 3X and clarified by centrifugation at 4,300Xg for 5 minutes.

Purification by CsCl centrifugation

[00455] Viral DNA was purified by CsCl centrifugation. Two discontinuous gradient runs were performed. The first to purify virus from cellular components and the second to further refine separation from cellular components and separate defective from infectious particles.

[00456] 10 mL of 1.2 (26.8g CsCl dissolved in 92 mL of 10 mM Tris pH 8.0) CsCl was added to polyallomer tubes. Then 8 mL of 1.4 CsCl (53g CsCl dissolved in 87 mL of 10 mM Tris pH 8.0) was carefully added using a pipette delivering to the bottom of the tube. The clarified virus was carefully layered on top of the 1.2 layer. If needed more 10 mM Tris was added to balance the tubes. The tubes were then placed in a SW-32Ti rotor and centrifuged for 2h 30 min at 10⁰C. The tube was then removed to a laminar flow cabinet and the virus band pulled using an 18 guage needle and a 10 mL syringe. Care was taken not to remove contaminating host cell DNA and protein. The band was then diluted at least 2X with 10 mM Tris pH 8.0 and layered as before on a discontinuous gradient as described above. The run was performed as described before except that this time the run was performed overnight. The next day the band was pulled with care to avoid pulling any of the defective particle band. The virus

was then dialyzed using a Slide-a-Lyzer™ Cassette (Pierce) against ARM buffer (20 mM Tris pH 8.0, 25 mM NaCl, 2.5% Glycerol). This was performed 3X, 1h per buffer exchange. The virus was then aliquoted for storage at -80°C.

Viral assays

[00457] VP concentration was performed by using an OD 260 assay based on the extinction coefficient of 1.1×10^{12} viral particles (VP) is equivalent to an Absorbance value of 1 at OD260 nm. Two dilutions (1:5 and 1:10) of adenovirus were made in a viral lysis buffer (0.1% SDS, 10 mM Tris pH 7.4, 1mM EDTA). OD was measured in duplicate at both dilutions and the VP concentration/ mL was measured by multiplying the OD260 value X dilution factor X 1.1×10^{12} VP.

[00458] An infectious unit (IU) titer was calculated by a limiting dilution assay of the viral stock. The virus was initially diluted 100X in DMEM/5% NS/ 1X PS and then subsequently diluted using 10-fold dilutions down to 1×10^{-7} . 100 μ L of these dilutions were then added to 293A cells that were seeded at least an hour before at 3×10^5 cells/ well of a 24 well plate. This was performed in duplicate. Plates were incubated for 48h in a CO2 (5%) incubator at 37 °C. The cells were then washed with 1XPBS and were then fixed with 100% cold methanol (-20 °C). The plates were then incubated at -20 °C for a minimum of 20 minutes. The wells were washed with 1XPBS then blocked in 1XPBS/0.1% BSA for 1 h at room temperature. A rabbit anti-Ad antibody (Abcam, Cambridge, MA) was added at 1:8,000 dilution in blocking buffer (0.25 ml per well) and incubated for 1 h at room temperature. The wells were washed 4X with 0.5 mL PBS per well. A HRP conjugated Goat anti-Rabbit antibody (Bethyl Labs, Montgomery Texas) diluted 1000X was added per well and incubated for 1h prior to a final round of washing. 5 PBS washes were performed and the plates were developed using DAB (Diaminobenzidine tetrahydrochloride) substrate in Tris buffered saline (0.67 mg/mL DAB in 50 mM Tris pH 7.5, 150 mM NaCl) with 0.01% H₂O₂. Wells were developed for 5 min prior to counting. Cells were counted under a 10X objective using a dilution that gave between 4-40 stained cells per field of view. The field of view that was used was a 0.32 mm² grid of which there are equivalent to 625 per field of view on a 24 well plate. The number of infectious viruses/ mL can be determined by the number of stained cells per grid multiplied by the number of grids per field of view multiplied by a dilution factor 10. Similarly, when working with GFP expressing cells florescent can be used rather than capsid staining to determine the number of GFP expressing virions per mL.

Immunizations

[00459] C57BL/6J female mice and Balb/c female mice were injected with 1×10^8 viral particles (VP) of ChAdV68.5WTnt.MAG25mer in 100 uL volume, bilateral intramuscular injection (50 uL per leg).

Splenocyte dissociation

[00460] Spleen and lymph nodes for each mouse were pooled in 3 mL of complete RPMI (RPMI, 10% FBS, penicillin/streptomycin). Mechanical dissociation was performed using the gentleMACS Dissociator (Miltenyi Biotec), following manufacturer's protocol. Dissociated cells were filtered through a 40 micron filter and red blood cells were lysed with ACK lysis buffer (150mM NH_4Cl , 10mM KHCO_3 , 0.1mM Na_2EDTA). Cells were filtered again through a 30 micron filter and then resuspended in complete RPMI. Cells were counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell were then adjusted to the appropriate concentration of live cells for subsequent analysis.

Ex vivo enzyme-linked immunospot (ELISPOT) analysis

[00461] ELISPOT analysis was performed according to ELISPOT harmonization guidelines {DOI: 10.1038/nprot.2015.068} with the mouse IFNg ELISpotPLUS kit (MABTECH). 5×10^4 splenocytes were incubated with 10uM of the indicated peptides for 16 hours in 96-well IFNg antibody coated plates. Spots were developed using alkaline phosphatase. The reaction was timed for 10 minutes and was terminated by running plate under tap water. Spots were counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation >50% were recorded as "too numerous to count". Samples with deviation of replicate wells > 10% were excluded from analysis. Spot counts were then corrected for well confluency using the formula: $\text{spot count} + 2 \times (\text{spot count} \times \% \text{confluence} / [100\% - \% \text{confluence}])$. Negative background was corrected by subtraction of spot counts in the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count were set to the highest observed corrected value, rounded up to the nearest hundred.

XV.B.2. Production of ChAdV68 Viral Delivery Particles after DNA Transfection

[00462] In one example, ChAdV68.4WTnt.GFP (Fig. 7) and ChAdV68.5WTnt.GFP (Fig. 8) DNA was transfected into HEK293A cells and virus replication (viral plaques) was observed 7-10 days after transfection. ChAdV68 viral plaques were visualized using light (Fig. 7A and 8A) and fluorescent microscopy (Fig. 7B-C and Fig. 8B-C). GFP denotes productive ChAdV68 viral delivery particle production.

XV.B.3. ChAdV68 Viral Delivery Particles Expansion

[00463] In one example, ChAdV68.4WTnt.GFP, ChAdV68.5WTnt.GFP, and ChAdV68.5WTnt.MAG25mer viruses were expanded in HEK293F cells and a purified virus stock produced 18 days after transfection (Fig. 9). Viral particles were quantified in the purified ChAdV68 virus stocks and compared to adenovirus type 5 (Ad5) and ChAdVY25 (a closely related ChAdV; Dicks, 2012, PloS ONE 7, e40385) viral stocks produced using the same protocol. ChAdV68 viral titers were comparable to Ad5 and ChAdVY25 (Table 7).

Table 7. Adenoviral vector production in 293F suspension cells

Construct	Average VP/cell+/- SD
Ad5-Vectors (Multiple vectors)	2.96e4+/- 2.26e4
Ad5-GFP	3.89e4
chAdY25-GFP	1.75e3+/-6.03e1
ChAdV68.4WTnt.GFP	1.2e4 +/-6.5e3
ChAdV68.5WTnt.GFP	1.8e3
ChAdV68.5WTnt.MAG25mer	1.39e3+/-1.1e3

*SD is only reported where multiple Production runs have been performed

XV.B.4. Evaluation of Immunogenicity in Tumor Models

[00464] C68 vector expressing mouse tumor antigens were evaluated in mouse immunogenicity studies to demonstrate the C68 vector elicits T-cell responses. T-cell responses to the MHC class I epitope SIINFELK were measured in C57BL/6J female mice and the MHC class I epitope AH1-A5 (Slansky et al., 2000, Immunity 13:529-538) measured in Balb/c mice. As shown in Fig. 15, strong T-cell responses relative to control were measured after immunization of mice with ChAdV68.5WTnt.MAG25mer. Mean cellular immune responses of 8957 or 4019 spot forming cells (SFCs) per 10⁶ splenocytes were observed in ELISpot assays when C57BL/6J or Balb/c mice were immunized with ChAdV68.5WTnt.MAG25mer, respectively, 10 days after immunization.

[00465] Tumor infiltrating lymphocytes were also evaluated in CT26 tumor model evaluating ChAdV and co-administration of a an anti-CTLA4 antibody. Mice were implanted with CT26 tumors cells and 7 days after implantation, were immunized with ChAdV vaccine and treated with anti-CTLA4 antibody (clone 9D9) or IgG as a control. Tumor infiltrating lymphocytes were analyzed 12 days after immunization. Tumors from each mouse were dissociated using the gentleMACS Dissociator (Miltenyi Biotec) and mouse tumor dissociation kit (Miltenyi Biotec). Dissociated cells were filtered through a 30 micron filter and resuspended in complete RPMI. Cells were counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell were then adjusted to the appropriate concentration of live cells for subsequent analysis. Antigen specific cells were identified by MHC-tetramer complexes and co-stained with anti-CD8 and a viability marker. Tumors were harvested 12 days after prime immunization.

[00466] Antigen-specific CD8⁺ T cells cells within the tumor comprised a median of 3.3%, 2.2%, or 8.1% of the total live cell population in ChAdV, anti-CTLA4, and ChAdV+anti-CTLA4 treated groups, respectively (Fig. 44 and Table 36). Treatment with anti-CTLA in combination with active ChAdV immunization resulted in a statistically significant increase in the antigen-specific CD8⁺ T cell frequency over both ChAdV alone and anti-CTLA4 alone demonstrating anti-CTLA4, when co-administered with the chAd68 vaccine, increased the number of infiltrating T cells within a tumor.

Table 36 - Tetramer⁺ infiltrating CD8 T cell frequencies in CT26 tumors

Treatment	Median % tetramer ⁺
ChAdV68.5WTnt.MAG25mer (ChAdV)	3.3
Anti-CTLA4	2.2
ChAdV68.5WTnt.MAG25mer (ChAdV) + anti-CTLA4	8.1

XVI. Alphavirus Antigen Cassette Delivery Vector

XVI.A. Alphavirus Delivery Vector Evaluation Materials and Methods

In vitro transcription to generate RNA

[00467] *For in vitro testing:* plasmid DNA was linearized by restriction digest with PmeI, column purified following manufacturer's protocol (GeneJet DNA cleanup kit, Thermo) and used as template. *In vitro* transcription was performed using the RiboMAX Large Scale RNA

production System (Promega) with the m⁷G cap analog (Promega) according to manufacturer's protocol. mRNA was purified using the RNeasy kit (Qiagen) according to manufacturer's protocol.

[00468] *For in vivo studies:* RNA was generated and purified by TriLink Biotechnologies and capped with Enzymatic Cap1.

Transfection of RNA

[00469] HEK293A cells were seeded at 6e4 cells/well for 96 wells and 2e5 cells/well for 24 wells, ~16 hours prior to transfection. Cells were transfected with mRNA using MessengerMAX lipofectamine (Invitrogen) and following manufacturer's protocol. For 96-wells, 0.15 uL of lipofectamine and 10 ng of mRNA was used per well, and for 24-wells, 0.75 uL of lipofectamine and 150 ng of mRNA was used per well. A GFP expressing mRNA (TriLink Biotechnologies) was used as a transfection control.

Luciferase assay

[00470] Luciferase reporter assay was performed in white-walled 96-well plates with each condition in triplicate using the ONE-Glo luciferase assay (Promega) following manufacturer's protocol. Luminescence was measured using the SpectraMax.

qRT-PCR

[00471] Transfected cells were rinsed and replaced with fresh media 2 hours post transfection to remove any untransfected mRNA. Cells were then harvested at various timepoints in RLT plus lysis buffer (Qiagen), homogenized using a QiaShredder (Qiagen) and RNA was extracted using the RNeasy kit (Qiagen), all according to manufacturer's protocol. Total RNA was quantified using a Nanodrop (Thermo Scientific). qRT-PCR was performed using the Quantitect Probe One-Step RT-PCR kit (Qiagen) on the qTower³ (Analytik Jena) according to manufacturer's protocol, using 20 ng of total RNA per reaction. Each sample was run in triplicate for each probe. Actin or GusB were used as reference genes. Custom primer/probes were generated by IDT (Table 8).

Table 8. qPCR primers/probes

Target		
Luci	Primer1	GTGGTGTGCAGCGAGAATAG
	Primer2	CGCTCGTTGTAGATGTCGTTAG
	Probe	/56-FAM/TTGCAGTTC/ZEN/TTCATGCCCGTGTTG/3IABkFQ/
GusB	Primer1	GTTTTTGATCCAGACCCAGATG
	Primer2	GCCCATTATTCAGAGCGAGTA
	Probe	/56-FAM/TGCAGGGTT/ZEN/TCACCAGGATCCAC/3IABkFQ/
ActB	Primer1	CCTTGCACATGCCGGAG
	Primer2	ACAGAGCCTCGCCTTTG
	Probe	/56-FAM/TCATCCATG/ZEN/GTGAGCTGGCGG/3IABkFQ/
MAG-25mer	Primer1	CTGAAAGCTCGGTTTGCTAATG
Set1	Primer2	CCATGCTGGAAGAGACAATCT
	Probe	/56-FAM/CGTTTCTGA/ZEN/TGGCGCTGACCGATA/3IABkFQ/
MAG-25mer	Primer1	TATGCCTATCCTGTCTCCTCTG
Set2	Primer2	GCTAATGCAGCTAAGTCCTCTC
	Probe	/56-FAM/TGTTTACCC/ZEN/TGACCGTGCCTTCTG/3IABkFQ/

B16-OVA tumor model

[00472] C57BL/6J mice were injected in the lower left abdominal flank with 10^5 B16-OVA cells/animal. Tumors were allowed to grow for 3 days prior to immunization.

CT26 tumor model

[00473] Balb/c mice were injected in the lower left abdominal flank with 10^6 CT26 cells/animal. Tumors were allowed to grow for 7 days prior to immunization.

Immunizations

[00474] For srRNA vaccine, mice were injected with 10 ug of RNA in 100 uL volume, bilateral intramuscular injection (50 uL per leg). For Ad5 vaccine, mice were injected with 5×10^{10} viral particles (VP) in 100 uL volume, bilateral intramuscular injection (50 uL per leg). Animals were injected with anti-CTLA-4 (clone 9D9, BioXcell), anti-PD-1 (clone RMP1-14, BioXcell) or anti-IgG (clone MPC-11, BioXcell), 250 ug dose, 2 times per week, via intraperitoneal injection.

In vivo bioluminescent imaging

[00475] At each timepoint mice were injected with 150 mg/kg luciferin substrate via intraperitoneal injection and bioluminescence was measured using the IVIS In vivo imaging system (PerkinElmer) 10-15 minutes after injection.

Splenocyte dissociation

[00476] Spleen and lymph nodes for each mouse were pooled in 3 mL of complete RPMI (RPMI, 10% FBS, penicillin/streptomycin). Mechanical dissociation was performed using the gentleMACS Dissociator (Miltenyi Biotec), following manufacturer's protocol. Dissociated cells were filtered through a 40 micron filter and red blood cells were lysed with ACK lysis buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA). Cells were filtered again through a 30 micron filter and then resuspended in complete RPMI. Cells were counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell were then adjusted to the appropriate concentration of live cells for subsequent analysis.

Ex vivo enzyme-linked immunospot (ELISPOT) analysis

[00477] ELISPOT analysis was performed according to ELISPOT harmonization guidelines {DOI: 10.1038/nprot.2015.068} with the mouse IFNg ELISpotPLUS kit (MABTECH). 5x10⁴ splenocytes were incubated with 10uM of the indicated peptides for 16 hours in 96-well IFNg antibody coated plates. Spots were developed using alkaline phosphatase. The reaction was timed for 10 minutes and was terminated by running plate under tap water. Spots were counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation >50% were recorded as "too numerous to count". Samples with deviation of replicate wells > 10% were excluded from analysis. Spot counts were then corrected for well confluency using the formula: spot count + 2 x (spot count x %confluence / [100% - %confluence]). Negative background was corrected by subtraction of spot counts in the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count were set to the highest observed corrected value, rounded up to the nearest hundred.

XVI.B. Alphavirus Vector

XVI.B.1. Alphavirus Vector *in vitro* Evaluation

[00478] In one implementation of the present invention, a RNA alphavirus backbone for the antigen expression system was generated from a Venezuelan Equine Encephalitis (VEE) (Kinney, 1986, Virology 152: 400-413) based self-replicating RNA (srRNA) vector. In one example, the sequences encoding the structural proteins of VEE located 3' of the 26S sub-

genomic promoter were deleted (VEE sequences 7544 to 11,175 deleted; numbering based on Kinney *et al* 1986; SEQ ID NO:6) and replaced by antigen sequences (SEQ ID NO:14 and SEQ ID NO:4) or a luciferase reporter (e.g., VEE-Luciferase, SEQ ID NO:15) (Fig. 10). RNA was transcribed from the srRNA DNA vector *in vitro*, transfected into HEK293A cells and luciferase reporter expression was measured. In addition, an (non-replicating) mRNA encoding luciferase was transfected for comparison. An ~30,000-fold increase in srRNA reporter signal was observed for VEE-Luciferase srRNA when comparing the 23 hour measurement vs the 2 hour measurement (Table 9). In contrast, the mRNA reporter exhibited a less than 10-fold increase in signal over the same time period (Table 9).

Table 9. Expression of luciferase from VEE self-replicating vector increases over time.

HEK293A cells transfected with 10 ng of VEE-Luciferase srRNA or 10 ng of non-replicating luciferase mRNA (TriLink L-6307) per well in 96 wells. Luminescence was measured at various times post transfection. Luciferase expression is reported as relative luminescence units (RLU). Each data point is the mean +/- SD of 3 transfected wells.

Construct	Timepoint (hr)	Mean RLU	Standard Dev (triplicate wells)
mRNA	2	878.6666667	120.7904522
mRNA	5	1847.333333	978.515372
mRNA	9	4847	868.3271273
mRNA	23	8639.333333	751.6816702
SRRNA	2	27	15
SRRNA	5	4884.333333	2955.158935
SRRNA	9	182065.5	16030.81784
SRRNA	23	783658.3333	68985.05538

[00479] In another example, replication of the srRNA was confirmed directly by measuring RNA levels after transfection of either the luciferase encoding srRNA (VEE-Luciferase) or an srRNA encoding a multi-epitope cassette (VEE-MAG25mer) using quantitative reverse transcription polymerase chain reaction (qRT-PCR). An ~150-fold increase in RNA was observed for the VEE-luciferase srRNA (Table 10), while a 30-50-fold increase in RNA was observed for the VEE-MAG25mer srRNA (Table 11). These data confirm that the VEE srRNA vectors replicate when transfected into cells.

Table 10. Direct measurement of RNA replication in VEE-Luciferase srRNA transfected cells. HEK293A cells transfected with VEE-Luciferase srRNA (150 ng per well, 24-well) and RNA levels quantified by qRT-PCR at various times after transfection. Each measurement was normalized based on the Actin reference gene and fold-change relative to the 2 hour timepoint is presented.

Timepoint (hr)	Luciferase Ct	Actin Ct	dCt	Ref dCt	ddCt	Relative Fold change
2	20.51	18.14	2.38	2.38	0.00	1.00
4	20.09	18.39	1.70	2.38	-0.67	1.59
6	15.50	18.19	-2.69	2.38	-5.07	33.51
8	13.51	18.36	-4.85	2.38	-7.22	149.43

Table 11. Direct measurement of RNA replication in VEE-MAG25mer srRNA transfected cells. HEK293 cells transfected with VEE-MAG25mer srRNA (150 ng per well, 24-well) and RNA levels quantified by qRT-PCR at various times after transfection. Each measurement was normalized based on the GusB reference gene and fold-change relative to the 2 hour timepoint is presented. Different lines on the graph represent 2 different qPCR primer/probe sets, both of which detect the epitope cassette region of the srRNA.

Primer/probe	Timepoint (hr)	Ct	GusB Ct	dCt	Ref dCt	ddCt	Relative Fold-Change
Set1	2	18.96	22.41	-3.45	-3.45	0.00	1.00
Set1	4	17.46	22.27	-4.81	-3.45	-1.37	2.58
Set1	6	14.87	22.04	-7.17	-3.45	-3.72	13.21
Set1	8	14.16	22.19	-8.02	-3.45	-4.58	23.86
Set1	24	13.16	22.01	-8.86	-3.45	-5.41	42.52
Set1	36	13.53	22.63	-9.10	-3.45	-5.66	50.45
Set2	2	17.75	22.41	-4.66	-4.66	0.00	1.00
Set2	4	16.66	22.27	-5.61	-4.66	-0.94	1.92
Set2	6	14.22	22.04	-7.82	-4.66	-3.15	8.90
Set2	8	13.18	22.19	-9.01	-4.66	-4.35	20.35
Set2	24	12.22	22.01	-9.80	-4.66	-5.13	35.10
Set2	36	13.08	22.63	-9.55	-4.66	-4.89	29.58

XVI.B.2. Alphavirus Vector *in vivo* Evaluation

[00480] In another example, VEE-Luciferase reporter expression was evaluated *in vivo*. Mice were injected with 10 ug of VEE-Luciferase srRNA encapsulated in lipid nanoparticle (MC3) and imaged at 24 and 48 hours, and 7 and 14 days post injection to determine

bioluminescent signal. Luciferase signal was detected at 24 hours post injection and increased over time and appeared to peak at 7 days after srRNA injection (Fig. 11).

XVI.B.3. Alphavirus Vector Tumor Model Evaluation

[00481] In one implementation, to determine if the VEE srRNA vector directs antigen-specific immune responses *in vivo*, a VEE srRNA vector was generated (VEE-UbAAY, SEQ ID NO:14) that expresses 2 different MHC class I mouse tumor epitopes, SIINFEDL and AH1-A5 (Slansky et al., 2000, Immunity 13:529-538). The SFL (SIINFEDL) epitope is expressed by the B16-OVA melanoma cell line, and the AH1-A5 (SPSYAYHQF; Slansky et al., 2000, Immunity) epitope induces T cells targeting a related epitope (AH1/ SPSYVYHQF; Huang et al., 1996, Proc Natl Acad Sci USA 93:9730-9735) that is expressed by the CT26 colon carcinoma cell line. In one example, for *in vivo* studies, VEE-UbAAY srRNA was generated by *in vitro* transcription using T7 polymerase (TriLink Biotechnologies) and encapsulated in a lipid nanoparticle (MC3).

[00482] A strong antigen-specific T-cell response targeting SFL, relative to control, was observed two weeks after immunization of B16-OVA tumor bearing mice with MC3 formulated VEE-UbAAY srRNA. In one example, a median of 3835 spot forming cells (SFC) per 10^6 splenocytes was measured after stimulation with the SFL peptide in ELISpot assays (Fig. 12A, Table 12) and 1.8% (median) of CD8 T-cells were SFL antigen-specific as measured by pentamer staining (Fig. 12B, Table 12). In another example, co-administration of an anti-CTLA-4 monoclonal antibody (mAb) with the VEE srRNA vaccine resulted in a moderate increase in overall T-cell responses with a median of 4794.5 SFCs per 10^6 splenocytes measured in the ELISpot assay (Fig. 12A, Table 12).

Table 12. Results of ELISPOT and MHCII-pentamer staining assays 14 days post VEE srRNA immunization in B16-OVA tumor bearing C57BL/6J mice.

Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)	Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)
Control	1	47	0.22	Vax	1	6774	4.92
	2	80	0.32		2	2323	1.34
	3	0	0.27		3	2997	1.52
	4	0	0.29		4	4492	1.86
	5	0	0.27		5	4970	3.7
	6	0	0.25		6		4.13
	7	0	0.23		7	3835	1.66
	8	87	0.25		8	3119	1.64
aCTLA4	1	0	0.24	Vax + aCTLA4	1	6232	2.16
	2	0	0.26		2	4242	0.82
	3	0	0.39		3	5347	1.57
	4	0	0.28		4	6568	2.33
	5	0	0.28		5	6269	1.55
	6	0	0.28		6	4056	1.74
	7	0	0.31		7	4163	1.14
	8	6	0.26		8	3667	1.01

* Note that results from mouse #6 in the Vax group were excluded from analysis due to high variability between triplicate wells.

[00483] In another implementation, to mirror a clinical approach, a heterologous prime/boost in the B16-OVA and CT26 mouse tumor models was performed, where tumor bearing mice were immunized first with adenoviral vector expressing the same antigen cassette (Ad5-UbAAY), followed by a boost immunization with the VEE-UbAAY srRNA vaccine 14 days after the Ad5-UbAAY prime. In one example, an antigen-specific immune response was induced by the Ad5-UbAAY vaccine resulting in 7330 (median) SFCs per 10^6 splenocytes measured in the ELISpot assay (Fig. 13A, Table 13) and 2.9% (median) of CD8 T-cells targeting the SFL antigen as measured by pentamer staining (Fig. 13C, Table 13). In another example, the T-cell response was maintained 2 weeks after the VEE-UbAAY srRNA boost in the B16-OVA model with 3960 (median) SFL-specific SFCs per 10^6 splenocytes measured in the ELISpot assay (Fig. 13B, Table 13) and 3.1% (median) of CD8 T-cells targeting the SFL antigen as measured by pentamer staining (Fig. 13D, Table 13).

Table 13. Immune monitoring of B16-OVA mice following heterologous prime/boost with Ad5 vaccine prime and srRNA boost.**Day 14**

Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)	Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)
Control	1	0	0.10	Vax	1	8514	1.87
	2	0	0.09		2	7779	1.91
	3	0	0.11		3	6177	3.17
	4	46	0.18		4	7945	3.41
	5	0	0.11		5	8821	4.51
	6	16	0.11		6	6881	2.48
	7	0	0.24		7	5365	2.57
	8	37	0.10		8	6705	3.98
aCTLA4	1	0	0.08	Vax + aCTLA4	1	9416	2.35
	2	29	0.10		2	7918	3.33
	3	0	0.09		3	10153	4.50
	4	29	0.09		4	7212	2.98
	5	0	0.10		5	11203	4.38
	6	49	0.10		6	9784	2.27
	7	0	0.10		8	7267	2.87
	8	31	0.14				

Day 28

Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)	Group	Mouse	SFC/1e6 splenocytes	Pentamer positive (% of CD8)
Control	2	0	0.17	Vax	1	5033	2.61
	4	0	0.15		2	3958	3.08
	6	20	0.17		4	3960	3.58
aCTLA4	1	7	0.23	Vax + aCTLA4	4	3460	2.44
	2	0	0.18		5	5670	3.46
	3	0	0.14				

[00484] In another implementation, similar results were observed after an Ad5-UbAAY prime and VEE-UbAAY srRNA boost in the CT26 mouse model. In one example, an AH1 antigen-specific response was observed after the Ad5-UbAAY prime (day 14) with a mean of 5187 SFCs per 10^6 splenocytes measured in the ELISpot assay (Fig. 14A, Table 14) and 3799 SFCs per 10^6 splenocytes measured in the ELISpot assay after the VEE-UbAAY srRNA boost (day 28) (Fig. 14B, Table 14).

Table 14. Immune monitoring after heterologous prime/boost in CT26 tumor mouse model.

Day 12			Day 21		
Group	Mouse	SFC/1e6 splenocytes	Group	Mouse	SFC/1e6 splenocytes
Control	1	1799	Control	9	167
	2	1442		10	115
	3	1235		11	347
aPD1	1	737	aPD1	8	511
	2	5230		11	758
	3	332	Vax	9	3133
Vax	1	6287		10	2036
	2	4086		11	6227
Vax + aPD1	1	5363	Vax + aPD1	8	3844
	2	6500		9	2071
				11	4888

XVII. ChAdV/srRNA Combination Tumor Model Evaluation

[00485] Various dosing protocols using ChAdV68 and self-replicating RNA (srRNA) were evaluated in murine CT26 tumor models.

XVII.A ChAdV/srRNA Combination Tumor Model Evaluation

Methods and Materials

Tumor Injection

[00486] Balb/c mice were injected with the CT26 tumor cell line. 7 days after tumor cell injection, mice were randomized to the different study arms (28-40 mice per group) and treatment initiated. Balb/c mice were injected in the lower left abdominal flank with 10^6 CT26 cells/animal. Tumors were allowed to grow for 7 days prior to immunization. The study arms are described in detail in Table 15.

Table 15 - ChAdV/srRNA Combination Tumor Model Evaluation Study Arms

Group	N	Treatment	Dose	Volume	Schedule	Route
1	40	chAd68 control	1e11 vp	2x 50 uL	day 0	IM
		srRNA control	10 ug	50 uL	day 14, 28, 42	IM
		Anti-PD1	250 ug	100 uL	2x / week (start day 0)	IP
2	40	chAd68 control	1e11 vp	2x 50 uL	day 0	IM
		srRNA control	10 ug	50 uL	day 14, 28, 42	IM
		Anti-IgG	250 ug	100 uL	2x / week (start day 0)	IP
3	28	chAd68 vaccine	1e11 vp	2x 50 uL	day 0	IM
		srRNA vaccine	10 ug	50 uL	day 14, 28, 42	IM
		Anti-PD1	250 ug	100 uL	2x / week (start day 0)	IP
4	28	chAd68 vaccine	1e11 vp	2x 50 uL	day 0	IM
		srRNA vaccine	10 ug	50 uL	day 14, 28, 42	IM
		Anti-IgG	250 ug	100 uL	2x / week (start day 0)	IP
5	28	srRNA vaccine	10 ug	50 uL	day 0, 28, 42	IM
		chAd68 vaccine	1e11 vp	2x 50 uL	day 14	IM
		Anti-PD1	250 ug	100 uL	2x / week (start day 0)	IP
6	28	srRNA vaccine	10 ug	50 uL	day 0, 28, 42	IM
		chAd68 vaccine	1e11 vp	2x 50 uL	day 14	IM
		Anti-IgG	250 ug	100 uL	2x / week (start day 0)	IP
7	40	srRNA vaccine	10 ug	50 uL	day 0, 14, 28, 42	IM
		Anti-PD1	250 ug	100 uL	2x / week (start day 0)	IP
8	40	srRNA vaccine	10 ug	50 uL	day 0, 14, 28, 42	IM
		Anti-IgG	250 ug	100 uL	2x / week (start day 0)	IP

Immunizations

[00487] For srRNA vaccine, mice were injected with 10 ug of VEE-MAG25mer srRNA in 100 uL volume, bilateral intramuscular injection (50 uL per leg). For C68 vaccine, mice were injected with 1×10^{11} viral particles (VP) of ChAdV68.5WTnt.MAG25mer in 100 uL volume, bilateral intramuscular injection (50 uL per leg). Animals were injected with anti-PD-1 (clone RMP1-14, BioXcell) or anti-IgG (clone MPC-11, BioXcell), 250 ug dose, 2 times per week, via intraperitoneal injection.

Splenocyte dissociation

[00488] Spleen and lymph nodes for each mouse were pooled in 3 mL of complete RPMI (RPMI, 10% FBS, penicillin/streptomycin). Mechanical dissociation was performed using the gentleMACS Dissociator (Miltenyi Biotec), following manufacturer's protocol. Dissociated

cells were filtered through a 40 micron filter and red blood cells were lysed with ACK lysis buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA). Cells were filtered again through a 30 micron filter and then resuspended in complete RPMI. Cells were counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell were then adjusted to the appropriate concentration of live cells for subsequent analysis.

Ex vivo enzyme-linked immunospot (ELISPOT) analysis

[00489] ELISPOT analysis was performed according to ELISPOT harmonization guidelines {DOI: 10.1038/nprot.2015.068} with the mouse IFN γ ELISpotPLUS kit (MABTECH). 5x10⁴ splenocytes were incubated with 10uM of the indicated peptides for 16 hours in 96-well IFN γ antibody coated plates. Spots were developed using alkaline phosphatase. The reaction was timed for 10 minutes and was terminated by running plate under tap water. Spots were counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation >50% were recorded as “too numerous to count”. Samples with deviation of replicate wells > 10% were excluded from analysis. Spot counts were then corrected for well confluency using the formula: spot count + 2 x (spot count x %confluence / [100% - %confluence]). Negative background was corrected by subtraction of spot counts in the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count were set to the highest observed corrected value, rounded up to the nearest hundred.

XVII.B ChAdV/srRNA Combination Evaluation in a CT26 Tumor Model

[00490] The immunogenicity and efficacy of the ChAdV68.5WTnt.MAG25mer/ VEE-MAG25mer srRNA heterologous prime/boost or VEE-MAG25mer srRNA homologous prime/boost vaccines were evaluated in the CT26 mouse tumor model. Balb/c mice were injected with the CT26 tumor cell line. 7 days after tumor cell injection, mice were randomized to the different study arms and treatment initiated. The study arms are described in detail in Table 15 and more generally in Table 16.

Table 16 – Prime/Boost Study Arms

Group	Prime	Boost
1	Control	Control
2	Control + anti-PD-1	Control + anti-PD-1
3	ChAdV68.5WTnt.MAG25mer	VEE-MAG25mer srRNA
4	ChAdV68.5WTnt.MAG25mer + anti-PD-1	VEE-MAG25mer srRNA + anti-PD-1
5	VEE-MAG25mer srRNA	ChAdV68.5WTnt.MAG25mer
6	VEE-MAG25mer srRNA + anti-PD-1	ChAdV68.5WTnt.MAG25mer + anti-PD-1
7	VEE-MAG25mer srRNA	VEE-MAG25mer srRNA
8	VEE-MAG25mer srRNA + anti-PD-1	VEE-MAG25mer srRNA + anti-PD-1

[00491] Spleens were harvested 14 days after the prime vaccination for immune monitoring. Tumor and body weight measurements were taken twice a week and survival was monitored. Strong immune responses relative to control were observed in all active vaccine groups.

[00492] Median cellular immune responses of 10,630, 12,976, 3319, or 3745 spot forming cells (SFCs) per 10^6 splenocytes were observed in ELISpot assays in mice immunized with ChAdV68.5WTnt.MAG25mer (ChAdV/group 3), ChAdV68.5WTnt.MAG25mer + anti-PD-1 (ChAdV + PD-1/group 4), VEE-MAG25mer srRNA (srRNA/median for groups 5 & 7 combined), or VEE-MAG25mer srRNA + anti-PD-1 (srRNA + PD-1/median for groups 6 & 8 combined), respectively, 14 days after the first immunization (Fig. 16 and Table 17). In contrast, the vaccine control (group 1) or vaccine control with anti-PD-1 (group 2) exhibited median cellular immune responses of 296 or 285 SFC per 10^6 splenocytes, respectively.

Table 17 – Cellular immune responses in a CT26 tumor model

Treatment	Median SFC/10⁶ Splenocytes
Control	296
PD1	285
ChAdV68.5WTnt.MAG25mer (ChAdV)	10630
ChAdV68.5WTnt.MAG25mer + PD1 (ChAdV + PD-1)	12976
VEE-MAG25mer srRNA (srRNA)	3319
VEE-MAG25mer srRNA + PD-1 (srRNA + PD1)	3745

[00493] Consistent with the ELISpot data, 5.6, 7.8, 1.8 or 1.9% of CD8 T cells (median) exhibited antigen-specific responses in intracellular cytokine staining (ICS) analyses for mice immunized with ChAdV68.5WTnt.MAG25mer (ChAdV/group 3), ChAdV68.5WTnt.MAG25mer + anti-PD-1 (ChAdV + PD-1/group 4), VEE-MAG25mer srRNA (srRNA/median for groups 5 & 7 combined), or VEE-MAG25mer srRNA + anti-PD-1 (srRNA + PD-1/median for groups 6 & 8 combined), respectively, 14 days after the first immunization (Fig. 17 and Table 18). Mice immunized with the vaccine control or vaccine control combined with anti-PD-1 showed antigen-specific CD8 responses of 0.2 and 0.1%, respectively.

Table 18 - CD8 T-Cell responses in a CT26 tumor model

Treatment	Median % CD8 IFN- gamma Positive
Control	0.21
PD1	0.1
ChAdV68.5WTnt.MAG25mer (ChAdV)	5.6
ChAdV68.5WTnt.MAG25mer + PD1 (ChAdV + PD-1)	7.8
VEE-MAG25mer srRNA (srRNA)	1.8
VEE-MAG25mer srRNA + PD-1 (srRNA + PD1)	1.9

[00494] Tumor growth was measured in the CT26 colon tumor model for all groups, and tumor growth up to 21 days after treatment initiation (28 days after injection of CT-26 tumor

cells) is presented. Mice were sacrificed 21 days after treatment initiation based on large tumor sizes ($>2500 \text{ mm}^3$); therefore, only the first 21 days are presented to avoid analytical bias. Mean tumor volumes at 21 days were 1129, 848, 2142, 1418, 2198 and 1606 mm^3 for ChAdV68.5WTnt.MAG25mer prime/ VEE-MAG25mer srRNA boost (group 3), ChAdV68.5WTnt.MAG25mer prime/ VEE-MAG25mer srRNA boost + anti-PD-1 (group 4), VEE-MAG25mer srRNA prime/ ChAdV68.5WTnt.MAG25mer boost (group 5), VEE-MAG25mer srRNA prime / ChAdV68.5WTnt.MAG25mer boost + anti-PD-1 (group 6), VEE-MAG25mer srRNA prime/ VEE-MAG25mer srRNA boost (group 7) and VEE-MAG25mer srRNA prime/ VEE-MAG25mer srRNA boost + anti-PD-1 (group 8), respectively (Fig. 18 and Table 19). The mean tumor volumes in the vaccine control or vaccine control combined with anti-PD-1 were 2361 or 2067 mm^3 , respectively. Based on these data, vaccine treatment with ChAdV68.5WTnt.MAG25mer / VEE-MAG25mer srRNA (group 3), ChAdV68.5WTnt.MAG25mer / VEE-MAG25mer srRNA + anti-PD-1 (group 4), VEE-MAG25mer srRNA/ ChAdV68.5WTnt.MAG25mer + anti-PD-1 (group 6) and VEE-MAG25mer srRNA/ VEE-MAG25mer srRNA + anti-PD-1 (group 8) resulted in a reduction of tumor growth at 21 days that was significantly different from the control (group 1).

Table 19 - Tumor size at day 21 measured in the CT26 model

Treatment	Tumor Size (mm^3)	SEM
Control	2361	235
PD1	2067	137
chAdV/srRNA	1129	181
chAdV/srRNA + PD1	848	182
srRNA/chAdV	2142	233
srRNA/chAdV + PD1	1418	220
srRNA	2198	134
srRNA + PD1	1606	210

[00495] Survival was monitored for 35 days after treatment initiation in the CT-26 tumor model (42 days after injection of CT-26 tumor cells). Improved survival was observed after vaccination of mice with 4 of the combinations tested. After vaccination, 64%, 46%, 41% and 36% of mice survived with ChAdV68.5WTnt.MAG25mer prime/ VEE-MAG25mer srRNA boost in combination with anti-PD-1 (group 4; $P < 0.0001$ relative to control group 1), VEE-

MAG25mer srRNA prime/ VEE-MAG25mer srRNA boost in combination with anti-PD-1 (group 8; P=0.0006 relative to control group 1), ChAdV68.5WTnt.MAG25mer prime/ VEE-MAG25mer srRNA boost (group 3; P=0.0003 relative to control group 1) and VEE-MAG25mer srRNA prime/ ChAdV68.5WTnt.MAG25mer boost in combination with anti-PD-1 (group 6; P=0.0016 relative to control group 1), respectively (Fig. 19 and Table 20). Survival was not significantly different from the control group 1 ($\leq 14\%$) for the remaining treatment groups [VEE-MAG25mer srRNAprime/ ChAdV68.5WTnt.MAG25mer boost (group 5), VEE-MAG25mer srRNA prime/ VEE-MAG25mer srRNA boost (group 7) and anti-PD-1 alone (group 2)].

