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(54) Title: ANTIGEN-ENCODING CASSETTES

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Dpagt1	Adpgk	Reps1																	

FIG. 1A

(57) Abstract: Disclosed herein are compositions that include antigen-encoding nucleic acid sequences having multiple repeats of distinct epitope-encoding sequences or having antigen-encoding nucleic acid sequences having less than 700 nucleotides and encoding multiple distinct epitope-encoding sequences. Also disclosed are nucleotides, cells, and methods associated with the compositions including their use as vaccines.



ANTIGEN-ENCODING CASSETTES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/013,466 filed April 21, 2020, 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 April 21, 2021, is named GSO_086WO_sequencelisting.txt and is 422,165 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] One question for antigen vaccine design in both cancer and infectious disease settings is which of the many coding mutations present generate the “best” therapeutic antigens, e.g., antigens that can elicit immunity.

[0005] In addition to the challenges of current antigen prediction methods certain challenges also exist with the available vector systems that can be used for antigen 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 antigen delivery in vaccination strategies, such as in cancer treatment or vaccinations against infectious diseases. While some progress has been made in vaccinations strategies addressing the above problems, improvements are still needed, particularly for clinical applications, such as improved vaccine potency and efficacy.

SUMMARY

[0006] Disclosed herein is: an antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N)_y)_z$$

wherein E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences, n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0, E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n, for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

[0007] In some aspects, for each iteration of z: x and y = 1, optionally except for the final iteration. In some aspects, x = at least 3, at least 4, at least 5, at least 6, or at least 7. In some aspects, for each iteration of z: x = 1, y = 1 optionally except for the final iteration, n = 2, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 , optionally except for the final iteration wherein the order is described by the formula: E- E^1 . In some aspects, z = at least 2, at least 3, at least 4, at least 5, at least 6, or at least 7. In some aspects, for each iteration z: x = 1, y = 1, n = 4, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 - E^3 - E^4 - E^5 . In some aspects, wherein z = at least 2, at least 3, or at least 4. In some aspects, each iteration z: x = 1, y = 1, n = 9, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 - E^3 - E^4 - E^5 - E^6 - E^7 - E^8 - E^9 . In some aspects, z = at least 2.

[0008] In some aspects, each E or E^N independently comprises a nucleotide sequence described, from 5' to 3', by the formula (L5_b-N_c-L3_d), wherein N comprises the distinct epitope-encoding nucleic acid sequence associated with each E or E^N , where c = 1, L5 comprises a 5' linker sequence, where b = 0 or 1, and L3 comprises a 3' linker sequence, where d = 0 or 1. In some aspects, each N encodes an epitope 7-15 amino acids in length, L5 is a native 5' linker sequence that encodes a native N-terminal amino acid sequence of the epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length,

and L3 is a native 3' linker sequence that encodes a native C-terminal amino acid sequence of the epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length.

[0009] In some aspects, each E and E^N encodes an epitope at least 7 amino acids in length. In some aspects, each E and E^N encodes an epitope 7-15 amino acids in length. In some aspects, each E and E^N is a nucleotide sequence at least 21 nucleotides in length. In some aspects, each E and E^N is a nucleotide sequence 75 nucleotides in length.

[0010] Also disclosed herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least two distinct epitope-encoding nucleic acid sequences.

[0011] Also disclosed herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises

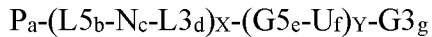
one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences linearly linked to each other, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences.

[0012] In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 3 distinct epitope-encoding nucleic acid sequences.

[0013] Also disclosed herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the vector backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector; and (b) a cassette, optionally wherein the cassette is integrated between a native promoter nucleotide sequence native to the vector backbone and a poly(A) sequence, optionally wherein the poly(A) sequence is native to the vector backbone, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least one epitope-encoding

nucleic acid sequence, optionally comprising at least two distinct epitope-encoding nucleic acid sequences linearly linked to each other, each epitope-encoding nucleic acid sequence optionally comprising: (A) a MHC class I epitope encoding nucleic acid sequence, wherein the MHC class 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 class 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 class I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and wherein the cassette is operably linked to the native promoter nucleotide sequence, wherein each of the epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length, and wherein each 3' end of each epitope-encoding nucleic acid sequence is linked to the 5' end of the following epitope-encoding nucleic acid sequence with the exception of the final epitope-encoding nucleic acid sequence in the cassette; and (ii) at least two MHC class II epitope-encoding nucleic acid sequences comprising: (I) a PADRE MHC class II sequence (SEQ ID NO:48), (II) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46), (III) 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, (IV) 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 epitope-encoding nucleic acid sequences to the epitope-encoding nucleic acid sequences, (V) 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 epitope-encoding nucleic acid sequences; (iii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the native promoter nucleotide sequence, and wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequence.

[0014] In some aspects, an ordered sequence of each element of the cassette is described in the formula, from 5' to 3', comprising:



wherein, P comprises the second promoter nucleotide sequence, where a = 0 or 1, N comprises one of the distinct epitope-encoding nucleic acid sequences, 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 epitope-encoding nucleic acid sequence, where f = 1, X = 1 to 400, where for each X the corresponding N_c is a epitope-encoding nucleic acid sequence, and Y = 0, 1, or 2, where for each Y the corresponding U_f is an MHC class II epitope-encoding nucleic acid sequence.

[0015] In some aspects, for each X the corresponding N_c is a distinct epitope-encoding nucleic acid sequence, except for the N_c corresponding to the at least two repeats of the distinct epitope-encoding nucleic acid sequence. In some aspects, for each Y the corresponding U_f is a distinct MHC class II epitope-encoding nucleic acid sequence. In some aspects, a = 0, b = 1, d = 1, e = 1, g = 1, h = 1, X = 20, Y = 2, the at least one promoter nucleotide sequence is a single native promoter nucleotide sequence native to the vector backbone, the at least one polyadenylation poly(A) sequence is a poly(A) sequence of at least 80 consecutive A nucleotides provided by the vector backbone, each N encodes an epitope 7-15 amino acids in length, L5 is a native 5' linker sequence that encodes a native N-terminal amino acid sequence of the epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length, L3 is a native 3' linker sequence that encodes a native C-terminal amino acid sequence of the 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 backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector, optionally wherein the native promoter nucleotide sequence is a subgenomic promoter when the vector backbone comprises an alphavirus vector, and each of the MHC class II epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length.

[0016] In some aspects, the at least two repeats is at least 3, at least 4, at least 5, at least 6, or at least 7 repeats. In some aspects, the at least two repeats is 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, or at least 20 repeats. In some aspects, the at least two repeats is between 2-3, between 2-4, between 2-5, between 2-6, or between 2-7 repeats. In some aspects, the at least two repeats is 7 repeats or less, 6 repeats or less, 5 repeats or less, 4 repeats or less, or 3 repeats or less.

[0017] In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least two distinct epitope-encoding nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 distinct epitope-encoding nucleic acid sequences. In some aspects, the at least two repeats are separated by at least one separate distinct epitope-encoding nucleic acid sequence. In some aspects, the at least two repeats are separated by at least 2 separate distinct epitope-encoding nucleic acid sequences. In some aspects, the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 75 nucleotides. In some aspects, the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 150 nucleotides, at least 300 nucleotides, or at least 675 nucleotides. In some aspects, the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 350 nucleotides, at least 400 nucleotides, at least 450 nucleotides, at least 500 nucleotides, at least 700 nucleotides, at least 750 nucleotides, at least 800 nucleotides, at least 900 nucleotides, or at least 1000 nucleotides. In some aspects, the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, at least 45 nucleotides, at least 50 nucleotides, at least 55 nucleotides, at least 60 nucleotides, at least 65 nucleotides, or at least 70 nucleotides.

[0018] In some aspects, the at least one antigen-encoding nucleic acid sequence is described, from 5' to 3', by the formula:

$$(E_x-(E^n)_y)_z$$

wherein, E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences, n represents the number of separate distinct epitope-

encoding nucleic acid sequences and is any integer including 0, E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n , for each iteration of z : $x = 0$ or 1 , $y = 0$ or 1 for each n , and at least one of x or $y = 1$, and $z = 2$ or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E , a given E^N , or a combination thereof.

[0019] In some aspects, for each iteration of z : x and $y = 1$, optionally except for the final iteration. In some aspects, $x =$ at least 3, at least 4, at least 5, at least 6, or at least 7. In some aspects, for each iteration of z : $x = 1$, $y = 1$ optionally except for the final iteration, $n = 2$, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: $E-E^1-E^2$, optionally except for the final iteration wherein the order is described by the formula: $E-E^1$. In some aspects, $z =$ at least 2, at least 3, at least 4, at least 5, at least 6, or at least 7. In some aspects, for each iteration z : $x = 1$, $y = 1$, $n = 4$, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: $E-E^1-E^2-E^3-E^4-E^5$. In some aspects, $z =$ at least 2, at least 3, or at least 4. In some aspects, for each iteration z : $x = 1$, $y = 1$, $n = 9$, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: $E-E^1-E^2-E^3-E^4-E^5-E^6-E^7-E^8-E^9$. In some aspects, $z =$ at least 2.

[0020] In some aspects, the at least two repeats comprises a number of repeats, or z comprises a number, sufficient to stimulate a greater immune response relative to an antigen-encoding nucleic acid sequence comprising a single iteration of the at least one epitope-encoding nucleic acid sequence. In some aspects, the at least two repeats comprises a number of repeats, or z comprises a number, sufficient to stimulate an immune response, and a single iteration of the at least one epitope-encoding nucleic acid sequence is insufficient to stimulate the immune response or insufficient to stimulate a detectable immune response. In some aspects, the immune response is an expansion of epitope-specific T cells following *in vivo* immunization with the composition for delivery of the antigen expression system. In some aspects, the immune response is increased activation of epitope-specific T cells and/or increased epitope-specific killing by epitope-specific T cells following *in vivo* immunization with the composition for delivery of the antigen expression system.

[0021] Also provided for herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii)

optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises; (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less.

[0022] Also provided for herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences linearly linked to each other, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises; (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii)

optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, and wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less.

[0023] In some aspects, the at least one antigen-encoding nucleic acid sequence comprises 3 distinct epitope-encoding nucleic acid sequences.

[0024] Also provided for herein is a composition for delivery of an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the vector backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector; and (b) a cassette, optionally wherein the cassette is integrated between a native promoter nucleotide sequence native to the vector backbone and a poly(A) sequence, optionally wherein the poly(A) sequence is native to the vector backbone, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least one epitope-encoding nucleic acid sequence, optionally comprising at least two distinct epitope-encoding nucleic acid sequences linearly linked to each other, each epitope-encoding nucleic acid sequence optionally comprising: (A) a MHC class I epitope encoding nucleic acid sequence, wherein the MHC class 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 class 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 class I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and wherein the cassette is operably linked to the native promoter nucleotide sequence, wherein each of the epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length, and wherein each 3' end of each epitope-encoding nucleic acid sequence is linked to the 5' end of the following epitope-encoding nucleic acid sequence with the exception of the final epitope-encoding nucleic acid sequence in the cassette; and (ii) at

least two MHC class II epitope-encoding nucleic acid sequences comprising: (I) a PADRE MHC class II sequence (SEQ ID NO:48), (II) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46), (III) 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, (IV) a second nucleic acid sequence encoding a GPGPG amino acid linker sequence linking the 5' end of the at least two MHC class II epitope-encoding nucleic acid sequences to the epitope-encoding nucleic acid sequences, (V) 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 epitope-encoding nucleic acid sequences, and (iii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the native promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less.

[0025] In some aspects, the cassette is between 375-700 nucleotides in length. In some aspects, the cassette is 600, 500, 400, 300, 200, or 100 nucleotides in length or less. In some aspects, the cassette is between 375-600, between 375-500, or between 375-400 nucleotides in length. In some aspects, one or more of the epitope-encoding nucleic acid sequences are derived from a tumor, an infection, or an infected cell of a subject.

[0026] In some aspects, each of the epitope-encoding nucleic acid sequences are derived from a tumor, an infection, or an infected cell of a subject. In some aspects, one or more of the epitope-encoding nucleic acid sequences are not derived from a tumor, an infection, or an infected cell of a subject. In some aspects, each of the epitope-encoding nucleic acid sequences are not derived from a tumor, an infection, or an infected cell of a subject.

[0027] In some aspects, the epitope-encoding nucleic acid sequence encodes an epitope known or suspected to be presented by MHC class I on a surface of a cell, optionally wherein the surface of the cell is a tumor cell surface or an infected cell surface, and optionally wherein the cell is a subject's cell. In some aspects, the cell is a tumor cell 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, or wherein the cell is an infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, a fungally

infected cell, and a parasitically infected cell. In some aspects, the virally infected cell is selected from the group consisting of: an HIV infected cell, a Severe acute respiratory syndrome-related coronavirus (SARS) infected cell, a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected cell, an Ebola infected cell, a Hepatitis B virus (HBV) infected cell, an influenza infected cell, a Hepatitis C virus (HCV) infected cell, a Human papillomavirus (HPV) infected cell, a Cytomegalovirus (CMV) infected cell, a Chikungunya virus infected cell, a Respiratory syncytial virus (RSV) infected cell, a Dengue virus infected cell, and an orthomyxoviridae family virus infected cell.

[0028] In some aspects, the composition further comprises a nanoparticulate delivery vehicle. In some aspects, the nanoparticulate delivery vehicle is 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 antigen expression system.

[0029] In some aspects, the cassette is integrated between the at least one promoter nucleotide sequence and the at least one poly(A) sequence. In some aspects, the second promoter is absent and the at least one promoter nucleotide sequence is operably linked to the antigen-encoding nucleic acid sequence.

[0030] In some aspects, the one or more vectors comprise one or more +-stranded RNA vectors. In some aspects, the one or more +-stranded RNA vectors comprise a 5' 7-methylguanosine (m7g) cap. In some aspects, the one or more +-stranded RNA vectors are produced by *in vitro* transcription. In some aspects, the one or more vectors are self-replicating within a mammalian cell. 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 subgenomic 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 subgenomic 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. In some aspects, the backbone does not encode structural virion proteins capsid, E2 and E1. In some aspects, the cassette is inserted in place of 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.

[0031] In some aspects, the Venezuelan equine encephalitis virus comprises the sequence of 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 comprises the sequence set forth in SEQ ID NO:6 or SEQ ID NO:7. In some aspects, the cassette is inserted at position 7544 to replace the deletion between base pairs 7544 and 11175 as set forth in the sequence of SEQ ID NO:3 or SEQ ID NO:5.

[0032] In some aspects, the insertion of the cassette provides for transcription of a polycistronic RNA comprising the nsP1-4 genes and the at least one antigen-encoding nucleic acid sequence, wherein the nsP1-4 genes and the at least one antigen-encoding nucleic acid sequence are in separate open reading frames.

[0033] In some aspects, the backbone comprises at least one nucleotide sequence of a chimpanzee adenovirus vector. In some aspects, the chimpanzee adenovirus vector is a ChAdV68 vector. In some aspects, the ChAdV vector comprises: (a) an ChAdV backbone, optionally wherein the ChAdV backbone comprises: (i) a modified ChAdV68 sequence comprising at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1, wherein the nucleotides 2 to 36,518 lack: (1) nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion; (2) nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion; and optionally (3) nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1 corresponding to a partial E4 deletion; (ii) optionally a CMV promoter nucleotide sequence; and (iii) optionally an SV40 polyadenylation (poly(A)) sequence; and (b) any of the antigen-encoding cassettes described herein, optionally wherein the antigen-encoding cassette is inserted within the E1 deletion and the cassette is operably linked to the CMV promoter nucleotide sequence and the SV40 poly(A) sequence.

[0034] In some aspects, the at least one promoter nucleotide sequence is the native subgenomic 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 subgenomic promoter nucleotide sequence. In some aspects, the second promoter nucleotide sequence comprises multiple subgenomic promoter nucleotide sequences, wherein each subgenomic promoter nucleotide sequence provides for transcription of one or more of the separate open reading frames.

[0035] In some aspects, the one or more vectors are each at least 300nt in size. In some aspects, the one or more vectors are each at least 1kb in size. In some aspects, the one or more vectors are each 2kb in size. In some aspects, the one or more vectors are each less than 5kb in size.

[0036] In some aspects, at least one of the at least one antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface.

[0037] In some aspects, each epitope-encoding nucleic acid sequence is linked directly to one another. In some aspects, at least one of the at least one epitope-encoding nucleic acid sequences is linked to a distinct epitope-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 GPGPG. In some aspects, at least one sequence of the at least one epitope-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 epitope-encoding nucleic acid sequences of epitope encoded therefrom. 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.

[0038] In some aspects, at least one of the at least one epitope-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 at least one epitope-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. In some aspects, at least one of the at least one epitope-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. In some aspects, 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.

[0039] 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, bladder cancer, brain cancer, B-cell lymphoma, acute myelogenous leukemia, adult acute lymphoblastic leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer, or the infectious disease organism is selected from the group consisting of: Severe acute respiratory syndrome-related coronavirus (SARS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Ebola, HIV, Hepatitis B virus (HBV), influenza, Hepatitis C virus (HCV), Human papillomavirus (HPV), Cytomegalovirus (CMV), Chikungunya virus, Respiratory syncytial virus (RSV), Dengue virus, an orthomyxoviridae family virus, and tuberculosis.