Table 20 - Survival in the CT26 model

Timepoint	Control	PD1	chAdV/ srRNA	chAdV/ srRNA + PD1	srRNA/ chAdV	srRNA/ chAdV + PD1	srRNA	srRNA + PD1
0	100	100	100	100.00	100.00	100	100	100
21	96	100	100	100	100	95	100	100
24	54	64	91	100	68	82	68	71
28	21	32	68	86	45	68	21	64
31	7	14	41	64	14	36	11	46
35	7	14	41	64	14	36	11	46

[00496] In conclusion, ChAdV68.5WTnt.MAG25mer and VEE-MAG25mer srRNA elicited strong T-cell responses to mouse tumor antigens encoded by the vaccines, relative to control. Administration of a ChAdV68.5WTnt.MAG25mer prime and VEE-MAG25mer srRNA boost with or without co-administration of anti-PD-1, VEE-MAG25mer srRNA prime and ChAdV68.5WTnt.MAG25mer boost in combination with anti-PD-1 or administration of VEE-MAG25mer srRNA as a homologous prime boost immunization in combination with anti-PD-1 to tumor bearing mice resulted in improved survival.

XVIII. Non-Human Primate Studies

[00497] Various dosing protocols using ChAdV68 and self-replicating RNA (srRNA) were evaluated in non-human primates (NHP).

Materials and Methods

[00498] A priming vaccine was injected intramuscularly (IM) in each NHP to initiate the study (vaccine prime). One or more boosting vaccines (vaccine boost) were also injected intramuscularly in each NHP. Bilateral injections per dose were administered according to groups outlined in tables and summarized below.

Immunizations

[00499] Mamu-A*01 Indian rhesus macaques were immunized bilaterally with 1×10^{12} viral particles (5×10^{11} viral particles per injection) of ChAdV68.5WTnt.MAG25mer, 30 ug of VEE-MAG25MER srRNA, 100 ug of VEE-MAG25mer srRNA or 300 ug of VEE-MAG25mer srRNA formulated in LNP-1 or LNP-2. Vaccine boosts of 30 ug, 100 ug or 300 ug VEE-MAG25mer srRNA were administered intramuscularly at the indicated time after prime vaccination.

Immune Monitoring

[00500] PBMCs were isolated at indicated times after prime vaccination using Lymphocyte Separation Medium (LSM, MP Biomedicals) and LeucoSep separation tubes (Greiner Bio-One) and resuspended in RPMI containing 10% FBS and penicillin/streptomycin. Cells were counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell were then adjusted to the appropriate concentration of live cells for subsequent analysis. For each monkey in the studies, T cell responses were measured using ELISpot or flow cytometry methods. T cell responses to 6 different rhesus macaque Mamu-A*01 class I epitopes encoded in the vaccines were monitored from PBMCs by measuring induction of cytokines, such as IFN-gamma, using ex vivo enzyme-linked immunospot (ELISpot) analysis. ELISpot analysis was performed according to ELISPOT harmonization guidelines {DOI: 10.1038/nprot.2015.068} with the monkey IFNg ELISpotPLUS kit (MABTECH). 200,000 PBMCs were incubated with 10uM of the indicated peptides for 16 hours in 96-well IFNg antibody coated plates. Spots were developed using alkaline phosphatase. The reaction was timed for 10 minutes and was terminated by running plate under tap water. Spots were counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation >50% were recorded as “too numerous to count”. Samples with deviation of replicate wells > 10% were excluded from analysis. Spot counts were then corrected for well confluency using the formula: $\text{spot count} + 2 \times (\text{spot count} \times \% \text{confluence} / [100\% - \% \text{confluence}])$. Negative background was corrected by subtraction of spot counts in

the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count were set to the highest observed corrected value, rounded up to the nearest hundred.

[00501] Specific CD4 and CD8 T cell responses to 6 different rhesus macaque Mamu-A*01 class I epitopes encoded in the vaccines were monitored from PBMCs by measuring induction of intracellular cytokines, such as IFN-gamma, using flow cytometry. The results from both methods indicate that cytokines were induced in an antigen-specific manner to epitopes.

Immunogenicity in rhesus macaques

[00502] This study was designed to (a) evaluate the immunogenicity and preliminary safety of VEE-MAG25mer srRNA 30 µg and 100 µg doses as a homologous prime/boost or heterologous prime/boost in combination with ChAdV68.5WTnt.MAG25mer; (b) compare the immune responses of VEE-MAG25mer srRNA in lipid nanoparticles using LNP1 versus LNP2; (c) evaluate the kinetics of T-cell responses to VEE-MAG25mer srRNA and ChAdV68.5WTnt.MAG25mer immunizations.

[00503] The study arm was conducted in Mamu-A*01 Indian rhesus macaques to demonstrate immunogenicity. Select antigens used in this study are only recognized in Rhesus macaques, specifically those with a Mamu-A*01 MHC class I haplotype. Mamu-A*01 Indian rhesus macaques were randomized to the different study arms (6 macaques per group) and administered an IM injection bilaterally with either ChAdV68.5WTnt.MAG25mer or VEE-MAG25mer srRNA vector encoding model antigens that includes multiple Mamu-A*01 restricted epitopes. The study arms were as described below.

[00504] **Table 21:** Non-GLP immunogenicity study in Indian Rhesus Macaques

Group	Prime	Boost 1	Boost 2
1	VEE-MAG25mer srRNA -LNP1(30 µg)	VEE-MAG25mer srRNA -LNP1 (30 µg)	VEE-MAG25mer srRNA -LNP1 (30 µg)
2	VEE-MAG25mer srRNA -LNP1 (100 µg)	VEE-MAG25mer srRNA -LNP1 (100 µg)	VEE-MAG25mer srRNA -LNP1 (100 µg)
3	VEE-MAG25mer srRNA -LNP2 (100 µg)	VEE-MAG25mer srRNA -LNP2 (100 µg)	VEE-MAG25mer srRNA -LNP2 (100 µg)
4	ChAdV68.5WTnt. MAG25mer	VEE-MAG25mer srRNA -LNP1 (100 µg)	VEE-MAG25mer srRNA -LNP1 (100 µg)

[00505] PBMCs were collected prior to immunization and on weeks 1, 2, 3, 4, 5, 6, 8, 9, and 10 after the initial immunization for immune monitoring.

Results

[00506] Antigen-specific cellular immune responses in peripheral blood mononuclear cells (PBMCs) were measured to six different Mamu-A*01 restricted epitopes prior to immunization and 1, 2, 3, 4, 5, 6, 8, 9, and 10 weeks after the initial immunization. Animals received a boost immunization with VEE-MAG25mer srRNA on weeks 4 and 8 with either 30 µg or 100 µg doses, and either formulated with LNP1 or LNP2, as described in Table 21. Combined immune responses to all six epitopes were plotted for each immune monitoring timepoint (FIG. 20A-D and Tables 22-25).

[00507] Combined antigen-specific immune responses were observed at all measurements with 170, 14, 15, 11, 7, 8, 14, 17, 12 SFCs per 10⁶ PBMCs (six epitopes combined) 1, 2, 3, 4, 5, 6, 8, 9, or 10 weeks after an initial VEE-MAG25mer srRNA-LNP1(30 µg) prime immunization, respectively (FIG. 20A). Combined antigen-specific immune responses were observed at all measurements with 108, -3, 14, 1, 37, 4, 105, 17, 25 SFCs per 10⁶ PBMCs (six epitopes combined) 1, 2, 3, 4, 5, 6, 8, 9, or 10 weeks after an initial VEE-MAG25mer srRNA-LNP1(100 µg) prime immunization, respectively (FIG. 20B). Combined antigen-specific immune responses were observed at all measurements with -17, 38, 14, -2, 87, 21, 104, 129, 89

SFCs per 10^6 PBMCs (six epitopes combined) 1, 2, 3, 4, 5, 6, 8, 9, or 10 weeks after an initial VEE-MAG25mer srRNA-LNP2(100 μ g) prime immunization, respectively (FIG. 20C).

Negative values are a result of normalization to pre-bleed values for each epitope/animal.

[00508] Combined antigen-specific immune responses were observed at all measurements with 1218, 1784, 1866, 973, 1813, 747, 797, 1249, and 547 SFCs per 10^6 PBMCs (six epitopes combined) 1, 2, 3, 4, 5, 6, 8, 9, or 10 weeks after an initial ChAdV68.5WTnt.MAG25mer prime immunization, respectively (FIG. 20D). The immune response showed the expected profile with peak immune responses measured ~2-3 weeks after the prime immunization followed by a contraction in the immune response after 4 weeks. Combined antigen-specific cellular immune responses of 1813 SFCs per 10^6 PBMCs (six epitopes combined) were measured 5 weeks after the initial immunization with ChAdV68.5WTnt.MAG25mer (i.e., 1 week after the first boost with VEE-MAG25mer srRNA). The immune response measured 1 week after the first boost with VEE-MAG25mer srRNA (week 5) was comparable to the peak immune response measured for the ChAdV68.5WTnt.MAG25mer prime immunization (week 3) (FIG. 20D). Combined antigen-specific cellular immune responses of 1249 SFCs per 10^6 PBMCs (six epitopes combined) was measured 9 weeks after the initial immunization with ChAdV68.5WTnt.MAG25mer, respectively (i.e., 1 week after the second boost with VEE-MAG25mer srRNA). The immune responses measured 1 week after the second boost with VEE-MAG25mer srRNA (week 9) was ~2-fold higher than that measured just before the boost immunization (FIG. 20D).

[00509] Table 22: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for VEE-MAG25mer srRNA-LNP1(30 μ g) (Group 1)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	39.7 $\pm$ 22.7	35.4 $\pm$ 25.1	3.2 $\pm$ 3.6	33 $\pm$ 28.1	30.9 $\pm$ 20.3	28.3 $\pm$ 17.5
3	2 $\pm$ 2.4	0.2 $\pm$ 1.8	1.8 $\pm$ 2.4	3.7 $\pm$ 1.9	1.7 $\pm$ 2.8	4.9 $\pm$ 2.3
4	1 $\pm$ 1.8	0.3 $\pm$ 1.2	5.5 $\pm$ 3.6	2.3 $\pm$ 2.2	5.7 $\pm$ 2.7	0.8 $\pm$ 0.8
5	0.5 $\pm$ 0.9	1.4 $\pm$ 3.8	3.1 $\pm$ 1.6	2.3 $\pm$ 2.7	1.9 $\pm$ 2	1.4 $\pm$ 1.2
6	1.9 $\pm$ 1.8	-0.3 $\pm$ 3	1.7 $\pm$ 1.2	1.4 $\pm$ 1.4	0.8 $\pm$ 1.1	1.1 $\pm$ 1
8	-0.4 $\pm$ 0.8	-0.9 $\pm$ 2.9	0.5 $\pm$ 1.3	3 $\pm$ 1.1	2.2 $\pm$ 2.1	3.7 $\pm$ 2
9	1 $\pm$ 1.7	1.2 $\pm$ 4.2	7.2 $\pm$ 3.9	0.5 $\pm$ 0.7	1.6 $\pm$ 3	3 $\pm$ 1
10	3.8 $\pm$ 1.8	11 $\pm$ 5	-1.1 $\pm$ 1.1	1.9 $\pm$ 0.9	1.3 $\pm$ 1.6	0.2 $\pm$ 0.5

[00510] Table 23: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for VEE-MAG25mer srRNA-LNP1(100 μ g) (Group 2)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	7.9 $\pm$ 17.2	23.2 $\pm$ 17.4	11.4 $\pm$ 4.9	41.7 $\pm$ 16.5	15 $\pm$ 13.5	8.9 $\pm$ 6.2
3	-3.1 $\pm$ 4.6	-7.2 $\pm$ 6.5	2.3 $\pm$ 2.3	-0.3 $\pm$ 2.7	2.7 $\pm$ 5.1	2.2 $\pm$ 1.4
4	1.9 $\pm$ 3.8	-6.2 $\pm$ 7.6	10.5 $\pm$ 4.1	1.2 $\pm$ 2.9	5.6 $\pm$ 4.9	1.1 $\pm$ 0.8
5	-2.6 $\pm$ 7	-8 $\pm$ 5.9	1.5 $\pm$ 1.7	6.4 $\pm$ 2.3	0.7 $\pm$ 4.3	3.3 $\pm$ 1.3
6	6.3 $\pm$ 6.3	4.4 $\pm$ 8.3	6.6 $\pm$ 4.4	5.2 $\pm$ 5.2	3.9 $\pm$ 5	10.8 $\pm$ 6.9
8	-3.6 $\pm$ 7.2	-6.8 $\pm$ 7.3	-0.8 $\pm$ 1.2	3.4 $\pm$ 4.2	6.4 $\pm$ 7.5	5.7 $\pm$ 2.7
9	8.1 $\pm$ 2.4	20.6 $\pm$ 23.4	18.9 $\pm$ 5.7	8.1 $\pm$ 8.9	9 $\pm$ 11.2	40 $\pm$ 17.6
10	3.1 $\pm$ 8	-3.9 $\pm$ 8.5	3.3 $\pm$ 1.8	0.6 $\pm$ 2.9	7.4 $\pm$ 6.4	6.1 $\pm$ 2.5

[00511] Table 24: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for VEE-MAG25mer srRNA-LNP2(100 μ g) (Group 3)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	-5.9 $\pm$ 3.8	-0.3 $\pm$ 0.5	-0.5 $\pm$ 1.5	-5.7 $\pm$ 6.1	-1 $\pm$ 1.3	-3.2 $\pm$ 5.5
3	0.7 $\pm$ 5.2	3.4 $\pm$ 2.4	4.2 $\pm$ 4.6	18.3 $\pm$ 15.5	11.9 $\pm$ 5.1	-0.4 $\pm$ 8.2
4	-3.8 $\pm$ 5.5	2.3 $\pm$ 1.8	11.3 $\pm$ 6.1	-3.1 $\pm$ 5.6	8.5 $\pm$ 4	-1.5 $\pm$ 6.1
5	-3.7 $\pm$ 5.7	-0.1 $\pm$ 0.7	-0.2 $\pm$ 1.6	3.4 $\pm$ 8.5	3 $\pm$ 3.1	-4.6 $\pm$ 5
6	12.3 $\pm$ 15	7.8 $\pm$ 4.9	24.7 $\pm$ 19.8	23.2 $\pm$ 22.5	18.7 $\pm$ 15.8	0.5 $\pm$ 6.2
8	5.9 $\pm$ 12.3	-0.1 $\pm$ 0.7	-0.5 $\pm$ 1.3	8.8 $\pm$ 14.4	8.7 $\pm$ 8	-1.3 $\pm$ 4
9	16.1 $\pm$ 13.4	16.5 $\pm$ 4	22.9 $\pm$ 4.2	13 $\pm$ 13.2	16.4 $\pm$ 7.8	19.6 $\pm$ 9.2
10	29.9 $\pm$ 21.8	22 $\pm$ 19.5	0.5 $\pm$ 2.6	22.2 $\pm$ 22.6	35.3 $\pm$ 15.8	19.4 $\pm$ 17.3

[00512] Table 25: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for ChAdV68.5WTnt.MAG25mer prime

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
1	178 $\pm$ 68.7	206.5 $\pm$ 94.8	221.2 $\pm$ 120	15.4 $\pm$ 16.7	33.3 $\pm$ 25.9	563.5 $\pm$ 174.4
2	311.2 $\pm$ 165.5	278.8 $\pm$ 100.9	344.6 $\pm$ 110.8	46.3 $\pm$ 13.5	181.6 $\pm$ 76.8	621.4 $\pm$ 220.9
3	277.3 $\pm$ 101.1	359.6 $\pm$ 90.5	468.2 $\pm$ 106.6	41.7 $\pm$ 11.1	169.8 $\pm$ 57.8	549.4 $\pm$ 115.7
4	140 $\pm$ 46.5	169.6 $\pm$ 46.8	239.4 $\pm$ 37	26.5 $\pm$ 11.4	75 $\pm$ 31.6	322.2 $\pm$ 50.7
5	155.6 $\pm$ 62.1	406.7 $\pm$ 96.4	542.7 $\pm$ 143.3	35.1 $\pm$ 16.6	134.2 $\pm$ 53.7	538.5 $\pm$ 91.9
6	78.9 $\pm$ 42.5	95.5 $\pm$ 29.4	220.9 $\pm$ 75.3	-1.4 $\pm$ 5.3	43.4 $\pm$ 19.6	308.1 $\pm$ 42.6
8	88.4 $\pm$ 30.4	162.1 $\pm$ 30.3	253.4 $\pm$ 78.6	21.4 $\pm$ 11.2	53.7 $\pm$ 22.3	217.8 $\pm$ 45.2
9	158.5 $\pm$ 69	322.3 $\pm$ 87.2	338.2 $\pm$ 137.1	5.6 $\pm$ 12.4	109.2 $\pm$ 17.9	314.8 $\pm$ 43.4
10	97.3 $\pm$ 32.5	133.2 $\pm$ 27	154.9 $\pm$ 59.2	10 $\pm$ 6	26 $\pm$ 16.7	125.5 $\pm$ 27.7

Non-GLP RNA dose ranging study (higher doses) in Indian rhesus macaques

[00513] This study was designed to (a) evaluate the immunogenicity of VEE-MAG25mer srRNA at a dose of 300 µg as a homologous prime/boost or heterologous prime/boost in combination with ChAdV68.5WTnt.MAG25mer; (b) compare the immune responses of VEE-MAG25mer srRNA in lipid nanoparticles using LNP1 versus LNP2 at the 300µg dose; and (c) evaluate the kinetics of T-cell responses to VEE-MAG25mer srRNA and ChAdV68.5WTnt.MAG25mer immunizations.

[00514] The study arm was conducted in Mamu-A*01 Indian rhesus macaques to demonstrate immunogenicity. Vaccine immunogenicity in nonhuman primate species, such as Rhesus, is the best predictor of vaccine potency in humans. Furthermore, select antigens used in this study are only recognized in Rhesus macaques, specifically those with a Mamu-A*01 MHC class I haplotype. Mamu-A*01 Indian rhesus macaques were randomized to the different study arms (6 macaques per group) and administered an IM injection bilaterally with either ChAdV68.5-WTnt.MAG25mer or VEE-MAG25mer srRNA encoding model antigens that includes multiple Mamu-A*01 restricted antigens. The study arms were as described below.

[00515] PBMCs were collected prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks after the initial immunization for immune monitoring for group 1 (heterologous prime/boost). PBMCs were collected prior to immunization and 4, 5, 7, 8, 10, 11, 12, 13, 14, or 15 weeks after the initial immunization for immune monitoring for groups 2 and 3 (homologous prime/boost).

[00516] **Table 26:** Non-GLP immunogenicity study in Indian Rhesus Macaques

Group	Prime	Boost 1	Boost 2	Boost 3
1	ChAdV68.5WTnt.MAG25mer	VEE-MAG25mer srRNA -LNP2 (300 µg)	VEE-MAG25mer srRNA -LNP2 (300 µg)	VEE-MAG25mer srRNA -LNP2 (300 µg)
2	VEE-MAG25mer srRNA -LNP2 (300 µg)	VEE-MAG25mer srRNA -LNP2 (300 µg)	VEE-MAG25mer srRNA -LNP2 (300 µg)	
3	VEE-MAG25mer srRNA -LNP1 (300 µg)	VEE-MAG25mer srRNA -LNP1 (300 µg)	VEE-MAG25mer srRNA -LNP1 (300 µg)	

Results

[00517] Mamu-A*01 Indian rhesus macaques were immunized with ChAdV68.5-WTnt.MAG25mer. Antigen-specific cellular immune responses in peripheral blood mononuclear cells (PBMCs) were measured to six different Mamu-A*01 restricted epitopes prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks after the initial immunization (FIG. 21 and Table 27). Animals received boost immunizations with VEE-MAG25mer srRNA using the LNP2 formulation on weeks 4, 12, and 20. Combined antigen-specific immune responses of 1750, 4225, 1100, 2529, 3218, 1915, 1708, 1561, 5077, 4543, 4920, 5820, 3395, 2728, 1996, 1465, 4730, 2984, 2828, or 3043 SFCs per 10^6 PBMCs (six epitopes combined) were measured 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks after the initial immunization with ChAdV68.5WTnt.MAG25mer (FIG. 21). Immune responses measured 1 week after the second boost immunization (week 13) with VEE-MAG25mer srRNA were ~3-fold higher than that measured just before the boost immunization (week 12). Immune responses measured 1 week after the third boost immunization (week 21) with VEE-MAG25mer srRNA, were ~3-fold higher than that measured just before the boost immunization (week 20), similar to the response observed for the second boost.

[00518] Mamu-A*01 Indian rhesus macaques were also immunized with VEE-MAG25mer srRNA using two different LNP formulations (LNP1 and LNP2). Antigen-specific cellular immune responses in peripheral blood mononuclear cells (PBMCs) were measured to six different Mamu-A*01 restricted epitopes prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, or 15 weeks after the initial immunization (FIGS. 22 and 23, Tables 28 and 29). Animals received boost immunizations with VEE-MAG25mer srRNA using the respective LNP1 or LNP2 formulation on weeks 4 and 12. Combined antigen-specific immune responses of 168, 204, 103, 126, 140, 145, 330, 203, and 162 SFCs per 10^6 PBMCs (six epitopes combined) were measured 4, 5, 7, 8, 10, 11, 13, 14, 15 weeks after the immunization with VEE-MAG25mer srRNA-LNP2 (FIG. 22). Combined antigen-specific immune responses of 189, 185, 349, 437, 492, 570, 233, 886, 369, and 381 SFCs per 10^6 PBMCs (six epitopes combined) were measured 4, 5, 7, 8, 10, 11, 12, 13, 14, 15 weeks after the immunization with VEE-MAG25mer srRNA-LNP1 (FIG. 23).

[00519] Table 27: Mean spot forming cells (SFC) per 10⁶ PBMCs for each epitope $\pm$ SEM for priming vaccination with ChAdV68.5WTnt.MAG25mer (Group 1)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	173 $\pm$ 41.6	373.5 $\pm$ 87.3	461.4 $\pm$ 74.2	38.4 $\pm$ 26.1	94.5 $\pm$ 26	609.2 $\pm$ 121.9
5	412.7 $\pm$ 138.4	987.8 $\pm$ 283.3	1064.4 $\pm$ 266.9	85.6 $\pm$ 31.2	367.2 $\pm$ 135.2	1306.8 $\pm$ 332.8
6	116.2 $\pm$ 41.2	231.1 $\pm$ 46.3	268.3 $\pm$ 90.7	86.1 $\pm$ 42	174.3 $\pm$ 61	223.9 $\pm$ 38.1
7	287.4 $\pm$ 148.7	588.9 $\pm$ 173.9	693.2 $\pm$ 224.8	92.1 $\pm$ 33.5	172.9 $\pm$ 55.6	694.6 $\pm$ 194.8
8	325.4 $\pm$ 126.6	735.8 $\pm$ 212	948.9 $\pm$ 274.5	211.3 $\pm$ 62.7	179.1 $\pm$ 50	817.3 $\pm$ 185.2
10	312 $\pm$ 129.7	543.2 $\pm$ 188.4	618.6 $\pm$ 221.7	-5.7 $\pm$ 4.1	136.5 $\pm$ 51.3	309.9 $\pm$ 85.6
11	248.5 $\pm$ 81.1	348.7 $\pm$ 129.8	581.1 $\pm$ 205.5	-3.1 $\pm$ 4.4	119 $\pm$ 51.2	413.7 $\pm$ 144.8
12	261.9 $\pm$ 68.2	329.9 $\pm$ 83	486.5 $\pm$ 118.6	-1.2 $\pm$ 5.1	132.8 $\pm$ 31.8	350.9 $\pm$ 69.3
13	389.3 $\pm$ 167.7	1615.8 $\pm$ 418.3	1244.3 $\pm$ 403.6	1.3 $\pm$ 8.1	522.5 $\pm$ 155	1303.3 $\pm$ 385.6
14	406.3 $\pm$ 121.6	1616 $\pm$ 491.7	1142.3 $\pm$ 247.2	6.6 $\pm$ 11.1	322.7 $\pm$ 94.1	1048.6 $\pm$ 215.6
15	446.8 $\pm$ 138.7	1700.8 $\pm$ 469.1	1306.3 $\pm$ 294.4	43 $\pm$ 24.5	421.2 $\pm$ 87.9	1001.5 $\pm$ 236.4
16	686.8 $\pm$ 268.8	1979.5 $\pm$ 541.7	1616.8 $\pm$ 411.8	2.4 $\pm$ 7.8	381.9 $\pm$ 116.4	1152.8 $\pm$ 352.7
17	375.8 $\pm$ 109.3	1378.6 $\pm$ 561.2	773.1 $\pm$ 210.3	-1.4 $\pm$ 4.3	177.6 $\pm$ 93.7	691.7 $\pm$ 245
18	255.9 $\pm$ 99.7	1538.4 $\pm$ 498.1	498.7 $\pm$ 152.3	-5.3 $\pm$ 3.3	26.2 $\pm$ 13.4	413.9 $\pm$ 164.8
19	133 $\pm$ 62.6	955.9 $\pm$ 456.8	491.1 $\pm$ 121.8	-5.7 $\pm$ 4.1	50.3 $\pm$ 25.4	371.2 $\pm$ 123.7
20	163.7 $\pm$ 55.8	641.7 $\pm$ 313.5	357.9 $\pm$ 91.1	2.6 $\pm$ 7.5	41.4 $\pm$ 24.2	257.8 $\pm$ 68.9
21	319.9 $\pm$ 160.5	2017.1 $\pm$ 419.9	1204.8 $\pm$ 335.2	-3.7 $\pm$ 5.1	268.1 $\pm$ 109.6	924.1 $\pm$ 301
22	244.7 $\pm$ 105.6	1370.9 $\pm$ 563.5	780.3 $\pm$ 390	-3.6 $\pm$ 5.1	118.2 $\pm$ 68.1	473.3 $\pm$ 249.3
23	176.7 $\pm$ 81.8	1263.7 $\pm$ 527.3	838.6 $\pm$ 367.9	-5.7 $\pm$ 4.1	73.6 $\pm$ 49	480.9 $\pm$ 163.9
24	236.5 $\pm$ 92	1324.7 $\pm$ 589.3	879.7 $\pm$ 321	-0.4 $\pm$ 5.7	104 $\pm$ 53.1	498 $\pm$ 135.8

[00520] Table 28: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for priming vaccination with VEE-MAG25mer srRNA-LNP2 (300 μ g) (Group 2)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	46 $\pm$ 27.1	18.4 $\pm$ 6.8	58.3 $\pm$ 45.8	29.9 $\pm$ 20.8	4.9 $\pm$ 2.3	10.7 $\pm$ 4
5	85.4 $\pm$ 54	5.2 $\pm$ 5.8	52.4 $\pm$ 51.2	34.5 $\pm$ 35	11.8 $\pm$ 12.2	14.4 $\pm$ 7.9
7	18.6 $\pm$ 32.5	1.9 $\pm$ 1.7	59.4 $\pm$ 55.7	9.3 $\pm$ 10.7	3.3 $\pm$ 3	10.7 $\pm$ 6.1
8	36.6 $\pm$ 39.4	6.3 $\pm$ 3.9	48.7 $\pm$ 39.9	13.5 $\pm$ 8.8	3.8 $\pm$ 3.6	17.2 $\pm$ 9.7
10	69.1 $\pm$ 59.1	4.4 $\pm$ 1.9	39.3 $\pm$ 38	14.7 $\pm$ 10.8	4.4 $\pm$ 5.3	8.5 $\pm$ 5.3
11	43 $\pm$ 38.8	22.6 $\pm$ 21.1	30.2 $\pm$ 26.2	3.3 $\pm$ 2.2	5.8 $\pm$ 3.5	40.3 $\pm$ 25.5
13	120.4 $\pm$ 78.3	68.2 $\pm$ 43.9	54.2 $\pm$ 36.8	21.8 $\pm$ 7.4	17.7 $\pm$ 6.1	47.4 $\pm$ 27.3
14	76 $\pm$ 44.8	28 $\pm$ 19.5	65.9 $\pm$ 64.3	-0.3 $\pm$ 1.3	2.5 $\pm$ 2	31.1 $\pm$ 26.5
15	58.9 $\pm$ 41.4	19.5 $\pm$ 15.1	55.4 $\pm$ 51	2.5 $\pm$ 2	5.5 $\pm$ 3.6	20.1 $\pm$ 15.7

[00521] Table 29: Mean spot forming cells (SFC) per 10^6 PBMCs for each epitope $\pm$ SEM for priming vaccination with VEE-MAG25mer srRNA-LNP1 (300 μ g) (Group 3)

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	19.5 $\pm$ 8.7	13.3 $\pm$ 13.1	16.5 $\pm$ 15.3	10.5 $\pm$ 7.3	35.9 $\pm$ 24.8	92.9 $\pm$ 91.6
5	87.9 $\pm$ 43.9	12.7 $\pm$ 11.7	37.2 $\pm$ 31.9	21.1 $\pm$ 23.8	13.2 $\pm$ 13.7	12.6 $\pm$ 13.7
7	21.1 $\pm$ 13.3	48.8 $\pm$ 48.4	51.7 $\pm$ 39.5	9.1 $\pm$ 10.5	58.6 $\pm$ 55.8	159.4 $\pm$ 159
8	47.7 $\pm$ 21.7	66.4 $\pm$ 52.2	59.8 $\pm$ 57.4	49.4 $\pm$ 28	79.4 $\pm$ 63	133.8 $\pm$ 132.1
10	49 $\pm$ 30.2	42.2 $\pm$ 41.1	139.3 $\pm$ 139.3	51.6 $\pm$ 51.2	78.2 $\pm$ 75.8	131.7 $\pm$ 131.6
11	42 $\pm$ 26.8	20.9 $\pm$ 21.4	177.1 $\pm$ 162	-6.3 $\pm$ 4.3	104.3 $\pm$ 104.1	231.5 $\pm$ 230.1
12	40.2 $\pm$ 19	20.3 $\pm$ 11.9	42.2 $\pm$ 46.7	3.7 $\pm$ 6.7	57 $\pm$ 44.7	70 $\pm$ 69.2
13	81.2 $\pm$ 48.9	38.2 $\pm$ 37.6	259.4 $\pm$ 222.2	-4 $\pm$ 4.1	164.1 $\pm$ 159.3	347.3 $\pm$ 343.5
14	34.5 $\pm$ 31.8	5.3 $\pm$ 11.6	138.6 $\pm$ 137.3	-4.7 $\pm$ 5.2	52.3 $\pm$ 52.9	142.6 $\pm$ 142.6
15	49 $\pm$ 24	6.7 $\pm$ 9.8	167.1 $\pm$ 163.8	-6.4 $\pm$ 4.2	47.8 $\pm$ 42.3	116.6 $\pm$ 114.5

srRNA Dose Ranging Study

[00522] In one implementation of the present invention, an srRNA dose ranging study can be conducted in mamu A01 Indian rhesus macaques to identify which srRNA dose to progress to NHP immunogenicity studies. In one example, Mamu A01 Indian rhesus macaques can be administered with an srRNA vector encoding model antigens that includes multiple mamu A01 restricted epitopes by IM injection. In another example, an anti-CTLA-4 monoclonal antibody can be administered SC proximal to the site of IM vaccine injection to target the vaccine draining lymph node in one group of animals. PBMCs can be collected every 2 weeks after the initial vaccination for immune monitoring. The study arms are described in below (Table 30).

Table 30: Non-GLP RNA dose ranging study in Indian Rhesus Macaques

Group	Prime	Boost 1	Boost 2
1	srRNA-LNP (Low Dose)	srRNA-LNP (Low Dose)	srRNA-LNP (Low Dose)
2	srRNA-LNP (Mid Dose)	srRNA-LNP (Mid Dose)	srRNA-LNP (Mid Dose)
3	srRNA-LNP (High Dose)	srRNA-LNP (High Dose)	srRNA-LNP (High Dose)
4	srRNA-LNP (High Dose) + anti-CTLA-4	srRNA-LNP (High Dose) + anti-CTLA-4	srRNA-LNP (High Dose) + anti-CTLA-4

* Dose range of srRNA to be determined with the high dose $\leq 300 \mu\text{g}$.

Immunogenicity Study in Indian Rhesus Macaques

[00523] Vaccine studies were conducted in mamu A01 Indian rhesus macaques (NHPs) to demonstrate immunogenicity using the antigen vectors. Fig. 34 illustrates the vaccination strategy. Three groups of NHPs were immunized with ChAdV68.5-WTnt.MAG25mer and either with the checkpoint inhibitor anti-CTLA-4 antibody Ipilimumab (Groups 5 & 6) or without the checkpoint inhibitor (Group 4). The antibody was administered either intravenously (group 5) or subcutaneously (group 6). Triangles indicate chAd68 vaccination (1×10^{12} vp/animal) at weeks 0 & 32. Circles represent alphavirus vaccination at weeks 0, 4, 12, 20, 28 and 32.

[00524] The time course of CD8⁺ anti-epitope responses in the immunized NHPs are presented for chAd-MAG immunization alone (Fig. 35 and Table 31A), chAd-MAG immunization with the checkpoint inhibitor delivered IV (Fig. 36 and Table 31B), and chAd-

MAG immunization with the checkpoint inhibitor delivered SC (Fig. 37 and Table 31C). The results demonstrate chAd68 vectors efficiently primed CD8⁺ responses in primates, alphavirus vectors efficiently boosted the chAd68 vaccine priming response, checkpoint inhibitor whether delivered IV or SC amplified both priming and boosting responses, and chAd vectors readministered post vaccination to effectively boosted the immune responses.

Table 31A: CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG (Group 4). Mean SFC/1e6 splenocytes +/- the standard error is shown

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	173±41.6	373.5±87.3	461.4±74.2	38.4±26.1	94.5±26	609.2±121.9
5	412.7±138.4	987.8±283.3	1064.4±266.9	85.6±31.2	367.2±135.2	1306.8±332.8
6	116.2±41.2	231.1±46.3	268.3±90.7	86.1±42	174.3±61	223.9±38.1
7	287.4±148.7	588.9±173.9	693.2±224.8	92.1±33.5	172.9±55.6	694.6±194.8
8	325.4±126.6	735.8±212	948.9±274.5	211.3±62.7	179.1±50	817.3±185.2
10	312±129.7	543.2±188.4	618.6±221.7	-5.7±4.1	136.5±51.3	309.9±85.6
11	248.5±81.1	348.7±129.8	581.1±205.5	-3.1±4.4	119±51.2	413.7±144.8
12	261.9±68.2	329.9±83	486.5±118.6	-1.2±5.1	132.8±31.8	350.9±69.3
13	389.3±167.7	1615.8±418.3	1244.3±403.6	1.3±8.1	522.5±155	1303.3±385.6
14	406.3±121.6	1616±491.7	1142.3±247.2	6.6±11.1	322.7±94.1	1048.6±215.6
15	446.8±138.7	1700.8±469.1	1306.3±294.4	43±24.5	421.2±87.9	1001.5±236.4
16	686.8±268.8	1979.5±541.7	1616.8±411.8	2.4±7.8	381.9±116.4	1152.8±352.7
17	375.8±109.3	1378.6±561.2	773.1±210.3	-1.4±4.3	177.6±93.7	691.7±245
18	255.9±99.7	1538.4±498.1	498.7±152.3	-5.3±3.3	26.2±13.4	413.9±164.8
19	133±62.6	955.9±456.8	491.1±121.8	-5.7±4.1	50.3±25.4	371.2±123.7
20	163.7±55.8	641.7±313.5	357.9±91.1	2.6±7.5	41.4±24.2	257.8±68.9
21	319.9±160.5	2017.1±419.9	1204.8±335.2	-3.7±5.1	268.1±109.6	924.1±301
22	244.7±105.6	1370.9±563.5	780.3±390	-3.6±5.1	118.2±68.1	473.3±249.3
23	176.7±81.8	1263.7±527.3	838.6±367.9	-5.7±4.1	73.6±49	480.9±163.9
24	236.5±92	1324.7±589.3	879.7±321	-0.4±5.7	104±53.1	498±135.8
25	136.4±52.6	1207.1±501.6	924±358.5	6.2±10.5	74.1±34.4	484.6±116.7
26	278.2±114.4	1645±661.7	1170.2±469.9	-2.9±5.7	80.6±55.8	784.4±214.1
27	159±56.8	961.7±547.1	783.6±366.4	-5±4.3	63.6±27.5	402.9±123.4
28	189.6±75.7	1073.1±508.8	668.3±312.5	-5.7±4.1	80.3±38.3	386.4±122
29	155.3±69.1	1102.9±606.1	632.9±235	34.5±24.2	80±35.5	422.5±122.9
30	160.2±59.9	859±440.9	455±209.1	-3±5.3	60.5±28.4	302.7±123.2
31	122.2±49.7	771.1±392.7	582.2±233.5	-5.7±4.1	55.1±27.3	295.2±68.3
32	119.3±28.3	619.4±189.7	566±222.1	-3.7±5.1	21.9±4.5	320.5±76.4
33	380.5±122	1636.1±391.4	1056.2±205.7	-5.7±4.1	154.5±38.5	988.4±287.7
34	1410.8±505.4	972.4±301.5	319.6±89.6	-4.8±4.2	141.1±49.8	1375.5±296.7
37	130.8±29.2	500±156.9	424.9±148.9	-3.5±4.7	77.7±24.6	207.1±42.4
38	167.7±54.8	1390.8±504.7	830.4±329.1	-5.5±4.1	111.8±43.2	516±121.7

Table 31B: CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered IV. (Group 5). Mean SFC/1e6 splenocytes +/- the standard error is shown

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	1848.1±432.2	1295.7±479.7	1709.8±416.9	513.7±219.8	838.5±221.1	2514.6±246.5
5	1844.1±410.2	2367.5±334.4	1983.1±370.7	732.1±249.4	1429.7±275.3	2517.7±286.5
6	822.4±216.7	1131.2±194.7	796.8±185.8	226.8±70	802.2±101.4	913.5±222.7
7	1147.2±332.9	1066±311.2	1149.8±467.3	267.4±162.6	621.5±283.2	1552.2±395.1
8	1192.7±188.8	1461.5±237.7	1566.9±310.5	522.5±118.6	662.3±142.4	1706±216.7
10	1249±220.3	1170.6±227.7	1297.3±264.7	-0.3±4.4	551.8±90.5	1425.3±142.6
11	934.2±221.7	808±191.3	1003.1±293.4	1.9±4.3	364.2±76.6	1270.8±191.6
12	1106.2±216.6	896.7±190.7	1020.1±243.3	1.3±3.9	436.6±90	1222±155.4
13	2023.8±556.3	3696.7±1.7	2248.5±436.4	-4.5±3.5	2614±406.1	3700±0
14	1278.7±240	2639.5±387	1654.6±381.1	-6±2.1	988.8±197.9	2288.3±298.7
15	1458.9±281.8	2932.5±488.7	1893.4±499	74.6±15.6	1657.8±508.9	2709.1±428.7
16	1556.8±243	2143.8±295.2	2082.8±234.2	-5.8±2.5	1014.6±161.4	2063.7±86.7
17	1527±495.1	2213±677.1	1767.7±391.8	15.1±5.9	633.8±133.9	2890.8±433.9
18	1068.2±279.9	1940.9±204.1	1114.1±216.1	-5.8±2.5	396.6±77.6	1659.4±171.7
19	760.7±362.2	1099.5±438.4	802.7±192.5	-2.4±3.3	262.2±62.2	1118.6±224.2
20	696.3±138.2	954.9±198	765.1±248.4	-1.4±4.4	279.6±89.3	1139±204.5
21	1201.4±327.9	3096±1.9	1901±412.1	-5.8±2.5	1676.3±311.5	2809.3±195.8
22	1442.5±508.3	2944.7±438.6	1528.4±349.6	2.8±5.1	940.7±160.5	2306.3±218.6
23	1400.4±502.2	2757.1±452.9	1604.2±450.1	-5.1±2.3	708.1±162.6	2100.4±362.9
24	1351±585.1	2264.5±496	1080.6±253.8	0.3±6.5	444.2±126.4	1823.7±306.5
25	1211.5±505.2	2160.4±581.8	970.8±235.9	2.5±3.8	450.4±126.9	1626.2±261.3
26	1443±492.5	2485±588	1252.5±326.4	-0.2±6	360.2±92.3	2081.9±331.1
27	896.2±413.3	1686±559.5	751±192.1	-3.7±2.5	247.4±82.8	1364.1±232
28	1147.8±456.9	1912.1±417.1	930.3±211.4	-5.8±2.5	423.9±79.6	1649.3±315
29	1038.5±431.9	1915.2±626.1	786.8±205.9	23.5±8.3	462.8±64	1441.5±249.7
30	730.5±259.3	1078.6±211.5	699.1±156.2	-4.4±2.7	234.4±43.9	1160.6±112.6
31	750.4±328.3	1431±549.9	650.6±141.1	-5.2±3	243.4±56.4	868.9±142.8
32	581.4±227.7	1326.6±505.2	573.3±138	-3.2±4.2	160.8±49.2	936.4±110.4
33	2198.4±403.8	3093.4±123.3	2391.8±378.4	7.1±8.5	1598.1±343.1	2827.5±289.5
34	2654.3±337	2709.9±204.3	1297.5±291.4	0.4±4.2	1091.8±242.9	1924±245.7
37	846.8±301.7	1706.9±196	973.6±149.3	50.5±45.2	777.3±140.2	1478.8±94.3

Table 31C: CD8⁺ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered SC (Group 6). Mean SFC/1e6 splenocytes +/- the standard error is shown

Wk	Antigen					
	Env CL9	Env TL9	Gag CM9	Gag LW9	Pol SV9	Tat TL8
4	598.3±157.4	923.7±306.8	1075.6±171.8	180.5±74.1	752.3±245.8	1955.3±444.4
5	842.2±188.5	1703.7±514.2	1595.8±348.4	352.7±92.3	1598.9±416.8	2163.7±522.1
6	396.4±45.3	728.3±232.7	503.8±151.9	282±69	463.1±135.7	555.2±191.5
7	584.2±177	838.3±254.9	1013.9±349.4	173.6±64.3	507.4±165.7	1222.8±368
8	642.9±134	1128.6±240.6	1259.1±163.8	366.1±72.8	726.7±220.9	1695.6±359.4
10	660.4±211.4	746.9±222.7	944.8±210.2	-1.2±1.9	523.4±230.7	787.3±308.3
11	571.2±162	609.4±194.3	937.9±186.5	-8.9±2.3	511.6±229.6	1033.3±315.7
12	485.3±123.7	489.4±142.7	919.3±214.1	-8.9±2.3	341.6±139.4	1394.7±432.1
13	986.9±154.5	2811.9±411.3	1687.7±344.3	-4.1±5.1	1368.5±294.2	2751±501.9
14	945.9±251.4	2027.7±492.8	1386.7±326.7	-5.7±2.8	708.9±277.1	1588.2±440.1
15	1075.2±322.4	2386±580.7	1606.3±368.1	-5.4±3.2	763.3±248.8	1896.5±507.8
16	1171.8±341.6	2255.1±439.6	1672.2±342.3	-7.8±2.4	1031.6±228.8	1896.4±419.9
17	1118.2±415.4	2156.3±476	1345.3±377.7	-1.1±6.7	573.7±118.8	1614.4±382.3
18	861.3±313.8	2668.2±366.8	1157.2±259.6	-8.9±2.3	481.2±164	1725.8±511.4
19	719.2±294.2	1447.2±285	968±294.5	-2.2±4.6	395.6±106.1	1199.6±289.2
20	651.6±184	1189.8±242.8	947.4±249.8	-8.9±2.3	355±106.3	1234.7±361.7
21	810.3±301.9	2576.2±283.7	1334±363.1	-8.9±2.3	892.2±305	1904.4±448.1
22	775±196.4	2949±409.7	1421.8±309.7	38±27.8	577±144.2	2330.6±572.3
23	584.9±240.2	1977.9±361.4	1209.8±405.1	-7.3±3.2	273.7±93.3	1430.6±363.9
24	485.4±194.4	1819.8±325.5	837.2±261.4	-3.4±4.1	234.4±71.1	943.9±243.3
25	452.3±175	2072±405.7	957.1±293.1	-8.9±2.3	163±43.2	1341.2±394.7
26	517.9±179.1	2616±567.5	1126.6±289	-8.3±2.3	199.9±89.2	1615.7±385.6
27	592.8±171.7	1838.3±372.4	749.3±170.4	-7.3±2.5	325.5±98.7	1110.7±308.8
28	793±228.5	1795.4±332.3	1068.7±210.3	2.5±4.1	553.1±144.3	1480.8±357.1
29	661.8±199.9	2140.6±599.3	1202.7±292.2	-8.7±2.8	558.9±279.2	1424.2±408.6
30	529.1±163.3	1528.2±249.8	840.5±218.3	-8.9±2.3	357.7±149.4	1029.3±335
31	464.8±152.9	1332.2±322.7	726.3±194.3	-8.9±2.3	354.3±158.6	884.4±282
32	612.9±175.3	1584.2±390.2	1058.3±219.8	-8.7±2.8	364.6±149.8	1388.8±467.3
33	1600.2±416.7	2597.4±367.9	2086.4±414.8	-6.3±3.3	893.8±266	2490.6±416.4
34	2814.6±376.2	2713.6±380.8	1888.8±499.4	-7.5±3.1	1288.9±438.9	2428.1±458.9
37	1245.9±471.7	1877.7±291.2	1606.6±441.9	14.2±13	1227.5±348.1	1260.7±342

Memory Phenotyping in Indian Rhesus Macaques

[00525] Rhesus macaque were immunized with ChAdV68.5WTnt.MAG25mer /VEE-MAG25mer srRNA heterologous prime/boost regimen with or without anti-CTLA4, and boosted again with ChAdV68.5WTnt.MAG25mer. Groups were assessed 11 months after the final ChAdV68 administration (study month 18). by ELISpot was performed as described. Fig. 38 and Table 43 shows cellular responses to six different Mamu-A*01 restricted epitopes as measured by ELISpot both pre-immunization (left panel) and after 18 months (right panel). The detection of responses to the restricted epitopes demonstrates antigen-specific memory responses were generated by ChAdV68/samRNA vaccine protocol.

[00526] To assess memory, CD8⁺ T-cells recognizing 4 different rhesus macaque Mamu-A*01 class I epitopes encoded in the vaccines were monitored using dual-color Mamu-A*01 tetramer labeling, with each antigen being represented by a unique double positive combination, and allowed the identification of all 4 antigen-specific populations within a single sample. Memory cell phenotyping was performed by co-staining with the cell surface markers CD45RA and CCR7. Fig. 39 and Table 44 shows the results of the combinatorial tetramer staining and CD45RA/CCR7 co-staining for memory T-cells recognizing four different Mamu-A*01 restricted epitopes. The T cell phenotypes were also assessed by flow cytometry. Fig. 40 shows the distribution of memory cell types within the sum of the four Mamu-A*01 tetramer+ CD8⁺ T-cell populations at study month 18. Memory cells were characterized as follows: CD45RA+CCR7+=naïve, CD45RA+CCR7-=effector (Teff), CD45RA-CCR7+=central memory (Tcm), CD45RA-CCR7-=effector memory (Tem). Collectively, the results demonstrate that memory responses were detected at least one year following the last boost indicating long lasting immunity, including effector, central memory, and effector memory populations.

Table 43 Mean spot forming cells (SFC) per 10⁶ PBMCs for each animal at both pre-prime and memory assessment time points (18 months).

Animal	Pre-prime baseline						18 months					
	Tat TL8	Gag CM9	Env TL9	Env CL9	Gag LW9	Pol SV9	Tat TL8	Gag CM9	Env TL9	Env CL9	Gag LW9	Pol SV9
1	1.7	0.0	0.0	5.0	0.0	13.7	683.0	499.2	1100.3	217.5	47.7	205.3
2	0.0	0.0	0.0	0.2	0.1	0.0	773.4	ND	1500.0	509.3	134.5	242.5
3	0.0	0.0	6.7	6.8	10.2	3.3	746.3	167.5	494.1	402.8	140.6	376.0
4	0.0	0.0	0.0	0.0	0.0	0.0	47.6	1023.9	85.1	44.2	44.2	47.6
5	15.3	6.7	18.6	15.6	5.2	12.1	842.4	467.7	1500.0	805.9	527.8	201.8
6	3.1	0.0	0.0	15.5	6.9	5.3	224.3	720.3	849.0	296.9	32.4	121.9

ND=not determined due to technical exclusion

Table 44 Percent Mamu-A*01 tetramer positive out of live CD8⁺ cells

Animal	Tat TL8	Gag CM9	Env TL9	Env CL9
1	0.42	0.11	0.19	0.013
2	0.36	0.048	0.033	0.00834
3	0.97	0.051	0.35	0.048
4	0.46	0.083	0.17	0.028
5	0.77	0.45	0.14	0.2
6	0.71	0.16	0.17	0.04

XIX. Co-Expression of an Anti-CTLA4 Immune Checkpoint Inhibitor

[00527] Vector(s) were engineered that co-expressed antigens and an immune checkpoint inhibitor.

Materials and Methods

[00528] In one example, a chimpanzee adenoviral viral vector was designed to express both model antigens and the immune checkpoint inhibitor, anti-CTLA4.

Vector Design

An E1/E3 deleted ChAdV68 viral vector was designed with an expression cassette in the following orientation from 5' to 3' introduced into the deleted E1 region in the following format (See FIG. 26): [CMV - model-antigens/GFP - IRES - Anti-CTLA4 - SV40]. Cassette expression was driven by a cytomegalovirus (CMV) promoter located 5' of the model antigen cassette (or GFP reporter) and the SV-40 polyadenylation signal 3' of the anti-CTLA4 antibody. The model antigen cassette was the MAG25mer cassette described above in Section XIV.B.4 (SEQ ID NO: 34). The antigen cassette (or GFP reporter) and the anti-CTLA4 antibody were separated by an IRES sequence, which enables separate translation of the model antigens and the anti-CTLA4 antibody from the same transcript. The anti-CTLA4 antibody nucleotide sequences were based on the anti-CTLA4 clone 9D9 sequences available on Genbank (NCBI ID LQ222660.1 and LQ222658.1) and described further in PCT publication WO2016025642, herein incorporated by reference for all it teaches. The anti-CTLA4 antibody expression cassette was designed in the following format: [Full length heavy chain - Furin cleavage site - T2A site - Full length light chain], as described (Fang J, Qian JJ, Yi S, Harding TC, Tu GH, Van Roey M, Jooss K. (2005). Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol.* 23(5):584-90), where the T2A site is a *Thosea asigna* virus 2A peptide. The variable regions were appended to the constant region of mouse IgG2b, corresponding to 9D9's original isotype. The nucleotide sequences were also codon optimized for expression relative to the Genbank sequences.

[00529] Two versions of the GFP reporter expressing construct were made: one with antibody leader sequences using the NCBI sequences (g9D9, SEQ ID NO: 60) and the other with the leader sequences based on the sequences predicted to be present in the original mouse 9D9 hybridoma by IgBLAST tool (o9D9, SEQ ID NO: 61).

[00530] The model antigen expressing construct was made with the "o9D9" antibody leader sequences described above. The full-length sequence for the chimpanzee C68 adenoviral

construct expressing the model antigens and anti-CTLA4 (“chAd-MAG-CTLA4”) is described below (SEQ ID NO: 57).

[00531] Additional checkpoint immune checkpoint inhibitor co-expression vectors were generated: a chimpanzee C68 adenoviral construct expressing the GFP reporter or model antigen cassette, as well as a sequence encoding anti-CTLA4 antibody Ipilimumab (chAd68-GFP-IRES-IPI “IPI-GFP” and chAd68-MAG-IRES-IPI “IPI-MAG”; SEQ ID NO: 70 and 71, respectively); a chimpanzee C68 adenoviral construct expressing the model antigen cassette and a sequence encoding anti-CTLA4 antibody Tremelimumab (chAd68-MAG-IRES-TREME; SEQ ID NO: 72).

Vector Production

[00532] The anti-CTLA4 expressing viral vectors were generated by transfecting PacI digested pA68-MAG-o9D9 (plasmid containing the ChAdV68 vector that expresses the model antigen cassette and the o9D9 version of anti-CTLA4), pA68-GFP-IRES-o9D9 (plasmid containing the ChAdV68 vector that expresses the GFP reporter and the o9D9 version of anti-CTLA4) and pA68-GFP-IRES-g9D9 (plasmid containing the ChAdV68 vector that expresses the GFP reporter and the g9D9 version of anti-CTLA4) into 293A cells using Fugene 6 (Promega). The viral vectors underwent expansion in 293 cells before large scale production (400 mL scale) in 293F suspension cells. 48h post infection cells were harvested, lysed and virus purified by two rounds of CsCl gradient. The virus was dialyzed into 20mM Tris pH 8.0, 25mM NaCl and 2.5% Glycerol. The purified viral vectors were aliquoted and stored at -80 C. The infectious unit titer of the purified viral vectors was determined using an anti-capsid assay. For in vitro and in vivo experiments, dosing was based on IU titers.

***In vitro* Expression**

[00533] 293F cells were infected with the viral vectors described below at an MOI of 1. Post infection, the supernatant was harvested, and the antibody recovered using Protein A beads. The antibody was eluted from the beads and separated on a 4-20% SDS-PAGE gel. The gel was subjected to Western Blot analysis using a goat anti-Mouse, HRP-conjugated antibody (Millipore, AP124P) at 1:2,500 dilution in TBST-5% dry milk, followed by chemiluminescent detection reagent (ThermoFisher, ECL Plus).

***In vivo* Evaluation**

[00534] C56F1 mice were dosed with the viral vectors described below at either 1.5e7 or 1.5e6 IU, delivered via bilateral intramuscular (IM) injections to the tibialis anterior muscles. Mice that were coadministered anti-CTLA4 were delivered the antibody via intraperitoneal (IP)

administration, 2x/week, at 250 ug. Groups, doses, routes and timing of injections are described below. Antigen-specific T-cells were measured by ELISpot and intracellular cytokine staining. Serum was obtained at various timepoints and anti-CTLA4 concentration was measured by ELISA.

Results

[00535] Virus was produced and quantified as described above. The titer data for the chAd68-o9D9 lot# CS110617E used in the in vivo studies is indicated below:

[00536] Table 32: Titer data of anti-CTLA4 clinical cassette virus (chAd68-MAG-o9D9)

Assay	Result
Infectious Unit Titer	3.06e8 IU/mL
Viral Particle Titer	3e12 VP/mL

Anti-CTLA4 *in vitro* Expression

[00537] 293F cells were infected with the following viral vectors: ChAdVC68-MAG25mer-IRES-o9D9 (“5WT-MAG-o9D9”), ChAdVC68-GFP-IRES-o9D9, and ChAdVC68-GFP-IRES-g9D9. Supernatant was collected at 7, 20, 30 and 48h hours post infection and, following antibody recovered using Protein A beads, was analyzed by Western. A commercial 9D9 antibody was used as a positive control (“(+) ctrl”). Supernatant from a viral vector encoding only GFP and not the anti-CTLA 9D9 antibody was used as a negative control (“(-) ctrl”). As shown in FIG. 27, bands correlating with the heavy and light chain of the 9D9 anti-CTLA4 positive control were present in the supernatants of the three vectors tested starting at 20h post infection, while no protein band was detected in the negative control lane, indicating each vector expressed the desired anti-CTLA4 antibodies. Protein was also not observed in the cell lysate only lane, *i.e.*, sample without antibody recovery using Protein A beads (lane 2), demonstrating detection of expressed antibody required antibody recovery.

[00538] Next, *in vitro* expression was assessed for chAd68-GFP-IRES-IPI and chAd68-MAG-IRES-IPI. Fig. 28A demonstrates expression of the heavy chain and light chain of Ipilimumab. Next, *in vitro* expression was assessed for chAd68-MAG-IRES-TREME. Fig. 28B demonstrates expression of the heavy chain and light chain of Tremelimumab.

In vivo evaluation of vectors co-expressing anti-CTLA4

[00539] The ChAdVC68-MAG25mer-IRES-o9D9 (“chAd-MAG-CTLA4”) co-expressing a model antigen cassette (MAG) and an anti-CTLA4 antibody (o9D9) was evaluated in mice for

cellular antigen-specific immune response and anti-CTLA4 expression in serum. The co-expression vector treatment was compared to mice receiving either a vector that expresses the same model antigen cassette but does not express an anti-CTLA4 antibody (“chAd-MAG”), or the vector that expresses the same model antigen cassette but does not express an anti-CTLA4 antibody while being co-administered the 9D9 anti-CTLA4 antibody (purchased from BioXcell). chAd viruses were dosed by the IM route at either 1.5e7 or 1.5e6 IU. Co-administered anti-CTLA4 antibody was injected IP at a dose of 250 ug. The various groups are described in detail in the table below:

[00540] Table 33. Design of in vivo evaluation of chAd-MAG-CTLA4 immunogenicity and expression

Group	N	Virus	Dose	Volume	Schedule	Route
1	8	chAd-MAG-CTLA4	1.5e7 IU	2 x 50 uL	Day 0	IM
2	8	chAd-MAG-CTLA4	1.5e6 IU	2 x 50 uL	Day 0	IM
3	8	chAd-MAG aCTLA4 antibody	1.5e7 IU 250 ug	2 x 50 uL 100 uL	Day 0 Day 0, 3, 6, 9	IM IP
4	8	chAd-MAG aCTLA4 antibody	1.5e6 IU 250 ug	2 x 50 uL 100 uL	Day 0 Day 0, 3, 6, 9	IM IP
5	8	chAd-MAG	1.5e7 IU	2 x 50 uL	Day 0	IM
6	8	chAd-MAG	1.5e6 IU	2 x 50 uL	Day 0	IM

[00541] Antigen-specific T-cells for MHC class I epitope AH1-A5 were measured by ELISpot and intracellular cytokine staining 12 days after vaccine administration. As shown in FIG. 41, the antigen-specific immune response measured by ELISpot at day 12 post immunization was equivalent for chAd-MAG-CTLA4, chAd-MAG alone, and chAd-MAG co-administered with anti-CTLA4 antibody delivered IP. The number of antigen-specific T-cells were similar for chAd-MAG-CTLA4, chAd-MAG + anti-CTLA4, and chAd-MAG, as measured by IFN γ -Elispot where median values were 11578, 12194, and 12945 SFC/1e6 splenocytes, respectively, for low dose of 1.5e6 IU (FIG. 41 left panel), and 18933, 17992, and 18766 SFC/1e6 splenocytes for high dose of 1.5e7 IU (FIG. 41 right panel).

[00542] As shown in FIG. 42, the three treatments also generated equivalent numbers of CD8⁺IFN γ ⁺ Antigen-specific T-cells for MHC class I epitope AH1-A5, as measured by ICS with median values of 3.7, 3.5 and 3.9 IFN γ ⁺ (% of CD8⁺) at 1.5e6 IU (FIG. 42 left panel), and 8.4, 7.3 and 8.2 at 1.5e7 IU (FIG. 42 right panel).

[00543] No statistically significant differences, as determined by ANOVA, for the ELISpot or ICS analyses were found between groups. The results demonstrate that the chAd-MAG-aCTLA4 vaccine is functional, and at least by these assays equivalent, in driving an antigen-specific T-cell response in mice.

[00544] The amount of anti-CTLA4 antibody in the serum of mice was evaluated by mesoscale discovery (MSD) ELISA at 1, 2, 3, 6, and 12 days post-immunization with chAd-MAG-CTLA4 (groups 1 and 2), or chAd-MAG co-administered with anti-CTLA4 antibody delivered IP. For groups 3 and 4, anti-CTLA4 mAb (250 µg, IP) was administered on days 0, 3, 6, and 9.

[00545] As shown in FIG. 43 and Table 34, anti-CTLA4 expression was detected in the serum of mice immunized with chAd-MAG-CTLA4 at both low (1.5e6 IU) and high (1.5e7 IU) doses. Expression peaked at day 2 post immunization and was maintained above baseline values (day 0) until the final measurement at day 12, without additional immunizations. A dose-response in expression was observed, with higher levels detected at all timepoints with the higher dose of chAd-MAG-CTLA4. Serum levels of anti-CTLA4 were significantly lower than with IP delivery of the anti-CTLA4 mAb, which had levels greater than the maximum range of the assay at all timepoints post administration. These results demonstrate that the ChAd-MAG-aCTLA4 vaccine expresses anti-CTLA4 antibody at low, relative to repeated IP administration, but durable levels in a dose-dependent manner.

Table 34: Anti-CTLA4 antibody levels in the serum of mice

Timepoint (weeks)	chAd-MAG-CTLA4, 1.5e7 IU		chAd-MAG-CTLA4, 1.5e6 IU		chAd-MAG, 1.5e7 IU + CTLA4 (250ug,IP)		chAd-MAG, 1.5e6 IU + CTLA4 (250ug,IP)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	591	512	345	77	821	692	681	862
1	29903	5455	1278	499	1723259	16272	1717583	33419
2	44656	13993	1756	933	1727280	11087	1717899	30496
3	39786	12746	1578	768	1724881	14540	1717923	30268
6	16757	5239	946	311	1724501	17319	1719999	32211
12	3536	1186	619	130	1666574	30887	1690529	36624

Mean ECL value for each group and timepoint.

Sequences

Full-Length ChAdVC68-MAG25mer-IRES-o9D9 sequence “chAd-MAG-CTLA4” (ChAdV68.5WTnt.MAG25mer (SEQ ID NO: 57); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,125-31,876) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at five positions; model

neoantigen cassette and anti-CTLA4 clone 9D9 connected by an IRES sequence under the control of the CMV promoter/enhancer inserted in place of deleted E1; SV40 poly A 3' of cassette.

CATCaTCAATAATATACCTCAAACCTTTTTGTGCGCTTAATATGCAAATGAGGCGTTTGAATTTGG
GGAGGAAGGGCGGTGATTGGTCGAGGGATGAGCGACCGTTAGGGGCGGGGCGAGTGACGTTTTG
ATGACGTGGTTGCGAGGAGGAGCCAGTTTGCAAGTTCTCGTGGGAAAAGTGACGTCAAACGAGGT
GTGGTTTGAACACGGAAATACTCAATTTCCCGCGCTCTCTGACAGGAAATGAGGTGTTTCTGGGC
GGATGCAAGTGAAAACGGGCCATTTTCGCGCGAAAACCTGAATGAGGAAGTGAAAATCTGAGTAA
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Kozak sequence

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9D9 heavy chain variable region (SEQ ID NO: 62)
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IgG2b Heavy chain Constant Region from Balb/C mice (SEQ ID NO: 63)
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9D9 Light chain sequence minus the leader sequence (SEQ ID NO: 68)
GAGCTAGATGCGATATCGTGATGACCCAGACAACACTGTCTCTGCCTGTGTCTCTGGGAGATCAGGCCTCTATCAGCTGTAGATCTAGCCAGAGCATTGTGCACTCTAACGGCAACACCTACCTGGAGTG GTACCTGCAGAAACCAGGACAAAGCCCTAAGCTGCTGATCTACAAAGTGAGCAACCGGTTTAGCGGCGTGCCCCGACAGATTTTTCTGGATCTGGCTCTGGCACCGATTTTACACTGAAGATCAGCAGAGTGGAGGCCGAGGATCTGGGAGTGTACTACTGCTTTCAGGGCTCTCATGTGCCTTACACATTTGGAGGAGGAACCAAGCTGGAGATCAAGcgggctgatgctgccaaactgtatccatcttcccacatccagtgagcagttaacatctggaggtgcctcagtcgtgtgtctcttgaacaacttctacccaaaagacatcaatgtcaagtggaagattgatggcagtgaaacgacaaaatggcgtctgaacagttggactgatcag gacagcaaaagacagcacctacagcatgagcagcacctcagcttgaccaagagcagatgaacgacataaacagtataacctgtgagccactcacaagacatcaactcacccattgtcaagagcttcaacaggaatgagtg
SV40 PolyA (SEQ ID NO: 69)
AGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACCTAGAATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTTCAGGGGGAGATGTGGGAGGTTTTTTAAAGCAAGTAAAACCTTACAAATGTGGTAAAA
chAd68.5WT-MAG-IRES-Ipilimumab (SEQ ID NO: 70); Ipilimumab sequence is in bold uppercase; GFP is italicized uppercase; IRES is italicized lowercase
ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACCGGCCACAAGTTCACGCTGTCCGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCCGCAAGCTGAGCTGCCCTGGCCCCACCTCGTGACCACCTGACCTACGGCGTGTCAGTGCTTCAGCCGCTACCCCGACCATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCCG

AGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGA
 AGTTCGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAAC
 ATCCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAG
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 CTACCAGCAGAAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCC
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CACCTGTCCACCTTGTCCCGCCCCCTGAGTTGCTCGGTGGACCATCAGTCTTCTTGTTCCTCC
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chAd68.5WT-MAG-IRES-Ipilimumab (SEQ ID NO: 71); *Ipilimumab sequence is in bold italic*

CATcTCAATAATATACCTCAAACCTTTTGTGCGCGTTAATATGCAAATGAGGCGTTTGAATTTGG
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chAd68.5WT-MAG-IRES- Tremelimumab (SEQ ID NO: 72); Tremelimumab sequence is in bold italic

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 CCAAAGTGGGAATAGTCAAGAGTCTGTACAGAGCAAGACAGTAAGGACTCTACATACAGTTTGTCA
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Tremelimumab nucleotide sequence (SEQ ID NO: 74)

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 AGTCTACAGCCGCTCTGGGCTGTCTGGTCAAGGACTACTTTCCTGAGCCTGTGACCGTGTCC
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 CTGAACAACCTTCTACCCCTCGGGAAGCCAAGGTGCAGTGGAAGTGGATAATGCCCTGCAGA
 GCGGCAACAGCCAAGAGAGCGTGACAGAGCAGGATAGCAAGGACAGCACCTATAGCCTGA
 GCAGCACACTGACCCTGAGCAAGGCCGACTACGAGAAGCACAAGGTGTACGCCTGCGAAGT
 GACACACCAGGACTGAGCAGCCCTGTGACCAAGAGCTTCAACAGAGGCGAGTGTGTA

Ipilimumab VL (SEQ ID NO: 74)

EIVLTQSPGTLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDRFSGSGSGT
 DFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKR

Ipilimumab VH (SEQ ID NO: 75)

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGNNKYYADSVKG RFTISRDN SKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDYWGQGT LVT
Ipilimumab VL CDR1 (SEQ ID NO: 76)
QSVGSSYL
Ipilimumab VL CDR2 (SEQ ID NO: 77)
GAFS
Ipilimumab VL CDR3 (SEQ ID NO: 78)
QQYGSSPWT
Ipilimumab VH CDR1 (SEQ ID NO: 79)
SVGSSYL
Ipilimumab VH CDR2 (SEQ ID NO: 80)
GAFS
Ipilimumab VH CDR3 (SEQ ID NO: 81)
QQYGSSPWT

XX. Co-Expression of an Anti-CTLA4 Immune Checkpoint Inhibitor Tumor Model Evaluation

[00546] Various dosing protocols using ChAdV68, with or without co-expression of anti-CTLA4 and optionally in combination with self-replicating RNA (srRNA), are evaluated in murine CT26 or B16-OVA tumor models.

Methods and Materials

Tumor Injection

[00547] For B16-OVA tumor models, Balb/c mice are injected in the lower left abdominal flank with 10^5 B16-OVA cells/animal. Tumors are allowed to grow for 3 days prior to immunization.

[00548] For CT26 tumor models, Balb/c mice are injected in the lower left abdominal flank with 10^6 CT26 cells/animal. Tumors are allowed to grow for 7 days prior to immunization.

Immunizations

[00549] Mice are immunized with ChAdVC68-MAG25mer-IRES-o9D9 co-expressing a model antigen cassette (MAG) and an anti-CTLA4 antibody (o9D9), a vector that expresses the same model antigen cassette but does not express an anti-CTLA4 antibody, and/or the vector that expresses the same model antigen cassette but does not express an anti-CTLA4 antibody while being co-administered the 9D9 anti-CTLA4 antibody (purchased from BioXcell). In various study arms, mice receive a boosting immunization with an alphavirus vaccine vector expressing the same model antigens.

Splenocyte dissociation

[00550] Spleen and lymph nodes for each mouse are pooled in 3 mL of complete RPMI (RPMI, 10% FBS, penicillin/streptomycin). Mechanical dissociation is performed using the gentleMACS Dissociator (Miltenyi Biotec), following manufacturer's protocol. Dissociated cells are filtered through a 40 micron filter and red blood cells are lysed with ACK lysis buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA). Cells are filtered again through a 30 micron filter and then resuspended in complete RPMI. Cells are counted on the Attune NxT flow cytometer (Thermo Fisher) using propidium iodide staining to exclude dead and apoptotic cells. Cell are then adjusted to the appropriate concentration of live cells for subsequent analysis.

Ex vivo enzyme-linked immunospot (ELISPOT) analysis

[00551] ELISPOT analysis is performed according to ELISPOT harmonization guidelines {DOI: 10.1038/nprot.2015.068} with the mouse IFN γ ELISpotPLUS kit (MABTECH). 5x10⁴ splenocytes are incubated with 10uM of the indicated peptides for 16 hours in 96-well IFN γ antibody coated plates. Spots are developed using alkaline phosphatase. The reaction is timed for 10 minutes and is terminated by running plate under tap water. Spots are counted using an AID vSpot Reader Spectrum. For ELISPOT analysis, wells with saturation >50% were recorded as "too numerous to count". Samples with deviation of replicate wells > 10% are excluded from analysis. Spot counts are then corrected for well confluency using the formula: spot count + 2 x (spot count x %confluence / [100% - %confluence]). Negative background is corrected by subtraction of spot counts in the negative peptide stimulation wells from the antigen stimulated wells. Finally, wells labeled too numerous to count are set to the highest observed corrected value, rounded up to the nearest hundred.

Results

[00552] Mice in the various study arms are evaluated for cellular antigen-specific immune responses by ELISpot and ICS, anti-CTLA4 antibody levels in the serum, tumor growth, and survival. Immunization with ChAdVC68-MAG25mer-IRES-o9D9 demonstrates an improved immune response..

XXI. Co-Expression of an Immune Checkpoint Inhibitor

[00553] In one example, a viral vector is designed to express both model antigens and an immune checkpoint inhibitor. The viral vector co-expressing the immune checkpoint inhibitor is compared to a vector that expresses the same model antigen cassette but does not express the

immune checkpoint inhibitor, and/or the vector that expresses the same model antigen cassette but does not express the immune checkpoint inhibitor while being co-administered the immune checkpoint inhibitor. Other immune modulators are also tested in the same manner.

Vector Design

[00554] In one example, an E1/E3 deleted ChAdV68 viral vector is designed with an expression cassette in the following orientation from 5' to 3' introduced into the deleted E1 region in the following format: [CMV - model-antigens/GFP - IRES – Immune Checkpoint Inhibitor - SV40]. Cassette expression is driven by a cytomegalovirus (CMV) promoter located 5' of the model antigen cassette (or GFP reporter) and the SV-40 polyadenylation signal 3' of the immune checkpoint inhibitor. The model antigen cassette is the MAG25mer cassette described above in Section XIV.B.4 (SEQ ID NO: 34). The antigen cassette (or GFP reporter) and the immune checkpoint inhibitor are separated by an IRES sequence, which enables separate translation of the model antigens and the are separated by an IRES sequence from the same transcript. The immune checkpoint inhibitor or immune modulator is based on available sequences, sequences obtained by peptide sequencing of available antibodies and subsequent design of nucleotide sequences for expression, or sequences obtained by BCR sequencing of hybridomas, for an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof. Immune checkpoint inhibitors and immune modulators that are available are described in Table 35 below. Sequences are also modified for expression, for example codon optimized or engineered for introduction into cloning vectors. The antibody encoding nucleotide sequence is designed in the following format: [heavy chain - Furin cleavage site - T2A site - light chain], as described (Fang J, Qian JJ, Yi S, Harding TC, Tu GH, Van Roey M, Jooss K. (2005). Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol.* 23(5):584-90), where the T2A site is a *Thosea asigna* virus 2A peptide. The variable regions are appended to the constant region of mouse Ig isotype.

[00555] Table 35: Available immune checkpoint inhibitors

Target	Clone	Source
4-1BB	3H3	Bio X Cell BE0239
4-1BB	LOB12.3	Bio X Cell BE0169
OX40	OX86	Bio X Cell BE0031
CTLA-4	9H10	Bio X Cell BE0131
PD-1	RMP1-14	Bio X Cell BE0146

[00556] In another example, an E1/E3 deleted ChAdV68 viral vector is designed with an expression cassette introduced into the deleted E1 region, as described above, and the immune checkpoint inhibitor is introduced into the deleted E3 region.

[00557] In another example, an alphavirus viral vector with the viral structural proteins deleted is designed with an expression cassette in the following orientation from 5' to 3' introduced into the deleted region in the following format: [model-antigens/GFP - IRES - Immune Checkpoint Inhibitor]. The various immune checkpoint inhibitors tested in the alphavirus vector are the same as those tested in the adenoviral vector described above.

Vector Production

[00558] The immune checkpoint inhibitor expressing viral vectors are generated by transfecting linearized vectors into 293A cells using Fugene 6 (Promega). The viral vectors undergo expansion in 293 cells before large scale production (400 mL scale) in 293F suspension cells. 48h post infection cells are harvested, lysed and virus purified by two rounds of CsCl gradient. The virus is dialyzed into 20mM Tris pH 8.0, 25mM NaCl and 2.5% Glycerol. The purified viral vectors is aliquoted and stored at -80 C. The infectious unit titer of the purified viral vectors is determined using an anti-capsid assay. For in vitro and in vivo experiments, dosing was based on IU titers.

In vitro Expression

[00559] 293F cells are infected with the various viral vectors at an MOI of 1. Post infection, the supernatant is harvested, and the antibody recovered using Protein A beads. The antibody is eluted from the beads and separated on a 4-20% SDS-PAGE gel. The gel is subjected to Western Blot analysis using a goat anti-Mouse, HRP-conjugated antibody (Millipore, AP124P)

at 1:2,500 dilution in TBST-5% dry milk, followed by chemiluminescent detection reagent (Thermofisher, ECL Plus).

In vivo evaluation

[00560] Mice are immunized with the various viral vectors, delivered via bilateral intramuscular (IM) injections to the tibialis anterior muscles. Mice in select groups receiving the viral vector that does not express the immune checkpoint inhibitor are co-administered the immune checkpoint inhibitor. Antigen-specific T-cells are measured by ELISpot and intracellular cytokine staining. Serum is obtained at various timepoints and the immune checkpoint inhibitor concentration is measured by ELISA.

Results

[00561] The various viral vectors, including those co-expressing the immune checkpoint inhibitor, are produced and quantified. The viral vectors encoding the immune checkpoint inhibitors express the immune checkpoint inhibitor at detectable levels in vitro and in serum.

[00562] The various viral vectors co-expressing the immune checkpoint inhibitor result in immune activation that is equivalent to or greater than that achieved by vector without checkpoint inhibitor.

Certain Sequences

[00563] Vectors, cassettes, and antibodies referred to herein are described below and referred to by SEQ ID NO.