[0040] In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-10, 2, 3, 4, 5, 6, 7, 8, 9, or 10 epitope-encoding nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 11-20, 15-

20, 11-100, 11-200, 11-300, 11-400, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 epitope-encoding nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 epitope-encoding nucleic acid sequences and wherein at least two of the epitope-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-10, 2, 3, 4, 5, 6, 7, 8, 9, or 10 antigen-encoding nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 11-20, 15-20, 11-100, 11-200, 11-300, 11-400, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 antigen-encoding nucleic acid sequences. In some aspects, the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 antigen-encoding 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 a cell surface, optionally a tumor cell surface or an infected cell surface. In some aspects, at least two of the epitope-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface.

[0041] In some aspects, when administered to the subject and translated, at least one of the epitopes encoded by the at least one epitope-encoding nucleic acid sequence are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on the tumor cell surface or the infected cell surface. In some aspects, the at least one antigen-encoding nucleic acid sequences when administered to the subject and translated, at least one of the MHC class I or class II epitopes are presented on antigen presenting cells resulting in an immune response targeting at least one of the epitopes on a tumor cell surface or the infected cell surface, and optionally wherein the expression of each of the at least one antigen-encoding nucleic acid sequences is driven by the at least one promoter nucleotide sequence.

[0042] In some aspects, each epitope-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.

[0043] In some aspects, the at least one MHC class II epitope-encoding nucleic acid sequence is present. In some aspects, the at least one MHC class II epitope-encoding nucleic acid sequence is present and comprises at least one MHC class II epitope-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 epitope-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 epitope-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.

[0044] In some aspects, the at least one promoter nucleotide sequence or the second promoter nucleotide sequence is inducible. In some aspects, the at least one promoter nucleotide sequence or the second promoter nucleotide sequence is non-inducible.

[0045] In some aspects, the at least one poly(A) sequence comprises a poly(A) sequence native to the backbone. In some aspects, the at least one poly(A) sequence comprises a poly(A) sequence exogenous to the backbone. In some aspects, the at least one poly(A) sequence is operably linked to at least one of the at least one antigen-encoding nucleic acid sequences. In some aspects, the at least one poly(A) sequence is at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 consecutive A nucleotides. In some aspects, the at least one poly(A) sequence is at least 80 consecutive A nucleotides.

[0046] In some aspects, the 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. In some aspects, the cassette further comprises a reporter gene, including but not limited to, green fluorescent protein (GFP), a GFP variant, secreted alkaline phosphatase, luciferase, a luciferase variant, or a detectable peptide or epitope. In some aspects, the detectable peptide or epitope is selected from the group consisting of an HA tag, a Flag tag, a His-tag, or a V5 tag.

[0047] In some aspects, the one or more vectors further comprises one or more nucleic acid sequences encoding at least one immune modulator. 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 or IRES; 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 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.

[0048] In some aspects, at least one epitope-encoding nucleic acid sequence is selected by performing the steps of: (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor, an infected cell, or an infectious disease organism, wherein the nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens; (b) 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 a cell surface, optionally a tumor cell surface or an infected cell surface, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and (c) 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 epitope-encoding nucleic acid sequence.

[0049] In some aspects, each of the epitope-encoding nucleic acid sequences is selected by performing the steps of: (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor, an infected cell, or an infectious disease organism, wherein the nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens; (b) 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 a cell surface, optionally a tumor cell surface or an infected cell surface, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and (c) 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 20 epitope-encoding nucleic acid sequences. In some aspects, a number of the set of selected

antigens is 2-20. In some aspects, the presentation model represents dependence between: (a) 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 (b) likelihood of presentation on a cell surface, optionally a tumor cell surface or an infected 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. In some aspects, selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being presented on the cell surface relative to unselected antigens based on the presentation model, optionally wherein the selected antigens have been validated as being presented by one or more specific HLA alleles. In some aspects, selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of stimulating a tumor-specific or infectious disease-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 stimulating 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 a tumor cell or tissue, an infected cell, or an infectious disease organism. In some aspects, the sequencing is next generation sequencing (NGS) or any massively parallel sequencing approach.

[0050] In some aspects, the cassette comprises junctional epitope sequences formed by adjacent sequences in the cassette. In some aspects, 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.

[0051] In some aspects, each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele present in at least 5% of a population. In some aspects, each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele, wherein each antigen/HLA pair has an antigen/HLA

prevalence of at least 0.01% in a population. In some aspects, each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele, wherein each antigen/HLA pair has an antigen/HLA prevalence of at least 0.1% in a population.

[0052] In some aspects, the 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 cassette.

[0053] In some aspects, the prediction is based on presentation likelihoods generated by inputting sequences of the non-therapeutic epitopes into a presentation model.

[0054] In some aspects, an order of the at least one antigen-encoding nucleic acid sequences in the cassette is determined by a series of steps comprising: (a) generating a set of candidate cassette sequences corresponding to different orders of the at least one antigen-encoding nucleic acid sequences; (b) determining, for each candidate cassette sequence, a presentation score based on presentation of non-therapeutic epitopes in the candidate cassette sequence; and (c) selecting a candidate cassette sequence associated with a presentation score below a predetermined threshold as the cassette sequence for an antigen vaccine.

[0055] Also provided for herein is a pharmaceutical composition comprising any of the compositions described 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, 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.

[0056] Also provided for herein is an isolated nucleotide sequence or set of isolated nucleotide sequences comprising the cassette of any of the compositions described herein and one or more elements obtained from the sequence of SEQ ID NO:3 or SEQ ID NO:5, optionally wherein the one or more elements are selected from the group consisting of the sequences necessary for nonstructural protein-mediated amplification, the subgenomic promoter nucleotide sequence, the poly(A) sequence, and the nsP1-4 genes of the sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, and optionally wherein the nucleotide sequence is cDNA. In some aspects, the sequence or set of isolated nucleotide sequences comprises the cassette of

any of the above composition claims inserted at position 7544 of the sequence set forth in SEQ ID NO:6 or SEQ ID NO:7. In some aspects, the composition further comprises: a) a T7 or SP6 RNA polymerase promoter nucleotide sequence 5' of the one or more elements obtained from the sequence of SEQ ID NO:3 or SEQ ID NO:5; and b) optionally, one or more restriction sites 3' of the poly(A) sequence. In some aspects, the cassette of any of the above composition claims is inserted at position 7563 of SEQ ID NO:8 or SEQ ID NO:9.

[0057] Also provided for herein is a vector or set of vectors comprising any of the nucleotide sequence described herein.

[0058] Also provided for herein is an isolated cell comprising any of the nucleotide sequences or set of isolated nucleotide sequences described herein, optionally wherein the cell is a BHK-21, CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.

[0059] Also provided for herein is a kit comprising any of the compositions described herein and instructions for use.

[0060] 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 antigen expression system.

[0061] In some aspects, any of the above compositions further comprise a plurality of LNPs, wherein the LNPs comprise: the antigen 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.

[0062] In some aspects, the non-cationic lipid is a mixture of (1) a phospholipid and (2) cholesterol or a cholesterol derivative.

[0063] 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 dialkylxypropyl (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.

[0064] In some aspects, the antigen expression system is fully encapsulated in the LNPs.

[0065] In some aspects, the non-lamellar morphology of the LNPs comprises an inverse hexagonal (H_{II}) or cubic phase structure.

[0066] 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.

[0067] 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.

[0068] 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.

[0069] 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.

[0070] 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.

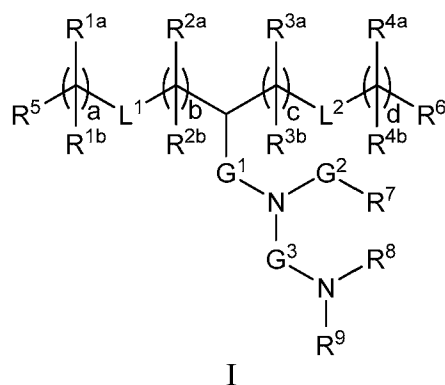
[0071] In some aspects, any of the above compositions further comprise a plurality of LNPs, wherein the LNPs comprise: a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the LNPs; a conjugated lipid that inhibits aggregation of LNPs comprising from 0.5 mol % to 2 mol % 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.

[0072] In some aspects, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

[0073] 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-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof. In some aspects, the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearoyloxypropyl (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.

[0074] In some aspects, the conjugated lipid comprises from 1 mol % to 2 mol % of the total lipid present in the LNPs.

[0075] In some aspects, the LNP comprises a compound having a structure of Formula I:

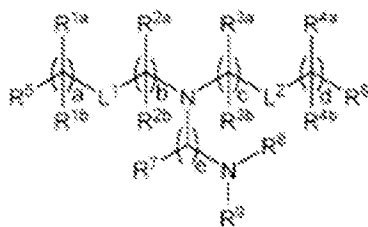


or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

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 $C1-C_{12}$ alkyl;

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 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 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.

[0076] 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

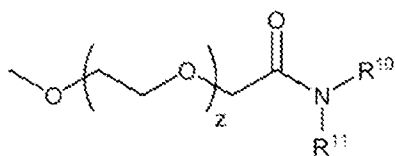
together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R^{4a} and R^{4b} are, at each occurrence, independently either (a) H or C₁-C₁₂ alkyl, or (b) R^{4a} is H or C₁-C₁₂ alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond; R⁵ and R⁶ are each independently methyl or cycloalkyl; R⁷ is, at each occurrence, independently H or C₁-C₁₂ alkyl; R⁸ and R⁹ are each independently unsubstituted C₁-C₁₂ alkyl; or R⁸ and R⁹, 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 L¹ or L² 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.

[0077] 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.

[0078] In some aspects, the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1.

[0079] 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.

[0080] 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 dialkoxypyrrolcarbamate. In some aspects, the pegylated lipid has the following structure III:



III

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein: R¹⁰ and R¹¹ 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, R¹⁰ and R¹¹ are each independently straight, saturated alkyl chains having 12 to 16 carbon atoms. In some aspects, the average z is about 45.

start here

[0081] 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 between 60nm and 140nm. 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.

[0082] Also provided for herein is a method for treating a subject with cancer, the method comprising administering to the subject any of the compositions or any of the pharmaceutical compositions described herein. In some aspects, the at least one epitope-encoding nucleic acid sequence is derived from the tumor of the subject with cancer or from a cell or sample of the infected subject. In some aspects, the at least one epitope-encoding nucleic acid sequence are not derived from the tumor of the subject with cancer or from a cell or sample of the infected subject.

[0083] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising administering to the subject any of the compositions or any of the pharmaceutical compositions described herein.

[0084] In some aspects, the subject expresses at least one HLA allele predicted or known to present the MHC class I epitope. In some aspects, the composition is administered intramuscularly (IM), intradermally (ID), subcutaneously (SC), or intravenously (IV). In some aspects, the composition is administered intramuscularly. In some aspects, the method further comprising administration of one or more immune modulators, optionally wherein the immune modulator is administered before, concurrently with, or after administration of the composition or pharmaceutical composition. In some aspects, the one or more immune modulators are selected from the group consisting of: 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, the subcutaneous administration is near the site of the composition or pharmaceutical composition administration or in close proximity to one or more vector or composition draining lymph nodes.

[0085] 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 any of the compositions or the pharmaceutical compositions described herein. In some aspects, the second vaccine composition is administered subsequent to the administration of any of the compositions or the pharmaceutical compositions described herein. In some aspects, the second vaccine composition is the same as any of the compositions or the pharmaceutical compositions described herein. In some aspects, the second vaccine composition is different any of the compositions or the pharmaceutical compositions described herein. In some aspects, the second vaccine composition comprises a chimpanzee adenovirus vector encoding at least one antigen-encoding nucleic acid sequence. In some aspects, the at least one antigen-encoding nucleic acid sequence encoded by the chimpanzee adenovirus vector is the same as the at least one antigen-encoding nucleic acid sequence of any of the above composition claims.

[0086] Also provided for herein is a method of manufacturing the one or more vectors of any of the above composition claims, the method comprising: (a) obtaining a linearized DNA sequence comprising the backbone and the cassette; (b) *in vitro* transcribing the linearized DNA sequence by addition of the linearized DNA sequence to an *in vitro* transcription reaction containing all the necessary components to transcribe the linearized DNA sequence into RNA, optionally further comprising *in vitro* addition of the m7g cap to the resulting RNA; and (c) isolating the one or more vectors from the *in vitro* transcription reaction. In some aspects, the linearized DNA sequence is generated by linearizing a DNA plasmid sequence or by amplification using PCR. In some aspects, the DNA plasmid sequence is generated using one of bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells. In some aspects, isolating the one or more vectors from the *in vitro* transcription reaction involves one or more of phenol chloroform extraction, silica column based purification, or similar RNA purification methods.

[0087] Also provided for herein is a method of manufacturing the composition of any of the above composition claims for delivery of the antigen expression system, the method comprising: (a) providing components for the nanoparticulate delivery vehicle; (b) providing the antigen expression system; and (c) providing conditions sufficient for the nanoparticulate

delivery vehicle and the antigen expression system to produce the composition for delivery of the antigen expression system. In some aspects, the conditions are provided by microfluidic mixing.

[0088] Also provided for herein is a method for treating a subject with a disease, optionally wherein the disease is cancer or an infection, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^n)_y)_z$$

wherein, E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences, n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0, E^n represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n, for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^n , or a combination thereof.

[0089] Also provided for herein is a method for treating a subject with a disease, optionally wherein the disease is cancer, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least one epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and wherein each of the epitope-encoding nucleic acid sequences comprises; (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the

antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences.

[0090] Also provided for herein is a method for treating a subject with a disease, optionally wherein the disease is cancer, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less.

[0091] In some aspects, the at least one epitope-encoding nucleic acid sequence is derived from a tumor of the subject with cancer or from a cell or sample of the infected subject. In some aspects, the at least one epitope-encoding nucleic acid sequence are not derived from a tumor of the subject with cancer or from a cell or sample of the infected subject.

[0092] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$

wherein, E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences, n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0, E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n, for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

[0093] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises: an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least one epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3'

linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences.

[0094] Also provided for herein is a method for treating a subject with a disease, optionally wherein the disease is cancer or an infection, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding

nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less.

[0095] In some aspects, the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence. In some aspects, the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence, and wherein the at least one epitope sequence comprises a an epitope known or suspected to be presented by MHC class I on a surface of a cell. In some aspects, the surface of the cell is a tumor cell surface. In some aspects, the cell is a tumor cell 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. In some aspects, the surface of the cell is an infected cell surface. In some aspects, the cell is an infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, a fungally infected cell, and a parasitically infected cell. In some aspects, the virally infected cell is selected from the group consisting of: an HIV infected cell, a Severe acute respiratory syndrome-related coronavirus (SARS) infected cell, a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected cell, an Ebola infected cell, a Hepatitis B virus (HBV) infected cell, an influenza infected cell, a Hepatitis C virus (HCV) infected cell, a Human papillomavirus (HPV) infected cell, a Cytomegalovirus (CMV) infected cell, a Chikungunya virus infected cell, a Respiratory syncytial virus (RSV) infected cell, a Dengue virus infected cell, and an orthomyxoviridae family virus infected cell.

[0096] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^n)_y)_z$$

wherein, E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences, n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0, E^n represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n, for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or

y = 1, and z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N, or a combination thereof.

[0097] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises: an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least one epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and wherein each of the epitope-encoding nucleic acid sequences comprises; (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences, and wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence.

[0098] Also provided for herein is a method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises:

an antigen expression system, comprising: the antigen expression system, wherein the antigen expression system comprises one or more vectors, the one or more vectors comprising: (a) a vector backbone, wherein the backbone comprises: (i) at least one promoter nucleotide sequence, and (ii) optionally, at least one polyadenylation (poly(A)) sequence; and (b) a cassette, wherein the cassette comprises: (i) at least one antigen-encoding nucleic acid sequence, comprising: (I) at least two epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and wherein each of the epitope-encoding nucleic acid sequences comprises: (A) optionally, a 5' linker sequence, and (B) optionally, a 3' linker sequence; (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence; (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and wherein the cassette is 700 nucleotides in length or less, and wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence.

[0099] In some aspects, the antigen expression system comprises any one of the antigen expression systems described herein. In some aspects, the antigen-based vaccine comprises any one of the pharmaceutical compositions described herein.

[00100] In some aspects, the antigen-based vaccine is administered as a priming dose. In some aspects, the antigen-based vaccine is administered as one or more boosting doses. In some aspects, the boosting dose is different than the priming dose. In some aspects, a) the priming dose comprises a chimpanzee adenovirus vector and the boosting dose comprises an alphavirus vector; or b) the priming dose comprises an alphavirus vector and the boosting dose comprises a chimpanzee adenovirus vector. In some aspects, the boosting dose is the same as

the priming dose. In some aspects, the injection site of the one or more boosting doses is as close as possible to the injection site of the priming dose.

[00101] In some aspects, the method further comprises determining or having determined the HLA-haplotype of the subject.