Tremelimumab VL (SEQ ID NO:16)
Tremelimumab VH (SEQ ID NO:17)
Tremelimumab VH CDR1 (SEQ ID NO:18)
Tremelimumab VH CDR2 (SEQ ID NO:19)
Tremelimumab VH CDR3 (SEQ ID NO:20)
Tremelimumab VL CDR1 (SEQ ID NO:21)
Tremelimumab VL CDR2 (SEQ ID NO:22)
Tremelimumab VL CDR3 (SEQ ID NO:23)
Durvalumab (MEDI4736) VL (SEQ ID NO:24)
MEDI4736 VH (SEQ ID NO:25)
MEDI4736 VH CDR1 (SEQ ID NO:26)
MEDI4736 VH CDR2 (SEQ ID NO:27)
MEDI4736 VH CDR3 (SEQ ID NO:28)
MEDI4736 VL CDR1 (SEQ ID NO:29)
MEDI4736 VL CDR2 (SEQ ID NO:30)
MEDI4736 VL CDR3 (SEQ ID NO:31)
UbA76-25merPDTT nucleotide (SEQ ID NO:32)
UbA76-25merPDTT polypeptide (SEQ ID NO:33)
MAG-25merPDTT nucleotide (SEQ ID NO:34)
MAG-25merPDTT polypeptide (SEQ ID NO:35)
Ub7625merPDTT NoSFL nucleotide (SEQ ID NO:36)
Ub7625merPDTT NoSFL polypeptide (SEQ ID NO:37)

ChAdV68.5WTnt.MAG25mer (SEQ ID NO:2); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,125- 31,825) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at five positions; model neoantigen cassette under the control of the CMV promoter/enhancer inserted in place of deleted E1; SV40 polyA 3' of cassette
Venezuelan equine encephalitis virus [VEE] (SEQ ID NO:3) GenBank: L01442.2
VEE-MAG25mer (SEQ ID NO:4); contains MAG-25merPDTT nucleotide (bases 30-1755)
Venezuelan equine encephalitis virus strain TC-83 [TC-83] (SEQ ID NO:5) GenBank: L01443.1
VEE Delivery Vector (SEQ ID NO:6); VEE genome with nucleotides 7544-11175 deleted [alphavirus structural proteins removed]
TC-83 Delivery Vector (SEQ ID NO:7); TC-83 genome with nucleotides 7544-11175 deleted [alphavirus structural proteins removed]
VEE Production Vector (SEQ ID NO:8); VEE genome with nucleotides 7544-11175 deleted, plus 5' T7-promoter, plus 3' restriction sites
TC-83 Production Vector (SEQ ID NO:9); TC-83 genome with nucleotides 7544-11175 deleted, plus 5' T7-promoter, plus 3' restriction sites
VEE-UbAAY (SEQ ID NO:14); VEE delivery vector with MHC class I mouse tumor epitopes SIINFEKL and AHI-A5 inserted
VEE-Luciferase (SEQ ID NO:15); VEE delivery vector with luciferase gene inserted at 7545
ubiquitin (SEQ ID NO:38)>UbG76 0-228
Ubiquitin A76 (SEQ ID NO:39)>UbA76 0-228
HLA-A2 (MHC class I) signal peptide (SEQ ID NO:40)>MHC SignalPep 0-78
HLA-A2 (MHC class I) Trans Membrane domain (SEQ ID NO:41)>HLA A2 TM Domain 0-201
IgK Leader Seq (SEQ ID NO:42)>IgK Leader Seq 0-60
Human DC-Lamp (SEQ ID NO:43)>HumanDCLAMP 0-3178
Mouse LAMP1 (SEQ ID NO:44)>MouseLamp1 0-1858
Human Lamp1 cDNA (SEQ ID NO:45)>Human Lamp1 0-2339
Tetanus toxoid nulceic acid sequence (SEQ ID NO:46)
Tetanus toxoid amino acid sequence (SEQ ID NO:47)
PADRE nulceotide sequence (SEQ ID NO:48)
PADRE amino acid sequence (SEQ ID NO:49)
WPPE (SEQ ID NO:50)>WPPE 0-593
IRES (SEQ ID NO:51)>eGFP_IRES_SEAP_Insert 1746-2335
GFP (SEQ ID NO:52)
SEAP (SEQ ID NO:53)
Firefly Luciferase (SEQ ID NO:54)
FMDV 2A (SEQ ID NO:55)

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CLAIMS

What is claimed is:

1. A vector system comprising an antigen cassette,

the antigen cassette comprising:

(1) at least one antigen-encoding nucleic acid sequence associated with a tumor present within a subject comprising:

at least one antigen-encoding nucleic acid sequence, optionally the at least one antigen-encoding nucleic acid sequence comprising an MHC class I antigen-encoding nucleic acid sequence, each comprising:

a. an epitope encoding nucleic acid sequence, optionally comprising at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence,

b. optionally a 5' linker sequence, and

c. optionally a 3' linker sequence;

(2) at least one promoter sequence operably linked to at least one antigen-encoding nucleic acid sequence,

(3) optionally, at least one MHC class II antigen-encoding nucleic acid sequence;

(4) optionally, at least one GP GPG linker sequence (SEQ ID NO:56);

(5) optionally, at least one polyadenylation sequence; and

the vector further comprising, optionally within the cassette, a nucleic acid sequence encoding at least one immune modulator, optionally wherein the nucleic acid sequence encoding the at least one immune modulator is transcribed on:

(1) the same transcript as the at least one antigen-encoding nucleic acid sequence with an internal ribosome entry sequence (IRES) sequence separating the sequences encoding the at least one immune modulator and the at least one antigen-encoding nucleic acid sequence, or

(2) a different transcript as the at least one antigen-encoding nucleic acid sequence, wherein at least one second promoter sequence is operably linked to the sequences encoding the at least one immune modulator.

2. A chimpanzee adenovirus vector comprising:

- a. a modified ChAdV68 sequence comprising the sequence of SEQ ID NO:1 with an E1 (nt 577 to 3403) deletion and an E3 (nt 27,125-31,825) deletion;
- b. a CMV promoter sequence;
- c. an SV40 polyadenylation signal nucleotide sequence;
- d. a nucleic acid sequence encoding an immune checkpoint inhibitor, and
- e. an antigen cassette, the antigen cassette comprising:

(1) at least one antigen-encoding nucleic acid sequences derived from a tumor present within a subject, the at least one antigen-encoding nucleic acid sequence comprising:

at least 10 tumor-specific and subject-specific MHC class I antigen-encoding nucleic acid sequences linearly linked to each other and each comprising:

(A) a MHC class I epitope encoding nucleic acid sequence with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, wherein the MHC I epitope encoding nucleic acid sequence encodes a MHC class I epitope 7-15 amino acids in length,

(B) a 5' linker sequence, wherein the 5' linker sequence encodes a native N-terminal amino acid sequence of the MHC I epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length,

(C) a 3' linker sequence, wherein the 3' linker sequence encodes a native C-terminal acid sequence of the MHC I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and

wherein each of the MHC class I antigen-encoding nucleic acid sequences encodes a polypeptide that is 25 amino acids in length, and wherein each 3' end of each MHC class I antigen-encoding nucleic acid sequence is linked to the 5' end of the

following MHC class I antigen-encoding nucleic acid sequence with the exception of the final MHC class I antigen-encoding nucleic acid sequence; and

(2) at least two MHC class II antigen-encoding nucleic acid sequences comprising:

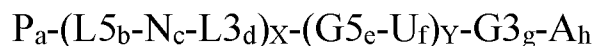
- (A) a PADRE MHC class II sequence (SEQ ID NO:48),
- (B) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46),
- (C) a first nucleic acid sequence encoding a GP GPG amino acid linker sequence linking the PADRE MHC class II sequence and the Tetanus toxoid MHC class II sequence,
- (D) a second nucleic acid sequence encoding a GP GPG amino acid linker sequence linking the 5' end of the at least two MHC class II antigen-encoding nucleic acid sequences to the at least 10 tumor-specific and subject-specific MHC class I neoantigen-encoding nucleic acid sequences,
- (E) optionally, a third nucleic acid sequence encoding a GP GPG amino acid linker sequence at the 3' end of the at least two MHC class II antigen-encoding nucleic acid sequences; and

wherein the antigen cassette is inserted within the E1 deletion and the CMV promoter sequence is operably linked to the antigen cassette, and wherein the nucleic acid sequence encoding the checkpoint inhibitor is transcribed:

(1) on the same transcript as the at least one antigen-encoding nucleic acid sequence with an internal ribosome entry sequence (IRES) sequence separating the sequences encoding the checkpoint inhibitor and the at least one antigen-encoding nucleic acid sequence, or

(2) on a different transcript as the at least one antigen-encoding nucleic acid sequences, optionally wherein a second CMV promoter sequence is operably linked to the sequences encoding the at least one immune modulator, or optionally wherein the at least one immune modulator is inserted within the E3 deletion.

3. The vector of claim 1, wherein an ordered sequence of each element of the vector is described in the formula, from 5' to 3', comprising:



wherein P comprises the at least one promoter sequence operably linked to at least one of the at least one antigen-encoding nucleic acid sequences, where $a = 1$,

N comprises one of the epitope encoding nucleic acid sequence with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by the wild-type nucleic acid sequence, where $c = 1$,

L5 comprises the 5' linker sequence, where $b = 0$ or 1 ,

L3 comprises the 3' linker sequence, where $d = 0$ or 1 ,

G5 comprises one of the at least one nucleic acid sequences encoding a GPGPG amino acid linker, where $e = 0$ or 1 ,

G3 comprises one of the at least one nucleic acid sequences encoding a GPGPG amino acid linker, where $g = 0$ or 1 ,

U comprises one of the at least one MHC class II antigen-encoding nucleic acid sequence, where $f = 1$,

A comprises the at least one polyadenylation sequence, where $h = 0$ or 1 ,

$X = 2$ to 400 , where for each X the corresponding N_c is an epitope encoding nucleic acid sequence, optionally wherein for each X the corresponding N_c is a distinct MHC class I epitope encoding nucleic acid sequence, and

$Y = 0$ - 2 , where for each Y the corresponding U_f is an antigen-encoding nucleic acid sequence, optionally wherein for each Y the corresponding U_f is a distinct MHC class II antigen-encoding nucleic acid sequence.

4. The vector of claim 3, wherein

$b = 1$, $d = 1$, $e = 1$, $g = 1$, $h = 1$, $X = 10$, $Y = 2$,

P is a CMV promoter sequence,

each N encodes a MHC class I epitope 7-15 amino acids in length,

L5 encodes a native N-terminal amino acid sequence of the MHC I epitope, and

wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length,

L3 encodes a native C-terminal amino acid sequence of the MHC I epitope, and

wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length,

- U is each of a PADRE class II sequence and a Tetanus toxoid MHC class II sequence, the vector comprises a modified ChAdV68 sequence comprising the sequence of SEQ ID NO:1 with an E1 (nt 577 to 3403) deletion and an E3 (nt 27,125- 31,825) deletion and the neoantigen cassette is inserted within the E1 deletion, and each of the MHC class I antigen-encoding nucleic acid sequences encodes a polypeptide that is 25 amino acids in length.
5. The vector of any of claims 1-4, wherein at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I on the tumor cell surface.
 6. The vector of any of the above claims except claim 2 or 4, wherein each antigen-encoding nucleic acid sequence is linked directly to one another.
 7. The vector of any of the above claims except claim 2 or 4, wherein at least one of the at least one antigen-encoding nucleic acid sequences is linked to a distinct antigen-encoding nucleic acid sequence with nucleic acid sequence encoding a linker.
 8. The vector of claim 7, wherein the linker links two MHC class I sequences or an MHC class I sequence to an MHC class II sequence.
 9. The vector of claim 8, wherein the linker is selected from the group consisting of: (1) consecutive glycine residues, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues in length; (2) consecutive alanine residues, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues in length; (3) two arginine residues (RR); (4) alanine, alanine, tyrosine (AAY); (5) a consensus sequence at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues in length that is processed efficiently by a mammalian proteasome; and (6) one or more native sequences flanking the antigen derived from the cognate protein of origin and that is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 2-20 amino acid residues in length.
 10. The vector of claim 7, wherein the linker links two MHC class II sequences or an MHC class II sequence to an MHC class I sequence.
 11. The vector of claim 10, wherein the linker comprises the sequence GPGPG.
 12. The vector of any of the above claims except claim 2 or 4, wherein at least one of the at least one antigen-encoding nucleic acid sequences is linked, operably or directly, to a separate or contiguous sequence that enhances the expression, stability, cell trafficking, processing and

presentation, and/or immunogenicity of the at least one antigen-encoding nucleic acid sequence.

13. The vector of claim 12, wherein the separate or contiguous sequence comprises at least one of: a ubiquitin sequence, a ubiquitin sequence modified to increase proteasome targeting (e.g., the ubiquitin sequence contains a Gly to Ala substitution at position 76), an immunoglobulin signal sequence (e.g., IgK), a major histocompatibility class I sequence, lysosomal-associated membrane protein (LAMP)-1, human dendritic cell lysosomal-associated membrane protein, and a major histocompatibility class II sequence; optionally wherein the ubiquitin sequence modified to increase proteasome targeting is A76.

14. The vector of any of the above claims, wherein at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has increased binding affinity to its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence.

15. The vector of any of the above claims, wherein at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has increased binding stability to its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence.

16. The vector of any of the above claims, wherein at least one of the antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that has an increased likelihood of presentation on its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence.

17. The vector of any of the above claims, wherein the at least one alteration comprises a point mutation, a frameshift mutation, a non-frameshift mutation, a deletion mutation, an insertion mutation, a splice variant, a genomic rearrangement, or a proteasome-generated spliced antigen.

18. The vector of any of the above claims, wherein the tumor is selected from the group consisting of: lung cancer, melanoma, breast cancer, ovarian cancer, prostate cancer, kidney cancer, gastric cancer, colon cancer, testicular cancer, head and neck cancer, pancreatic cancer, brain cancer, B-cell lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer.

19. The vector of any of the above claims except claim 2 or 4, wherein the expression of each of the at least one antigen-encoding nucleic acid sequences is driven by the at least one promoter.

20. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleic acid sequences.

21. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequence the comprises at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 nucleic acid sequences.

22. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 nucleic acid sequences and wherein at least two of the antigen-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC class I on the tumor cell surface.

23. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 nucleic acid sequences and wherein, when administered to the subject and translated, at least one of the antigens are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on the tumor cell surface.

24. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 MHC class I and/or class II antigen-encoding nucleic acid sequences, wherein, when administered to the subject and translated, at least one of the MHC class I or class II antigens are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on the tumor cell surface, and optionally wherein the expression of each of the at least 2-400 MHC class I or class II antigen-encoding nucleic acid sequences is driven by the at least one promoter.

25. The vector of any of the above claims except claim 2 or 4, wherein each MHC class I antigen-encoding nucleic acid sequence encodes a polypeptide sequence between 8 and 35 amino acids in length, optionally 9-17, 9-25, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 amino acids in length.

26. The vector of any of the above claims except claim 2 or 4, wherein the at least one MHC class II antigen-encoding nucleic acid sequence is present.
27. The vector of any of the above claims except claim 2 or 4, wherein the at least one MHC class II antigen-encoding nucleic acid sequence is present and comprises at least one MHC class II antigen-encoding nucleic acid sequence that comprises at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence.
28. The vector of any of the above claims except claim 2 or 4, wherein the at least one MHC class II antigen-encoding nucleic acid sequence is 12-20, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 20-40 amino acids in length.
29. The vector of any of the above claims except claim 2 or 4, wherein the at least one MHC class II antigen-encoding nucleic acid sequence is present and comprises at least one universal MHC class II antigen-encoding nucleic acid sequence, optionally wherein the at least one universal sequence comprises at least one of Tetanus toxoid and PADRE.
30. The vector of any of the above claims except claim 2 or 4, wherein the at least one promoter sequence is inducible.
31. The vector of any of the above claims except claim 2 or 4, wherein the at least one promoter sequence is non-inducible.
32. The vector of any of the above claims except claim 2 or 4, wherein the at least one promoter sequence is a CMV, SV40, EF-1, RSV, PGK, HSA, MCK, or EBV promoter sequence.
33. The vector of any of the above claims, wherein the antigen cassette further comprises at least one poly-adenylation (polyA) sequence operably linked to at least one of the at least one antigen-encoding nucleic acid sequences, optionally wherein the polyA sequence is located 3' of the at least one antigen-encoding nucleic acid sequence.
34. The vector of claim 33, wherein the polyA sequence comprises an or Bovine Growth Hormone (BGH) SV40 polyA sequence.
35. The vector of any of the above claims, wherein the antigen cassette further comprises at least one of: an intron sequence, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence, an internal ribosome entry sequence (IRES) sequence, a nucleotide

sequence encoding a 2A self cleaving peptide sequence, a nucleotide sequence encoding a Furin cleavage site, or a sequence in the 5' or 3' non-coding region known to enhance the nuclear export, stability, or translation efficiency of mRNA that is operably linked to at least one of the at least one antigen-encoding nucleic acid sequences.

36. The vector of any of the above claims, wherein the antigen cassette further comprises a reporter gene, including but not limited to, green fluorescent protein (GFP), a GFP variant, secreted alkaline phosphatase, luciferase, or a luciferase variant.

37. The vector of any of the above claims, wherein the at least one immune modulator inhibits an immune checkpoint molecule.

38. The vector of claim 37, wherein the immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof.

39. The vector of claim 38, wherein the antibody or antigen-binding fragment thereof is a Fab fragment, a Fab' fragment, a single chain Fv (scFv), a single domain antibody (sdAb) either as single specific or multiple specificities linked together (e.g., camelid antibody domains), or full-length single-chain antibody (e.g., full-length IgG with heavy and light chains linked by a flexible linker).

40. The vector of claim 38 or 39, wherein the heavy and light chain sequences of the antibody are a contiguous sequence separated by either a self-cleaving sequence such as 2A, optionally wherein the self-cleaving sequence has a Furin cleavage site sequence 5' of the self-cleaving sequence, or an IRES sequence; or the heavy and light chain sequences of the antibody are linked by a flexible linker such as consecutive glycine residues.

41. The vector of claim 37, wherein the immune modulator is a cytokine.

42. The vector of claim 41, wherein the cytokine is at least one of IL-2, IL-7, IL-12, IL-15, or IL-21 or variants thereof of each.

43. The vector of any of the above claims except claim 2 or 4, wherein the vector is a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a

ChAdV68 vector, or an srRNA vector, optionally wherein the srRNA vector is a Venezuelan equine encephalitis virus srRNA vector.

44. The vector of any of the above claims except claim 2 or 4, wherein the vector comprises the sequence set forth in SEQ ID NO:1.

45. The vector of any of the above claims except claim 2 or 4, wherein the vector comprises the sequence set forth in SEQ ID NO:1, except that the sequence is fully deleted or functionally deleted in at least one gene selected from the group consisting of the chimpanzee adenovirus E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1, optionally wherein the sequence is fully deleted or functionally deleted in: (1) E1A and E1B; (2) E1A, E1B, and E3; or (3) E1A, E1B, E3, and E4 of the sequence set forth in SEQ ID NO: 1.

46. The vector of any of the above claims except claim 2 or 4, wherein the vector comprises a gene or regulatory sequence obtained from the sequence of SEQ ID NO: 1, optionally wherein the gene is selected from the group consisting of the chimpanzee adenovirus inverted terminal repeat (ITR), E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1.

47. The vector of any of the above claims except claim 2 or 4, wherein the antigen cassette is inserted in the vector at the E1 region, E3 region, and/or any deleted AdV region that allows incorporation of the antigen cassette.

48. The vector of any of the above claims except claim 2 or 4, wherein the vector is generated from one of a first generation, a second generation, or a helper-dependent adenoviral vector.

49. The vector of any of the above claims except claim 2 or 4, wherein the vector comprises one or more deletions between base pair number 577 and 3403 or between base pair 456 and 3014, and optionally wherein the vector further comprises one or more deletions between base pair 27,125 and 31,825 or between base pair 27,816 and 31,333 of the sequence set forth in SEQ ID NO:1.

50. The vector of any of the above claims except claim 2 or 4, wherein the vector further comprises one or more deletions between base pair number 3957 and 10346, base pair number 21787 and 23370, and base pair number 33486 and 36193 of the sequence set forth in SEQ ID NO:1.

51. The vector of any of the above claims except claim 2 or 4, wherein the at least one antigen-encoding nucleic acid sequences are selected by performing the steps of:

obtaining at least one of exome, transcriptome, or whole genome tumor nucleotide sequencing data from the tumor, wherein the tumor nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens;

inputting the peptide sequence of each antigen into a presentation model to generate a set of numerical likelihoods that each of the antigens is presented by one or more of the MHC alleles on the tumor cell surface of the tumor, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and

selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens which are used to generate the at least one antigen-encoding nucleic acid sequences.

52. The vector of claim 2, wherein each of the MHC class I epitope encoding nucleic acid sequences are selected by performing the steps of:

obtaining at least one of exome, transcriptome, or whole genome tumor nucleotide sequencing data from the tumor, wherein the tumor nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens;

inputting the peptide sequence of each antigen into a presentation model to generate a set of numerical likelihoods that each of the antigens is presented by one or more of the MHC alleles on the tumor cell surface of the tumor, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and

selecting a subset of the set of antigens based on the set of numerical likelihoods to generate a set of selected antigens which are used to generate the at least two MHC class I antigen-encoding nucleic acid sequences.

53. The vector of claim 51, wherein a number of the set of selected antigens is 2-20.

54. The vector of claim 51 or 52, wherein the presentation model represents dependence between:

presence of a pair of a particular one of the MHC alleles and a particular amino acid at a particular position of a peptide sequence; and

likelihood of presentation on the tumor cell surface, by the particular one of the MHC alleles of the pair, of such a peptide sequence comprising the particular amino acid at the particular position.

55. The vector of claim 51 or 52, wherein selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being presented on the tumor cell surface relative to unselected antigens based on the presentation model.

56. The vector of claim 51 or 52, wherein selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of inducing a tumor-specific immune response in the subject relative to unselected antigens based on the presentation model.

57. The vector of claim 51 or 52, wherein selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of being presented to naïve T cells by professional antigen presenting cells (APCs) relative to unselected antigens based on the presentation model, optionally wherein the APC is a dendritic cell (DC).

58. The vector of claim 51 or 52, wherein selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being subject to inhibition via central or peripheral tolerance relative to unselected antigens based on the presentation model.

59. The vector of claim 51 or 52, wherein selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being capable of inducing an autoimmune response to normal tissue in the subject relative to unselected antigens based on the presentation model.

60. The vector of claim 51 or 52, wherein exome or transcriptome nucleotide sequencing data is obtained by performing sequencing on the tumor tissue.

61. The vector of claim 51 or 52, wherein the sequencing is next generation sequencing (NGS) or any massively parallel sequencing approach.

62. The vector of any of the above claims, wherein the antigen cassette comprises junctional epitope sequences formed by adjacent sequences in the antigen cassette.

63. The vector of claim 62, wherein at least one or each junctional epitope sequence has an affinity of greater than 500 nM for MHC.

64. The vector of claim 62 or 63, wherein each junctional epitope sequence is non-self.

65. The vector of any of the above claims, wherein the antigen cassette does not encode a non-therapeutic MHC class I or class II epitope nucleic acid sequence comprising a translated, wild-type nucleic acid sequence, wherein the non-therapeutic epitope is predicted to be displayed on an MHC allele of the subject.

66. The vector of claim 65, wherein the non-therapeutic predicted MHC class I or class II epitope sequence is a junctional epitope sequence formed by adjacent sequences in the antigen cassette.

67. The vector of claims 62 or 66, wherein the prediction is based on presentation likelihoods generated by inputting sequences of the non-therapeutic epitopes into a presentation model.

68. The vector of any one of claims 62-67, wherein an order of the at least one antigen-encoding nucleic acid sequences in the antigen cassette is determined by a series of steps comprising:

1. generating a set of candidate antigen cassette sequences corresponding to different orders of the at least one antigen-encoding nucleic acid sequences;
2. determining, for each candidate antigen cassette sequence, a presentation score based on presentation of non-therapeutic epitopes in the candidate antigen cassette sequence; and
3. selecting a candidate cassette sequence associated with a presentation score below a predetermined threshold as the antigen cassette sequence for an antigen vaccine.

69. A pharmaceutical composition comprising the vector of any of the above claims and a pharmaceutically acceptable carrier.

70. The pharmaceutical composition of claim 69, wherein the composition further comprises an adjuvant.

71. The pharmaceutical composition of claim 69 or 70, wherein the composition further comprises an immune modulator.

72. The pharmaceutical composition of claim 71, wherein the immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an

anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof.

73. An isolated nucleotide sequence comprising the antigen cassette of any of the above vector claims and a gene obtained from the sequence of SEQ ID NO: 1, optionally wherein the gene is selected from the group consisting of the chimpanzee adenovirus ITR, E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence set forth in SEQ ID NO: 1, and optionally wherein the nucleotide sequence is cDNA.

74. An isolated cell comprising the nucleotide sequence of claim 73, optionally wherein the cell is a CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.

75. A vector comprising the nucleotide sequence of claim 73.

76. A kit comprising the vector of any of the above vector claims and instructions for use.

77. A method for treating a subject with cancer, the method comprising administering to the subject the vector of any of the above vector claims or the pharmaceutical composition of any of claims 69-70.

78. The method of claim 77, wherein the vector or composition is administered intramuscularly (IM), intradermally (ID), or subcutaneously (SC).

79. The method of claim 77 or 78, further comprising administering to the subject an immune modulator, optionally wherein the immune modulator is administered before, concurrently with, or after administration of the vector or pharmaceutical composition.

80. The method of claim 79, wherein the immune modulator is an anti-CTLA4 antibody or an antigen-binding fragment thereof, an anti-PD-1 antibody or an antigen-binding fragment thereof, an anti-PD-L1 antibody or an antigen-binding fragment thereof, an anti-4-1BB antibody or an antigen-binding fragment thereof, or an anti-OX-40 antibody or an antigen-binding fragment thereof.

81. The method of claim 79, wherein the immune modulator is administered intravenously (IV), intramuscularly (IM), intradermally (ID), or subcutaneously (SC).

82. The method of claim 81, wherein the subcutaneous administration is near the site of the vector or composition administration or in close proximity to one or more vector or composition draining lymph nodes.

83. The method of any one of claims 77-82, further comprising administering to the subject a second vaccine composition.

84. The method of claim 83, wherein the second vaccine composition is administered prior to the administration of the vector or the pharmaceutical composition of any one of claims 77-82.

85. The method of claim 83, wherein the second vaccine composition is administered subsequent to the administration of the vector or the pharmaceutical composition of any one of claims 77-82.

86. The method of claim 84 or 85, wherein the second vaccine composition is the same as the vector or the pharmaceutical composition of any one of claims 77-82.

87. The method of claim 84 or 85, wherein the second vaccine composition is different from the vector or the pharmaceutical composition of any one of claims 77-82.

88. The method of claim 87, wherein the second vaccine composition comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an srRNA vector, optionally wherein the srRNA vector is a Venezuelan equine encephalitis virus vector, and

optionally wherein the chimpanzee adenovirus vector or the srRNA vector comprises a nucleic acid sequence encoding at least one immune modulator.

89. The method of claim 88, wherein the at least one antigen-encoding nucleic acid sequence encoded by the chimpanzee adenovirus vector or the srRNA vector is the same as the at least one antigen-encoding nucleic acid sequences of any of the above vector claims, and optionally wherein the nucleic acid sequence encoding the at least one immune modulator encoded by the chimpanzee adenovirus vector or the srRNA vector is the same as the the at least one immune modulator of any of the above claims.

90. A method of manufacturing the vector of any of the above vector claims, the method comprising:

obtaining a plasmid sequence comprising the at least one promoter sequence and the antigen cassette;

transfecting the plasmid sequence into one or more host cells; and

isolating the vector from the one or more host cells.

91. The method of manufacturing of claim , wherein isolating comprises:

lysing the one or more host cells to obtain a cell lysate comprising the vector; and

purifying the vector from the cell lysate and optionally also from media used to culture the one or more host cells.

92. The method of manufacturing of claim 90 or 91, wherein the plasmid sequence is generated using one of the following; DNA recombination or bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells.

93. The method of manufacturing of any of claims 90-92, wherein the one or more host cells are at least one of CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, and AE1-2a cells.

94. The method of manufacturing of any of claims 91-93, wherein purifying the vector from the cell lysate involves one or more of chromatographic separation, centrifugation, virus precipitation, and filtration.

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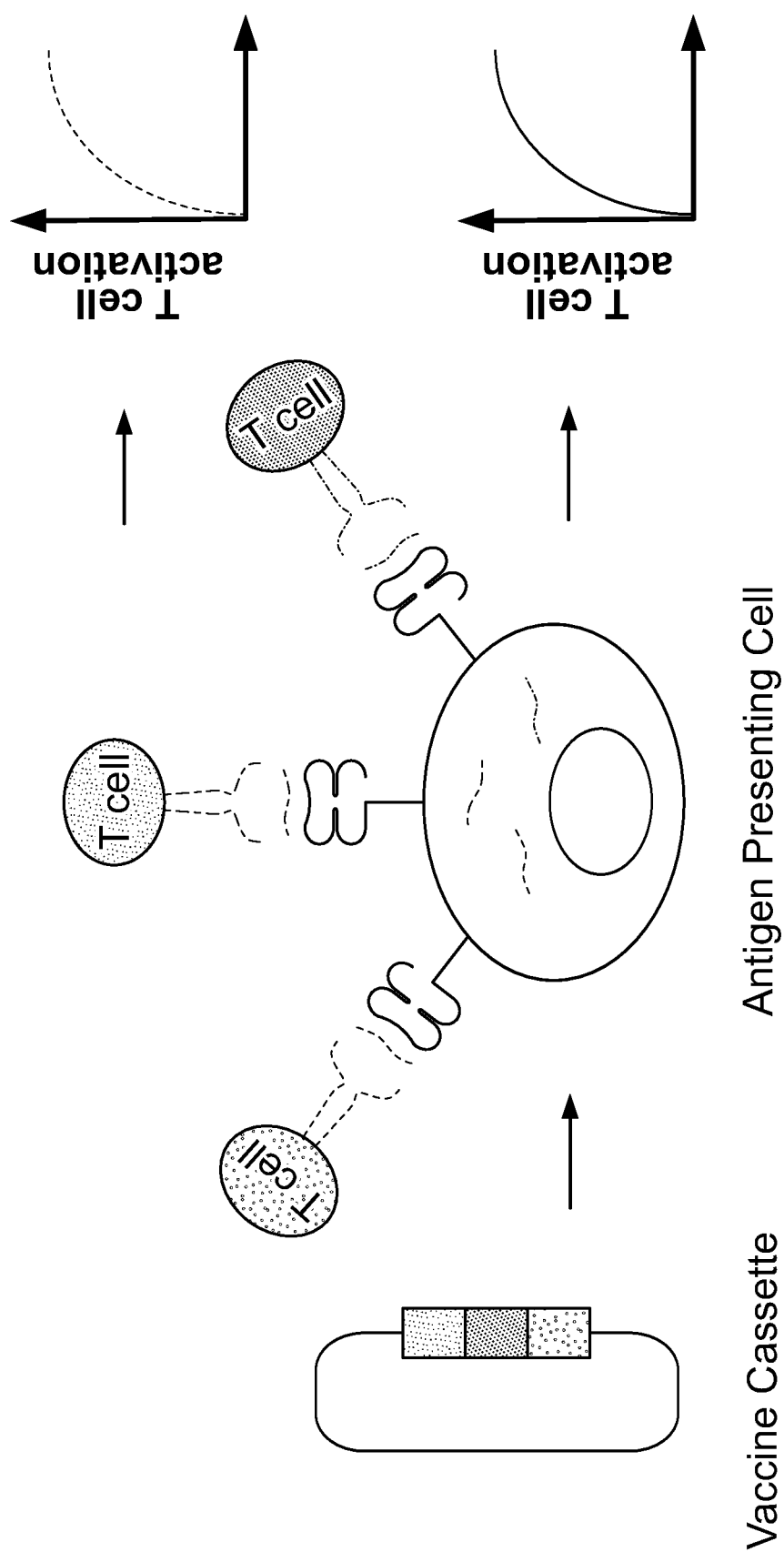


FIG. 1

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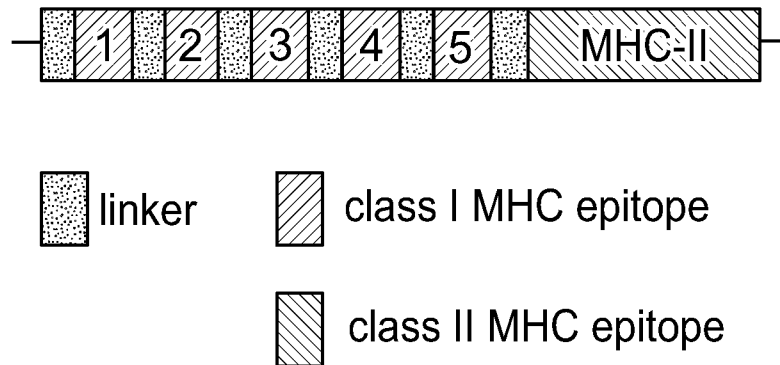


FIG. 2A

#	HLA	Sequence	Origin
1	A*0201	NLVPMVATV	HCMV pp65 (495– 503)
2	A*0201	CLGGLLTMV	EBV LMP2A(426-434)
3	A*0201	GLCTLVAML	EBV BMLF1 (280–288)
4	A*0201	LLFGYPVYV	HTLV-1 Tax (11-19)
5	A*0201	GILGFVFTL	Influenza A Matrix 1 (58– 66)
	MHC-II	AKFVAAWTLKAAA	PADRE (artificial seq)
	MHC-II	QYIKANSKFIGITE	Tetanus toxoid (830–844)

FIG. 2B

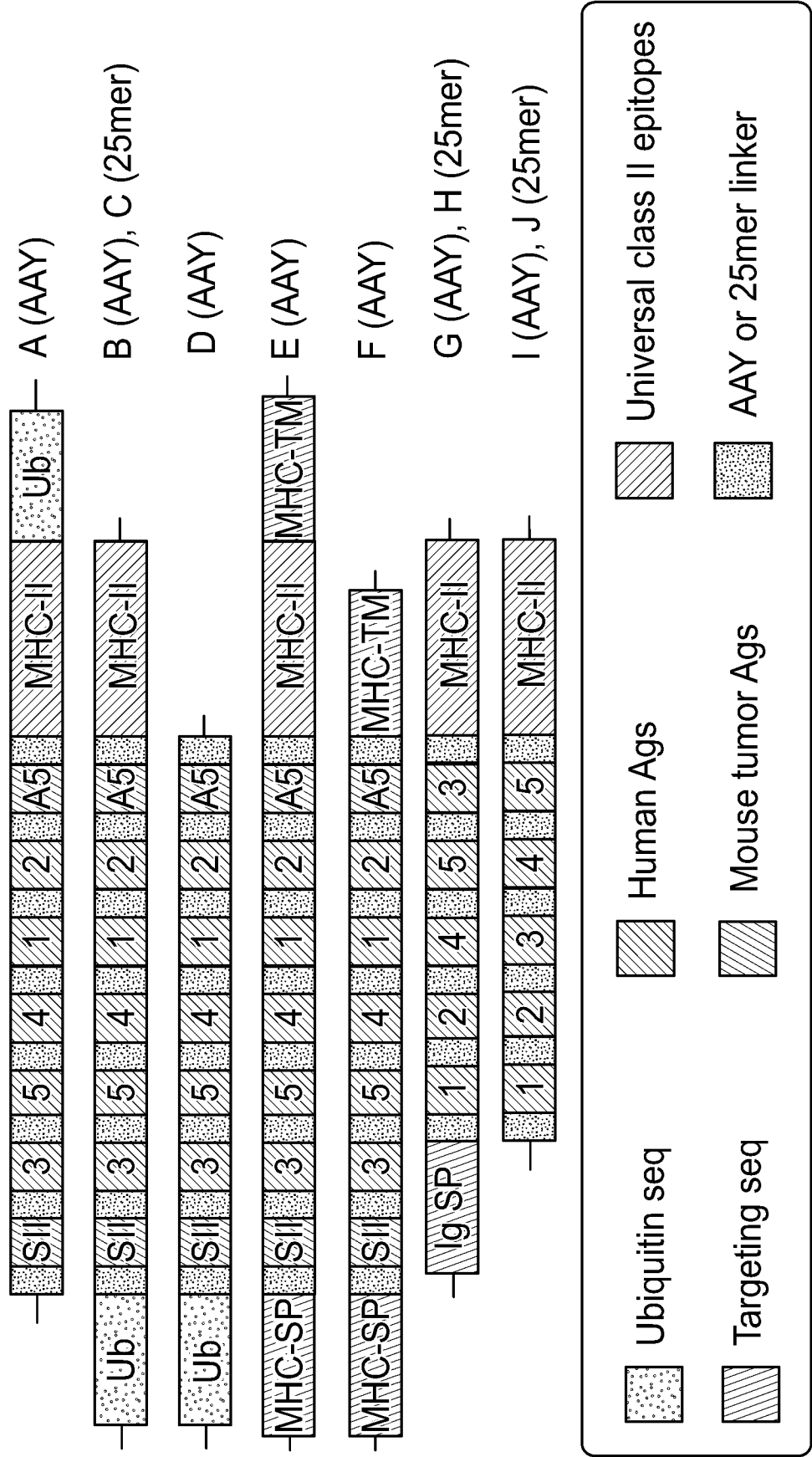


FIG. 3

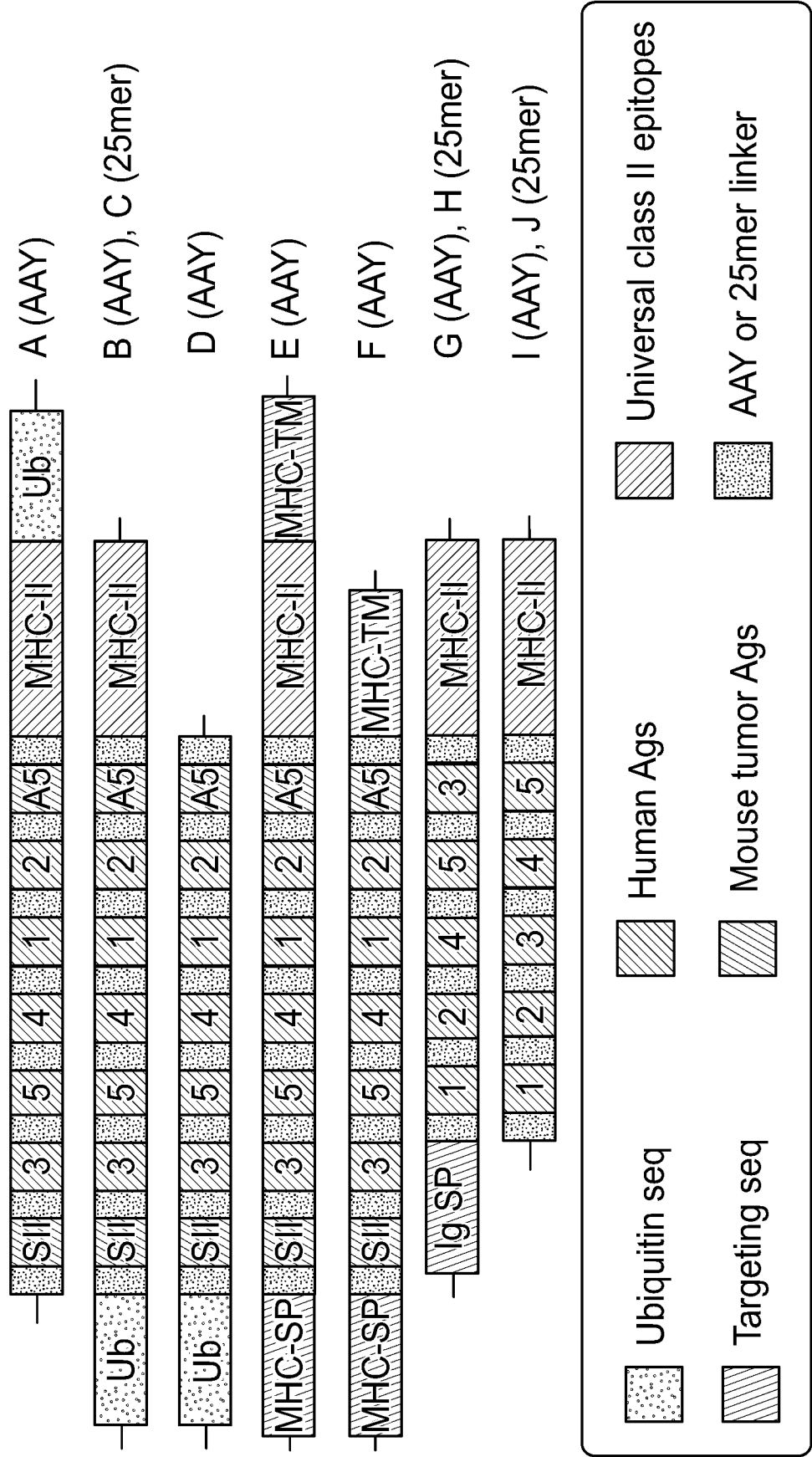


FIG. 4

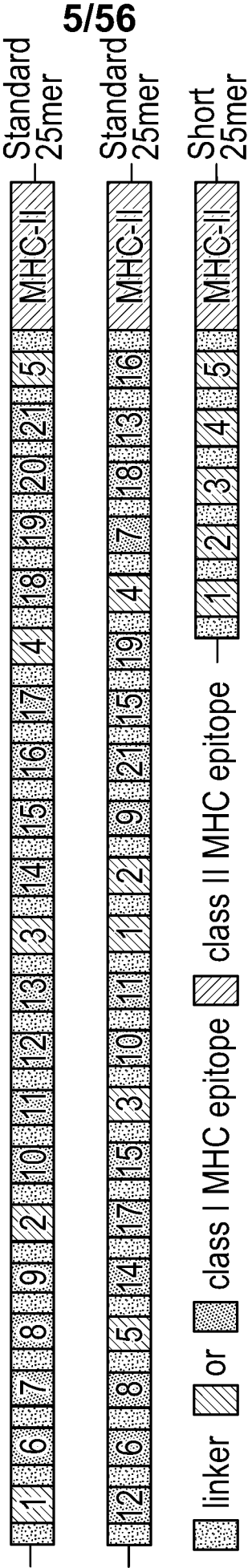


FIG. 5A

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#	HLA	Sequence	Origin
1	A*02:01	NLVPMVATV	HCMV pp65 495-504
2	A*02:01	CLGGLLTMV	EBV LMP-2 426-434
3	A*02:01	GLCTLVAML	EBV BMLF-1 259-267
4	A*02:01	LLFGYPVYV	HTLV1 Tax 11-19
5	A*02:01	GILGFVFTL	Influenza A MP 58-66
6	A*02:01	DLMGYIPAV	HCV core 132-140
7	A*02:01	FLPSDFFPSV	HBV core antigen 18-27
8	A*02:01	FLLTRILT	HBV envelope 183-191
9	A*02:01	WLSLLVPFV	HBV surface antigen 172-181
10	A*02:01	FLLSLGIHL	HBV polymerase 573-581
11	A*02:01	ILKEPVHGV	HIV-1 RT 476-484
12	A*02:01	YMLDLQPETT	HPV 16 E7 11-20
13	A*02:01	CINGVCWTV	HCV NS3 1073-1081
14	A*02:01	YLLPRRGPR	HCV core 35-44
15	A*02:01	FLYALALL	EBV LMP-2 356-364
16	A*02:01	AAGIGILTV	MELAN-A/MART-1 (27-35)
17	A*02:01	SLLMWITQV	NY-ESO-1(157-165) C9V
18	A*03:01	KLGGALQAK	CVM-IE1
19	A*03:01	RLRAEAQVK	EBV-EBNA-3a
20	B*44:05	EENLLDFVRF	EBV EBNA3C (281-290)
21	B*44:05	EEYLQAFTY	Self ABCD3 protein