[00102] In some aspects, the antigen-based vaccine is administered intramuscularly (IM), intradermally (ID), subcutaneously (SC), or intravenously (IV). In some aspects, the antigen-based vaccine is administered intramuscularly (IM). In some aspects, the IM administration is administered at separate injection sites. In some aspects, the separate injection sites are in opposing deltoid muscles. In some aspects, the separate injection sites are in gluteus or rectus femoris sites on each side.

[00103] Also disclosed herein is a pharmaceutical composition comprising any of the compositions disclosed herein (such as an alphavirus-based or ChAd-based vector disclosed herein) and a pharmaceutically acceptable carrier. In some aspects, the pharmaceutical composition further comprises an adjuvant. In some aspects, the pharmaceutical composition further comprises an immune modulator. 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.

[00104] Also disclosed herein is a vector comprising an isolated nucleotide sequence disclosed herein.

[00105] Also disclosed herein is a kit comprising a vector or a composition disclosed herein and instructions for use.

[00106] Also disclosed herein is a method for treating a subject, the method comprising administering to the subject a vector disclosed herein or a pharmaceutical composition disclosed herein. Also disclosed herein is a method for stimulating an immune response in a subject, the method comprising administering to the subject any of the compositions, vectors, or pharmaceutical compositions described herein. In some aspects, the subject expresses at least one HLA allele predicted or known to present the MHC class I epitope. In some aspects, the vector or composition is administered intramuscularly (IM), intradermally (ID), or subcutaneously (SC), or intravenously (IV).

[00107] Also disclosed herein is a method of manufacturing the one or more vectors of any of the above compositions, the method comprising: obtaining a linearized DNA sequence

comprising the backbone and the antigen cassette; *in vitro* transcribing the linearized DNA sequence by addition of the linearized DNA sequence to an *in vitro* transcription reaction containing all the necessary components to transcribe the linearized DNA sequence into RNA, optionally further comprising *in vitro* addition of the m7g cap to the resulting RNA; and isolating the one or more vectors from the *in vitro* transcription reaction. In some aspects, the linearized DNA sequence is generated by linearizing a DNA plasmid sequence or by amplification using PCR. In some aspects, the DNA plasmid sequence is generated using one of bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells. In some aspects, the isolating the one or more vectors from the *in vitro* transcription reaction involves one or more of phenol chloroform extraction, silica column based purification, or similar RNA purification methods.

[00108] Also disclosed herein is a method of manufacturing any of the compositions disclosed herein, the method comprising: providing components for the nanoparticulate delivery vehicle; providing the antigen expression system; and providing conditions sufficient for the nanoparticulate delivery vehicle and the antigen expression system to produce the composition for delivery of the antigen expression system. In some aspects, the conditions are provided by microfluidic mixing.

[00109] Also disclosed herein is a method of manufacturing a adenovirus 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 adenovirus vector from the one or more host cells.

[00110] In some aspects, isolating comprises: lysing the host cell to obtain a cell lysate comprising the adenovirus vector; and purifying the adenovirus vector from the cell lysate.

[00111] In some aspects, the plasmid sequence is generated using one of 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 adenovirus 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

[00112] 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:

[00113] **Figure (FIG.) 1A** presents an illustration of a 6x/7x epitope repeat cassette design in reference to a 1x design. The 1x cassette encodes 20 unique 25 amino acid epitopes, including the three epitopes measured here (Dpagt1, Adpgk, Reps1). The other 17 epitopes encode other mouse, human and primate antigens. The 6x/7x cassette encodes the three epitopes measured here, each repeated 6 or 7 times, as depicted in the schematic.

[00114] **FIG. 1B** demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Dpagt1. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00115] **FIG. 1C** demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Adpgk. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median. Dashed line represent samples that were too numerous to count (TNTC).

[00116] **FIG. 1D** demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Reps1. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median. Dashed line represent samples that were too numerous to count (TNTC).

[00117] **FIG. 2A** presents an illustration of a 4x epitope repeat cassette design in reference to a 1x design. The 1x cassette encodes 20 unique 25 amino acid epitopes, including the two epitopes measured here (gp100, Trp2). The other 18 epitopes encode other mouse, human and

primate antigens. The 4x cassette encodes the two epitopes measured here, each repeated 4 times, , as well as three additional mouse antigens, as depicted in the schematic.

[00118] FIG. 2B demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen gp100. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median. Dashed line represent samples that were too numerous to count (TNTC).

[00119] FIG. 2C demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Trp2. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median. Dashed line represent samples that were too numerous to count (TNTC).

[00120] FIG. 3A presents an illustration of 1x, 2x, 4x, and 6x/7x epitope repeat cassette designs. The 1x cassette encodes 20 unique 25 amino acid epitopes, including the three epitopes measured here (gp100, Trp1, Trp2). The other 17 epitopes encode other mouse, human and primate antigens. The 2x, 4x, and 6x/7x cassettes encodes the three epitopes measured here, each repeated the indicated number of times, as depicted in the schematic.

[00121] FIG. 3B demonstrates repeating epitopes up to 4 times increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen gp100. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00122] FIG. 3C demonstrates repeating epitopes up to 4 times increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Trp1. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data

presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00123] FIG. 3D demonstrates repeating epitopes up to 4 times increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Trp2. C57Bl6 mice ($n = 8$ per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00124] FIG. 4A presents an illustration of 1x, 5x, and 6x/7x epitope repeat cassette designs. The 1x cassette encodes 20 unique 25 amino acid epitopes, including the three epitopes measured here (Dpagt1, Adpgk, Reps1). The other 17 epitopes encode other mouse, human and primate antigens. The 5x cassette encodes the three epitopes measured here, each repeated five times, as depicted in the schematic. The 6x/7x cassette encodes the three epitopes measured here, each repeated 6 or 7 times, as depicted in the schematic.

[00125] FIG. 4B demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Dpagt1. C57Bl6 mice ($n = 8$ per group) were immunized with 2×10^8 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00126] FIG. 4C demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Adpgk. C57Bl6 mice ($n = 8$ per group) were immunized with 2×10^8 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00127] FIG. 4D demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Reps1. C57Bl6 mice ($n = 8$ per group) were immunized with 2×10^8 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFNg ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

[00128] FIG. 4E demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Dpagt1. C57Bl6 mice (n = 8 per group) were immunized with 1e9 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1x10⁶ splenocytes for each animal. Bar represents the median.

[00129] FIG. 4F demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Adpgk. C57Bl6 mice (n = 8 per group) were immunized with 1e9 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1x10⁶ splenocytes for each animal. Bar represents the median.

[00130] FIG. 4G demonstrates repeating epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the repeated antigen Reps1. C57Bl6 mice (n = 8 per group) were immunized with 1e9 VP ChAdV and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1x10⁶ splenocytes for each animal. Bar represents the median.

[00131] FIG. 5A presents an illustration of the standard 20 epitope slot cassette design encoding 20 unique 25 amino acid epitopes ("20 Epitope") and two "short" insert cassette designs encoding only three total epitopes Dpagt1, Adpgk, and Reps1 ("SHORT-1") or gp100, Trp1, and Trp2 ("SHORT-2").

[00132] FIG. 5B demonstrates "short" epitope cassettes encoding only three total epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are IFN γ ICS results for the antigens Adpgk, and Reps1, gp100, and Trp2. C57Bl6 mice (n = 8 per group) were immunized with 10 ug of SAM-LNP and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by intracellular cytokine staining for IFN γ , following 6-hour stimulation with the specified antigen peptide. Data presented as IFN γ ⁺ cells as a percent of CD8⁺ cells, background signal with negative control peptide is subtracted. Bar represents the median.

[00133] FIG. 5C demonstrates "short" epitope cassettes encoding only three total epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are IFN γ ICS results for the antigens gp100 and Trp2. C57Bl6 mice (n = 8 per group) were immunized with 1 x 10¹⁰ VP

of ChAd68 and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by intracellular cytokine staining for IFN γ , following 6-hour stimulation with the specified antigen peptide. Data presented as IFN γ ⁺ cells as a percent of CD8⁺ cells, background signal with negative control peptide is subtracted. Bar represents the median.

[00134] FIG. 5D demonstrates “short” epitope cassettes encoding only three total epitopes increases vaccine stimulated antigen-specific T-cell response. Shown are ELISpot results for the antigens gp100 and Trp2. C57Bl6 mice (n = 8 per group) were immunized with 1×10^{10} VP of ChAd68 and splenocytes isolated 12 days post-immunization. The number of antigen-specific T-cells were measured by IFN γ ELISpot following overnight stimulation with the specified antigen peptide. Data presented as spot forming colonies (SFC) per 1×10^6 splenocytes for each animal. Bar represents the median.

DETAILED DESCRIPTION

I. Definitions

[00135] 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.

[00136] As used herein the term “antigen” is a substance that stimulates 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.

[00137] 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 non-frameshift 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. The subject can be identified for administration through the use of various diagnostic methods, e.g., patient selection methods described further below.

[00138] As used herein the term “tumor antigen” is an 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.

[00139] 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.

[00140] As used herein the term “candidate antigen” is a mutation or other aberration giving rise to a sequence that may represent an antigen.

[00141] As used herein the term “coding region” is the portion(s) of a gene that encode protein.

[00142] As used herein the term “coding mutation” is a mutation occurring in a coding region.

[00143] As used herein the term “ORF” means open reading frame.

[00144] As used herein the term “NEO-ORF” is a tumor-specific ORF arising from a mutation or other aberration such as splicing.

[00145] As used herein the term “missense mutation” is a mutation causing a substitution from one amino acid to another.

[00146] 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.

[00147] As used herein the term “frameshift mutation” is a mutation causing a change in the frame of the protein.

[00148] As used herein the term “indel” is an insertion or deletion of one or more nucleic acids.

[00149] 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.

[00150] 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).

[00151] 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*).

[00152] 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.

[00153] As used herein the term “non-stop or read-through” is a mutation causing the removal of the natural stop codon.

[00154] As used herein the term “epitope” is the specific portion of an antigen typically bound by an antibody or T cell receptor.

[00155] As used herein the term “immunogenic” is the ability to stimulate an immune response, e.g., via T cells, B cells, or both.

[00156] As used herein the term “HLA binding affinity” “MHC binding affinity” means affinity of binding between a specific antigen and a specific MHC allele.

[00157] As used herein the term “bait” is a nucleic acid probe used to enrich a specific sequence of DNA or RNA from a sample.

[00158] As used herein the term “variant” is a difference between a subject’s nucleic acids and the reference human genome used as a control.

[00159] As used herein the term “variant call” is an algorithmic determination of the presence of a variant, typically from sequencing.

[00160] As used herein the term “polymorphism” is a germline variant, i.e., a variant found in all DNA-bearing cells of an individual.

[00161] As used herein the term “somatic variant” is a variant arising in non-germline cells of an individual.

[00162] 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.

[00163] As used herein the term “HLA type” is the complement of HLA gene alleles.

[00164] 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.

[00165] 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.

[00166] 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.

[00167] 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.

[00168] 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.

[00169] 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.

[00170] As used herein the term “proteome” is the set of all proteins expressed and/or translated by a cell, group of cells, or individual.

[00171] 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, or the infectious disease peptidome, meaning the union of the peptidomes of all cells that are infected by the infectious disease).

[00172] 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.

[00173] As used herein the term “dextramers” is a dextran-based peptide-MHC multimers used for antigen-specific T-cell staining in flow cytometry.

[00174] 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.

[00175] 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).

[00176] 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.

[00177] 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.

[00178] 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.

[00179] 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.

[00180] 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, infection type, infection sub-type, and smoking history.

[00181] The term “antigen-encoding nucleic acid sequences derived from a tumor” refers to nucleic acid sequences obtained 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.

[00182] The term “antigen-encoding nucleic acid sequences derived from an infection” refers to nucleic acid sequences obtained from infected cells or an infectious disease organism, e.g. via RT-PCR; or sequence data obtained by sequencing the infected cell or infectious disease organism and then synthesizing the nucleic acid sequences using the sequencing data, e.g., via various synthetic or PCR-based methods known in the art. Derived sequences can include nucleic acid sequence variants, such as sequence-optimized nucleic acid sequence variants (e.g., codon-optimized and/or otherwise optimized for expression), that encode the same polypeptide sequence as the corresponding native infectious disease organism nucleic acid sequence. Derived sequences can include nucleic acid sequence variants that encode a modified infectious disease organism polypeptide sequence having one or more (e.g., 1, 2, 3, 4, or 5) mutations relative to a native infectious disease organism polypeptide sequence. For example, a modified polypeptide sequence can have one or more missense mutations relative to the native polypeptide sequence of an infectious disease organism protein.

[00183] 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.

[00184] 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 subgenomic promoter element.

[00185] 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, a subgenomic promoter sequence (e.g., a 26S subgenomic promoter sequence), a 19-nt CSE, and an alphavirus 3' UTR.

[00186] 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.

[00187] 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.

[00188] 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

[00189] 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.

[00190] 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.

[00191] 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.

[00192] 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.

[00193] 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. Antigen Identification

[00194] 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. The research methods described can also be applied to identification of antigens in other settings, such as identification of identifying antigens from an infectious disease organism, an infection in a subject, or an infected cell of a subject. Examples of optimizations are known to those skilled in the art, for example the methods described in more detail in US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, US App. No. 16/606,577, and international patent application publications WO2020181240A1, WO/2018/195357 and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00195] Methods for identifying antigens (*e.g.*, antigens derived from a tumor or an infectious disease organism) include identifying antigens that are likely to be presented on a cell surface (*e.g.*, presented by MHC on a tumor cell, an infected cell, or an immune cell,

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 nucleotide sequencing and/or expression data from a tumor, an infected cell, or an infectious disease organism, wherein the nucleotide sequencing data and/or expression data is used to obtain data representing peptide sequences of each of a set of antigens (*e.g.*, antigens derived from a tumor or an infectious disease organism); 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 a cell surface, such as a tumor cell or an infected 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.

III. Identification of Tumor Specific Mutations in Neoantigens

[00196] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, and international patent application publications WO/2018/195357 and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00197] 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 non-

frameshift indel, missense or nonsense substitution, splice site alteration, genomic rearrangement or gene fusion, or any genomic or expression alteration giving rise to a neoORF.

[00198] 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.

[00199] 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.

[00200] 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.

[00201] 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.

[00202] 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.

[00203] 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.

[00204] 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.

[00205] 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)).

[00206] 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 adds 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.

[00207] 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.

[00208] 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.

[00209] 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.

[00210] 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.

[00211] 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.

[00212] 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.

[00213] 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

[00214] 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.

[00215] 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.

[00216] 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. Tumor antigens (e.g., shared tumor antigens and tumor neoantigens) can include, but are not limited to, those described in US App. No. 17/058,128, herein incorporated by reference for all purposes.

[00217] Also disclosed herein are peptides derived from any polypeptide associated with an infectious disease organism, an infection in a subject, or an infected cell of a subject. Antigens can be derived from nucleotide sequences or polypeptide sequences of an infectious disease organism. Polypeptide sequences of an infectious disease organism include, but are not limited to, a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and/or a parasite-derived peptide. Infectious disease organism include, but are not limited to, Severe acute respiratory syndrome-related coronavirus (SARS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Ebola, HIV, Hepatitis B virus (HBV), influenza, Hepatitis C virus (HCV), Human papillomavirus (HPV), Cytomegalovirus (CMV), Chikungunya virus, Respiratory syncytial virus (RSV), Dengue virus, an orthomyxoviridae family virus, and tuberculosis.

[00218] Disclosed herein are isolated peptides that comprise infectious disease organism specific antigens or epitopes identified by the methods disclosed herein, peptides that comprise known infectious disease organism specific antigens or epitopes, and mutant polypeptides or

fragments thereof identified by methods disclosed herein. Antigen peptides can be described in the context of their coding sequence where an antigen includes the nucleotide sequence (e.g., DNA or RNA) that codes for the related polypeptide sequence.

[00219] Vectors and associated compositions described herein can be used to deliver antigens from any organism, including their toxins or other by-products, to prevent and/or treat infection or other adverse reactions associated with the organism or its by-product.