FIG. 5B

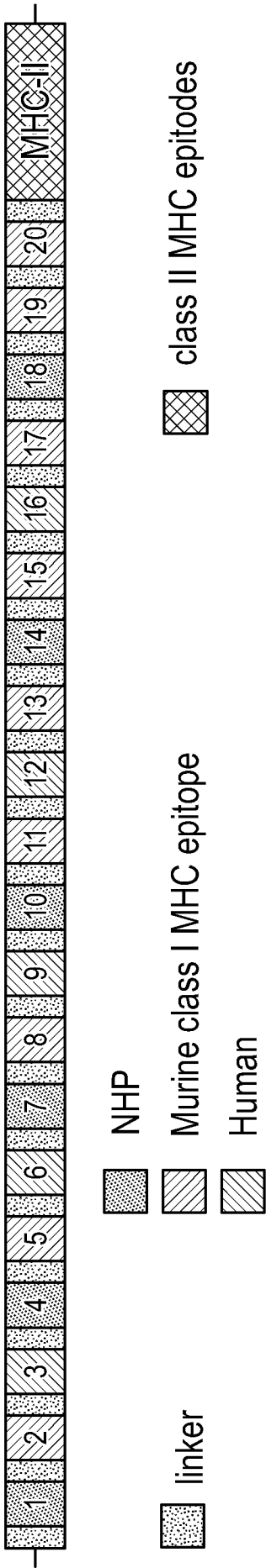


FIG. 6A

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NHP Epitopes

 MHC	Sequence
1 Mamu*01	CTPYDINQM
4 Mamu*01	TTPESANL
7 Mamu*01	CAPPGALL
10 Mamu*01	SGPKTNIIV
14 Mamu*01	LSPRTLNAW
18 Mamu*01	TVPWPNASL

Murine MHC-I Epitopes

 MHC	Sequence
2 H-2Kb	SIINFEKL
5 H-2Ld	SPSYAYHQF
8 H-2Db	EGPRNQDWL
11 H-2Kb	DWENVSPDL
13 H-2Kb	SIIVFNLL
15 H-2Db	ASMTNMELM
17 H-2Db	AQLANDVVL
19 H-2Kb	SVYDFFVWL
20 H-2Ld	MNKYAYHML

Human Epitopes

 HLA	Sequence
3 A*02:01	GILGFVFTL
6 A*02:01	LLFGYPVYV
9 A*02:01	GLCTLVAML
12 A*02:01	NLVPMVATV
16 A*02:01	CLGGLTMV

Universal MHC-II Epitopes

 HLA	Sequence
1 MHC-II	AKFVAAWTLKAAA
2 MHC-II	QYIKANSKFIGITEL

FIG. 6B

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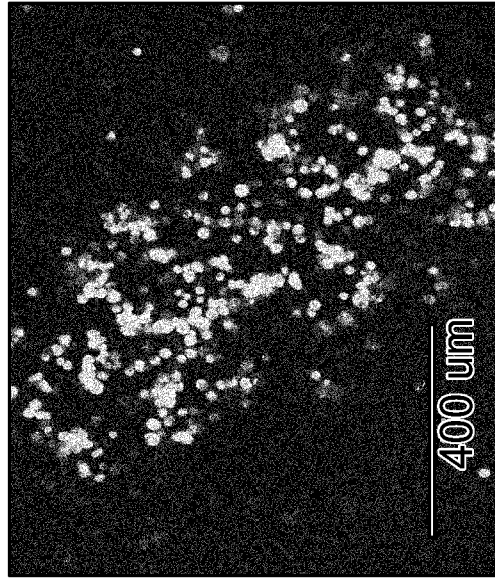


FIG. 7C

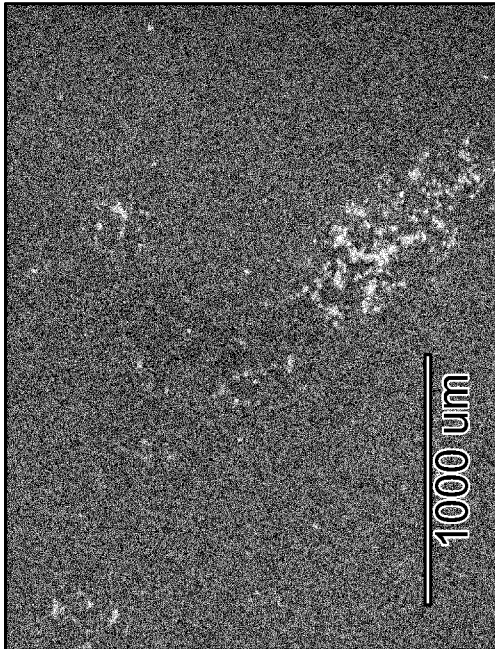


FIG. 7B

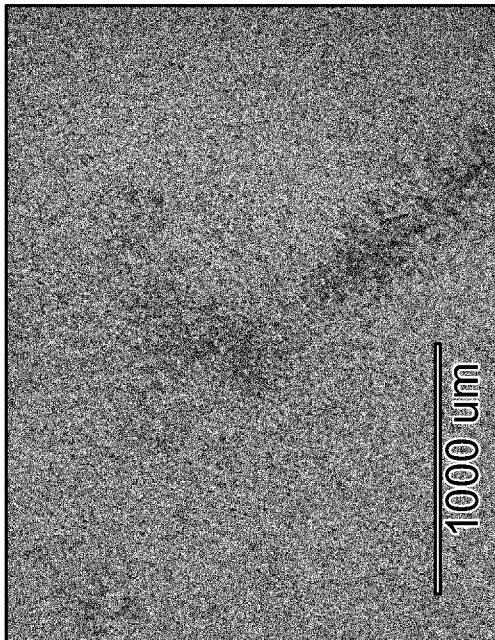


FIG. 7A

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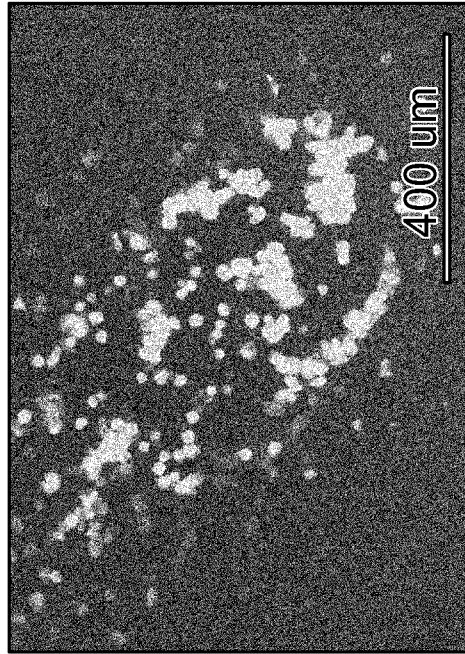


FIG. 8C

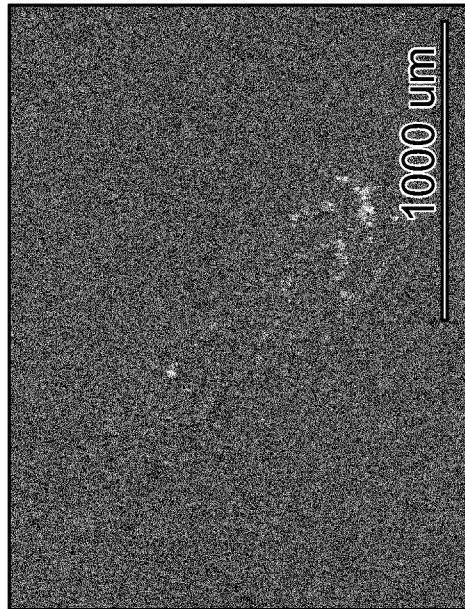


FIG. 8B

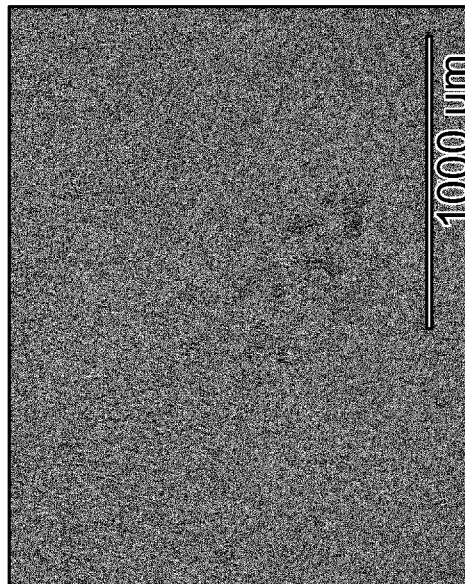
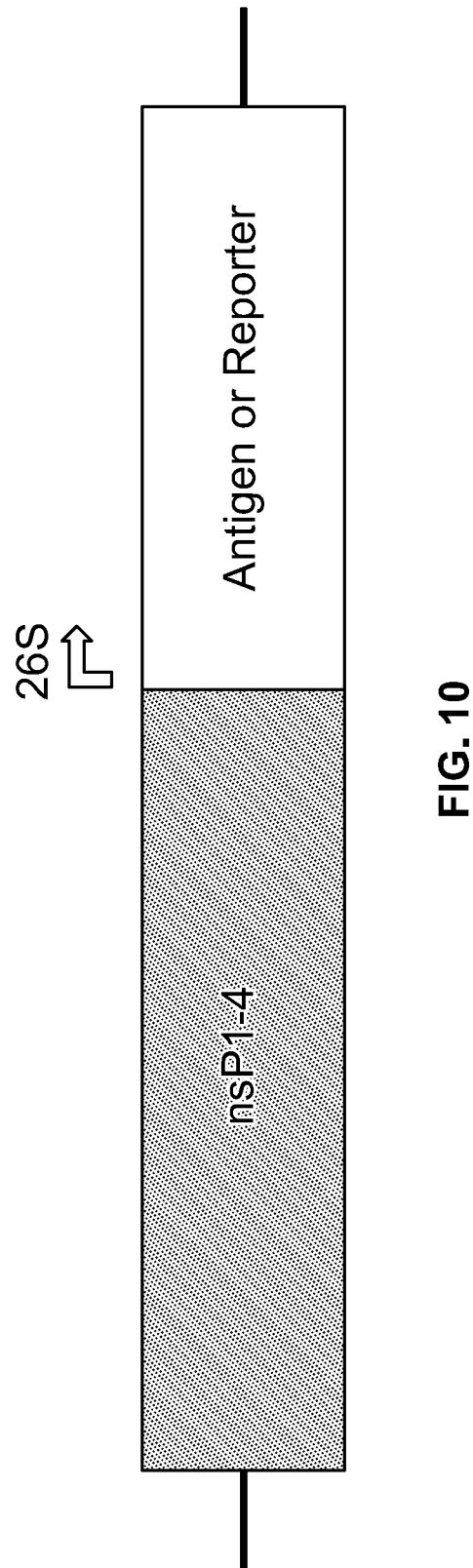


FIG. 8A

**FIG. 9**

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14 days

7 days

48 h

24 h

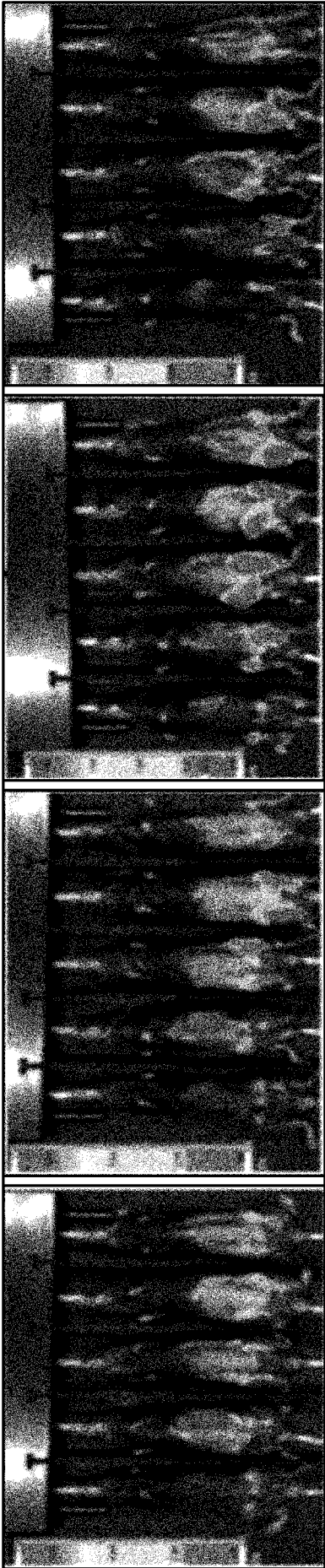
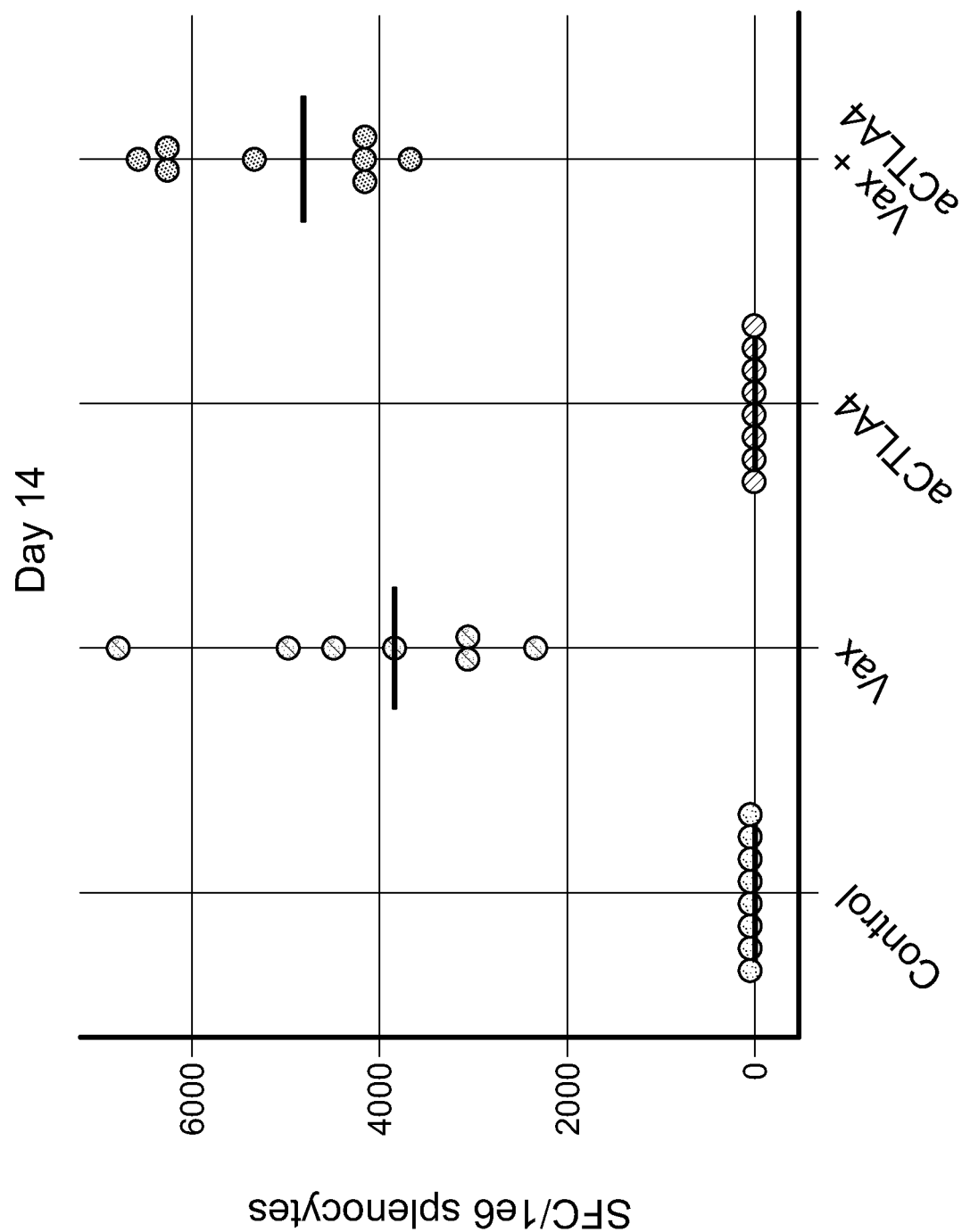
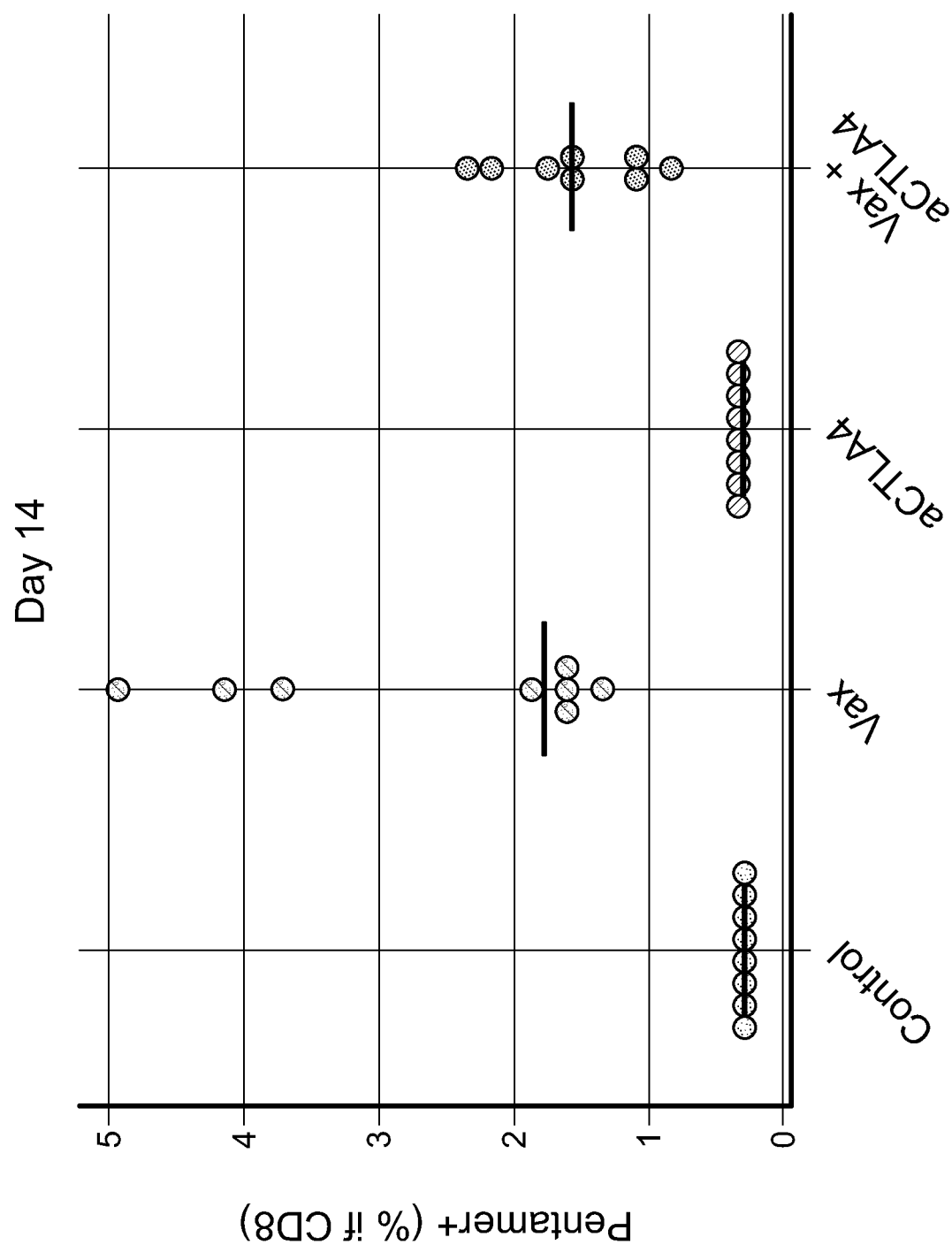
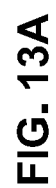


FIG. 11







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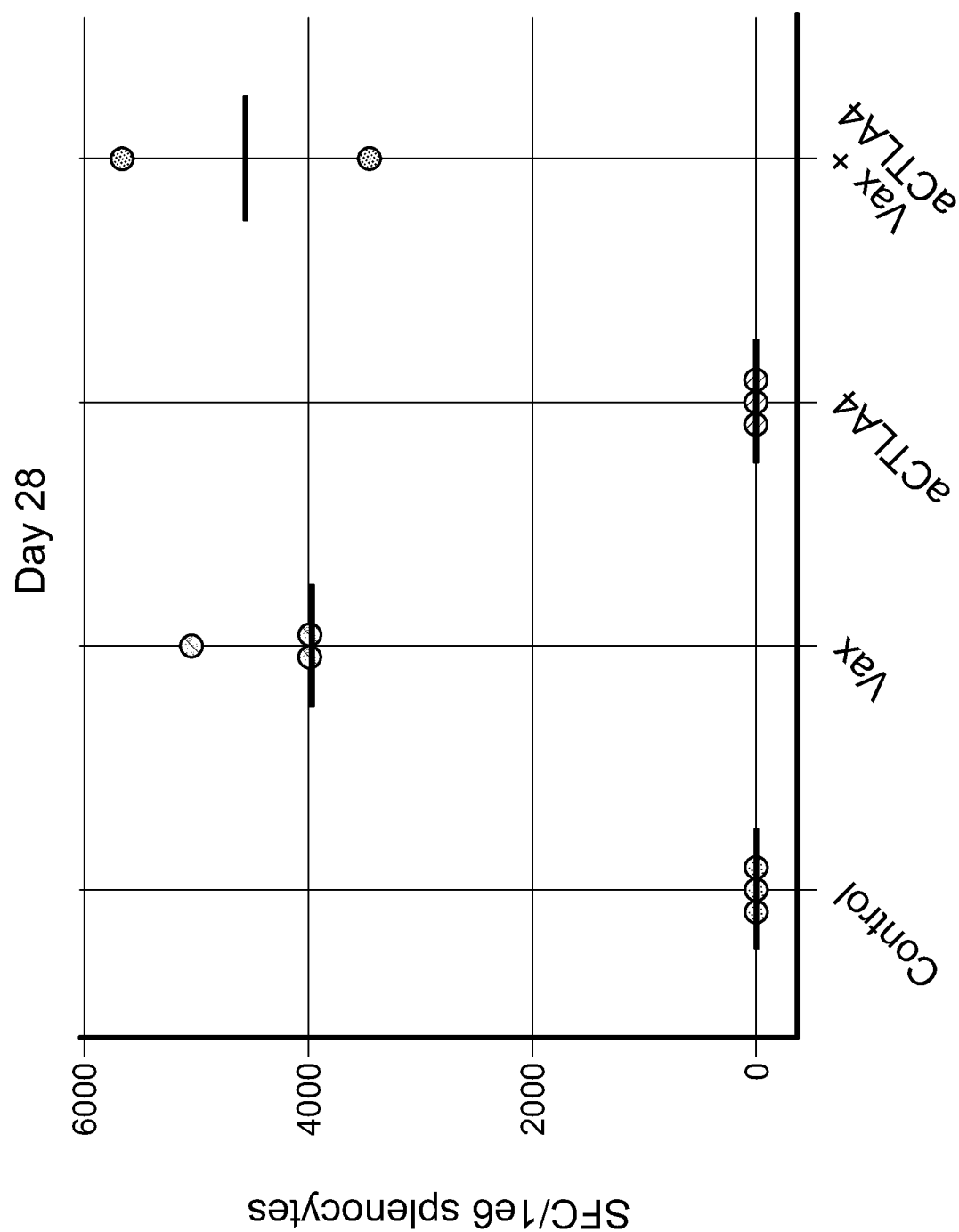


FIG. 13B

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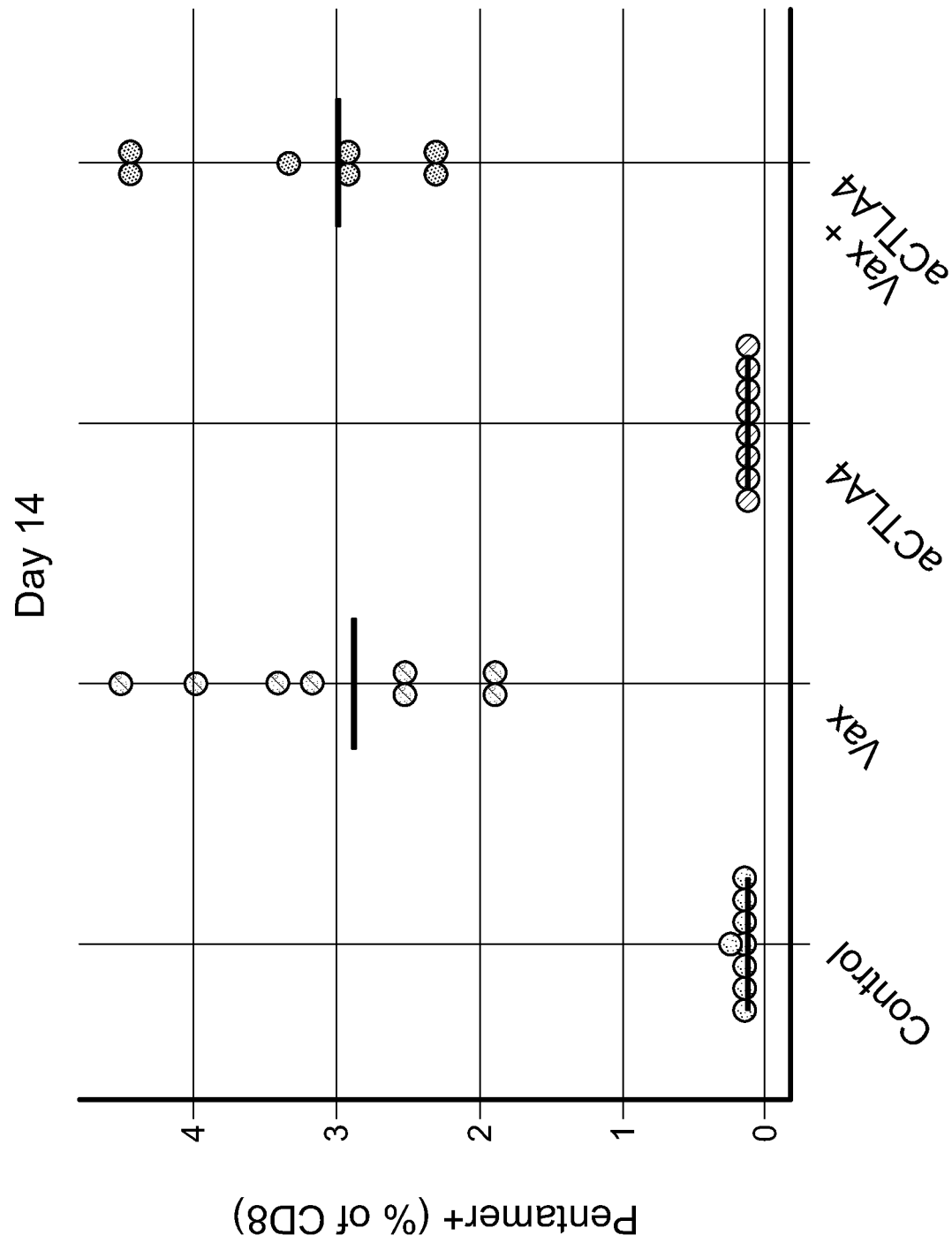


FIG. 13C

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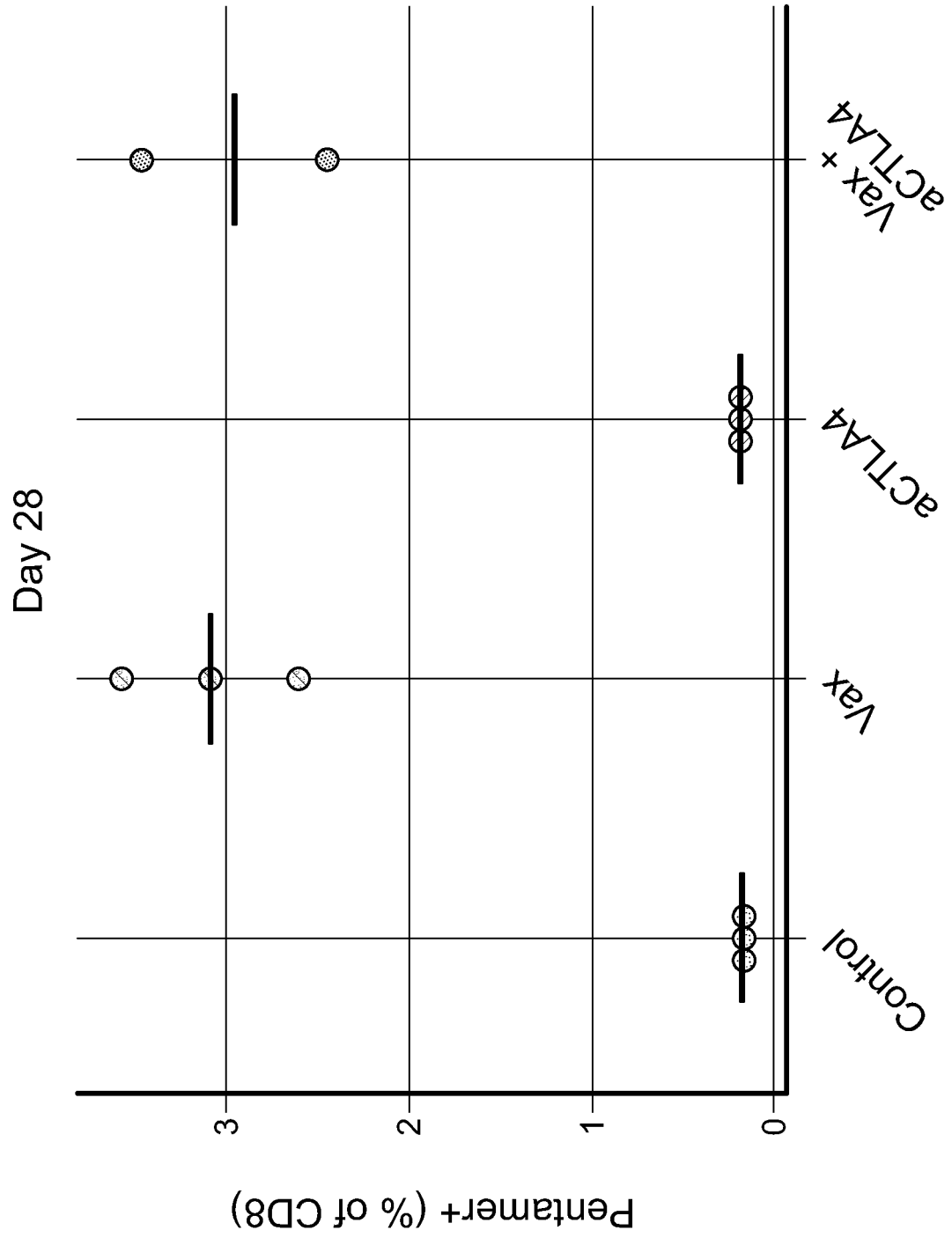


FIG. 13D

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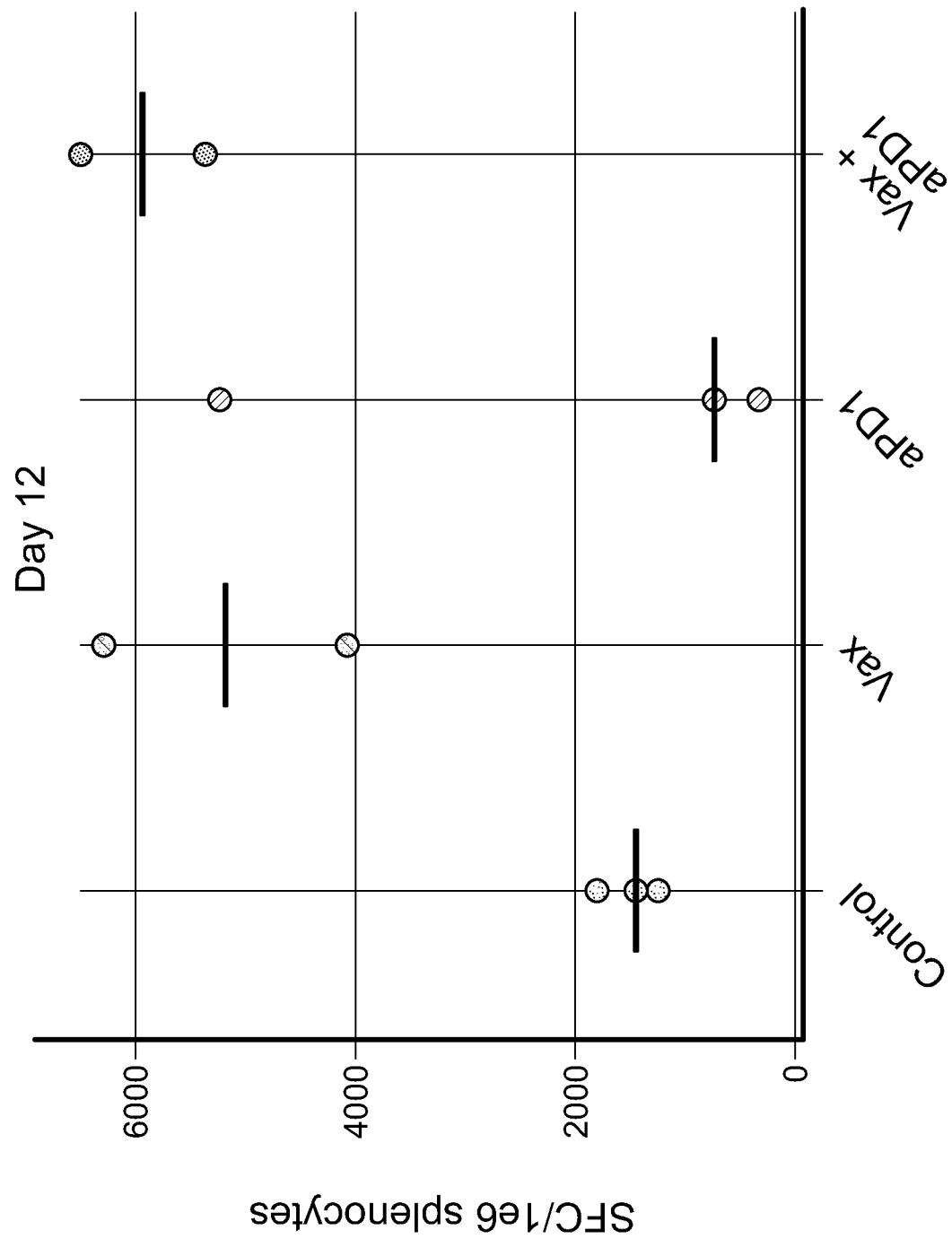


FIG. 14A

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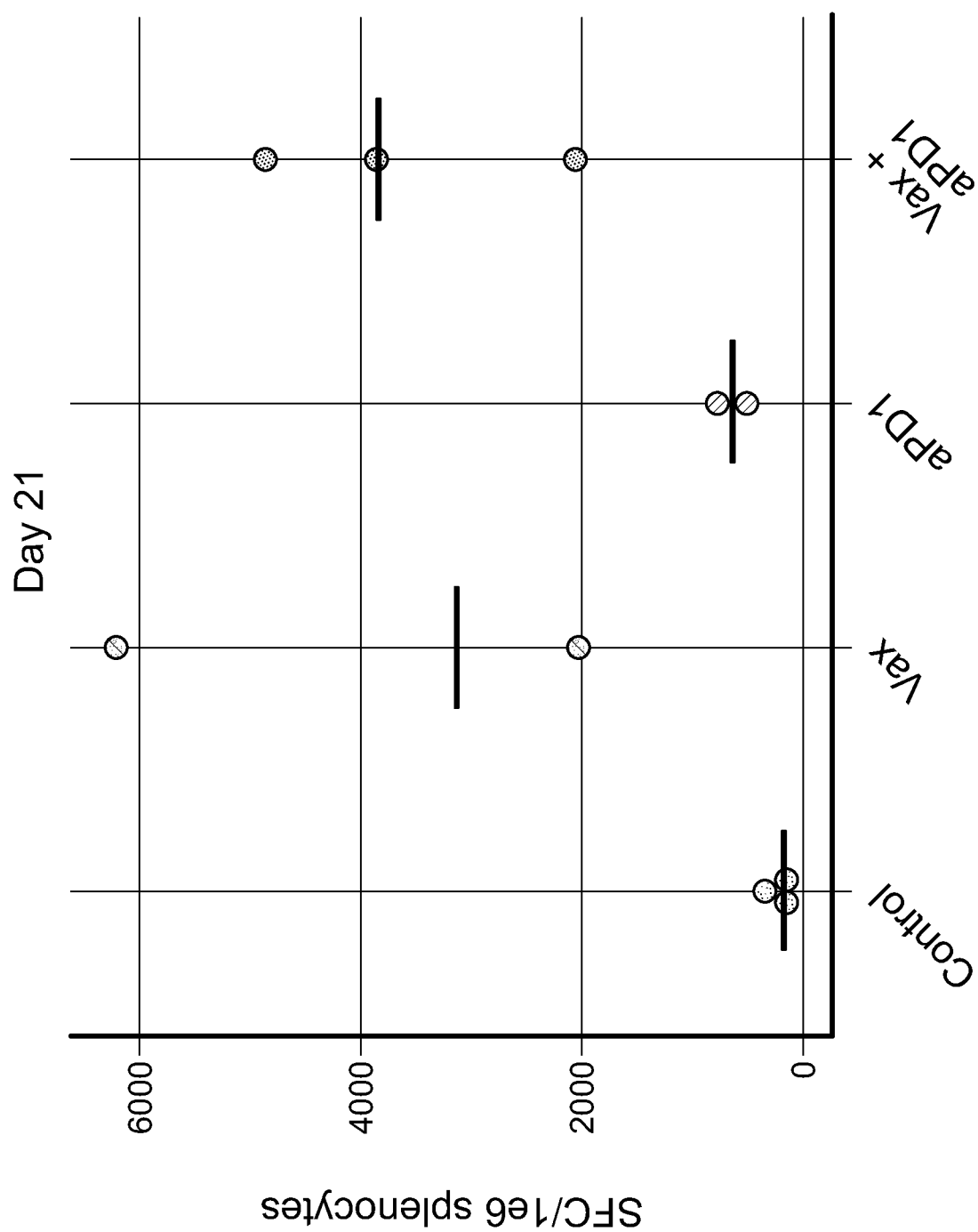


FIG. 14B

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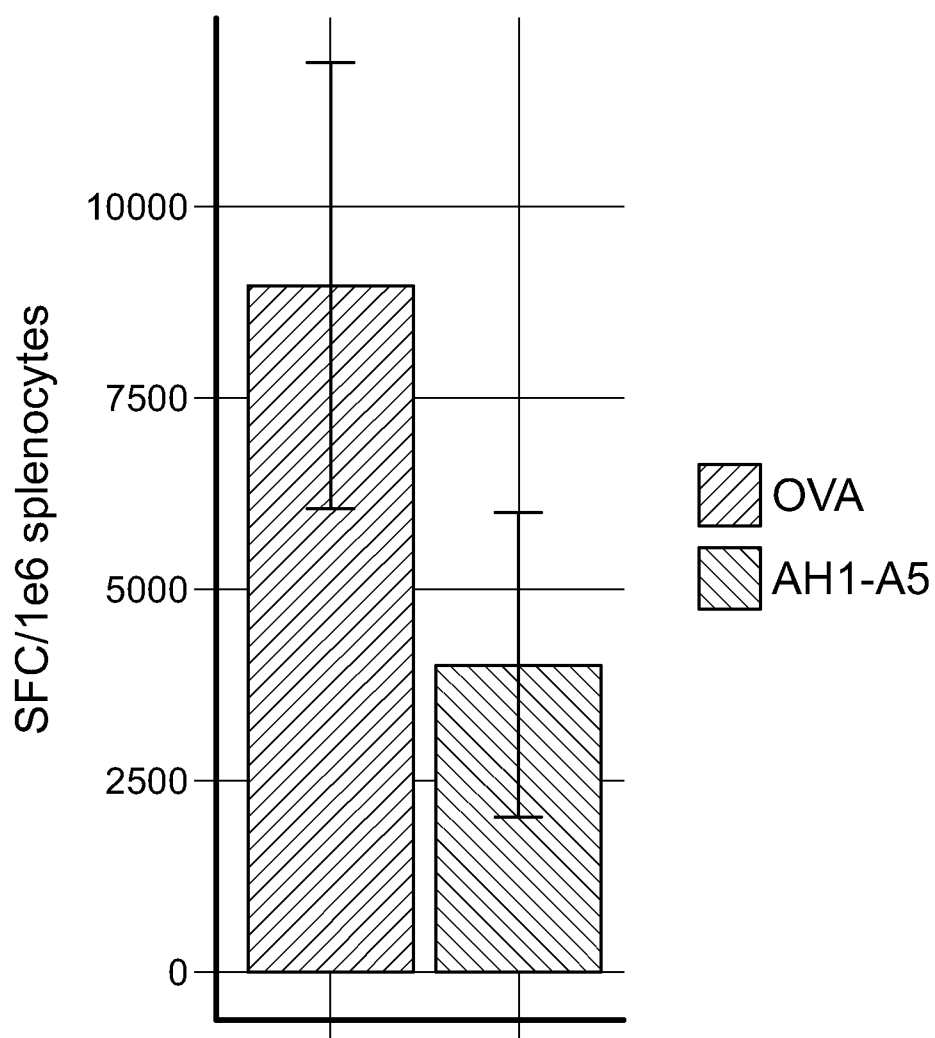


FIG. 15

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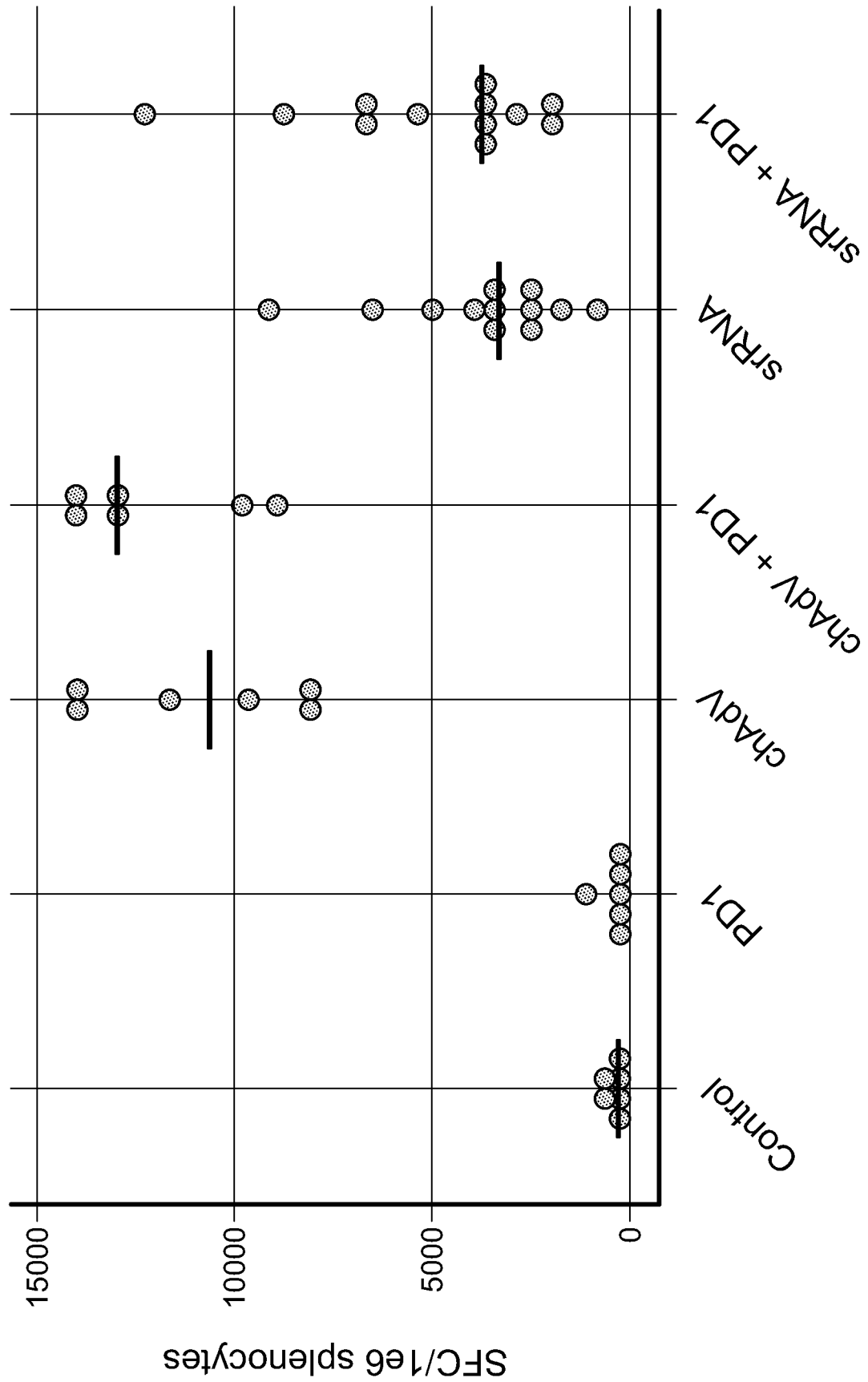


FIG. 16

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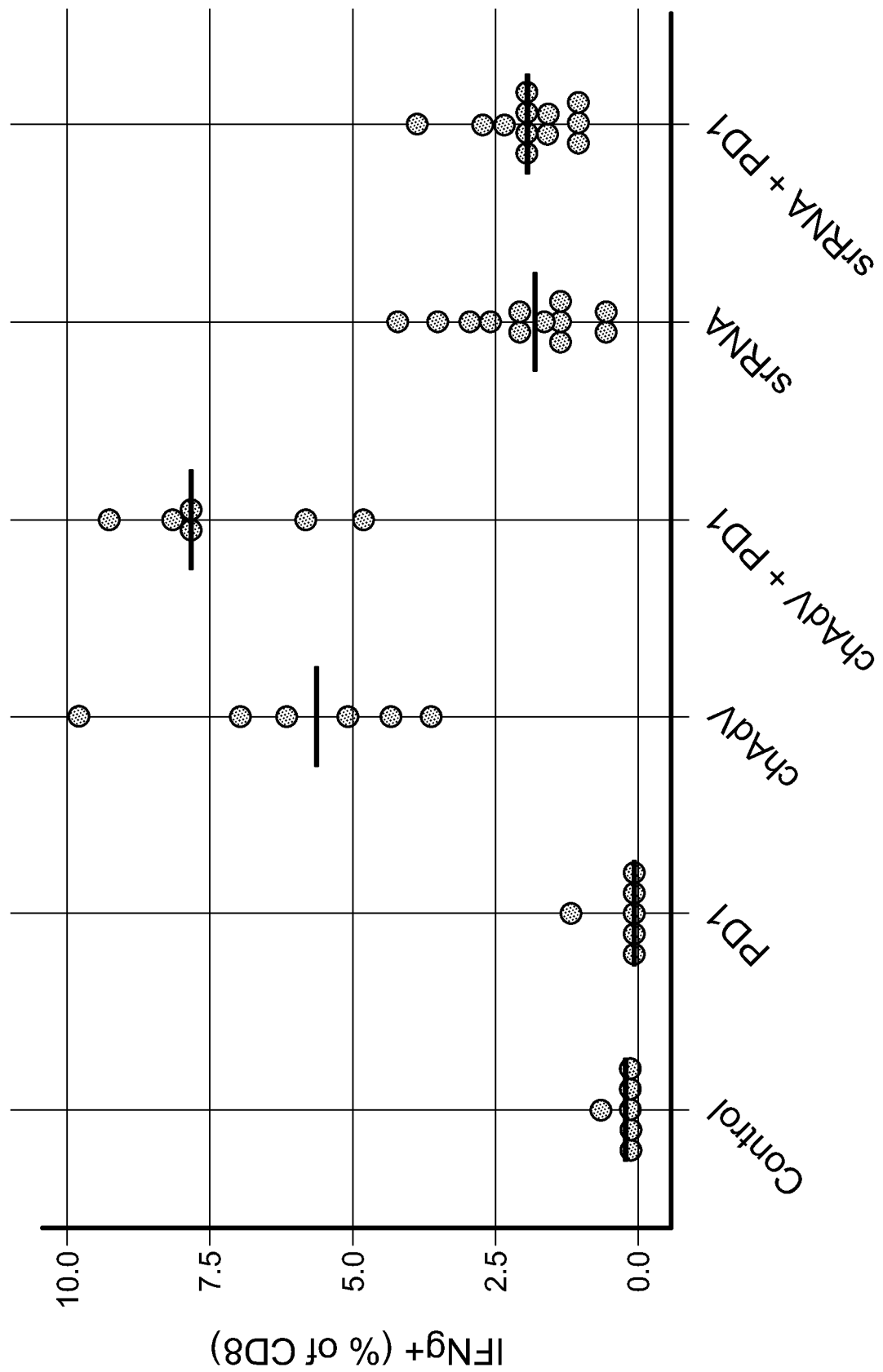


FIG. 17

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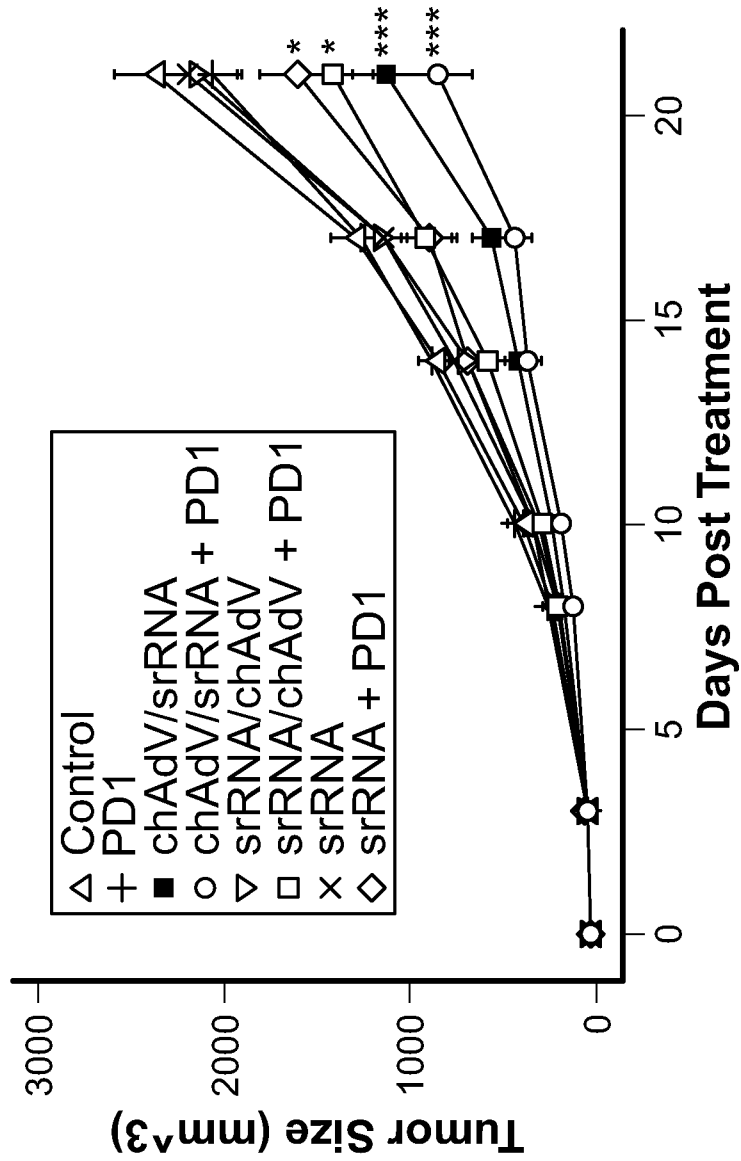


FIG. 18

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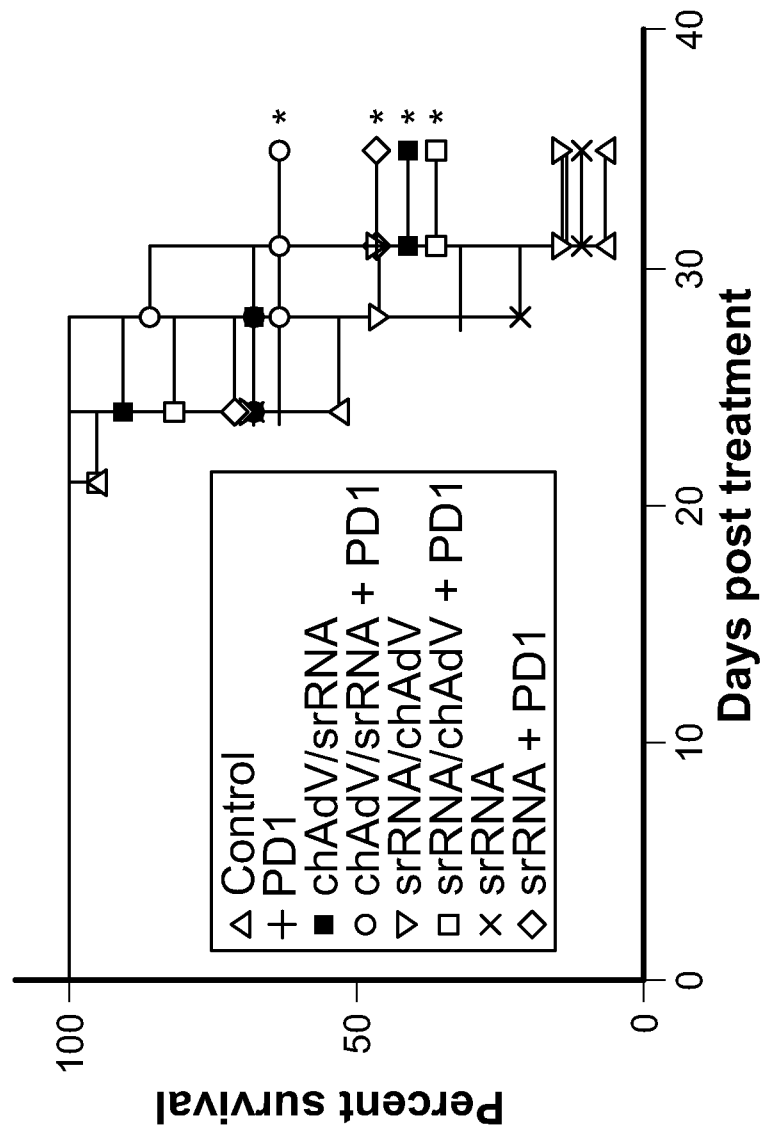


FIG. 19

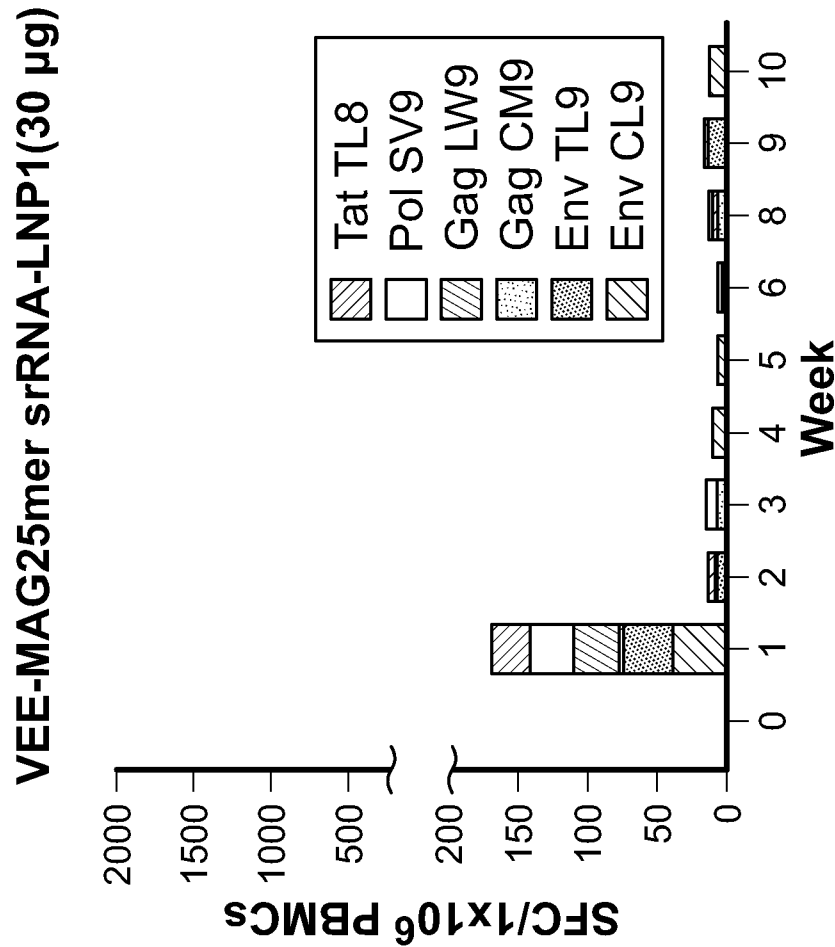


FIG. 20A

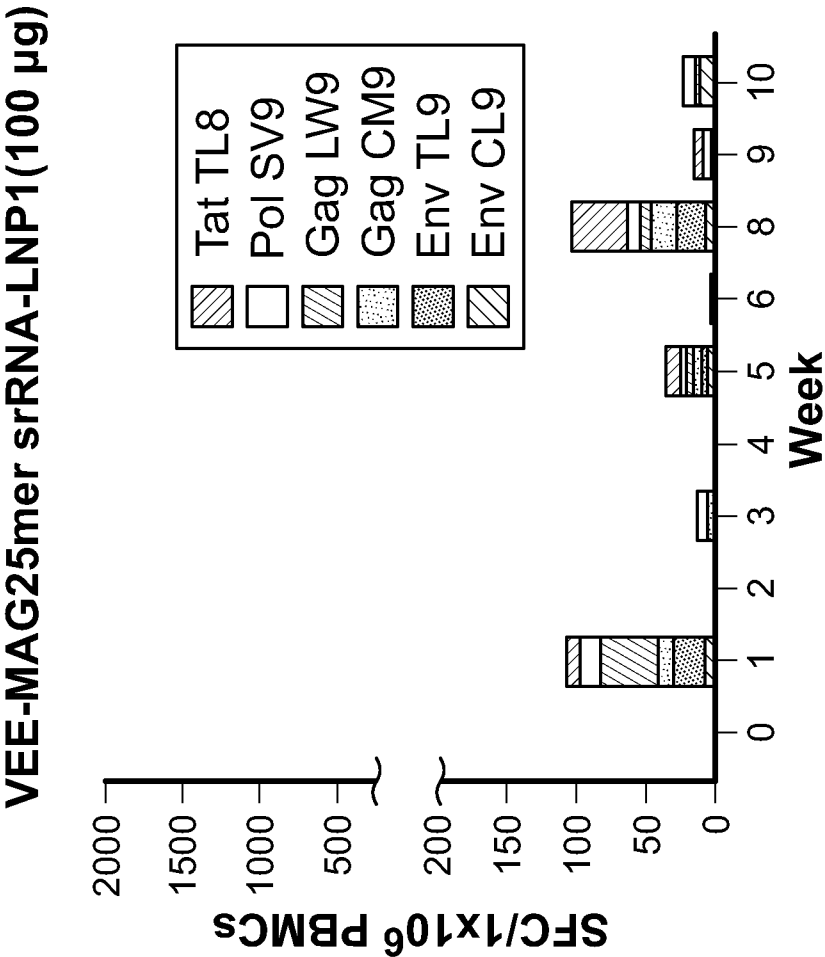
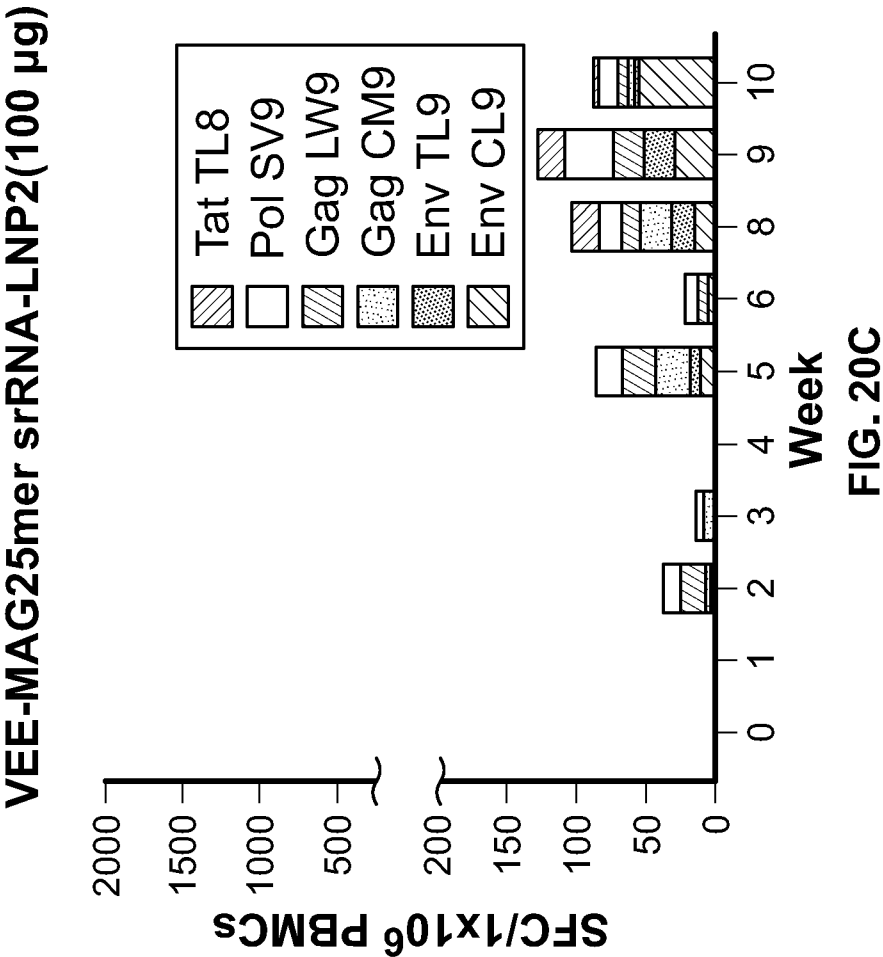
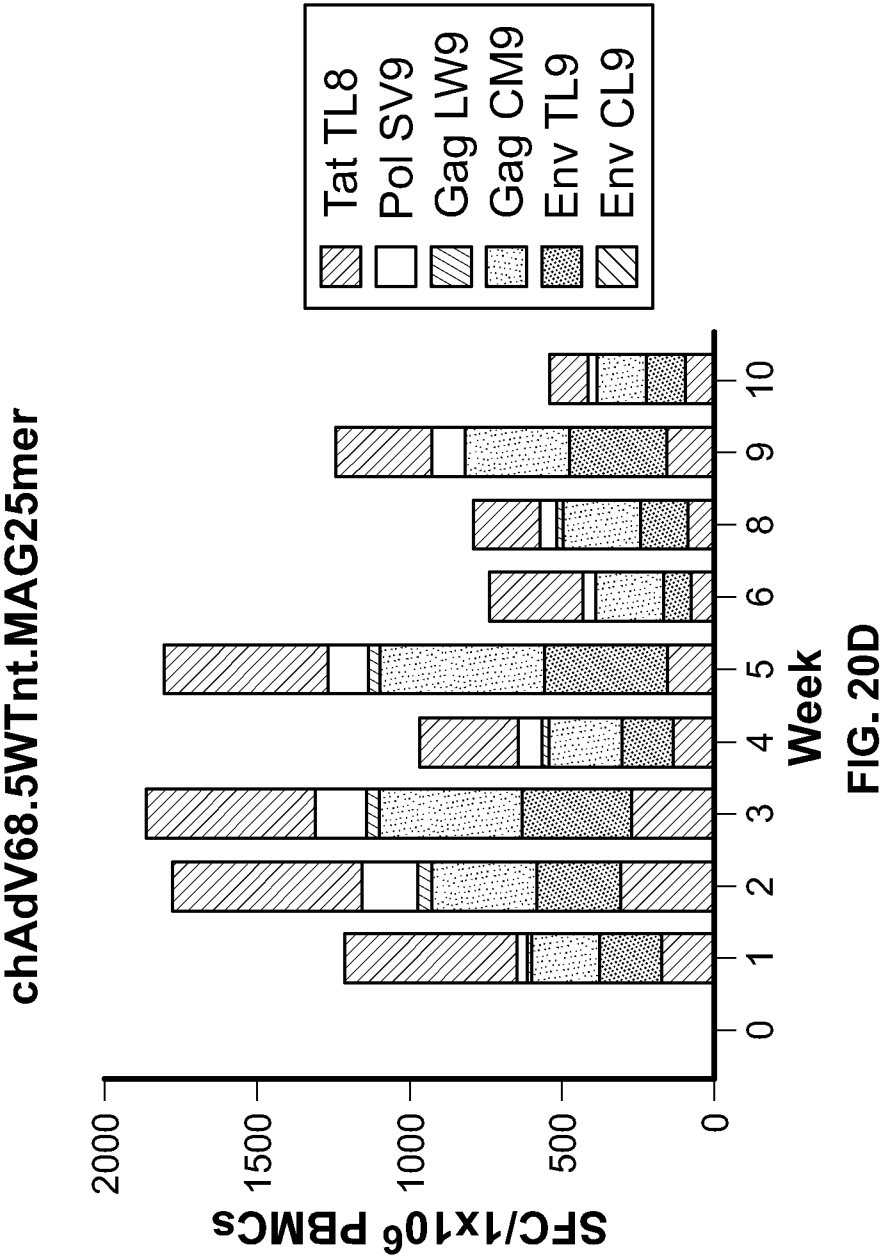


FIG. 20B





chAdV

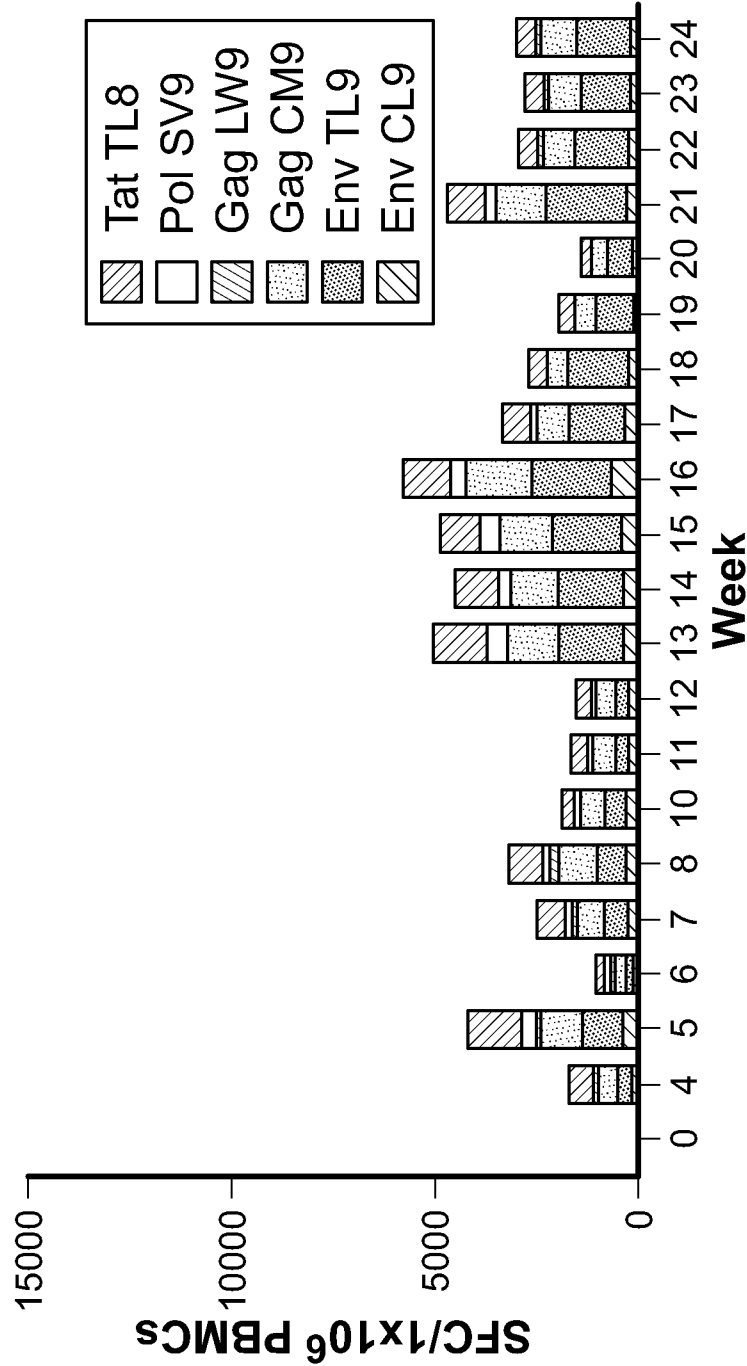
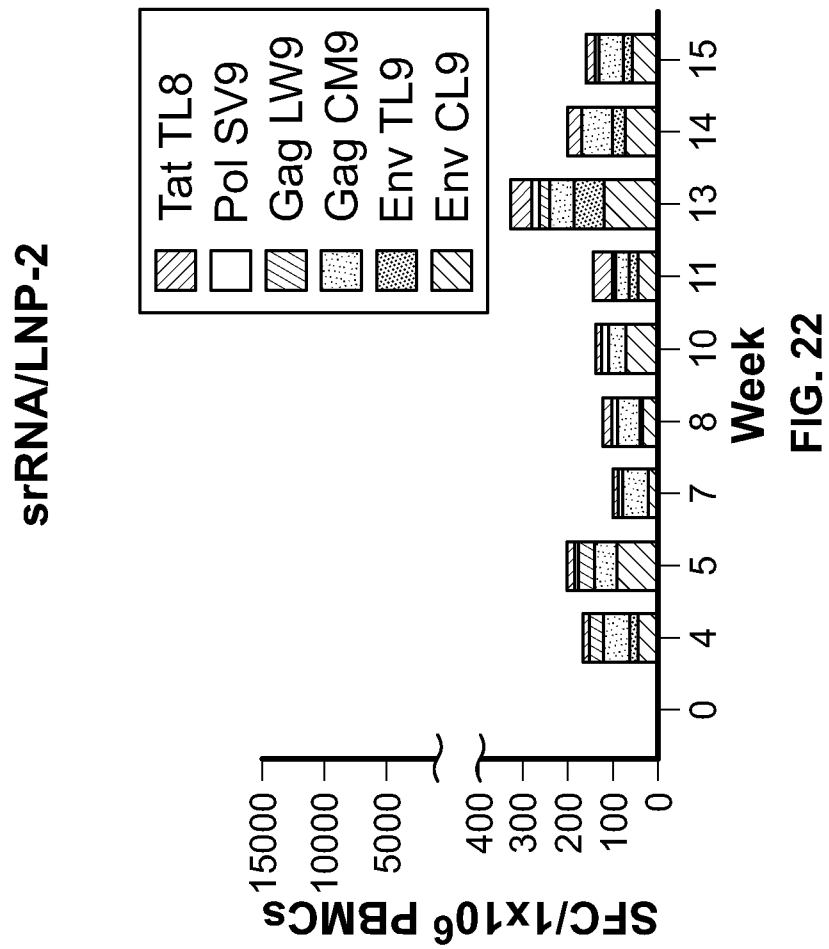
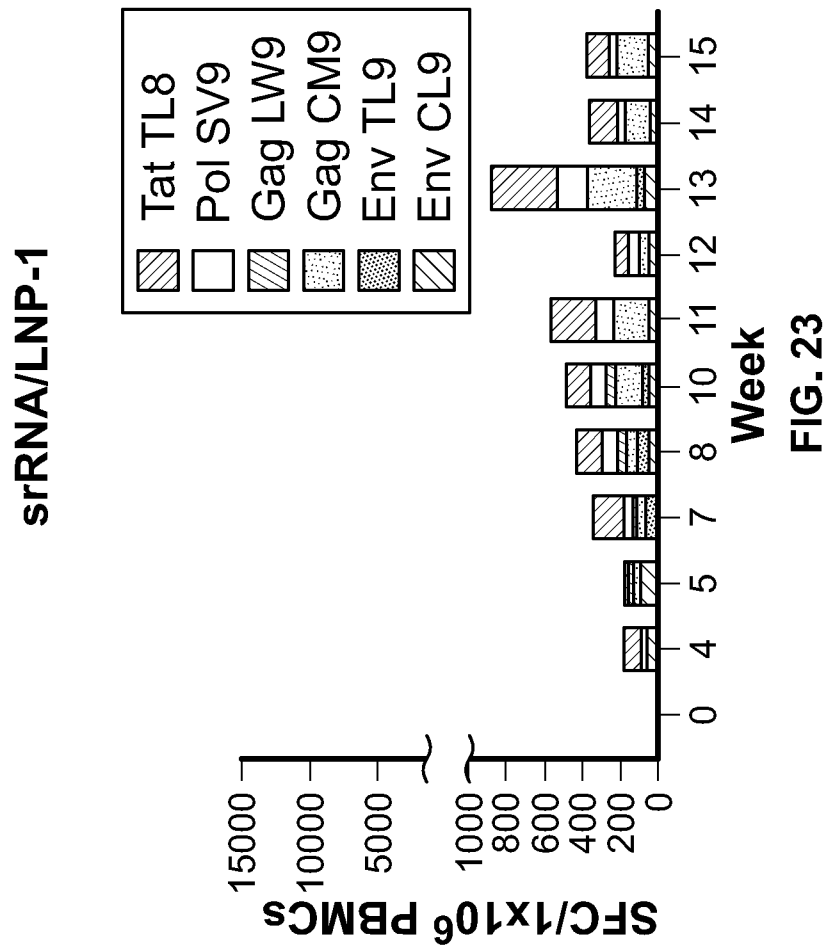