[00220] Antigens that can be incorporated into a vaccine (*e.g.*, encoded in a cassette) include immunogens which are useful to immunize a human or non-human animal against viruses, such as pathogenic viruses which infect human and non-human vertebrates. Antigens may be selected from a variety of viral families. Example of desirable viral families against which an immune response would be desirable include, the picornavirus family, which includes the genera rhinoviruses, which are responsible for about 50% of cases of the common cold; the genera enteroviruses, which include polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus; and the genera aphthoviruses, which are responsible for foot and mouth diseases, primarily in non-human animals. Within the picornavirus family of viruses, target antigens include the VP1, VP2, VP3, VP4, and VPG. Another viral family includes the calcivirus family, which encompasses the Norwalk group of viruses, which are an important causative agent of epidemic gastroenteritis. Still another viral family desirable for use in targeting antigens for stimulating immune responses in humans and non-human animals is the togavirus family, which includes the genera alphavirus, which include Sindbis viruses, Ross River virus, and Venezuelan, Eastern & Western Equine encephalitis, and rubivirus, including Rubella virus. The Flaviviridae family includes dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses. Other target antigens may be generated from the Hepatitis C or the coronavirus family, which includes a number of non-human viruses such as infectious bronchitis virus (poultry), porcine transmissible gastroenteric virus (pig), porcine hemagglutinating encephalomyelitis virus (pig), feline infectious peritonitis virus (cats), feline enteric coronavirus (cat), canine coronavirus (dog), and human respiratory coronaviruses, which may cause the common cold and/or non-A, B or C hepatitis. Within the coronavirus family, target antigens include the E1 (also called M or matrix protein), E2 (also called S or Spike protein), E3 (also called HE or hemagglutinin-esterase) glycoprotein (not present in all coronaviruses), or N (nucleocapsid). Still other antigens may be targeted against the rhabdovirus family, which includes the genera vesiculovirus (*e.g.*, Vesicular Stomatitis Virus), and the general lyssavirus (*e.g.*, rabies). Within the rhabdovirus

family, suitable antigens may be derived from the G protein or the N protein. The family filoviridae, which includes hemorrhagic fever viruses such as Marburg and Ebola virus, may be a suitable source of antigens. The paramyxovirus family includes parainfluenza Virus Type 1, parainfluenza Virus Type 3, bovine parainfluenza Virus Type 3, rubulavirus (mumps virus), parainfluenza Virus Type 2, parainfluenza virus Type 4, Newcastle disease virus (chickens), rinderpest, morbillivirus, which includes measles and canine distemper, and pneumovirus, which includes respiratory syncytial virus (e.g., the glyco-(G) protein and the fusion (F) protein, for which sequences are available from GenBank). Influenza virus is classified within the family orthomyxovirus and can be suitable source of antigens (e.g., the HA protein, the N1 protein). The bunyavirus family includes the genera bunyavirus (California encephalitis, La Crosse), phlebovirus (Rift Valley Fever), hantavirus (puremala is a hemahagin fever virus), nairovirus (Nairobi sheep disease) and various unassigned bungaviruses. The arenavirus family provides a source of antigens against LCM and Lassa fever virus. The reovirus family includes the genera reovirus, rotavirus (which causes acute gastroenteritis in children), orbiviruses, and cultivirus (Colorado Tick fever, Lebombo (humans), equine encephalosis, blue tongue). The retrovirus family includes the sub-family oncorivirinal which encompasses such human and veterinary diseases as feline leukemia virus, HTLV I and HTLV II, lentivirinal (which includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus, and spumavirinal). Among the lentiviruses, many suitable antigens have been described and can readily be selected. Examples of suitable HIV and SIV antigens include, without limitation the gag, pol, Vif, Vpx, VPR, Env, Tat, Nef, and Rev proteins, as well as various fragments thereof. For example, suitable fragments of the Env protein may include any of its subunits such as the gp120, gp160, gp41, or smaller fragments thereof, e.g., of at least about 8 amino acids in length. Similarly, fragments of the tat protein may be selected. [See, U.S. Pat. No. 5,891,994 and U.S. Pat. No. 6,193,981.] See, also, the HIV and SIV proteins described in D. H. Barouch et al, *J. Virol.*, 75(5):2462-2467 (March 2001), and R. R. Amara, et al, *Science*, 292:69-74 (6 Apr. 2001). In another example, the HIV and/or SIV immunogenic proteins or peptides may be used to form fusion proteins or other immunogenic molecules. See, e.g., the HIV-1 Tat and/or Nef fusion proteins and immunization regimens described in WO 01/54719, published Aug. 2, 2001, and WO 99/16884, published Apr. 8, 1999. The invention is not limited to the HIV and/or SIV immunogenic proteins or peptides described herein. In addition, a variety of modifications to these proteins have been described or could readily be made by one of skill in the art. See, e.g.,

the modified gag protein that is described in U.S. Pat. No. 5,972,596. Further, any desired HIV and/or SIV immunogens may be delivered alone or in combination. Such combinations may include expression from a single vector or from multiple vectors. The papovavirus family includes the sub-family polyomaviruses (BKU and JCU viruses) and the sub-family papillomavirus (associated with cancers or malignant progression of papilloma). The adenovirus family includes viruses (EX, AD7, ARD, O.B.) which cause respiratory disease and/or enteritis. The parvovirus family feline parvovirus (feline enteritis), feline panleucopeniavirus, canine parvovirus, and porcine parvovirus. The herpesvirus family includes the sub-family alphaherpesvirinae, which encompasses the genera simplexvirus (HSV1, HSV2), varicellovirus (pseudorabies, varicella zoster) and the sub-family betaherpesvirinae, which includes the genera cytomegalovirus (Human CMV), muromegalovirus) and the sub-family gammaherpesvirinae, which includes the genera lymphocryptovirus, EBV (Burkitt's lymphoma), infectious rhinotracheitis, Marek's disease virus, and rhadinovirus. The poxvirus family includes the sub-family chordopoxvirinae, which encompasses the genera orthopoxvirus (Variola (Smallpox) and Vaccinia (Cowpox)), parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, and the sub-family entomopoxvirinae. The hepadnavirus family includes the Hepatitis B virus. One unclassified virus which may be suitable source of antigens is the Hepatitis delta virus. Still other viral sources may include avian infectious bursal disease virus and porcine respiratory and reproductive syndrome virus. The alphavirus family includes equine arteritis virus and various Encephalitis viruses.

[00221] Antigens that can be incorporated into a vaccine (*e.g.*, encoded in a cassette) also include immunogens which are useful to immunize a human or non-human animal against pathogens including bacteria, fungi, parasitic microorganisms or multicellular parasites which infect human and non-human vertebrates. Examples of bacterial pathogens include pathogenic gram-positive cocci include pneumococci; staphylococci; and streptococci. Pathogenic gram-negative cocci include meningococcus; gonococcus. Pathogenic enteric gram-negative bacilli include enterobacteriaceae; *pseudomonas*, *acinetobacteria* and *eikenella*; melioidosis; *salmonella*; *shigella*; *haemophilus* (*Haemophilus influenzae*, *Haemophilus somnus*); *moraxella*; *H. ducreyi* (which causes chancroid); *brucella*; *Fransisella tularensis* (which causes tularemia); *yersinia (pasteurella)*; *streptobacillus moniliformis* and *spirillum*. Gram-positive bacilli include *listeria monocytogenes*; *erysipelotheix rhusiopathiae*; *Corynebacterium diphtheria* (diphtheria); cholera; *B.*

anthracis (anthrax); donovanosis (granuloma inguinale); and bartonellosis. Diseases caused by pathogenic anaerobic bacteria include tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Examples of specific bacterium species are, without limitation, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Moraxella catarrhalis*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Bordetella pertussis*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare* complex, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Clostridium tetani*, *Leptospira interrogans*, *Borrelia burgdorferi*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Mycoplasma gallisepticum*. Pathogenic spirochetal diseases include syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include actinomycosis; nocardiosis; cryptococcosis (*Cryptococcus*), blastomycosis (*Blastomyces*), histoplasmosis (*Histoplasma*) and coccidioidomycosis (*Coccidioides*); candidiasis (*Candida*), aspergillosis (*Aspergillus*), and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include Typhus fever, Rocky Mountain spotted fever, Q fever, and Rickettsialpox. Examples of mycoplasma and chlamydial infections include: *mycoplasma pneumoniae*; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes encompass pathogenic protozoans and helminths and infections produced thereby include: amebiasis; malaria; leishmaniasis (e.g., caused by *Leishmania major*); trypanosomiasis; toxoplasmosis (e.g., caused by *Toxoplasma gondii*); *Pneumocystis carinii*; Trichans; *Toxoplasma gondii*; babesiosis; giardiasis (e.g., caused by *Giardia*); trichinosis (e.g., caused by *Trichomonas*); filariasis; schistosomiasis (e.g., caused by *Schistosoma*); nematodes; trematodes or flukes; and cestode (tapeworm) infections. Other parasitic infections may be caused by *Ascaris*, *Trichuris*, *Cryptosporidium*, and *Pneumocystis carinii*, among others.

[00222] Also disclosed herein are peptides derived from any polypeptide associated with an infectious disease organism, an infection in a subject, or an infected cell of a subject. Antigens can be derived from nucleic acid sequences or polypeptide sequences of an infectious disease organism. Polypeptide sequences of an infectious disease organism include, but are not limited to, a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-

derived peptide, and/or a parasite-derived peptide. Infectious disease organism include, but are not limited to, Severe acute respiratory syndrome-related coronavirus (SARS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Ebola, HIV, Hepatitis B virus (HBV), influenza, Hepatitis C virus (HCV), Human papillomavirus (HPV), Cytomegalovirus (CMV), Chikungunya virus, Respiratory syncytial virus (RSV), Dengue virus, an orthomyxoviridae family virus, and tuberculosis.

[00223] Antigens can be selected that are predicted to be presented on the cell surface of a cell, such as a tumor cell, an infected cell, or an immune cell, including professional antigen presenting cells such as dendritic cells. Antigens can be selected that are predicted to be immunogenic.

[00224] 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.

[00225] One or more antigens can be presented on the surface of a tumor. One or more antigens can be presented on the surface of an infected cell.

[00226] One or more antigens can be immunogenic in a subject having a tumor, e.g., capable of stimulating a T cell response and/or a B cell response in the subject. One or more antigens can be immunogenic in a subject having or suspected to have an infection, e.g., capable of stimulating a T cell response and/or a B cell response in the subject. One or more antigens can be immunogenic in a subject at risk of an infection, e.g., capable of stimulating a T cell response and/or a B cell response in the subject that provides immunological protection (*i.e.*, immunity) against the infection, *e.g.*, such as stimulating the production of memory T cells, memory B cells, and/or antibodies specific to the infection.

[00227] One or more antigens can be capable of stimulating a B cell response, such as the production of antibodies that recognize the one or more antigens (*e.g.*, antibodies that recognize an infectious disease antigen). Antibodies can recognize linear polypeptide sequences or recognize secondary and tertiary structures. Accordingly, B cell antigens can include linear polypeptide sequences or polypeptides having secondary and tertiary structures, including, but

not limited to, full-length proteins, protein subunits, protein domains, or any polypeptide sequence known or predicted to have secondary and tertiary structures. Antigens capable of stimulating a B cell response to an infection can be an antigen found on the surface of an infectious disease organism. Antigens capable of eliciting a B cell response to an infection can be an intracellular antigen expressed in an infectious disease organism.

[00228] One or more antigens can include a combination of antigens capable of stimulating a T cell response (*e.g.*, peptides including predicted T cell epitope sequences) and distinct antigens capable of stimulating a B cell response (*e.g.*, full-length proteins, protein subunits, protein domains).

[00229] One or more antigens that stimulate an autoimmune response in a subject can be excluded from consideration in the context of vaccine generation for a subject.

[00230] The size of at least one antigenic peptide molecule (*e.g.*, an epitope sequence) 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.

[00231] 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.

[00232] 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 or infectious disease-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 stimulation of T cell responses. Longer peptides can also include a full-length protein, a protein subunit, a protein domain, and combinations thereof of a peptide, such as those expressed in an infectious disease organism. Longer peptides (*e.g.*, full-length protein, protein subunit, or protein domain) and combinations thereof can be included to stimulate a B cell response.

[00233] 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, an 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.

[00234] In some aspects, antigenic peptides and polypeptides do not stimulate an autoimmune response and/or invoke immunological tolerance when administered to a subject.

[00235] 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. Tumor-specific peptides can be 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. Peptides can be derived from any polypeptide known to or suspected to be associated with an infectious disease organism, or peptides derived from any polypeptide known to or have been found to have altered expression in an infected cell in comparison to a normal cell or tissue (*e.g.*, an infectious disease polynucleotide or polypeptide, including infectious disease polynucleotides or polypeptides with expression restricted to a host cell). 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. A peptide can include a tumor-specific mutation. In some aspects the tumor specific mutation is a driver mutation for a particular cancer type.

[00236] 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).

[00237] 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.

[00238] 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 stimulate CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of stimulating 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.

[00239] An 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.

[00240] 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.

[00241] In a further aspect an antigen includes a nucleic acid (e.g. polynucleotide) that encodes an 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 phosphorothioate backbone, or combinations thereof and it may or may not contain introns. A polynucleotide sequence encoding an antigen can be sequence-optimized to improve expression, such as through improving transcription, translation, post-transcriptional processing, and/or RNA stability. For example, polynucleotide sequence encoding an antigen can be codon-optimized. "Codon-optimization" herein refers to replacing infrequently used codons, with respect to codon bias of a given organism, with frequently used synonymous codons. Polynucleotide sequences can be

optimized to improve post-transcriptional processing, for example optimized to reduce unintended splicing, such as through removal of splicing motifs (*e.g.*, canonical and/or cryptic/non-canonical splice donor, branch, and/or acceptor sequences) and/or introduction of exogenous splicing motifs (*e.g.*, splice donor, branch, and/or acceptor sequences) to bias favored splicing events. Exogenous intron sequences include, but are not limited to, those derived from SV40 (*e.g.*, an SV40 mini-intron) and derived from immunoglobulins (*e.g.*, human β -globin gene). Exogenous intron sequences can be incorporated between a promoter/enhancer sequence and the antigen(s) sequence. Exogenous intron sequences for use in expression vectors are described in more detail in Callendret et al. (Virology. 2007 Jul 5; 363(2): 288–302), herein incorporated by reference for all purposes. Polynucleotide sequences can be optimized to improve transcript stability, for example through removal of RNA instability motifs (*e.g.*, AU-rich elements and 3' UTR motifs) and/or repetitive nucleotide sequences. Polynucleotide sequences can be optimized to improve accurate transcription, for example through removal of cryptic transcriptional initiators and/or terminators. Polynucleotide sequences can be optimized to improve translation and translational accuracy, for example through removal of cryptic AUG start codons, premature polyA sequences, and/or secondary structure motifs. Polynucleotide sequences can be optimized to improve nuclear export of transcripts, such as through addition of a Constitutive Transport Element (CTE), RNA Transport Element (RTE), or Woodchuck Posttranscriptional Regulatory Element (WPRE). Nuclear export signals for use in expression vectors are described in more detail in Callendret et al. (Virology. 2007 Jul 5; 363(2): 288–302), herein incorporated by reference for all purposes. Polynucleotide sequences can be optimized with respect to GC content, for example to reflect the average GC content of a given organism. Sequence optimization can balance one or more sequence properties, such as transcription, translation, post-transcriptional processing, and/or RNA stability. Sequence optimization can generate an optimal sequence balancing each of transcription, translation, post-transcriptional processing, and RNA stability. Sequence optimization algorithms are known to those of skill in the art, such as GeneArt (Thermo Fisher), Codon Optimization Tool (IDT), Cool Tool (University of Singapore), SGI-DNA (La Jolla California). One or more regions of an antigen-encoding protein can be sequence-optimized separately.

[00242] 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

[00243] 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 or an infectious disease organism-specific immune response. Vaccine compositions typically comprise one or a plurality of antigens, e.g., selected using a method described herein, or selected from a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and/or a parasite-derived peptide. Vaccine compositions can also be referred to as vaccines.

[00244] 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.

[00245] A vaccine can contain between 1 and 30 antigen-encoding nucleic acid 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-encoding nucleic acid sequences, 6, 7, 8, 9, 10, 11, 12, 13, or 14 different antigen-encoding nucleic acid sequences, or 12, 13 or 14 different antigen-encoding nucleic acid sequences. Antigen-encoding nucleic acid sequences can refer to the antigen encoding portion of an “antigen cassette.” Features of an antigen cassette are described in greater detail herein. An antigen-encoding nucleic acid sequence can contain one or more epitope-encoding nucleic acid sequences (*e.g.*, an antigen-encoding nucleic acid sequence encoding concatenated T cell epitopes).

[00246] A vaccine can contain between 1 and 30 distinct epitope-encoding nucleic acid 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 distinct epitope-encoding nucleic acid sequences, 6, 7, 8, 9, 10, 11, 12, 13, or 14 distinct epitope-encoding nucleic acid sequences, or 12, 13 or 14 distinct epitope-encoding nucleic acid sequences. Epitope-encoding nucleic acid sequences can refer to sequences for individual epitope sequences, such as each of the T cell epitopes in an antigen-encoding nucleic acid sequence encoding concatenated T cell epitopes.