FIG. 21

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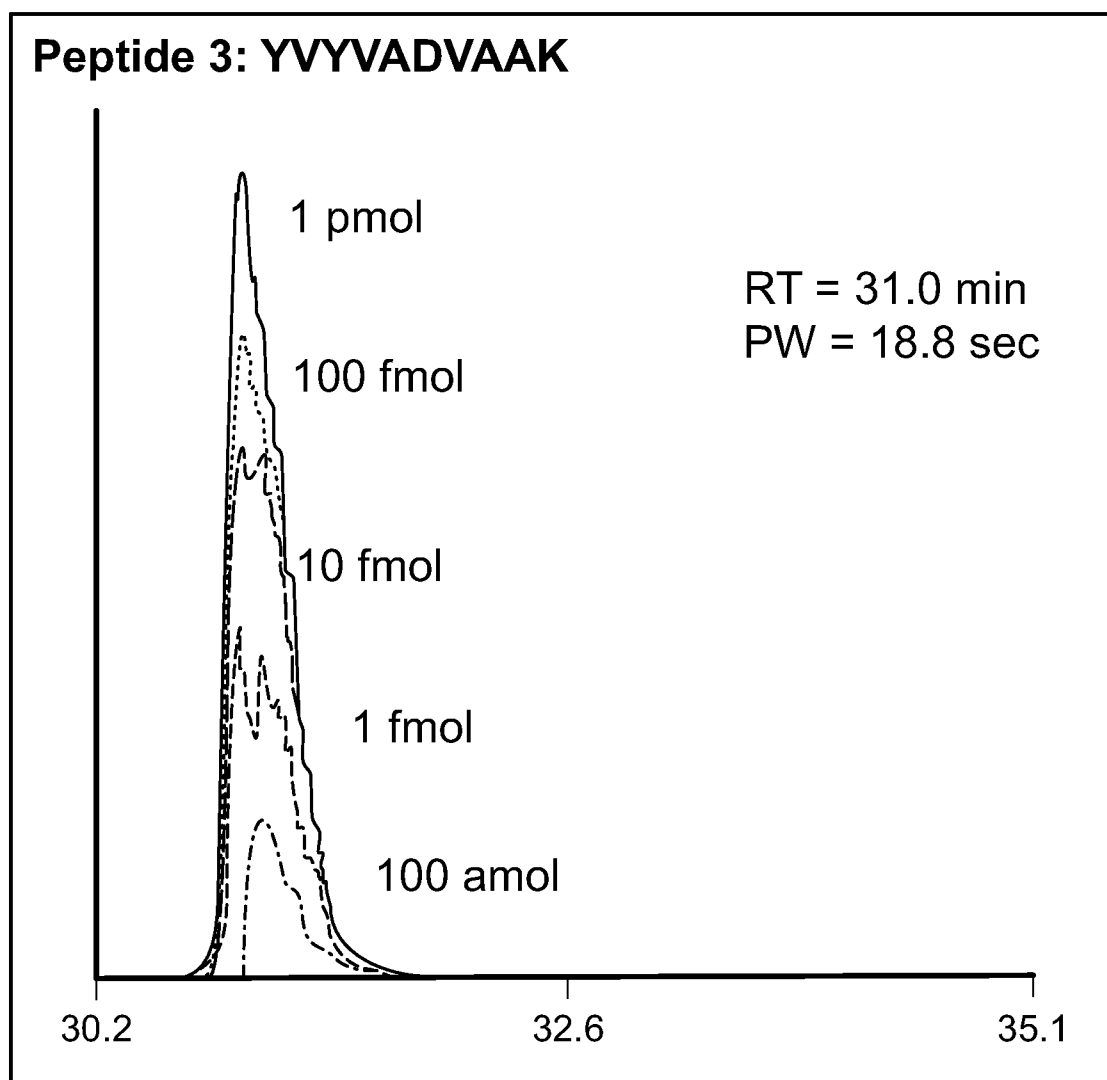


FIG. 24A

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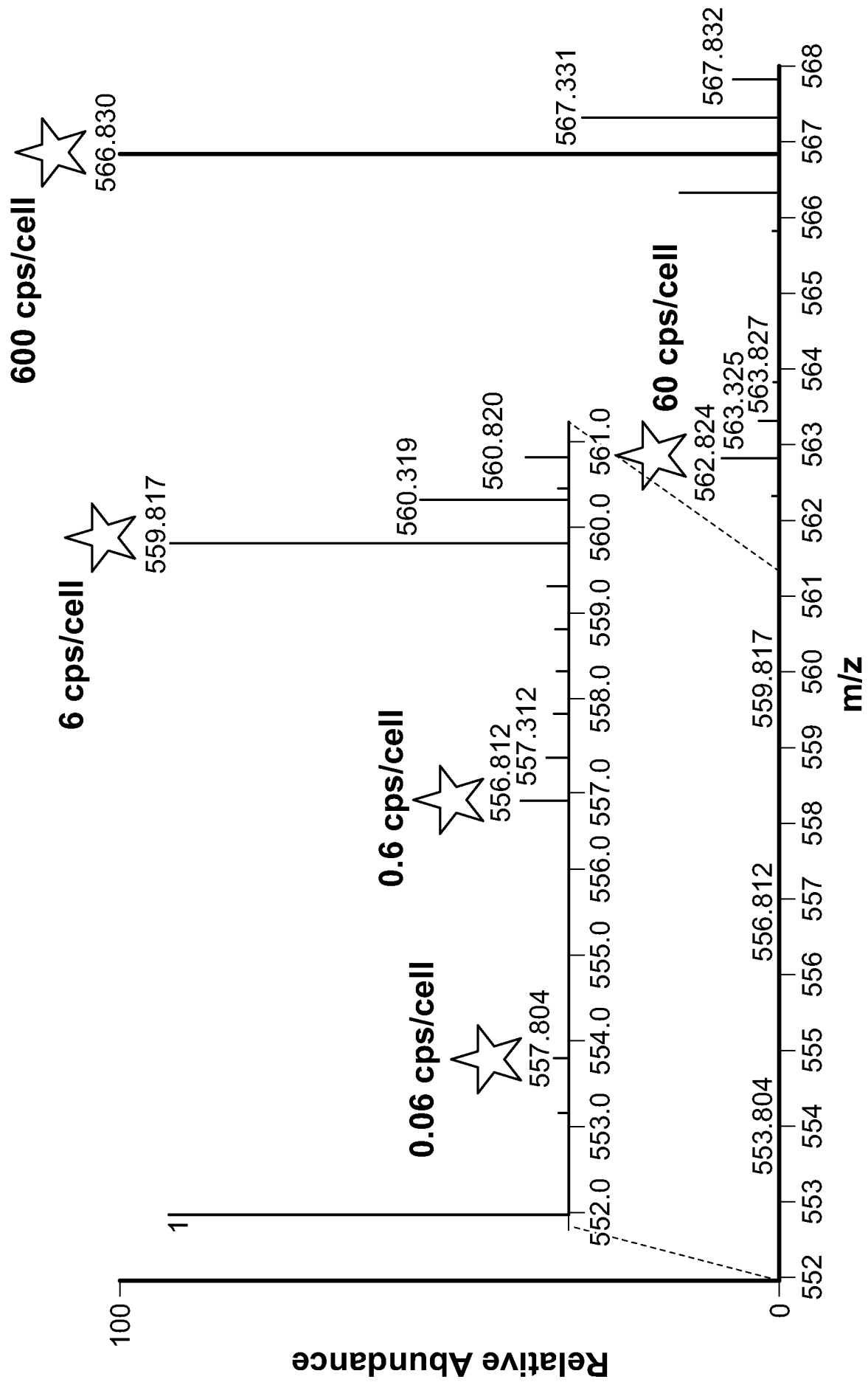


FIG. 24B

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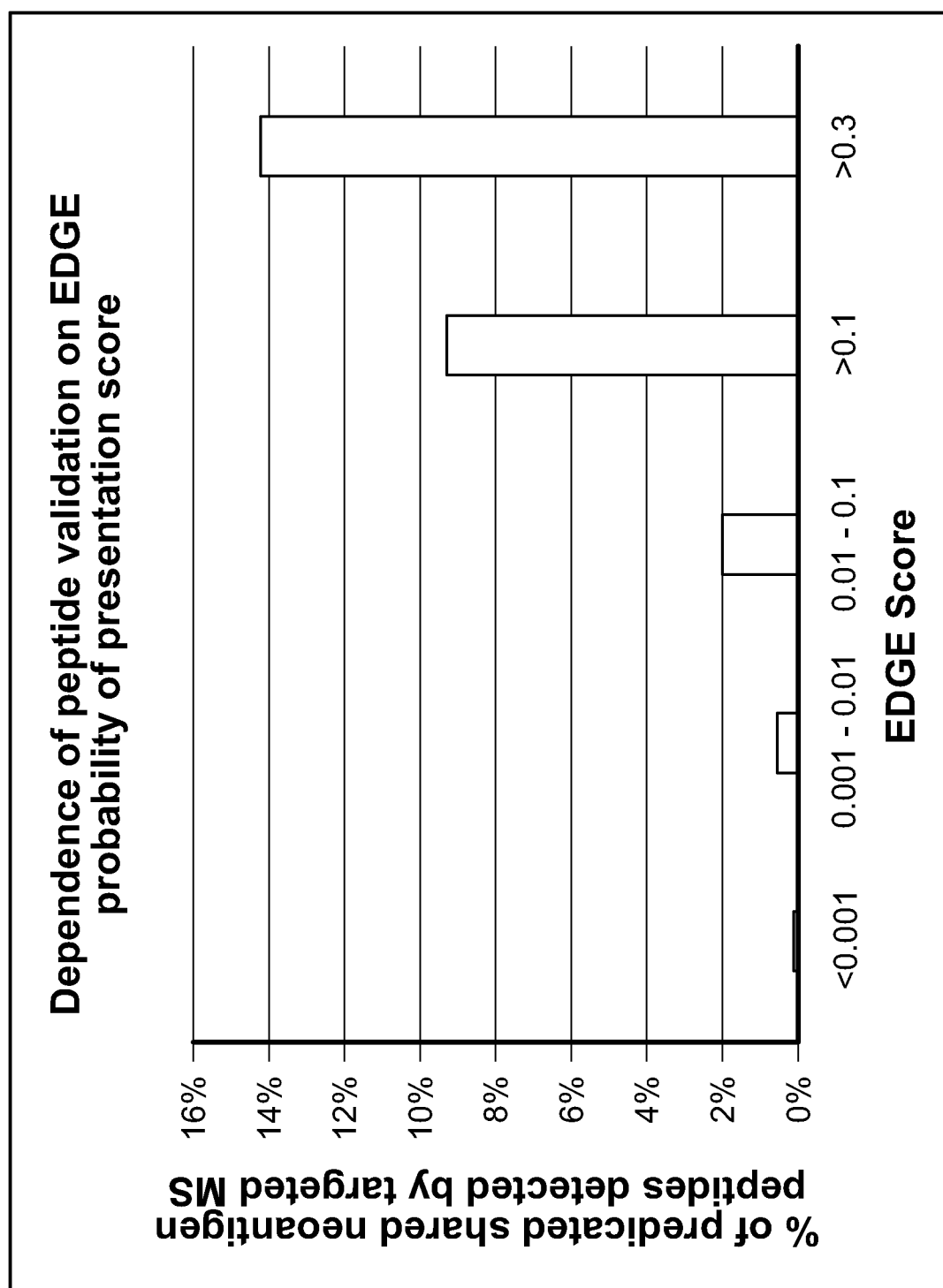


FIG. 25

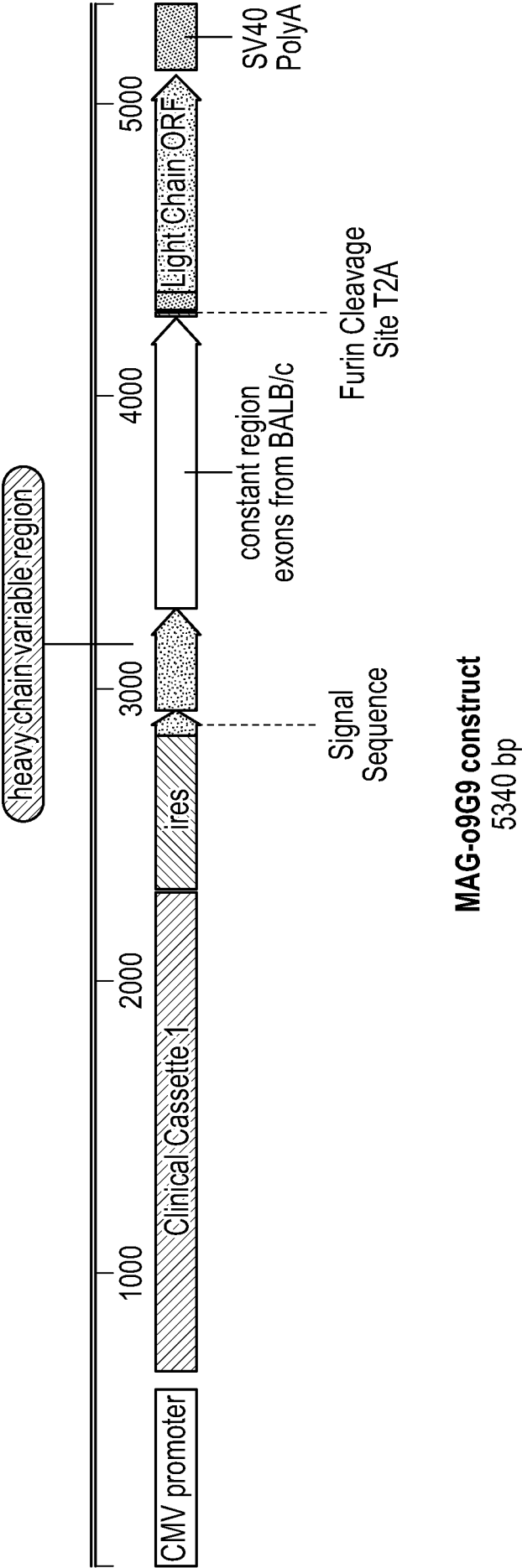


FIG. 26

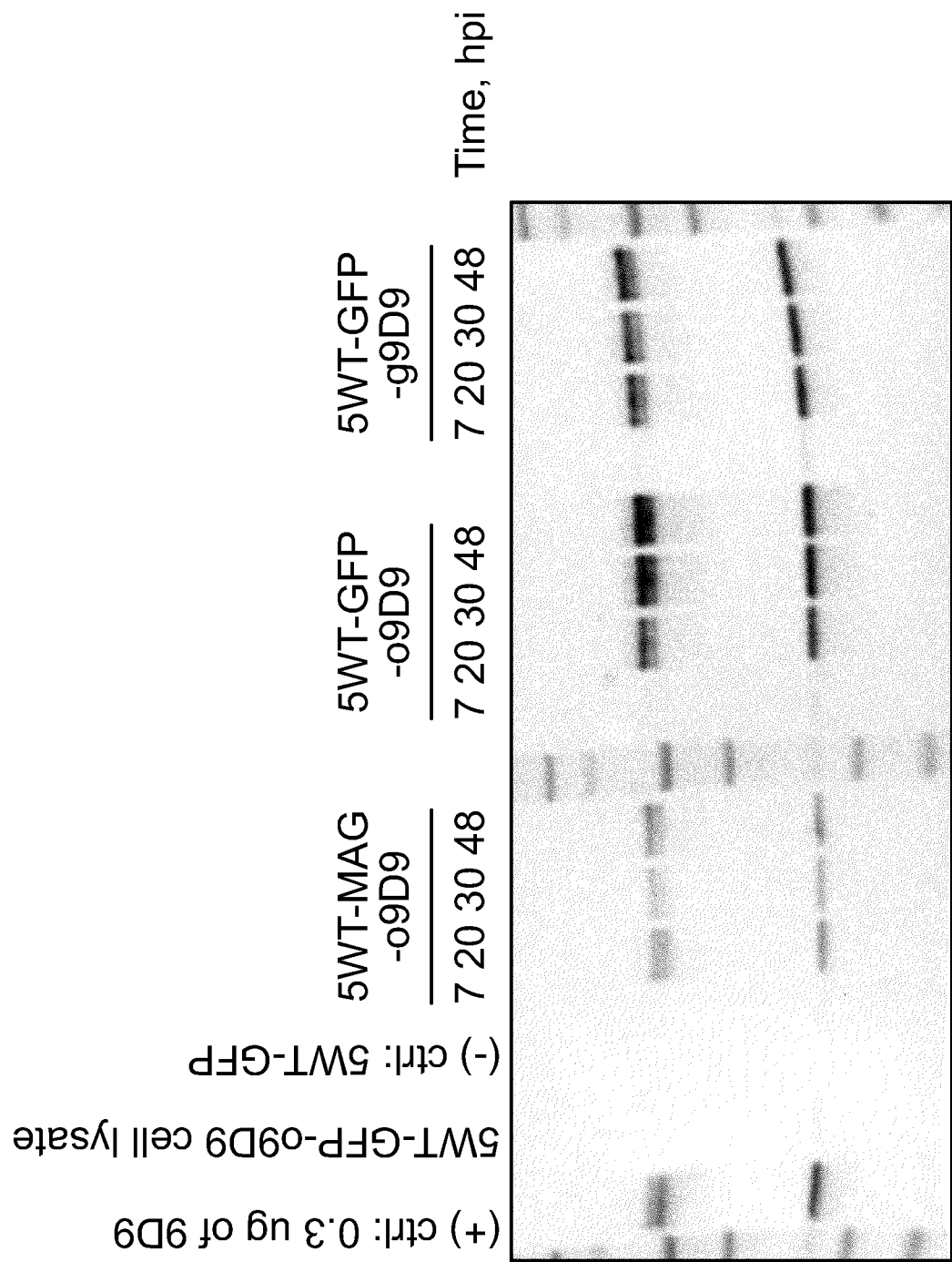


FIG. 27

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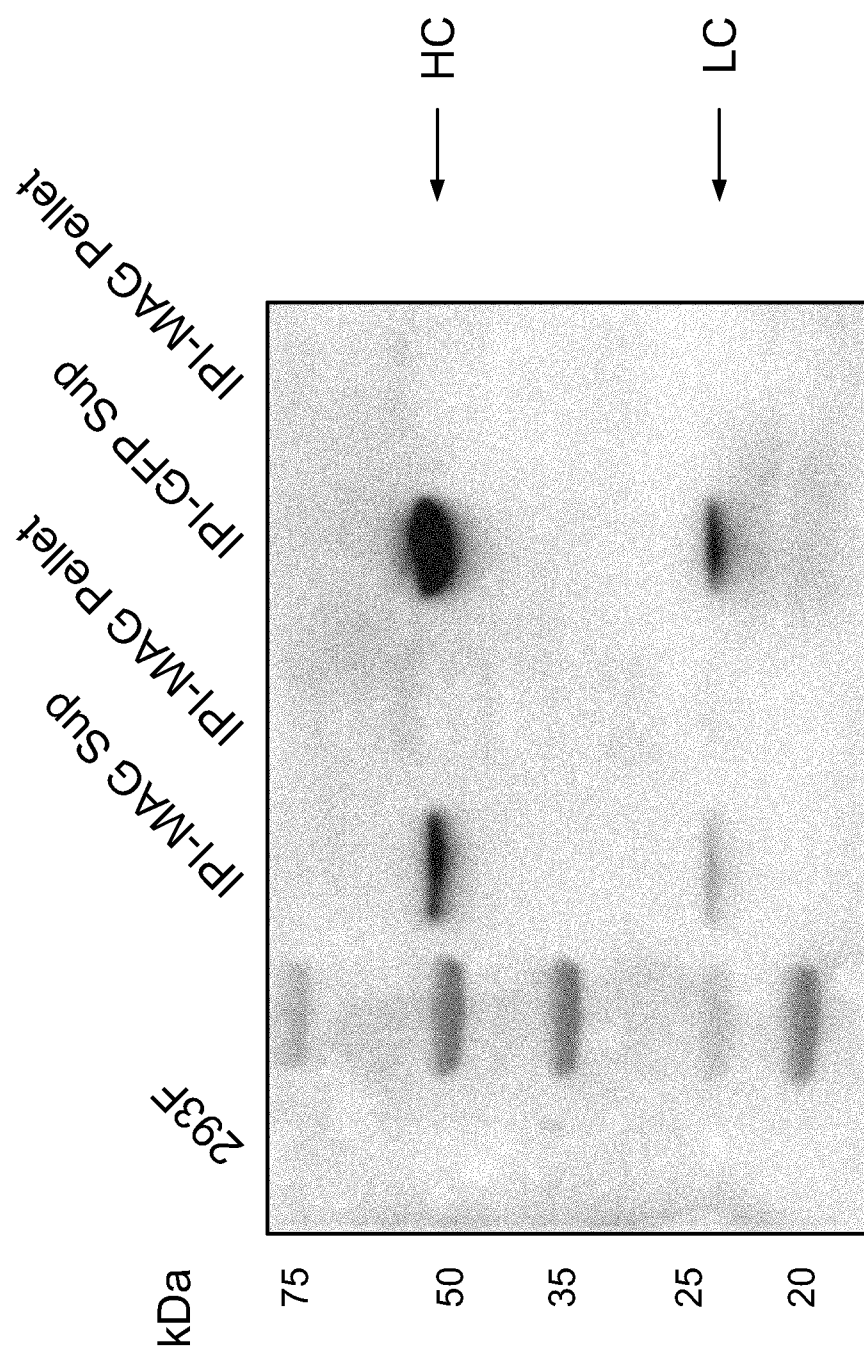


FIG. 28A

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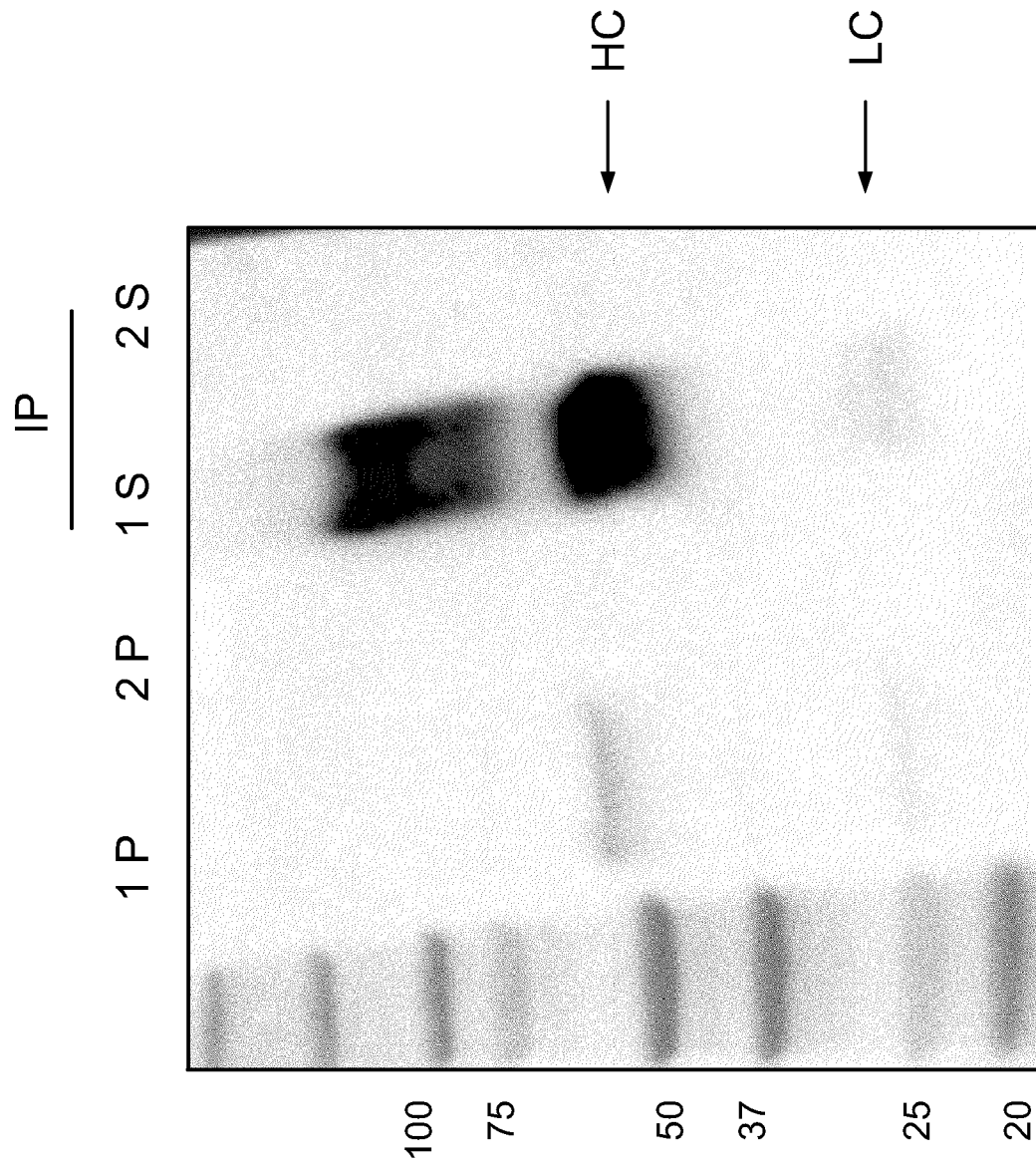


FIG. 28B

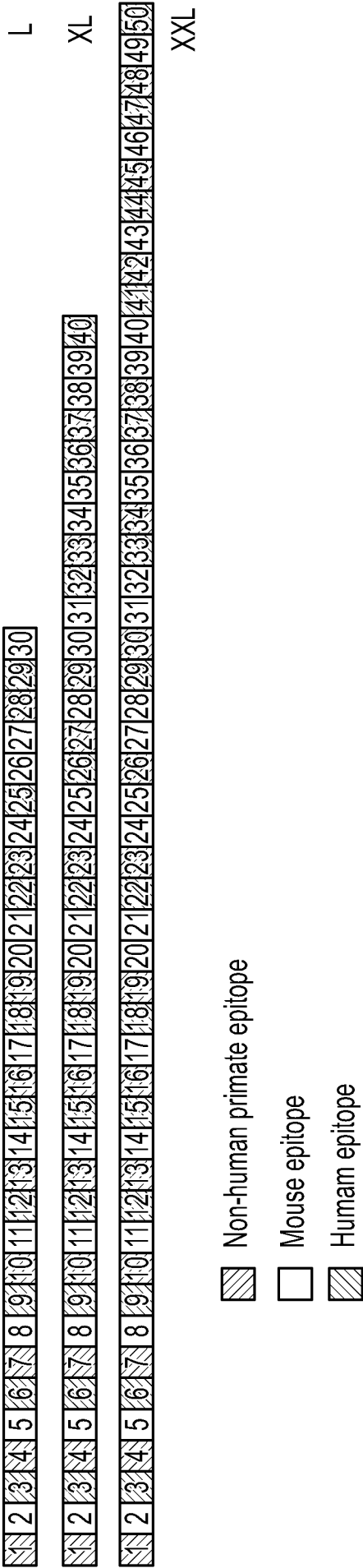


FIG. 29

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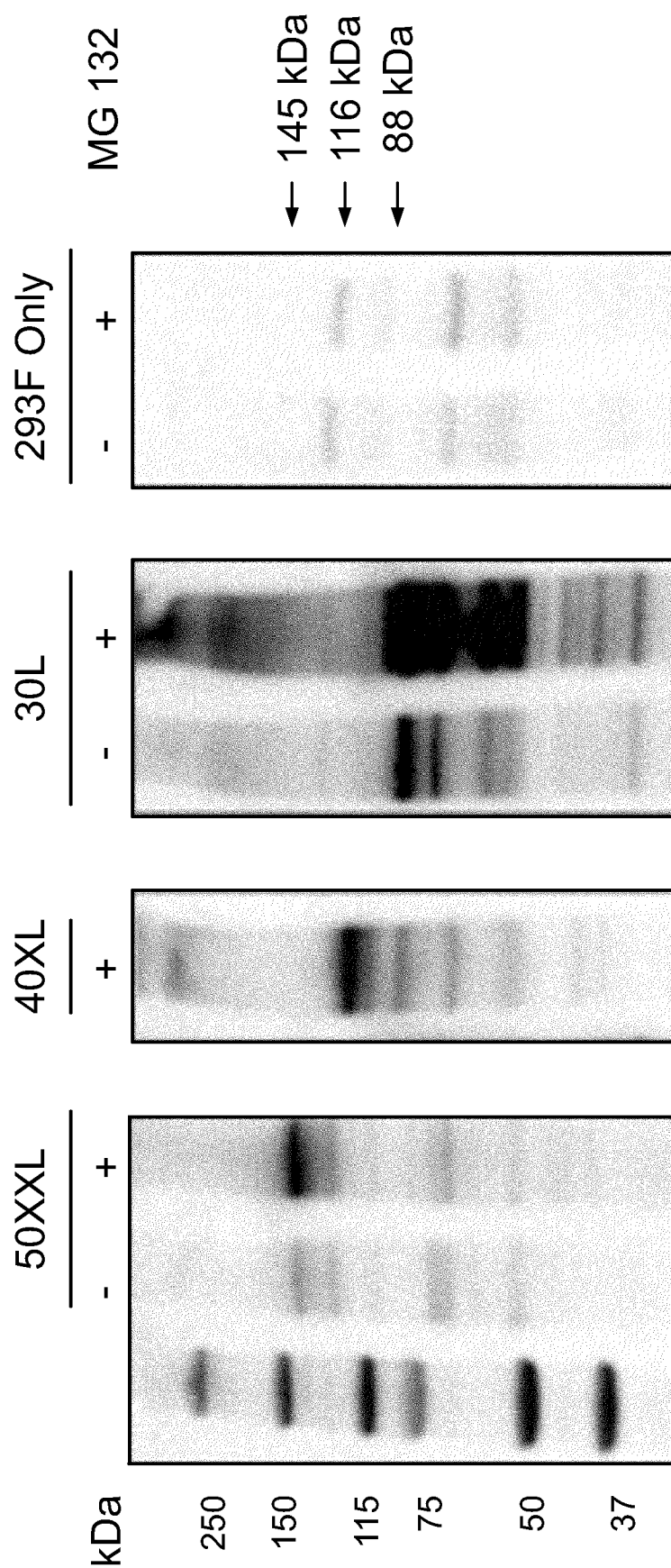
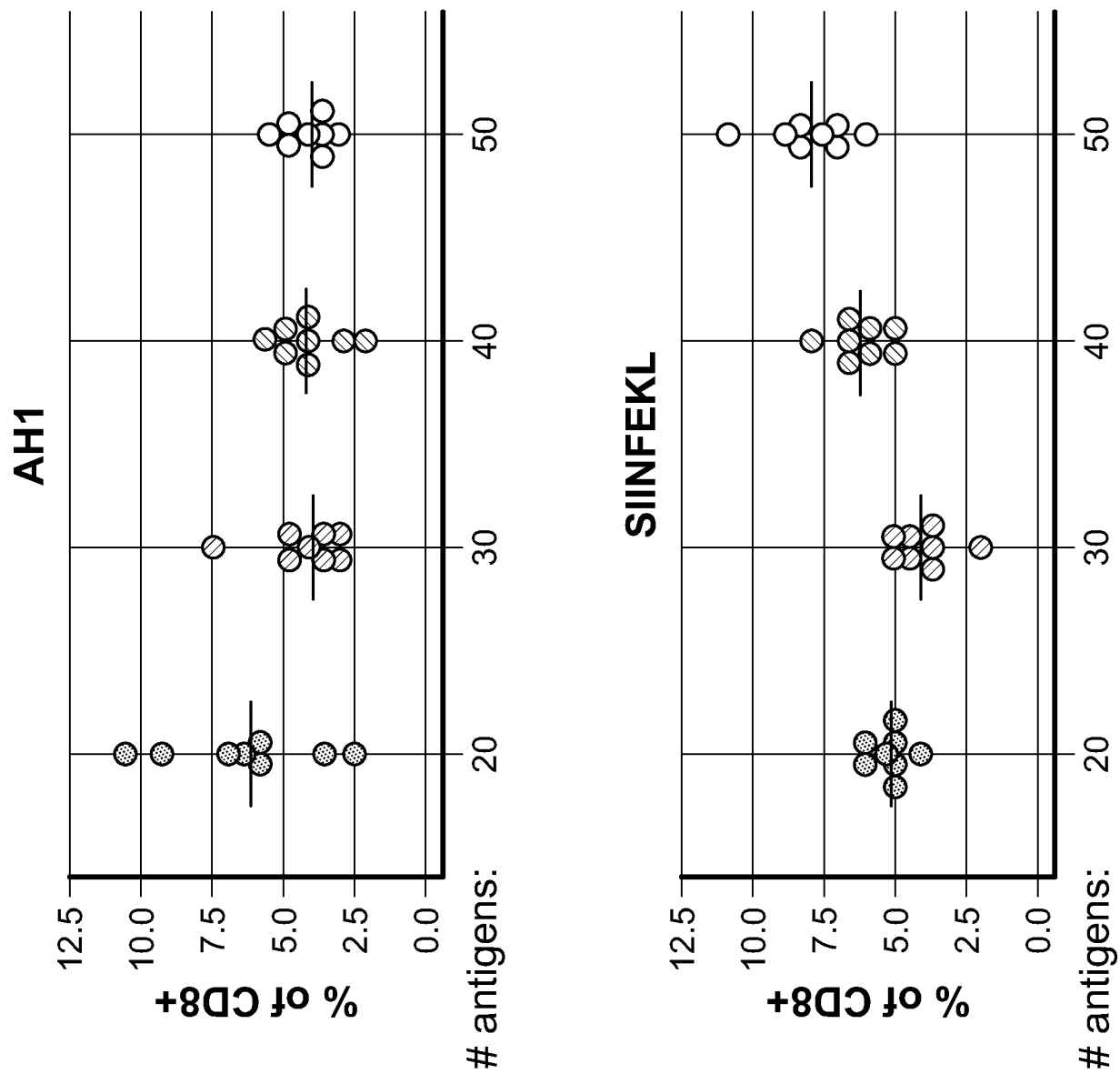


FIG. 30



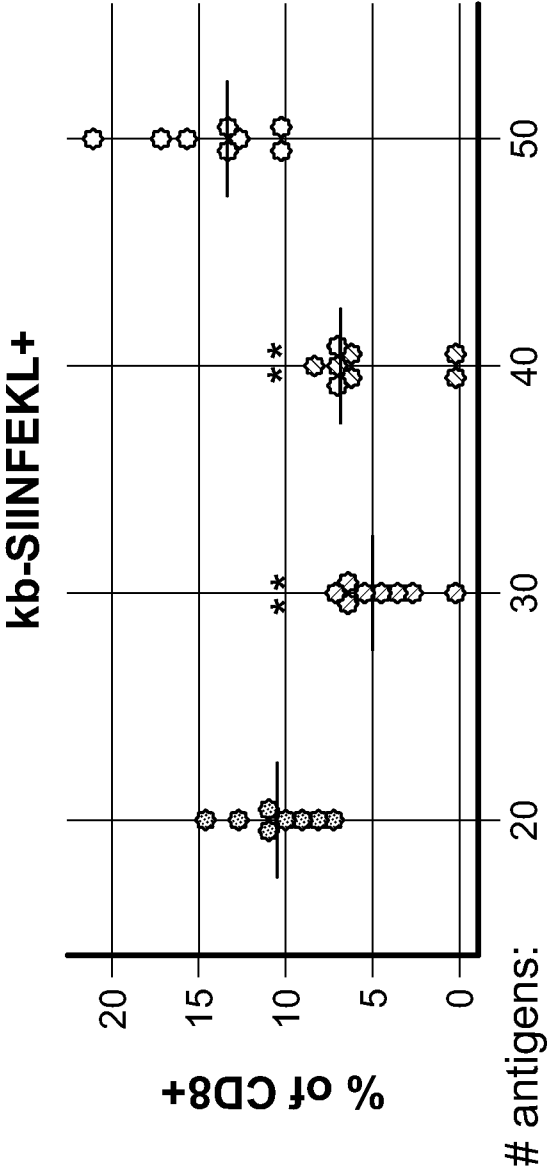
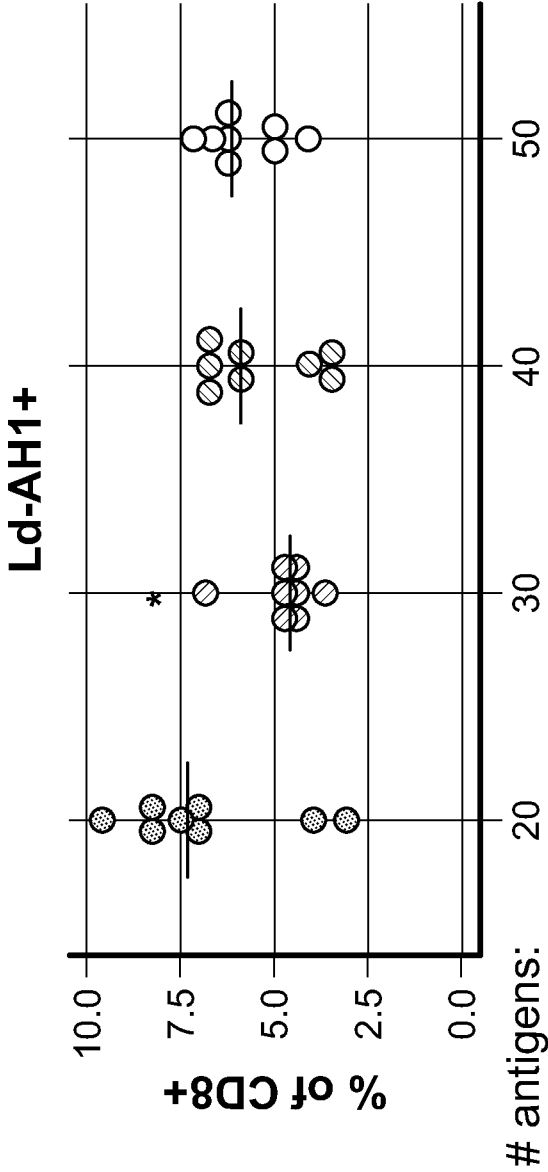


FIG. 32

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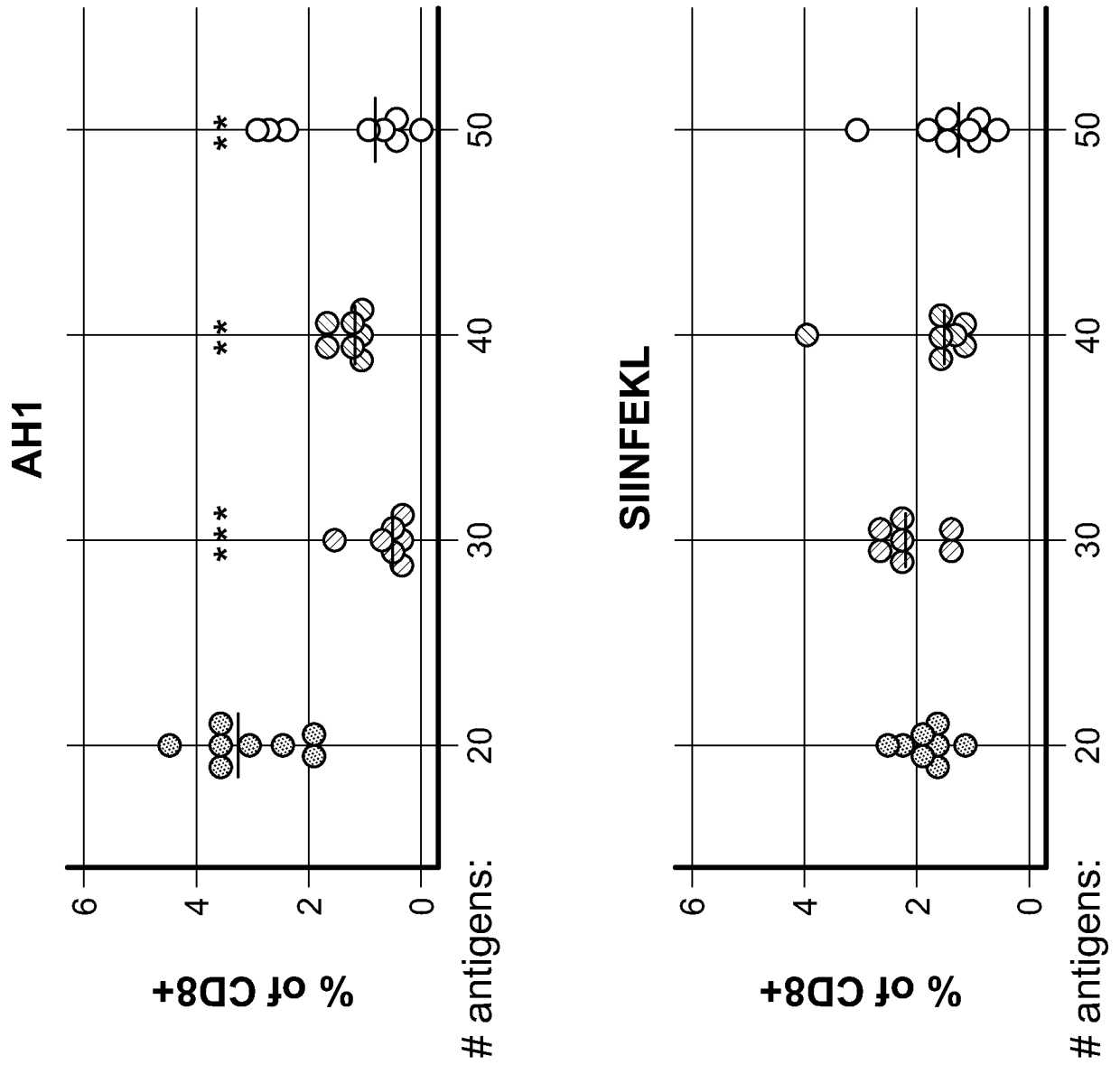