[00247] A vaccine can contain at least two repeats of an epitope-encoding nucleic acid sequence. As used herein, a “repeat” refers to two or more iterations of an identical nucleic acid epitope-encoding nucleic acid sequence (inclusive of the optional 5’ linker sequence and/or the optional 3’ linker sequences described herein) within an antigen-encoding nucleic acid sequence. In one example, the antigen-encoding nucleic acid sequence portion of a cassette encodes at least two repeats of an epitope-encoding nucleic acid sequence. In further non-limiting examples, the antigen-encoding nucleic acid sequence portion of a cassette encodes more than one distinct epitope, and at least one of the distinct epitopes is encoded by at least two repeats of the nucleic acid sequence encoding the distinct epitope (*i.e.*, at least two distinct epitope-encoding nucleic acid sequences). In illustrative non-limiting examples, an antigen-encoding nucleic acid sequence encodes epitopes A, B, and C encoded by epitope-encoding nucleic acid sequences epitope-encoding sequence A (E_A), epitope-encoding sequence B (E_B), and epitope-encoding sequence C (E_C), and exemplary antigen-encoding nucleic acid

sequences having repeats of at least one of the distinct epitopes are illustrated by, but is not limited to, the formulas below:

- Repeat of one distinct epitope (repeat of epitope A):

$E_A-E_B-E_C-E_A$; or

$E_A-E_A-E_B-E_C$

- Repeat of multiple distinct epitopes (repeats of epitopes A, B, and C):

$E_A-E_B-E_C-E_A-E_B-E_C$; or

$E_A-E_A-E_B-E_B-E_C-E_C$

- Multiple repeats of multiple distinct epitopes (repeats of epitopes A, B, and C):

$E_A-E_B-E_C-E_A-E_B-E_C-E_A-E_B-E_C$; or

$E_A-E_A-E_A-E_B-E_B-E_B-E_C-E_C-E_C$

[00248] The above examples are not limiting and the antigen-encoding nucleic acid sequences having repeats of at least one of the distinct epitopes can encode each of the distinct epitopes in any order or frequency. For example, the order and frequency can be a random arrangement of the distinct epitopes, *e.g.*, in an example with epitopes A, B, and C, by the formula $E_A-E_B-E_C-E_C-E_A-E_B-E_A-E_C-E_A-E_C-E_C-E_B$.

[00249] Also provided for herein is an antigen-encoding cassette, the antigen-encoding cassette having at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$

where E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,

n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,

E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,

for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and

z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

[00250] Each E or E^N can independently comprise any epitope-encoding nucleic acid sequence described herein (*e.g.*, a peptide encoding an infectious disease T cell epitope and/or a neoantigen epitope). For example, Each E or E^N can independently comprises a nucleotide sequence described, from 5' to 3', by the formula (L5_b-N_c-L3_d), where N comprises the distinct

epitope-encoding nucleic acid sequence associated with each E or E^N, where c = 1, L5 comprises a 5' linker sequence, where b = 0 or 1, and L3 comprises a 3' linker sequence, where d = 0 or 1. Epitopes and linkers that can be used are further described herein.

[00251] Repeats of an epitope-encoding nucleic acid sequences (inclusive of optional 5' linker sequence and/or the optional 3' linker sequences) can be linearly linked directly to one another (*e.g.*, E_A-E_A-... as illustrated above). Repeats of an epitope-encoding nucleic acid sequences can be separated by one or more additional nucleotides sequences. In general, repeats of an epitope-encoding nucleic acid sequences can be separated by any size nucleotide sequence applicable for the compositions described herein. In one example, repeats of an epitope-encoding nucleic acid sequences can be separated by a separate distinct epitope-encoding nucleic acid sequence (*e.g.*, E_A-E_B-E_C-E_A..., as illustrated above). In examples where repeats are separated by a single separate distinct epitope-encoding nucleic acid sequence, and each epitope-encoding nucleic acid sequences (inclusive of optional 5' linker sequence and/or the optional 3' linker sequences) encodes a peptide 25 amino acids in length, the repeats can be separated by 75 nucleotides, such as in antigen-encoding nucleic acid represented by E_A-E_B-E_A..., E_A is separated by 75 nucleotides. In an illustrative example, an antigen-encoding nucleic acid having the sequence

VTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDT encoding repeats of 25mer antigens Trp1 (VTNTEMFVTAPDNLGYMYEVQWPGQ) and Trp2

(TQPQIANCSVYDFFVWLHYYSVRDT), the repeats of Trp1 are separated by the 25mer Trp2 and thus the repeats of the Trp1 epitope-encoding nucleic acid sequences are separated the 75 nucleotide Trp2 epitope-encoding nucleic acid sequence. In examples where repeats are separated by 2, 3, 4, 5, 6, 7, 8, or 9 separate distinct epitope-encoding nucleic acid sequence, and each epitope-encoding nucleic acid sequences (inclusive of optional 5' linker sequence and/or the optional 3' linker sequences) encodes a peptide 25 amino acids in length, the repeats can be separated by 150, 225, 300, 375, 450, 525, 600, or 675 nucleotides, respectively.

[00252] 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.

[00253] The vaccine composition can be capable of stimulating a specific cytotoxic T-cell response and/or a specific helper T-cell response. The vaccine composition can be capable of stimulating a specific cytotoxic T-cell response and a specific helper T-cell response.

[00254] The vaccine composition can be capable of stimulating a specific B-cell response (*e.g.*, an antibody response).

[00255] The vaccine composition can be capable of stimulating a specific cytotoxic T-cell response, a specific helper T-cell response, and/or a specific B-cell response. The vaccine composition can be capable of stimulating a specific cytotoxic T-cell response and a specific B-cell response. The vaccine composition can be capable of stimulating a specific helper T-cell response and a specific B-cell response. The vaccine composition can be capable of stimulating a specific cytotoxic T-cell response, a specific helper T-cell response, and a specific B-cell response.

[00256] 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.

[00257] Adjuvants are any substance whose admixture into a vaccine composition increases or otherwise modifies the immune response to an 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.

[00258] 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.

[00259] Suitable adjuvants include, but are not limited to 1018 ISS, alum, aluminum 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).

[00260] 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.

[00261] 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).

[00262] 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.

[00263] 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.

[00264] 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.

[00265] 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 stimulate 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

[00266] The methods employed for the selection of one or more antigens, the cloning and construction of an “antigen cassette” and its insertion into a viral vector are within the skill in the art given the teachings provided herein. By “antigen cassette” or “cassette” is meant the combination of a selected antigen or plurality of antigens (e.g., antigen-encoding nucleic acid sequences) and the other regulatory elements necessary to transcribe the antigen(s) and express the transcribed product. The selected antigen or plurality of antigens can refer to distinct epitope sequences, e.g., an antigen-encoding nucleic acid sequence in the cassette can encode an epitope-encoding nucleic acid sequence (or plurality of epitope-encoding nucleic acid sequences) such that the epitopes are transcribed and expressed. An 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. A cassette can include one or more antigens, such as one or more pathogen-derived peptides, virus-derived peptides, bacteria-derived peptides, fungus-derived peptides, parasite-derived peptides, and/or tumor-derived peptides. A cassette can have one or more antigen-encoding nucleic acid sequences, such as a cassette containing multiple antigen-encoding nucleic acid sequences each independently operably linked to separate promoters and/or linked together

using other multicistronic systems, such as 2A ribosome skipping sequence elements (*e.g.*, E2A, P2A, F2A, or T2A sequences) or Internal Ribosome Entry Site (IRES) sequence elements. A linker can also have a cleavage site, such as a TEV or furin cleavage site. Linkers with cleavage sites can be used in combination with other elements, such as those in a multicistronic system. In a non-limiting illustrative example, a furin protease cleavage site can be used in conjunction with a 2A ribosome skipping sequence element such that the furin protease cleavage site is configured to facilitate removal of the 2A sequence following translation. In a cassette containing more than one antigen-encoding nucleic acid sequences, each antigen-encoding nucleic acid sequence can contain one or more epitope-encoding nucleic acid sequences (*e.g.*, an antigen-encoding nucleic acid sequence encoding concatenated T cell epitopes).

[00267] 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.

[00268] 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. An 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.

[00269] An 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.

[00270] As described elsewhere herein, the antigen cassette can be located in the site of any selected deletion in a viral vector, such as the deleted structural proteins of a VEE backbone or the site of the E1 gene region deletion or E3 gene region deletion of a ChAd-based vector, among others which may be selected.

[00271] The antigen cassette can be described using the following formula to describe the ordered sequence of each element, from 5' to 3':

$$(P_a-(L5_b-N_c-L3_d)_X)_Z-(P2_h-(G5_e-U_f)_Y)_W-G3_g$$

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 a MHC class II epitope-encoding nucleic acid sequence (*e.g.*, universal MHC class II epitope-encoding nucleic acid sequence). A universal sequence can comprise at least one of Tetanus toxoid and PADRE. A universal sequence can comprise a Tetanus toxoid peptide. A universal sequence can comprise a PADRE peptide. A universal sequence can comprise a Tetanus toxoid and PADRE peptides. 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.

[00272] 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 (*e.g.*, only the promoter nucleotide sequence provided by a vector backbone, such as an RNA alphavirus backbone, is present), 10 MHC class I epitopes 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.*, an RNA alphavirus backbone). Examples of linking the 3' end of the antigen cassette to a vector backbone (*e.g.*, an RNA alphavirus backbone) include linking directly to the 3' UTR elements

provided by the vector backbone, such as a 3' 19-nt CSE. Examples of linking the 5' end of the antigen cassette to a vector backbone (*e.g.*, an RNA alphavirus backbone) include linking directly to a promoter or 5' UTR element of the vector backbone, such as a subgenomic promoter sequence (*e.g.*, a 26S subgenomic promoter sequence), an alphavirus 5' UTR, a 51-nt CSE, or a 24-nt CSE.

[00273] Other examples include: where $a = 1$ describing where a promoter other than the promoter nucleotide sequence provided by a vector backbone (*e.g.*, an RNA alphavirus backbone) is present; where $a = 1$ and Z is greater than 1 where multiple promoters other than the promoter nucleotide sequence provided by the vector 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 epitope-encoding nucleic acid sequences; and where $g = 0$ describing the MHC class II epitope-encoding nucleic acid sequence, if present, is directly linked to a vector backbone (*e.g.*, an RNA alphavirus backbone).

[00274] 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.

[00275] 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.

[00276] Other examples include where each antigen that is present can have a 5' linker, a 3' linker, neither, or both. In examples where more than one antigen is present in the same antigen cassette, some antigens may have both a 5' linker and a 3' linker, while other antigens may have either a 5' linker, a 3' linker, or neither. In other examples where more than one antigen is present in the same antigen cassette, some antigens may have either a 5' linker or a 3' linker, while other antigens may have either a 5' linker, a 3' linker, or neither.

[00277] The promoter nucleotide sequences P and/or P2 can be the same as a promoter nucleotide sequence provided by a vector backbone, such as an RNA alphavirus backbone. For example, the promoter sequence provided by the vector backbone, Pn and P2, can each comprise a subgenomic promoter sequence (*e.g.*, a 26S subgenomic promoter sequence) or a CMV promoter. The promoter nucleotide sequences P and/or P2 can be different from the promoter nucleotide sequence provided by a vector backbone (*e.g.*, an RNA alphavirus backbone), as well as can be different from each other.

[00278] 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.

[00279] 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.

[00280] 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.

[00281] For each X, each N can encode a MHC class I epitope, a MHC class II epitope, an epitope/antigen capable of stimulating a B cell response, or a combination thereof. For each X, each N can encode a combination of a MHC class I epitope, a MHC class II epitope, and an epitope/antigen capable of stimulating a B cell response. For each X, each N can encode a combination of a MHC class I epitope and a MHC class II epitope. For each X, each N can encode a combination of a MHC class I epitope and an epitope/antigen capable of stimulating a B cell response. For each X, each N can encode a combination of a MHC class II epitope and an epitope/antigen capable of stimulating a B cell response. For each X, each N can encode a MHC class II epitope. For each X, each N can encode an epitope/antigen capable of stimulating a B cell response. For each X, each N can encode 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.

[00282] The cassette encoding the one or more antigens can be 700 nucleotides or less. The cassette encoding the one or more antigens can be 700 nucleotides or less and encode 2 distinct epitope-encoding nucleic acid sequences (*e.g.*, encode 2 distinct infectious disease or tumor derived nucleic acid sequences encoding an immunogenic polypeptide). The cassette encoding the one or more antigens can be 700 nucleotides or less and encode at least 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 700 nucleotides or less and encode 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 700 nucleotides or less and encode at least 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 700 nucleotides or less and include 1-10, 1-5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antigens.

[00283] The cassette encoding the one or more antigens can be between 375-700 nucleotides in length. The cassette encoding the one or more antigens can be between 375-700 nucleotides

in length and encode 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-700 nucleotides in length and encode at least 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-700 nucleotides in length and encode 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens be between 375-700 nucleotides in length and encode at least 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-700 nucleotides in length and include 1-10, 1-5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antigens.

[00284] The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less. The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less and encode 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less and encode at least 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less and encode 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less and encode at least 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be 600, 500, 400, 300, 200, or 100 nucleotides in length or less and include 1-10, 1-5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antigens.

[00285] The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length. The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length and encode 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length and encode at least 2 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length and encode 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length and encode at least 3 distinct epitope-encoding nucleic acid sequences. The cassette encoding the one or more antigens can be between 375-600, between 375-500, or between 375-400 nucleotides in length and include 1-10, 1-5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antigens.

V.B. Immune Modulators

[00286] 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. An immune modulator can include a binding molecule (e.g., an antibody such as an scFv) which binds to and blocks the activity of an immune checkpoint molecule. An immune modulator can include a cytokine, such as IL-2, IL-7, IL-12 (including IL-12 p35, p40, p70, and/or p70-fusion constructs), IL-15, or IL-21. An immune modulator can include a modified cytokine (e.g., pegIL-2). Vectors can comprise an antigen cassette and one or more nucleic acid molecules encoding an immune modulator.

[00287] 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.

V.C. Additional Considerations for Vaccine Design and Manufacture

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

Subclones

[00288] Truncal peptides, meaning those presented by all or most tumor subclones, can be prioritized for inclusion into a vaccine. 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 a vaccine.

V.C.2. Antigen Prioritization

[00289] 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, an infectious disease, and/or an infected cell 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 or infectious disease escape)

[00290] 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 or infected cell. 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

[00291] 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).

[00292] 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.

[00293] 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). Encapsidation 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

[00294] 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 mRNA (SAM) 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 stimulate 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 an antigen expression vector described herein can utilize an alphavirus backbone that allows for a high level of antigen expression, stimulates a robust immune response to antigen, does not stimulate an immune response to the vector itself, and can be used in a safe manner. Furthermore, the antigen expression cassette can be designed to stimulate 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.

[00295] 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.

[00296] 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 an antigen expression vector described herein can utilize an alphavirus backbone wherein the structural proteins are replaced by an 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*

[00297] 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 (*e.g.*, chemical and/or enzymatic 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 or column purification (*e.g.*, chromatography-based purification).

V.D.4. Delivery via lipid nanoparticle

[00298] 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.

[00299] 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.

[00300] 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.

[00301] 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.

[00302] 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

[00303] Vaccine compositions for delivery of one or more antigens (e.g., via an antigen cassette) 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. ChAdV68-based vectors and delivery systems are described in detail in US App. Pub. No. US20200197500A1 and international patent application publication WO2020243719A1, each of which is herein incorporated by reference for all purposes.

[00304] In a further aspect, provided herein is a recombinant adenovirus comprising the DNA sequence of a chimpanzee adenovirus such as C68 and an 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. An antigen cassette can be inserted into any of these sites of gene deletion. The antigen cassette can include an antigen against which a primed immune response is desired.

[00305] In another aspect, provided herein is a mammalian cell infected with a chimpanzee adenovirus such as C68.

[00306] 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.

[00307] In still a further aspect, provided herein is a method for delivering an 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.

[00308] Still another aspect provides a method for stimulating 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 an antigen cassette that encodes one or more antigens from the tumor against which the immune response is targeted.

[00309] Still another aspect provides a method for stimulating an immune response in a mammalian host to treat or prevent a disease in a subject, such as an infectious disease. The method can comprise the step of administering to the host an effective amount of a recombinant chimpanzee adenovirus, such as C68, comprising an antigen cassette that encodes one or more antigens, such as from the infectious disease against which the immune response is targeted.

[00310] 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.

[00311] 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.

[00312] Also disclosed is a vector comprising a chimpanzee adenovirus DNA sequence obtained from SEQ ID NO: 1 and an 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.

[00313] Also disclosed herein is an adenovirus vector comprising: a partially deleted E4 gene comprising a deleted or partially-deleted E4orf2 region and a deleted or partially-deleted E4orf3 region, and optionally a deleted or partially-deleted E4orf4 region. The partially deleted E4 can comprise an E4 deletion of at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1, and wherein the vector comprises at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1. The partially deleted E4 can comprise an E4 deletion of at least a partial deletion of nucleotides 34,916 to 34,942 of the sequence shown in SEQ ID NO:1, at least a partial deletion of nucleotides 34,952 to 35,305 of the sequence shown in SEQ ID NO:1, and at least a partial deletion of nucleotides 35,302 to 35,642 of the sequence shown in SEQ ID NO:1, and wherein the vector comprises at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1. The partially deleted E4 can comprise an E4 deletion of at least nucleotides 34,980 to 36,516 of the sequence shown in SEQ ID NO:1, and wherein the

vector comprises at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1. The partially deleted E4 can comprise an E4 deletion of at least nucleotides 34,979 to 35,642 of the sequence shown in SEQ ID NO:1, and wherein the vector comprises at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1. The partially deleted E4 can comprise an E4 deletion of at least a partial deletion of E4Orf2, a fully deleted E4Orf3, and at least a partial deletion of E4Orf4. The partially deleted E4 can comprise an E4 deletion of at least a partial deletion of E4Orf2, at least a partial deletion of E4Orf3, and at least a partial deletion of E4Orf4. The partially deleted E4 can comprise an E4 deletion of at least a partial deletion of E4Orf1, a fully deleted E4Orf2, and at least a partial deletion of E4Orf3. The partially deleted E4 can comprise an E4 deletion of at least a partial deletion of E4Orf2 and at least a partial deletion of E4Orf3. The partially deleted E4 can comprise an E4 deletion between the start site of E4Orf1 to the start site of E4Orf5. The partially deleted E4 can be an E4 deletion adjacent to the start site of E4Orf1. The partially deleted E4 can be an E4 deletion adjacent to the start site of E4Orf2. The partially deleted E4 can be an E4 deletion adjacent to the start site of E4Orf3. The partially deleted E4 can be an E4 deletion adjacent to the start site of E4Orf4. The E4 deletion can be at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1000, at least 1100, at least 1200, at least 1300, at least 1400, at least 1500, at least 1600, at least 1700, at least 1800, at least 1900, or at least 2000 nucleotides. The E4 deletion can be at least 700 nucleotides. The E4 deletion can be at least 1500 nucleotides. The E4 deletion can be 50 or less, 100 or less, 200 or less, 300 or less, 400 or less, 500 or less, 600 or less, 700 or less, 800 or less, 900 or less, 1000 or less, 1100 or less, 1200 or less, 1300 or less, 1400 or less, 1500 or less, 1600 or less, 1700 or less, 1800 or less, 1900 or less, or 2000 or less nucleotides. The E4 deletion can be 750 nucleotides or less. The E4 deletion can be at least 1550 nucleotides or less.

[00314] The partially deleted E4 gene can be the E4 gene sequence shown in SEQ ID NO:1 that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1. The partially deleted E4 gene can be the E4 gene sequence shown in SEQ ID NO:1 that lacks the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,916 to 34,942, nucleotides 34,952 to 35,305 of the sequence shown in SEQ ID NO:1, and nucleotides 35,302 to 35,642 of the sequence shown in SEQ ID NO:1. The partially deleted E4 gene can be the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,980 to 36,516 of the sequence shown in SEQ ID NO:1. The partially deleted E4 gene can be the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,979 to 35,642 of the

sequence shown in SEQ ID NO:1. The adenovirus vector having the partially deleted E4 gene can have a cassette, wherein the cassette comprises at least one payload nucleic acid sequence, and wherein the cassette comprises at least one promoter sequence operably linked to the at least one payload nucleic acid sequence. The adenovirus vector having the partially deleted E4 gene can have one or more genes or regulatory sequences of the ChAdV68 sequence shown in SEQ ID NO: 1, optionally wherein the one or more genes or regulatory sequences comprise at least one of the chimpanzee adenovirus inverted terminal repeat (ITR), E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence shown in SEQ ID NO: 1. The adenovirus vector having the partially deleted E4 gene can have nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1, wherein the partially deleted E4 gene is 3' of the nucleotides 2 to 34,916, and optionally the nucleotides 2 to 34,916 additionally lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion and/or lack nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion. The adenovirus vector having the partially deleted E4 gene can have nucleotides 35,643 to 36,518 of the sequence shown in SEQ ID NO:1, and wherein the partially deleted E4 gene is 5' of the nucleotides 35,643 to 36,518. The adenovirus vector having the partially deleted E4 gene can have nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1, wherein the partially deleted E4 gene is 3' of the nucleotides 2 to 34,916, the nucleotides 2 to 34,916 additionally lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion and lack nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion. The adenovirus vector having the partially deleted E4 gene can have nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1, wherein the partially deleted E4 gene is 3' of the nucleotides 2 to 34,916, the nucleotides 2 to 34,916 additionally lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion and lack nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion, and have nucleotides 35,643 to 36,518 of the sequence shown in SEQ ID NO:1, and wherein the partially deleted E4 gene is 5' of the nucleotides 35,643 to 36,518.

[00315] The partially deleted E4 gene can be the E4 gene sequence shown in SEQ ID NO:1 that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1, nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1, wherein the partially deleted E4 gene is 3' of the nucleotides 2 to 34,916, the nucleotides 2 to 34,916 additionally lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1

deletion and lack nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion, and have nucleotides 35,643 to 36,518 of the sequence shown in SEQ ID NO:1, and wherein the partially deleted E4 gene is 5' of the nucleotides 35,643 to 36,518.

[00316] Also disclosed herein is a host cell transfected with a vector disclosed herein such as a C68 vector engineered to expression an 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.

[00317] Also disclosed herein is a method for delivering an 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.

[00318] Also disclosed herein is a method for producing an 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

[00319] 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.

[00320] 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.

[00321] 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.

[00322] 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.

[00323] 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

[00324] 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 an 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. An 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.

[00325] 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.

[00326] 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

[00327] 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].

[00328] In the construction of useful chimpanzee adenovirus C68 vectors for delivery of an 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

[00329] 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

[00330] 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.

[00331] 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.

[00332] 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.

[00333] 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.

[00334] 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.

[00335] 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.

[00336] 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

[00337] 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.

[00338] 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.

[00339] 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.

[00340] 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

[00341] 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.

[00342] 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.

[00343] The resulting recombinant chimpanzee C68 adenoviruses are useful in transferring an 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

[00344] 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.

[00345] The above-described recombinant vectors are administered to humans according to published methods for gene therapy. A chimpanzee viral vector bearing an 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.

[00346] 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.

[00347] 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.

[00348] 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 an 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.

[00349] 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.

[00350] 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

[00351] Also provided is a method of stimulating 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.

[00352] Also provided is a method of stimulating an infectious disease organism-specific immune response in a subject, vaccinating against an infectious disease organism, treating and/or alleviating a symptom of an infection associated with an infectious disease organism in a subject by administering to the subject one or more antigens such as a plurality of antigens identified using methods disclosed herein.

[00353] 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.

[00354] In some aspects, a subject has been diagnosed with an infection or is at risk of an infection, such as age, geographical/travel, and/or work-related increased risk of or predisposition to an infection, or at risk to a seasonal and/or novel disease infection.

[00355] An antigen can be administered in an amount sufficient to stimulate a CTL response. An antigen can be administered in an amount sufficient to stimulate a T cell response. An antigen can be administered in an amount sufficient to stimulate a B cell response.

[00356] An antigen can be administered alone or in combination with other therapeutic agents. Therapeutic agents can include those that target an infectious disease organism, such as an anti-viral or antibiotic agent.

[00357] 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.

[00358] The optimum amount of each antigen to be included in a vaccine composition and the optimum dosing regimen can be determined. For example, an 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.

[00359] A vaccine can be compiled so that the selection, number and/or amount of antigens present in the composition is/are tissue, cancer, infectious disease, 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 or disease status of a patient. The selection can be dependent on the specific type of cancer, the specific infectious disease (*e.g.* a specific infectious disease isolate/strain the subject is infected with or at risk for infection by), the status of the disease, the goal of the vaccination (*e.g.*, preventative or targeting an ongoing 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.

[00360] 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. Patient selection can involve identifying the infectious disease of an ongoing infection. Patient selection can involve identifying risk of an infection by an infectious disease. 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.

[00361] For a composition to be used as a vaccine for cancer or an infectious disease, 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 or infected cell of a patient expresses high amounts of a certain antigen, the respective pharmaceutical composition for treatment of this cancer or infection can be present in high amounts and/or more than one antigen specific for this particularly antigen or pathway of this antigen can be included.

[00362] Compositions comprising an antigen can be administered to an individual already suffering from cancer or an infection. In therapeutic applications, compositions are administered to a patient in an amount sufficient to stimulate an effective CTL response to the tumor antigen or infectious disease organism 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 a cancer has metastasized or an infectious disease organism has induced organ damage and/or other immune pathology. 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.

[00363] For therapeutic use, administration can begin at the detection or surgical removal of tumors, or begin at the detection or treatment of an infection. This can be followed by boosting doses until at least symptoms are substantially abated and for a period thereafter, or immunity is considered to be provided (e.g., a memory B cell or T cell population, or antigen specific B cells or antibodies are produced).

[00364] The pharmaceutical compositions (e.g., vaccine compositions) for therapeutic treatment are intended for parenteral, topical, nasal, oral or local administration. A pharmaceutical compositions can be administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. The compositions can be administered at a site of surgical excision to stimulate a local immune response to a tumor. The compositions can be administered to target specific infected tissues and/or cells of a subject. 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.

[00365] 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.

[00366] 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.

[00367] 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.

[00368] 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).

[00369] 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 stimulate 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.

[00370] 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.

[00371] 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.

[00372] Also disclosed is a method of manufacturing a vaccine, comprising performing the steps of a method disclosed herein; and producing a vaccine comprising a plurality of antigens or a subset of the plurality of antigens.

[00373] 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.

[00374] 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 an 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

[00375] 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.

[00376] A priming vaccine, can be based on C68 (e.g., the sequences shown in SEQ ID NO:1 or 2) or SAM (e.g., the sequences shown in SEQ ID NO:3 or 4). A boosting vaccine can also be based on C68 (e.g., the sequences shown in SEQ ID NO:1 or 2) or SAM (e.g., the sequences shown in SEQ ID NO:3 or 4).

[00377] Each vector in a prime/boost strategy typically includes a cassette that includes antigens. Cassettes can include about 1-50 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) an immune modulator. 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. Each vaccine dose can be administered to the subject in conjunction with (e.g., concurrently, before, or after) a cytokine, such as IL-2, IL-7, IL-12 (including IL-12 p35, p40, p70, and/or p70-fusion constructs), IL-15, or IL-21. Each vaccine dose can be administered to the subject in conjunction with (e.g., concurrently, before, or after) a modified cytokine (e.g., pegIL-2).

[00378] 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 SAM vectors 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 SAM vectors at high vaccine dose selected from the range 1 to 100 ug RNA, in particular 10 or 100 ug can be used.

[00379] 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 SAM vectors 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 SAM vectors at high vaccine dose selected from the range 1 to 100 ug RNA, in particular 10 or 100 ug can be used.

[00380] 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 SAM 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.

[00381] 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.

[00382] Immune monitoring can be performed before, during, and/or after vaccine administration. Such monitoring can inform safety and efficacy, among other parameters.

[00383] 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).

[00384] Immune responses, such as T cell responses and B cell responses, can be assessed as part of an immune monitoring protocol. For example, the ability of a vaccine composition described herein to stimulate an immune response can be monitored and/or assessed. As used herein, "stimulate an immune response" refers to any increase in a immune response, such as

initiating an immune response (*e.g.*, a priming vaccine stimulating the initiation of an immune response in a naïve subject) or enhancement of an immune response (*e.g.*, a boosting vaccine stimulating the enhancement of an immune response in a subject having a pre-existing immune response to an antigen, such as a pre-existing immune response initiated by a priming vaccine). 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- γ , 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- γ , 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.

[00385] B cell responses can be measured using one or more methods known in the art such as assays used to determine B cell differentiation (*e.g.*, differentiation into plasma cells), B cell or plasma cell proliferation, B cell or plasma cell activation (*e.g.*, upregulation of costimulatory markers such as CD80 or CD86), antibody class switching, and/or antibody production (*e.g.*, an ELISA). Antibodies can also be assessed for function, such as assessed for neutralizing ability.

VIII. Isolation and Detection of HLA Peptides

[00386] 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.

[00387] 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
SPVL3	Class II – HLA - DQ
B7/21	Class II – HLA - DP

[00388] 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. HLA IPs can also be performed in 96well plate format using plates that contain filter bottoms. Use of the plates allows for multiple IPs to be performed in tandem.

[00389] 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.

[00390] 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

[00391] 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}).

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

[00392] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1 and US20110293637, and international patent application publications WO/2018/195357, WO/2018/208856, and WO2016187508, each herein incorporated by reference, in their entirety, for all purposes.

X. Training Module

[00393] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, and international patent

application publications 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

[00394] 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, infected cells patients, or infectious disease organisms themselves. 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 are pathogen-derived peptides, virally-derived peptides, bacterially-derived peptides, fungally-derived peptides, and parasitically-derived peptides, such as by comparing sequence data extracted from normal tissue cells of a patient with the sequence data extracted from infected cells of the patient to identify portions containing one or more infectious disease organism associated antigens. 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. A prediction module may identify candidate antigens that are expressed in an infected cell or infected 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 infected tissue cells of the patient to identify expressed candidate antigens (*e.g.*, identifying expressed polynucleotides and/or polypeptides specific to an infectious disease).

[00395] 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 or infected cell 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 stimulate immune responses.

XI.B.Cassette Design Module

XI.B.1 Overview

[00396] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, and international patent application publications WO/2018/195357 and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00397] 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.

[00398] 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.

[00399] 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.

[00400] 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

[00401] 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.

[00402] 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.

[00403] 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.

[00404] 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.

[00405] 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.

[00406] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, and international patent application publications WO/2018/195357 and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

[00407] Shared (neo)antigen sequences for inclusion in a shared antigen vaccine and appropriate patients for treatment with such vaccine can be chosen by one of skill in the art, *e.g.*, as described in US App. No. 17/058,128, herein incorporated by reference for all purposes. Mass spectrometry (MS) validation of candidate shared (neo)antigens can be performed as part of the selection process.

XIII. Example Computer

[00408] 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 US Pat No. 10,055,540, US Application Pub. No. US20200010849A1, and international patent application publications WO/2018/195357 and WO/2018/208856, each herein incorporated by reference, in their entirety, for all purposes.

XIV. Examples

[00409] 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.

[00410] 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. Evaluation of Cassettes with Repeated Epitopes or Short Inserts Overview

[00411] Through vaccination, multiple class I MHC restricted antigens that stimulate the corresponding cellular immune response(s) can be delivered. Several vaccine cassettes were engineered to encode multiple epitopes as a single gene product where the epitopes were embedded within their natural, surrounding peptide sequence. Various cassettes designs feature multiple copies of one or more epitopes. Various cassettes designs feature short cassettes of less than 700 nucleotides encoding two or more epitopes.

XVI.B. Repeated Epitope and Short Cassette Evaluation Materials and Methods

Antigens

[00412] The epitope-containing 25mer amino acid sequences (*i.e.*, epitope flanked by native N and C terminal amino acid linkers) used in the examples below are presented in **Table 2A**. were used as described below. The antigen-encoding sequences of the cassettes for the various constructs were constructed by directly linking each 25mer sequence to each other (*i.e.*, no additional amino acids in between consecutive 25mer sequences) in the order and number

described in the examples below, *e.g.*, see **FIGs. 1A, 2A, 3A, and 4A**. The cassettes containing the full-length antigen-encoding sequences containing the multiple distinct epitopes linked together, as well as universal MHC class II antigens tetanus toxoid and PADRE (bolded sequence), are presented in **Table 2B**. The complete exogenous nucleotide insertions into the vectors include from 5' to 3': a Kozak sequence (GCCACC), nucleotides encoding three amino acids MAG (ATGGCCGGG), one of the cassette sequences of **Table 2B**, and two stop codons (TAATGA).