FIG. 33

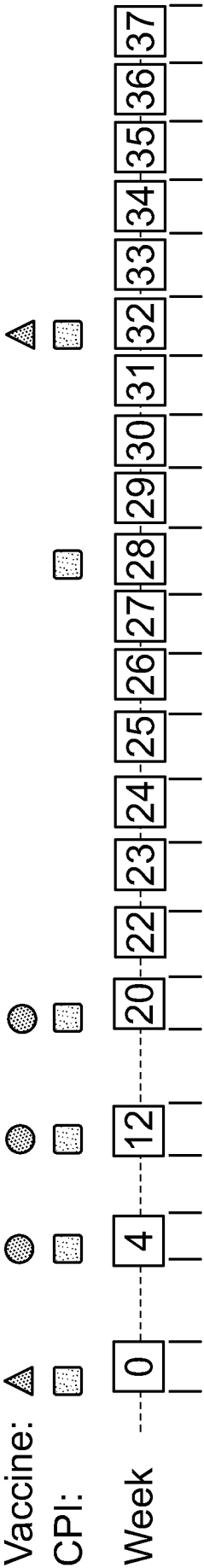


FIG. 34

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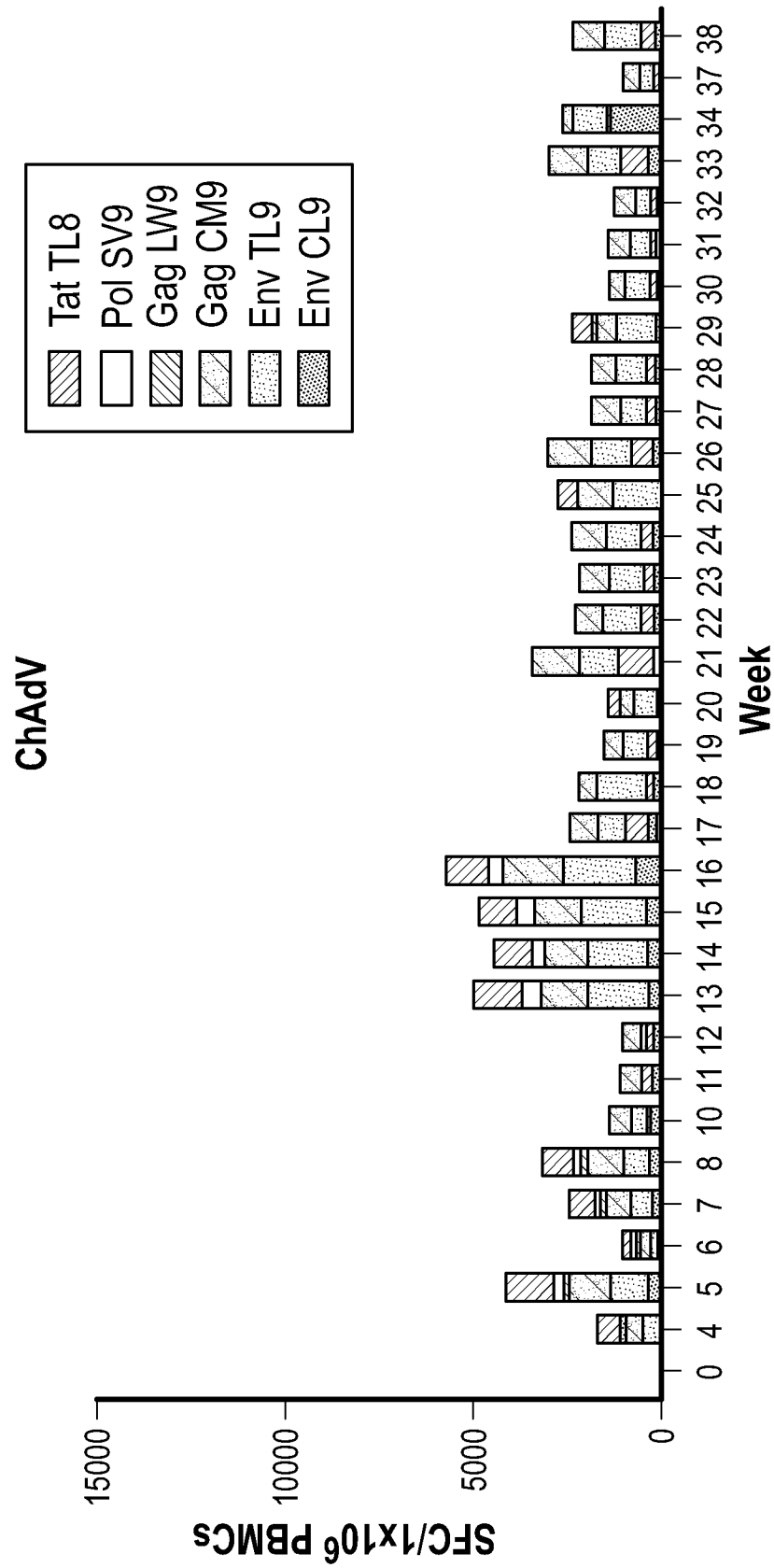


FIG. 35

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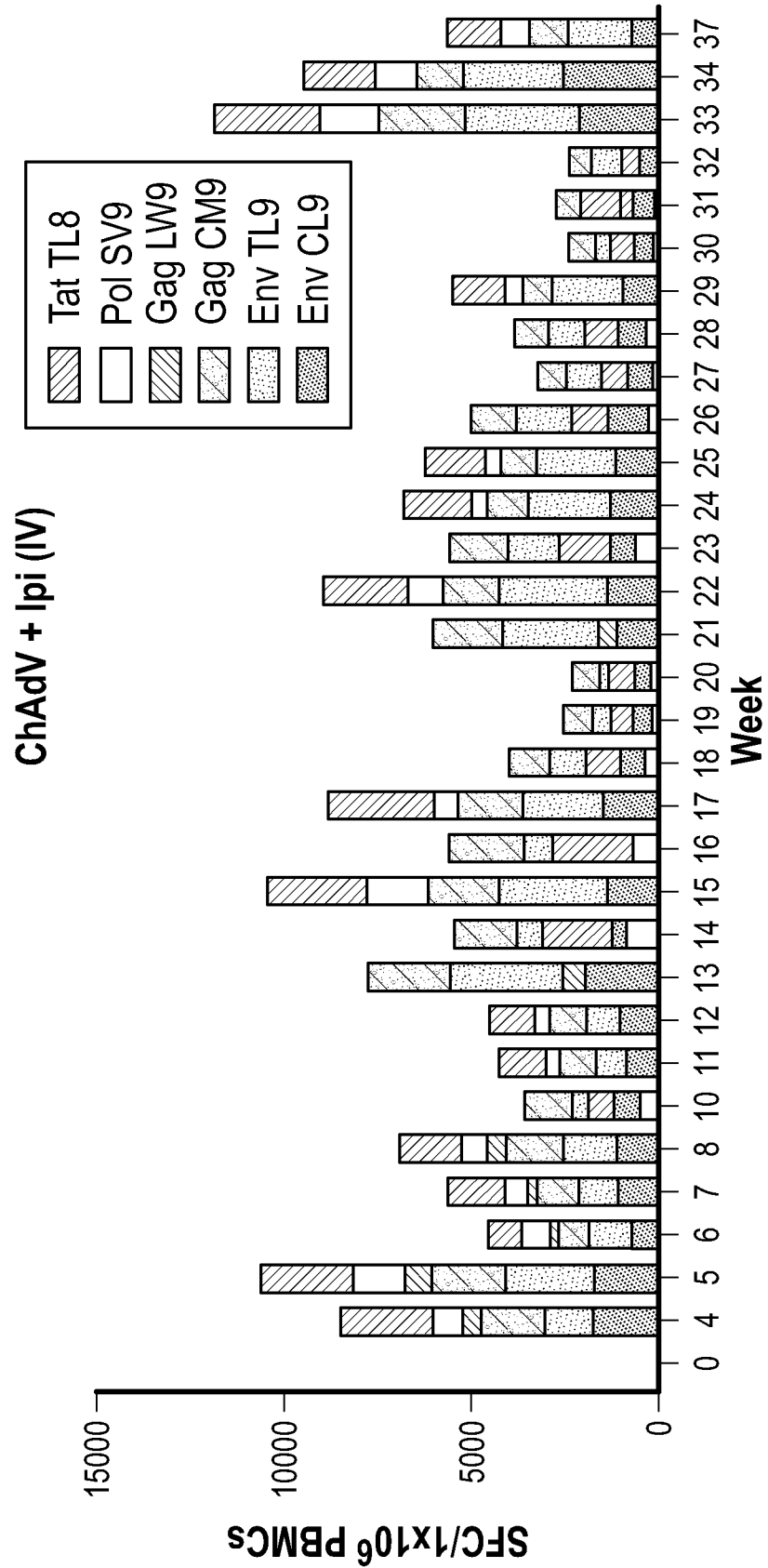
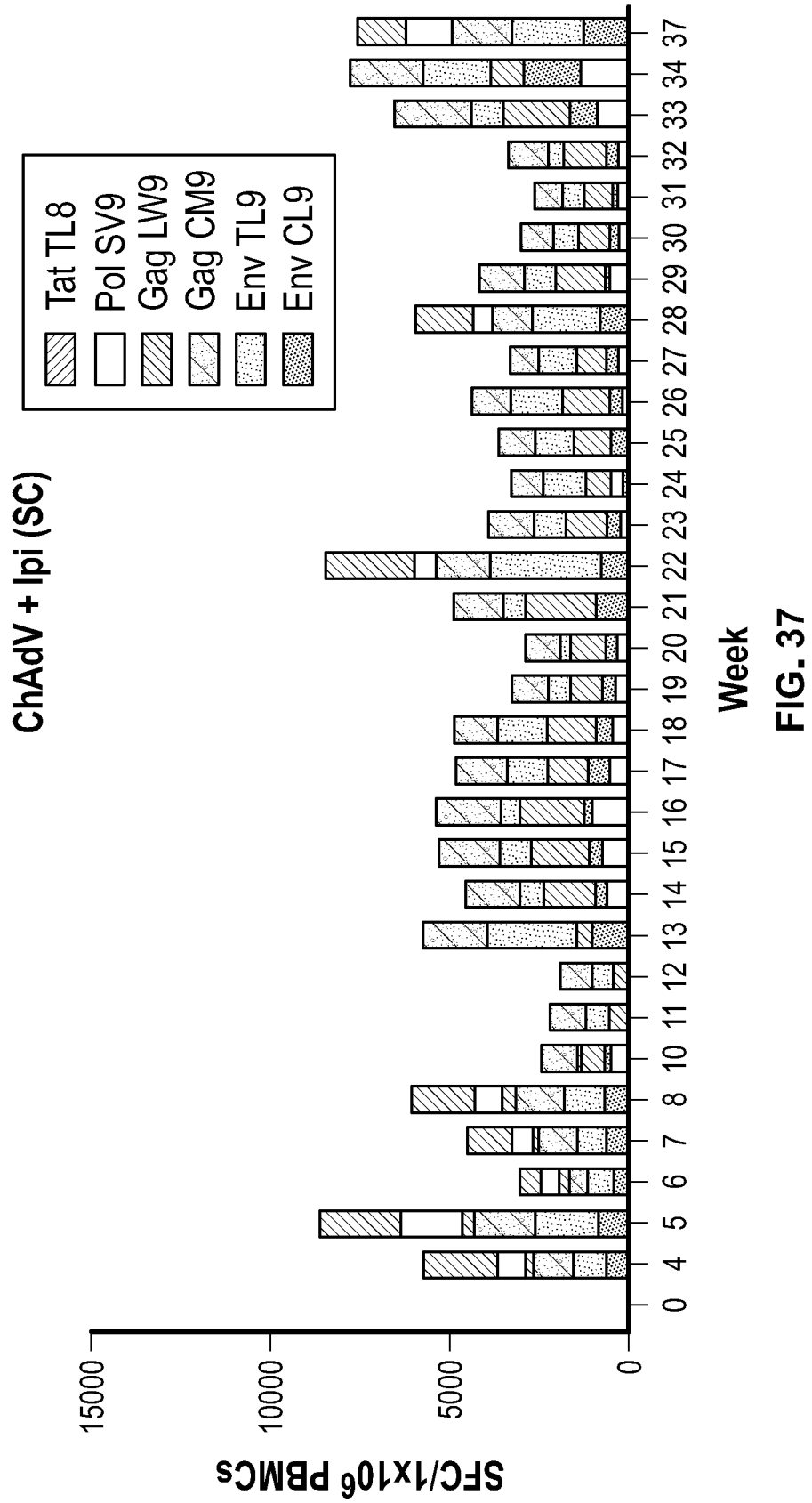


FIG. 36

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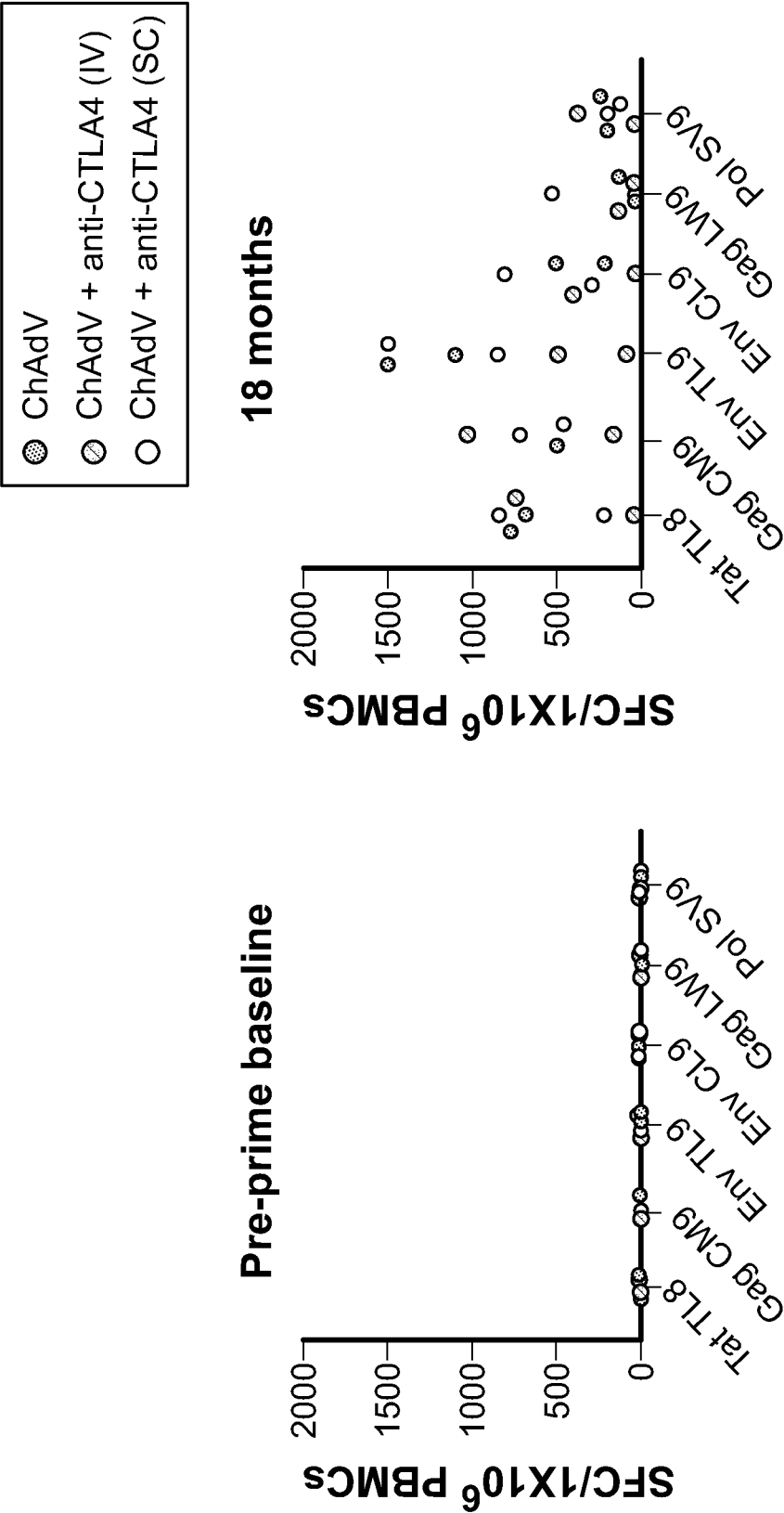


FIG. 38

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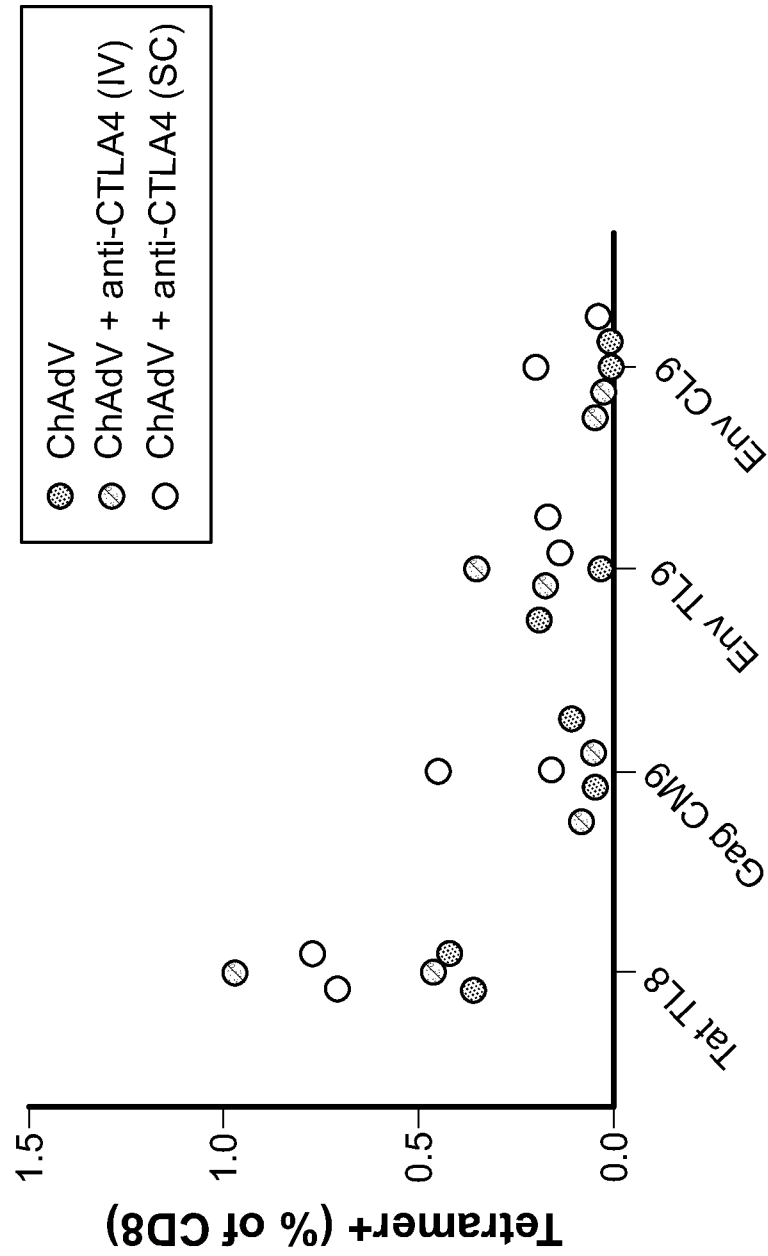


FIG. 39

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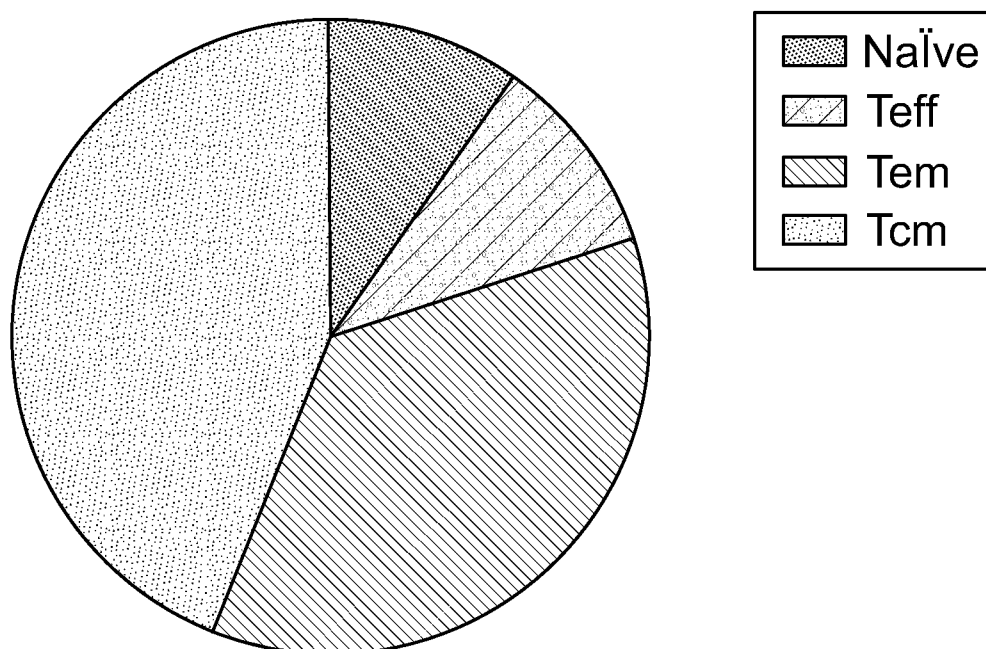


FIG. 40

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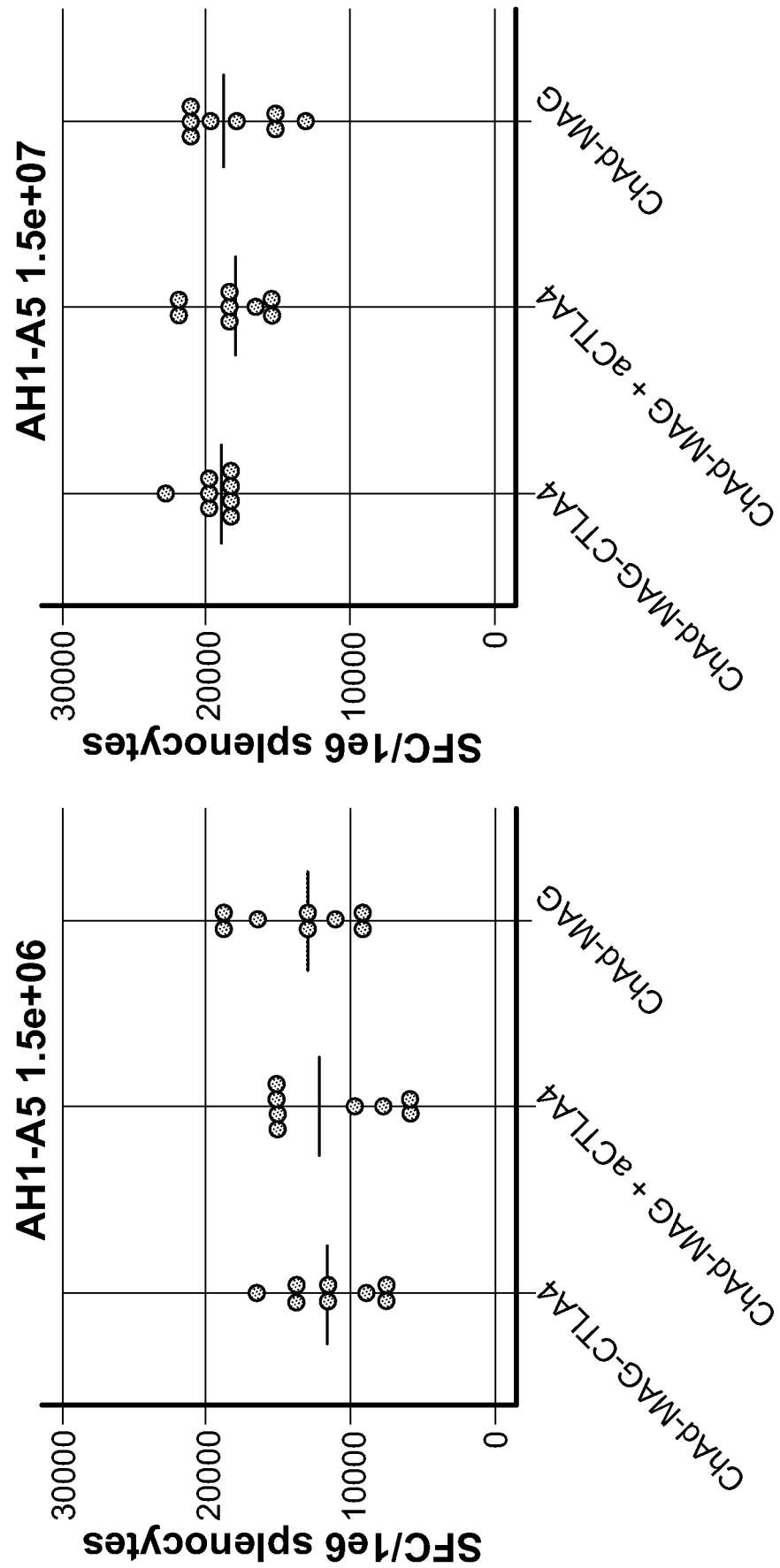


FIG. 41

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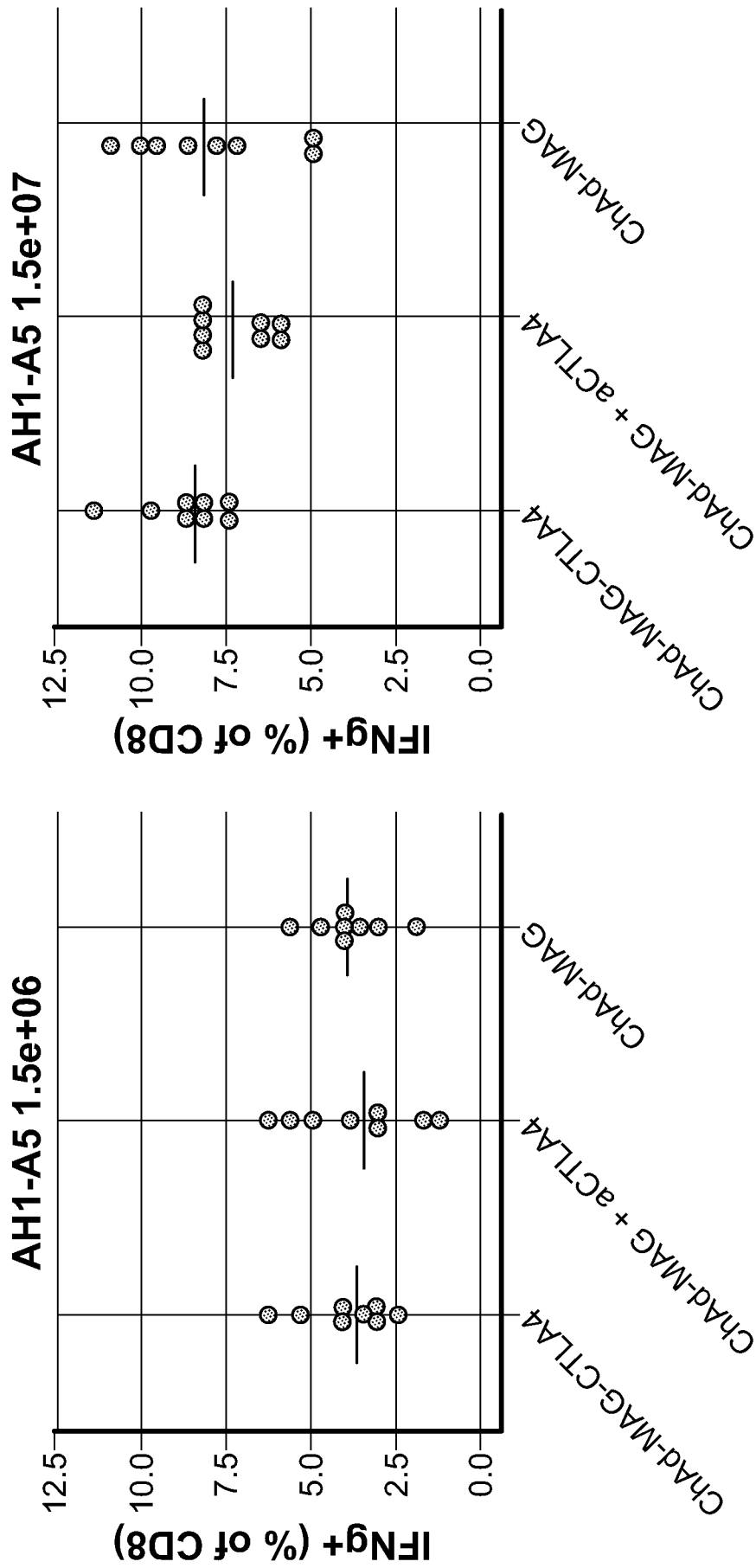


FIG. 42

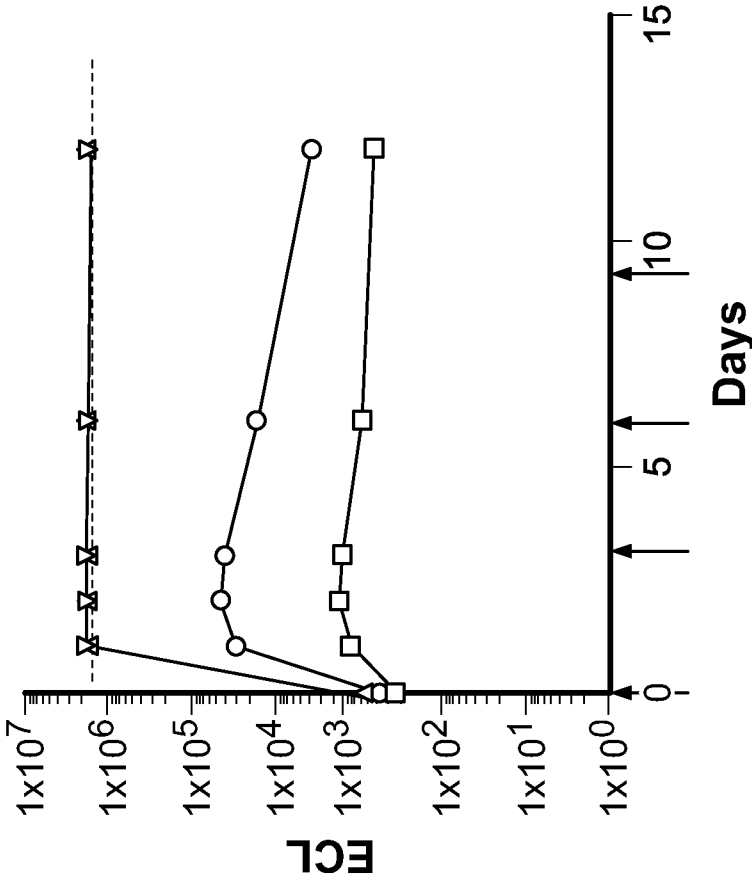


FIG. 43

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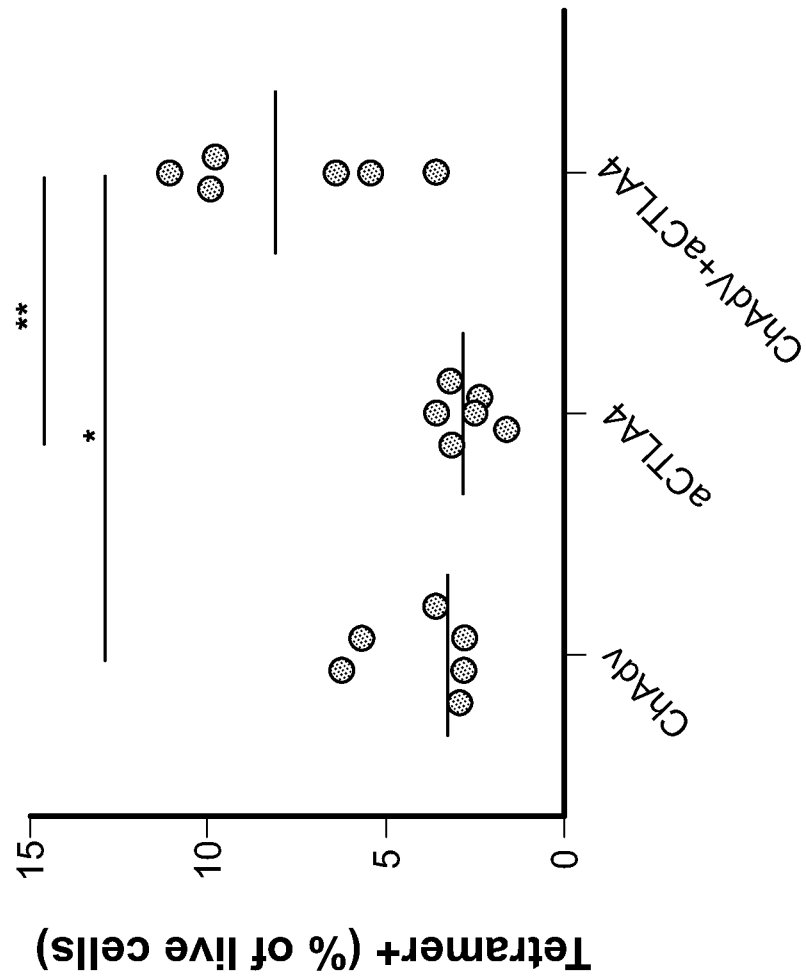


FIG. 44

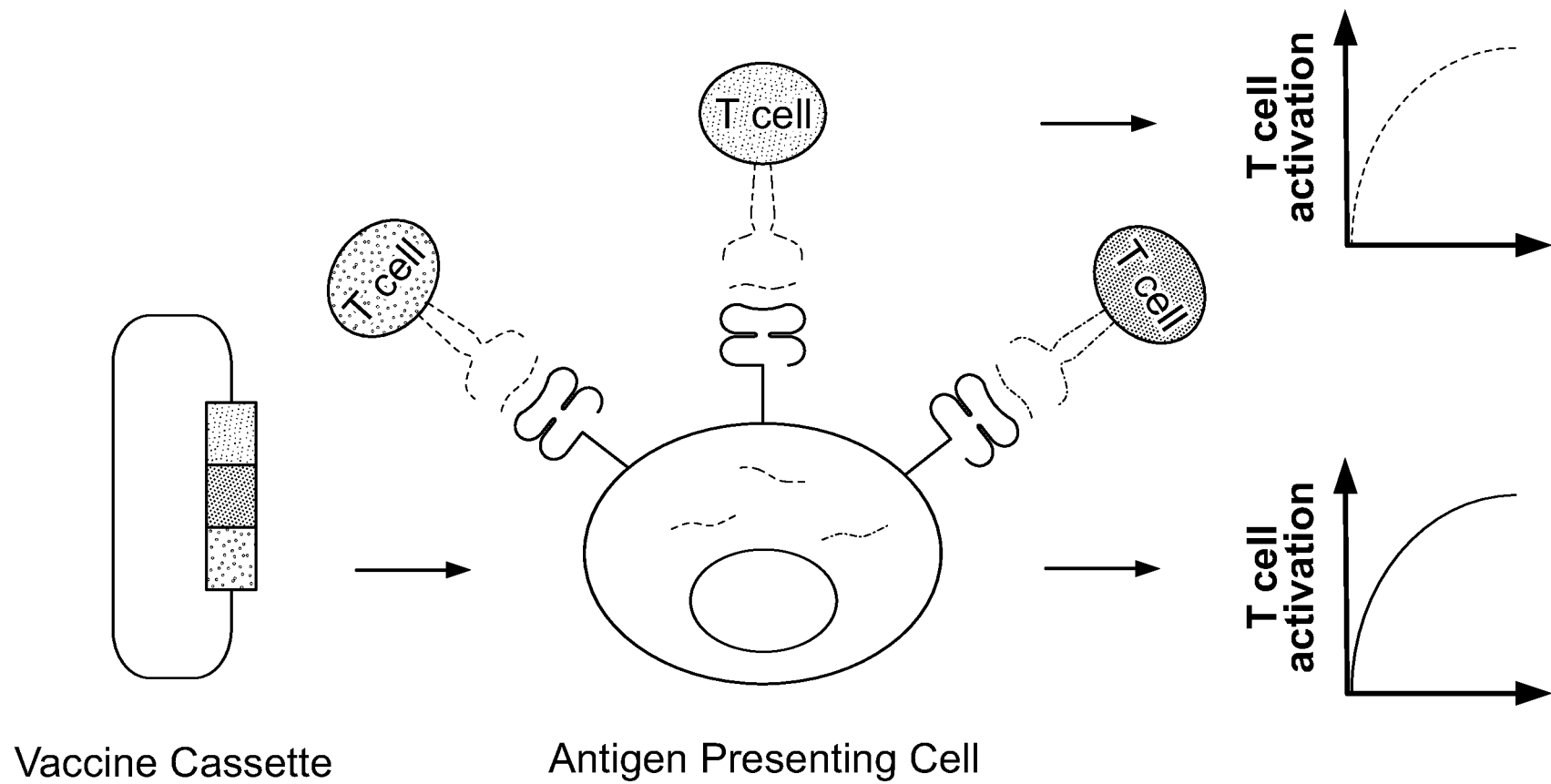


FIG. 1