Table 2A – 25mer Epitope Containing Sequences

Antigen Name	25mer Sequence	SEQ ID NO:
Dpagt1	GQSLVISASIIVFNLLELEGDYRDD	57
Adpgk	GIPVHLELASMTNMELMSSIVHQQV	58
Reps1	RVLELFRAAQLANDVVLQIMELCGA	59
gp100	ALLAVGALEGPRNQDWLGVPRLVT	60
Trp1	VTNTEMFVTAPDNLGYMYEVQWPGQ	61
Trp2	TQPQIANCSVYDFFVWLHYYSVRDT	62

Table 2B – Full-Length Multi-Epitope Sequences in Cassettes

1X Dpagt1/Adpgk/Reps1/gp100/Trp2 Cassette (SEQ ID NO: 63)
MFQALSEGCTPYDINQMLNVLGDHGVSGLEQLESIIINFEKLTWTSSNVMPILSPLTK GILGFVFTLTVPSEGLSCISEADATTPESANLGEEILSQLYLWPRVTYHSPSYAYHQF ERRAKYKRHFPGFGQSLLFGYPVYVFGDCVQGDWDAIRFRYCAPPGYALLRCNDT NYSALLAVGALEGPRNQDWLGVPRLVTRMQAIQNAGLCTLVAMLEETIFWLQAF LMALTDSPGPKTNIIVDSQYVMGISKPSFQEFVDWENVSPELNSTDQPFWQAGILARN LVPMVATVQGQNLKYQGQSLVISASIIVFNLLELEGDYRDDGDNVWVHTPLSPRTL N AWVKAVEEKKGIPVHLELASMTNMELMSSIVHQQVVRTYGPVFMCLGGLLTMVAG AVWLTVRVLELFRAAQLANDVVLQIMELCGAAFRQVCHTTVPWPNASLTPKWNNE TTQPQIANCSVYDFFVWLHYYSVRDTLWPRVTYHMNKYAYHMLERRAKYKR GPG PGAKFVAAWTLKAAAGPGPGQYIKANSKFIGITELGPGPG
6X/7X Dpagt1/Adpgk/Reps1 Cassette (SEQ ID NO: 64)
GQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRA AQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNME LMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYR DDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSL VISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLA NDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELSSI VHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGI PVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISA SIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQV GPGPGAKFVAAWTL KAAAGPGPGQYIKANSKFIGITELGPGPG

5X Dpagt1/Adpgk/Reps1 Cassette (SEQ ID NO: 65)
GQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGAGP GPGAKFVAAWTLKAAAGP GPGQYIKANSKFIGITELGPGPG
1X gp100/Trp1/Trp2 Cassette (SEQ ID NO: 66)
ALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGANEIPFTYEQLSIFKHKLDKTYPQGYSRASLLGPGFVLIWIPALLPALRLSNWHKGWNWSSGHNECPVGASCHPFTKTLLKVSKGQKMNAQAIALVACYLRMFQALSEGCTPYDINQMLNVLGDHQCISEADATTPESANLGEELISQLYWDAIRFRYCAPPGYALLRCNDTNYS AFLMALTD SGPKTNIIVDSQYVMGIGNVWVHTPLSPRTLNAWVKA VEEKKAFRQVCHTTVPWPNASLTPKWNNETWQAGILARNLVPMVATVQGQNLKYQRTYGPVFMCLGGLLTMVAGAVWLTVHFPGFGQSLFLGYPVYVFGDCVQGDPLSPLTKGILGFVFTLTVPSERGL GPGPAKFVAAWTLKAAAGP GPGQYIKANSKFIGITELGPGPG
2X gp100/Trp1/Trp2 Cassette (SEQ ID NO: 67)
ALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTGQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRAAQLANDVVLQIMELCGANEIPFTYEQLSIFKHKLDKTYPQGYSRASLLGPGFVLIWIPALLPALRLSNWHKGWNWSSGHNECPVGASCHPFTKTLLKVSKGQKMNAQAIALVACYLRG GPGPAKFVAAWTLKAAAGP GPGQYIKANSKFIGITELGPGPG
4X gp100/Trp1/Trp2 Cassette (SEQ ID NO: 68)
ALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTPSKPSFQEFVDWENVSPELNSTDQPFLEFKHIKAFDRTFANNP GPMVVFATPGMALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTPSKPSFQEFVDWENVSPELNSTDQPFLEFKHIKAFDRTFANNP GPMVVFATPGMALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTPSKPSFQEFVDWENVSPELNSTDQPFLEFKHIKAFDRTFANNP GPMVVFATPGMAL LAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTPSKPSFQEFVDWENVSPELNSTDQPFLEFKHIKAFDRTFANNP GPMVVFATPGMGPGPAKFVAAWTLKAAAGP GPGQYIKANSKFIGITELGPGPG

6X/7x gp100/Trp1/Trp2 Cassette (SEQ ID NO: 69)
ALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQ IANCSVYDFFVWLHYYSVRDTALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVT APDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTALLAVGALEGPRN QDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWL HYYSVRDTALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQ WPGQTQPQIANCSVYDFFVWLHYYSVRDTALLAVGALEGPRNQDWLGVPRLVTV TNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCSVYDFFVWLHYYSVRDTALLAV GALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQIANCS VYDFFVWLHYYSVRDTALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDN LGMYEVQWPGQGPGPGAKFVAAWTLKAAAGPGPGQYIKANSKFIGITELGPG PG
Short Dpagt1/Adpgk/Reps1 Cassette (SEQ ID NO: 70)
GQSLVISASIIVFNLLELEGDYRDDGIPVHLELASMTNMELMSSIVHQQVRVLELFRA AQLANDVVLQIMELCGAGPGPGAKFVAAWTLKAAAGPGPGQYIKANSKFIGITE LGPGPG
Short gp100/Trp1/Trp2 Cassette (SEQ ID NO: 71)
ALLAVGALEGPRNQDWLGVPRLVTVTNTEMFVTAPDNLGYMYEVQWPGQTQPQ IANCSVYDFFVWLHYYSVRDTGPGPGAKFVAAWTLKAAAGPGPGQYIKANSKFI GITELGPGPG

SAM Vectors

[00413] In one implementation of the present invention, an RNA alphavirus backbone for the antigen expression system was generated from a self-replicating Venezuelan Equine Encephalitis (VEE) virus (Kinney, 1986, Virology 152: 400-413) by deleting the structural proteins of VEE located 3' of the 26S subgenomic promoter, except the last 50 amino acids of E1 (VEE sequences 7544 to 11,175 deleted; numbering based on Kinney *et al* 1986; SEQ ID NO:6). To generate the self-amplifying mRNA ("SAM") vector, the deleted sequences were replaced by antigen sequences. A representative SAM vector containing 20 model antigens is "VEE-MAG25mer" (SEQ ID NO:4). The vectors featuring the antigen cassettes described below having the MAG25mer cassette can be replaced by the antigen cassettes described above.

In vitro transcription to generate SAM

[00414] *For in vivo studies:* SAM vectors were generated and purified by TriLink Biotechnologies. A modified T7 RNA polymerase promoter (TAATACGACTCACTATA), which lacks the canonical 3' dinucleotide GG, was added to the 5' end of the SAM vector to generate the *in vitro* transcription template DNA (SEQ ID NO:57; 7544 to 11,175 deleted without an inserted antigen cassette). Reaction conditions are described below:

- 1x transcription buffer (40 mM Tris, 10 mM dithiothreitol, 2 mM spermidine, 0.002% Triton X-100, and 27 mM magnesium chloride) using final concentrations of 1x T7 RNA polymerase mix (E2040S); 0.025 mg/mL DNA transcription template (linearized by restriction digest); 8 mM CleanCap Reagent AU (Cat. No. N-7114) and 10 mM each of ATP, cytidine triphosphate (CTP), GTP, and uridine triphosphate (UTP)
- Transcription reactions were incubated at 37°C for 2 hr and treated with final 2 U DNase I (AM2239) /0.001 mg DNA transcription template in DNase I buffer for 1 hr at 37°C.
- SAM was purified by RNeasy Maxi (QIAGEN, 75162)

Adenoviral Vectors

[00415] A modified ChAdV68 vector for the antigen expression system 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 (Independently sequenced Full-Length VR-594 C68 SEQ ID NO:10) nucleotides substituted at five positions. The full-length ChAdVC68 AC_000011.1 sequence with corresponding ATCC VR-594 nucleotides substituted at five positions is referred to as “ChAdV68.5WTnt” (SEQ ID NO:1). Antigen cassettes under the control of the CMV promoter/enhancer were inserted in place of deleted E1 sequences. A representative ChAdV68 vector containing 20 model antigens in an antigen cassette is “ChAdV68.5WTnt.MAG25mer” (SEQ ID NO:2). The vectors featuring antigen cassettes described below having the MAG25mer cassette can be replaced by the antigen cassettes described above, e.g., in Table 2B.

Adenoviral Production in 293F cells

[00416] 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 10⁶ 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.

Adenoviral Purification by CsCl centrifugation

[00417] 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.

[00418] 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 gauge 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-LyzerTM 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.

Adenoviral Viral Assays

[00419] 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.

[00420] 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 fluorescent can be used rather than capsid staining to determine the number of GFP expressing virions per mL.

Immunizations

[00421] For SAM vaccines, C57BL/6J mice were injected with 10 ug of RNA-LNP complexes in 100 uL volume, bilateral intramuscular injection (50 uL per leg).

[00422] For ChAdV68 vaccines, C57BL/6J mice were injected with 2×10^8 viral particles (VP) or 1×10^8 VP, as indicated, in 100 uL volume, bilateral intramuscular injection (50 uL per leg).

Splenocyte dissociation

[00423] Splenocytes were isolated 12 days post-immunization. Spleens 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 Cytoflex LX (Beckman Coulter) 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

[00424] 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 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.

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

[00425] Freshly isolated lymphocytes at a density of $2-5 \times 10^6$ cells/mL were incubated with 10uM of the indicated peptides for 2 hours. After two hours, brefeldin A was added to a concentration of 5ug/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 Cytoflex LX (Beckman Coulter). Flow cytometry data was plotted and analyzed using FlowJo. To assess degree of antigen-specific response, the percent IFN γ ⁺ of CD8⁺ cells was calculated in response to each peptide stimulant.

XVI.C. Repeated Epitope Evaluation Results

[00426] Vaccine efficacy with cassettes having repeated epitopes was compared against cassettes having only a single copy of a given epitope. Mice were immunized using the delivery vectors described below and efficacy was assessed by ELISpot.

[00427] As illustrated in **FIG. 1A**, a series of SAM-LNP delivery vectors were designed to assess efficacy of cassettes featuring either a single copy of the antigens Dpagt1, Adpgk, and Repls1 (“1x”; cassette = SEQ ID NO: 63) or 7 repeats of the antigens Dpagt1 and Adpgk together with 6 repeats of the antigen Repls1 (“6x/7x”; cassette = SEQ ID NO: 64). As shown in **FIGs. 1B-D** and presented in **Table 3**, vaccination with vectors featuring multiple repeats of the antigens demonstrated increased spot forming colonies (SFC), indicating repetition of antigen encoding sequences in a cassette leads to an increased antigen-specific immune response using a SAM-LNP delivery system.

Table 3 – ELISpot data for Dpagt1, Adpgk, and Reps1 (1x vs 6x/7x): SAM-LNP

No. Repeats	Antigen	Mean	SD	Median
1x	Adpgk	2841	2193	2779
7x	Adpgk	6054	411	6080
1x	Dpagt1	933	1351	401
7x	Dpagt1	5097	765	5220
1x	Reps1	1499	1372	1700
6x	Reps1	6462	531	6480

[00428] As illustrated in **FIG. 2A**, another series of SAM-LNP delivery vectors were designed to assess efficacy of cassettes featuring either a single copy of the antigens gp100 and Trp2 (“1x”; cassette = SEQ ID NO: 63) or 4 repeats of each antigen (“4x”; cassette = SEQ ID NO: 68). As shown in **FIGs. 2B** and **2C** and presented in **Table 4**, vaccination with vectors featuring multiple repeats of the antigens demonstrated increased spot forming colonies (SFC), indicating repetition of antigen encoding sequences in a cassette leads to an increased antigen-specific immune response using a SAM-LNP delivery system.

Table 4 – ELISpot data for gp100 and Trp2 (1x vs 4x): SAM-LNP

No. Repeats	Antigen	Mean	SD	Median
1x	gp100	1892	1477	1407
4x	gp100	6091	1972	7100
1x	Trp2	185	372	0
4x	Trp2	5535	1366	5868

[00429] As illustrated in **FIG. 3A**, another series of SAM-LNP delivery vectors were designed to assess efficacy of cassettes featuring either a single copy of the antigens gp100, Trp1, and Trp2 (“1x”; cassette = SEQ ID NO: 66), 2 repeats of each antigen (“2x”; cassette = SEQ ID NO: 67), 4 repeats of each antigen (“4x”; cassette = SEQ ID NO: 68), or 7 repeats of the antigens gp100 and Trp1 together with 6 repeats of the antigen Trp2 (“6x/7x”; cassette = SEQ ID NO: 69). As shown in **FIGs. 3B-D** and presented in **Table 5**, vaccination with vectors featuring 2 or 4 repeats of the antigens demonstrated increased spot forming colonies (SFC), with Trp1 and Trp2 demonstrating a further increase from 2x to 4x. However, 6 or 7 repeats did not demonstrate a further SFC increase over the 4x constructs, and in some instances reduced SFC numbers, including at 1x levels (gp100; **FIG. 3B**). The data indicates repetition of antigen encoding sequences in a cassette leads to an increased antigen-specific immune

response using a SAM-LNP delivery system. However, beyond a certain number of repeats, additional repetitions of certain antigens may saturate the benefit.

Table 5 – ELISpot data for gp100, Trp1, and Trp2 (1x, 2x, 4x, 6/7x): SAM-LNP

No. Repeats	Antigen	Mean	SD	Median
1x	gp100	2412	1368	2195
2x	gp100	4249	1343	4031
4x	gp100	3934	1017	3911
7x	gp100	1864	792	1726
1x	Trp1	1128	422	1146
2x	Trp1	2093	793	2153
4x	Trp1	3893	952	4042
7x	Trp1	3533	591	3725
1x	Trp2	591	489	555
2x	Trp2	1061	541	1243
4x	Trp2	1991	880	2055
7x	Trp2	1551	510	1606

[00430] To understand whether the above results with the SAM vector system also occur in a different vector system, a series of ChAdV68 delivery vectors were designed to assess efficacy of cassettes featuring either a single copy of the antigens Dpagt1, Adpgk, and Repl1 (“1x”; cassette = SEQ ID NO: 63), 5 repeats of each antigen (“5x”; cassette = SEQ ID NO: 65), or 7 repeats of the antigens Dpagt1 and Adpgk together with 6 repeats of the antigen Repl1 (“6x/7x”; cassette = SEQ ID NO: 64). *See FIG. 4A.* As shown in **FIGs. 4B-D** and presented in **Table 6**, vaccination with vectors featuring multiple repeats of the antigens demonstrated increased spot forming colonies (SFC). In a further series of experiments, the ChAdV68 vaccination dose was then increased 5-fold to 1e9 VP ChAdV68. As shown in **FIGs. 4E-G** and presented in **Table 7**, vaccination with vectors featuring multiple repeats of the antigens at the higher dose also demonstrated increased spot forming colonies (SFC). The results indicate repetition of antigen encoding sequences in a cassette leads to an increased antigen-specific immune response using a ChAdV68 system. However, beyond a certain number of repeats, additional repetitions of certain antigens may saturate the benefit.

Table 6 – ELISpot data for Dpagt1, Adpgk, and Reps1 (1x, 5x, 6x/7x): ChAdV68 (2e8 VP)

No. Repeats	Antigen	Mean	SD	Median
1x	Adpgk	614	551	403
5x	Adpgk	10902	1713	11504
7x	Adpgk	9569	2185	9316
1x	Dpagt1	85	64	74
5x	Dpagt1	875	788	660
7x	Dpagt1	1232	535	1219
1x	Reps1	19	28	7
5x	Reps1	1109	547	1001
6x	Reps1	1869	1399	1316

Table 7 – ELISpot data for Dpagt1, Adpgk, and Reps1 (1x, 5x, 6x/7x): ChAdV68 (1e9 VP)

No. Repeats	Antigen	Mean	SD	Median
1x	Adpgk	1850	1952	1124
5x	Adpgk	17691	3476	17370
7x	Adpgk	20067	3382	20813
1x	Dpagt1	53	70	30
5x	Dpagt1	2303	1602	1730
7x	Dpagt1	4447	1741	4844
1x	Reps1	37	99	0
5x	Reps1	3696	2381	3081
6x	Reps1	7237	2933	8627

XIV.D. Short Cassette Insert Evaluation Results

[00431] Vaccine efficacy with cassettes having short epitope encoding inserts was compared against cassettes having a larger insert, specifically a shorter 375nt antigen-encoding insert in comparison to a larger antigen-encoding insert of 1,623 nucleotides. Mice were immunized using the delivery vectors described below and efficacy was assessed by IFN γ ICS.

[00432] As illustrated in **FIG. 5A**, a series of delivery vectors were designed to assess efficacy of cassettes featuring either a short insert encoding only single copies of the three distinct antigens Dpagt1, Adpgk, and Reps1 (“SHORT-1”; cassette = SEQ ID NO: 70), a short insert encoding only single copies of the three distinct antigens gp100, Trp1, and Trp2 (“SHORT-2”; cassette = SEQ ID NO: 71), or a longer insert encoding only single copies of 20 distinct antigens, including Dpagt1, Adpgk, Reps1, gp100, and Trp2 (“1x”; cassette = SEQ ID NO: 63).

[00433] A series of SAM-LNP constructs containing the short cassettes or longer cassettes (see **FIG. 5A**) were evaluated. As shown in **FIGs. 5B** and presented in **Table 8**, vaccination with vectors featuring shorter antigen encoding inserts demonstrated an increased percentage of IFN γ + T cells by ICS, indicating that shorter antigen-encoding sequences and/or fewer distinct antigens encoded in a cassette can lead to an increased antigen-specific immune response using a SAM-LNP system.

[00434] A series of ChAdV vectors containing the short cassettes or longer cassettes (see **FIG. 5A**) were also evaluated. As shown in **FIGs. 5C** and **5D** and presented in **Tables 9** and **10**, vaccination with vectors featuring shorter antigen encoding inserts demonstrated an increased percentage of IFN γ + T cells by ICS and increased spot forming colonies (SFC), respectively, indicating that shorter antigen-encoding sequences and/or fewer distinct antigens encoded in a cassette can lead to an increased antigen-specific immune response using a ChAdV68 system.

Table 8 – IFN γ ICS data for Adpgk, Repls1, gp100, and Trp2 (Long vs Short): SAM-LNP

Cassette Antigen	20mer				Short-1		Short-2	
	Adpgk	Repls1	gp100	Trp2	Adpgk	Repls1	gp100	Trp2
	2.39	1.35	1.4	0.36	4.758	3.668	2.11	1.13
	1.68	1.2	1.21	0.37	4.549	2.239	3.827	1.267
	1.59	1.46	0.97	0.18	5.46	3.81	4.921	0.931
	0.67	1.35	1.63	0.22	3.878	4.178	2.76	0.8
	1.078	0.878	0.868	0.118	5.044	5.364	1.552	0.942
	0.68	1.56	1.4	0.47	4.78	6.41	1.84	0.79
	0.96	1.25	0.88	0.01	6.06	2.23	2.026	0.956
	3.16	0.78	1.08	0.18	4.937	4.637	1.26	1.8
Mean	1.53	1.23	1.18	0.24	4.93	4.07	2.54	1.08
Median	1.33	1.30	1.15	0.20	4.86	3.99	2.07	0.95
SD	0.88	0.27	0.28	0.15	0.64	1.44	1.25	0.33

Table 9 – IFN γ ICS data for Adpgk, Reps1, gp100, and Trp2 (Long vs Short): SAM-LNP

Antigen Cassette	Gp100		trp2	
	20mer	Short	20mer	Short
	0.178	6.75	0.228	0.32
	0.331	7.669	0.012	0.379
	0.37	11.246	0	0.346
	0.277	6.265	0.137	0.385
	0.216	7.205	0.136	0.225
	0.297	6.69	0.177	0.52
	0.212	8.512	0.102	0.052
	0.319	12.241	0.169	0.03
Mean	0.28	8.32	0.12	0.28
Median	0.29	7.44	0.14	0.33
St Dev	0.07	2.24	0.08	0.17

Table 10 – ELISpot data for gp100 and Trp2 (Long vs Short): ChAdV68

Antigen Cassette	Gp100		trp2	
	20mer	Short	20mer	Short
	894	16209	502	1588
	885	17226	27	1395
	1342	17673	149	947
	522	12185	110	1149
	517	15711	409	1313
	1146	14671	777	1754
	997	16882	409	991
	450	21260	316	644
Mean	844	16477	337	1222
Median	889	16546	362	1231
St Dev	323	2601	243	363

Certain Sequences

[00435] 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)
UbA76-25merPDDTT nucleotide (SEQ ID NO:32)
UbA76-25merPDDTT polypeptide (SEQ ID NO:33)
MAG-25merPDDTT nucleotide (SEQ ID NO:34)
MAG-25merPDDTT polypeptide (SEQ ID NO:35)
Ub7625merPDDTT NoSFL nucleotide (SEQ ID NO:36)
Ub7625merPDDTT 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 poly A 3' of cassette
Venezuelan equine encephalitis virus [VEEV] (SEQ ID NO:3) GenBank: L01442.2
VEE-MAG25mer (SEQ ID NO:4); contains MAG-25merPDDTT nucleotide (bases 30-1755)
Venezuelan equine encephalitis virus strain TC-83 [TC-83](SEQ ID NO:5) GenBank: L01443.1
VEEV Delivery Vector (SEQ ID NO:6); VEEV genome with nucleotides 7544-11175 deleted Except the last 50 amino acids of E1 [alphavirus structural proteins removed]
TC-83 Delivery Vector(SEQ ID NO:7); TC-83 genome with nucleotides 7544-11175 deleted [alphavirus structural proteins removed]
VEEV Production Vector (SEQ ID NO:8); VEEV 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); VEEV delivery vector with MHC class I mouse tumor epitopes SIINFEKL and AH1-A5 inserted
VEE-Luciferase (SEQ ID NO:15); VEEV 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)
WPRE (SEQ ID NO:50)>WPRE 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)
GPGPG linker (SEQ ID NO:56)
SAM <i>in vitro</i> transcription template DNA (SEQ ID NO:57); VEEV genome with nucleotides 7544-11175 deleted, plus minimal 5' T7-promoter (Bold Italic)
TAATACGACTCACTATAATG ggcggcgcatgagagaagcccagaccaattacctacccaaaATG Gagaaagttcacgttgacatcgagga agacagcccattcctcagagctttgcagcggagcttcccgcagtttgaggtagaagccaagcaggtcactgataatgacctgctaagccagagcgtttcgcacat ctggctcaaaactgatcgaacggaggtggaccatccgacacgatccttgacattggaagtgcgcccggccgcagaatgtattctaagcacaagatcattgta tctgtccgatgagatgtgcggaagatccggacagattgtataagatgcaactaagctgaagaaaaactgtaaggaataactgataagggaattggacaagaaat gaaggagctcgccgccgtcatgagcgaccctgacctggaactgagactatgtgcctccacgacgacgagtcgtgtcgtacgaagggaagtcgctgtttacc aggatgtatacgcggttgacggaccgacaagctctatcaccaagccaataaggaggttagagtcgcctactggataggcttgacaccacccctttatgtttaag aacttgctggagcatatccatcactctaccaactgggccgacgaaccgtgttaacggctcgttaacataggcctatgcagctctgacgttatggagcgggtcac

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CLAIMS

What is claimed is:

1. An antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$
 wherein E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,
 n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,
 E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,
 for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and
 z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N, or a combination thereof.
2. The composition of claim 1, wherein for each iteration of z: x and y = 1, optionally except for the final iteration.
3. The composition of claim 2, wherein x = at least 3, at least 4, at least 5, at least 6, or at least 7.
4. The composition of claim 1, wherein for each iteration of z: x = 1, y = 1 optionally except for the final iteration, n = 2, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E-E¹-E², optionally except for the final iteration wherein the order is described by the formula: E-E¹.
5. The composition of claim 4, wherein z = at least 2, at least 3, at least 4, at least 5, at least 6, or at least 7.
6. The composition of claim 1, wherein for each iteration z: x = 1, y = 1, n = 4, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E-E¹-E²-E³-E⁴-E⁵.
7. The composition of claim 6, wherein z = at least 2, at least 3, or at least 4.

8. The composition of claim 1, for each iteration z : $x = 1$, $y = 1$, $n = 9$, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: $E-E^1-E^2-E^3-E^4-E^5-E^6-E^7-E^8-E^9$.
9. The composition of claim 8, wherein $z =$ at least 2.
10. The composition of any one of claims 1-9, wherein each E or E^N independently comprises a nucleotide sequence described, from 5' to 3', by the formula $(L5_b-N_c-L3_d)$, wherein N comprises the distinct epitope-encoding nucleic acid sequence associated with each E or E^N , where $c = 1$,
 $L5$ comprises a 5' linker sequence, where $b = 0$ or 1, and
 $L3$ comprises a 3' linker sequence, where $d = 0$ or 1.
11. The composition of claim 10, wherein
each N encodes an epitope 7-15 amino acids in length,
 $L5$ is a native 5' linker sequence that encodes a native N-terminal amino acid sequence of the epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length, and
 $L3$ is a native 3' linker sequence that encodes a native C-terminal amino acid sequence of the epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length.
12. The composition of any one of claims 1-11, wherein each E and E^N encodes an epitope at least 7 amino acids in length.
13. The composition of any one of claims 1-11, wherein each E and E^N encodes an epitope 7-15 amino acids in length.
14. The composition of any one of claims 1-13, wherein each E and E^N is a nucleotide sequence at least 21 nucleotides in length.
15. The composition of any one of claims 1-13, wherein each E and E^N is a nucleotide sequence 75 nucleotides in length.
16. A composition for delivery of an antigen expression system, comprising:
the antigen expression system,
wherein the antigen expression system comprises one or more vectors,
the one or more vectors comprising:
(a) a vector backbone, wherein the backbone comprises:
(i) at least one promoter nucleotide sequence, and

- (ii) optionally, at least one polyadenylation (poly(A)) sequence; and
 - (b) a cassette, wherein the cassette comprises:
 - (i) at least one antigen-encoding nucleic acid sequence, comprising:
 - (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and
 - wherein each of the epitope-encoding nucleic acid sequences comprises:
 - (A) optionally, a 5' linker sequence, and
 - (B) optionally, a 3' linker sequence;
 - (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and
 - (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence;
 - (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and
 - (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,
- wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and
- wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least two distinct epitope-encoding nucleic acid sequences.

17. A composition for delivery of an antigen expression system, comprising:
the antigen expression system,
wherein the antigen expression system comprises one or more vectors,
the one or more vectors comprising:
- (a) a vector backbone, wherein the backbone comprises:
 - (i) at least one promoter nucleotide sequence, and
 - (ii) at least one polyadenylation (poly(A)) sequence; and
 - (b) a cassette, wherein the cassette comprises:
 - (i) at least one antigen-encoding nucleic acid sequence, comprising:
 - (I) at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences linearly linked to each other,
optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and
wherein each of the epitope-encoding nucleic acid sequences comprises:
 - (A) optionally, a 5' linker sequence, and
 - (B) optionally, a 3' linker sequence;
 - (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence;
 - (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence;
 - (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences.

18. The composition of claim 17, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 3 distinct epitope-encoding nucleic acid sequences.

19. A composition for delivery of an antigen expression system, comprising:
the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the vector backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector; and

(b) a cassette, optionally wherein the cassette is integrated between a native promoter nucleotide sequence native to the vector backbone and a poly(A) sequence, optionally wherein the poly(A) sequence is native to the vector backbone, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least one epitope-encoding nucleic acid sequence, optionally comprising at least two distinct epitope-encoding nucleic acid sequences linearly linked to each other, each epitope-encoding nucleic acid sequence optionally comprising:

- (A) a MHC class I epitope encoding nucleic acid sequence, wherein the MHC class 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 class 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 class I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and

wherein the cassette is operably linked to the native promoter nucleotide sequence, wherein each of the epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length, and wherein each 3' end of each epitope-encoding nucleic acid sequence is linked to the 5' end of the following epitope-encoding nucleic acid sequence with the exception of the final epitope-encoding nucleic acid sequence in the cassette; and

(ii) at least two MHC class II epitope-encoding nucleic acid sequences comprising:

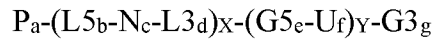
- (I) a PADRE MHC class II sequence (SEQ ID NO:48),
- (II) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46),
- (III) a first nucleic acid sequence encoding a GPGPG amino acid linker sequence linking the PADRE MHC class II sequence and the Tetanus toxoid MHC class II sequence,
- (IV) a second nucleic acid sequence encoding a GPGPG amino acid linker sequence linking the 5' end of the at least two MHC class II epitope-encoding nucleic acid sequences to the epitope-encoding nucleic acid sequences,
- (V) 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 epitope-encoding nucleic acid sequences;

(iii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the native promoter nucleotide sequence, and

wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequence.

20. The composition of any of claims 16-18, wherein an ordered sequence of each element of the cassette is described in the formula, from 5' to 3', comprising:



wherein,

P comprises the second promoter nucleotide sequence, where $a = 0$ or 1 ,

N comprises one of the distinct epitope-encoding nucleic acid sequences, 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 epitope-encoding nucleic acid sequence, where $f = 1$,

$X = 1$ to 400 , where for each X the corresponding N_c is a epitope-encoding nucleic acid sequence, and

$Y = 0, 1$, or 2 , where for each Y the corresponding U_f is an MHC class II epitope-encoding nucleic acid sequence.

21. The composition of claim 20, wherein for each X the corresponding N_c is a distinct epitope-encoding nucleic acid sequence, except for the N_c corresponding to the at least two repeats of the distinct epitope-encoding nucleic acid sequence.
22. The composition of claim 20 or 21, wherein for each Y the corresponding U_f is a distinct MHC class II epitope-encoding nucleic acid sequence.

23. The composition of any one of claims 20-22, wherein
- $a = 0, b = 1, d = 1, e = 1, g = 1, h = 1, X = 20, Y = 2,$
- the at least one promoter nucleotide sequence is a single native promoter nucleotide sequence native to the vector backbone,
- the at least one polyadenylation poly(A) sequence is a poly(A) sequence of at least 80 consecutive A nucleotides provided by the vector backbone,
- each N encodes an epitope 7-15 amino acids in length,
- L5 is a native 5' linker sequence that encodes a native N-terminal amino acid sequence of the epitope, and wherein the 5' linker sequence encodes a peptide that is at least 3 amino acids in length,
- L3 is a native 3' linker sequence that encodes a native C-terminal amino acid sequence of the 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 backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector,
- optionally wherein the native promoter nucleotide sequence is a subgenomic promoter when the vector backbone comprises an alphavirus vector, and
- each of the MHC class II epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length.
24. The composition of any one of claims 16-23, wherein the at least two repeats is at least 3, at least 4, at least 5, at least 6, or at least 7 repeats.
25. The composition of any one of claims 16-23, wherein the at least two repeats is at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, 1 at least 4, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 repeats.
26. The composition of any one of claims 16-23, wherein the at least two repeats is between 2-3, between 2-4, between 2-5, between 2-6, or between 2-7 repeats.
27. The composition of any one of claims 16-23, wherein the at least two repeats is 7 repeats or less, 6 repeats or less, 5 repeats or less, 4 repeats or less, or 3 repeats or less.

28. The composition of any one of claims 16-27, wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least two distinct epitope-encoding nucleic acid sequences.
29. The composition of any one of claims 16-27, wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 distinct epitope-encoding nucleic acid sequences.
30. The composition of any one of claims 16-29, wherein the at least two repeats are separated by at least one separate distinct epitope-encoding nucleic acid sequence.
31. The composition of any one of claims 16-29, wherein the at least two repeats are separated by at least 2 separate distinct epitope-encoding nucleic acid sequences.
32. The composition of any one of claims 16-29, wherein the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 75 nucleotides.
33. The composition of any one of claims 16-29, wherein the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 150 nucleotides, at least 300 nucleotides, or at least 675 nucleotides.
34. The composition of any one of claims 16-29, wherein the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 350 nucleotides, at least 400 nucleotides, at least 450 nucleotides, at least 500 nucleotides, at least 700 nucleotides, at least 700 nucleotides, at least 750 nucleotides, at least 800 nucleotides, at least 900 nucleotides, or at least 1000 nucleotides.
35. The composition of any one of claims 16-29, wherein the at least two repeats, inclusive of the optional 5' linker sequence and/or the optional 3' linker sequence, are separated by at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, at least 45 nucleotides, at least 50 nucleotides, at least 55 nucleotides, at least 60 nucleotides, at least 65 nucleotides, or at least 70 nucleotides.

36. The composition of any one of claims 16-35, wherein the at least one antigen-encoding nucleic acid sequence is described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$

wherein,

E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,

n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,

E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,

for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and

z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

37. The composition of claim 36, wherein for each iteration of z: x and y = 1, optionally except for the final iteration.
38. The composition of claim 37, wherein x = at least 3, at least 4, at least 5, at least 6, or at least 7.
39. The composition of claim 36, wherein for each iteration of z: x = 1, y = 1 optionally except for the final iteration, n = 2, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 , optionally except for the final iteration wherein the order is described by the formula: E- E^1 .
40. The composition of claim 39, wherein z = at least 2, at least 3, at least 4, at least 5, at least 6, or at least 7.
41. The composition of claim 36, wherein for each iteration z: x = 1, y = 1, n = 4, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 - E^3 - E^4 - E^5 .
42. The composition of claim 41, wherein z = at least 2, at least 3, or at least 4.
43. The composition of claim 36, for each iteration z: x = 1, y = 1, n = 9, and wherein the order of the three distinct epitope-encoding nucleic acid sequences for each iteration of z is described by the formula: E- E^1 - E^2 - E^3 - E^4 - E^5 - E^6 - E^7 - E^8 - E^9 .
44. The composition of claim 43, wherein z = at least 2.

45. The composition of any one of claims 1-44, wherein the at least two repeats comprises a number of repeats, or z comprises a number, sufficient to stimulate a greater immune response relative to an antigen-encoding nucleic acid sequence comprising a single iteration of the at least one epitope-encoding nucleic acid sequence.
46. The composition of any one of claims 1-45, wherein the at least two repeats comprises a number of repeats, or z comprises a number, sufficient to stimulate an immune response, and a single iteration of the at least one epitope-encoding nucleic acid sequence is insufficient to stimulate the immune response or insufficient to stimulate a detectable immune response.
47. The composition of claims 45 or 46, wherein the immune response is an expansion of epitope-specific T cells following *in vivo* immunization with the composition for delivery of the antigen expression system.
48. The composition of claims 45 or 46, wherein the immune response is increased activation of epitope-specific T cells and/or increased epitope-specific killing by epitope-specific T cells following *in vivo* immunization with the composition for delivery of the antigen expression system.
49. A composition for delivery of an antigen expression system, comprising:
the antigen expression system,
wherein the antigen expression system comprises one or more vectors,
the one or more vectors comprising:
(a) a vector backbone, wherein the backbone comprises:
(i) at least one promoter nucleotide sequence, and
(ii) optionally, at least one polyadenylation (poly(A)) sequence; and
(b) a cassette, wherein the cassette comprises:
(i) at least one antigen-encoding nucleic acid sequence, comprising:
(I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a

bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the cassette is 700 nucleotides in length or less.

50. A composition for delivery of an antigen expression system, comprising: the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

(ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct epitope-encoding nucleic acid sequences linearly linked to each other,

optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone, and

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the cassette is 700 nucleotides in length or less.

51. The composition of claim 50, wherein the at least one antigen-encoding nucleic acid sequence comprises 3 distinct epitope-encoding nucleic acid sequences.

52. A composition for delivery of an antigen expression system, comprising:
the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the vector backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector, or an alphavirus vector, optionally wherein the alphavirus vector is a Venezuelan equine encephalitis virus vector; and

(b) a cassette, optionally wherein the cassette is integrated between a native promoter nucleotide sequence native to the vector backbone and a poly(A) sequence, optionally wherein the poly(A) sequence is native to the vector backbone, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least one epitope-encoding nucleic acid sequence, optionally comprising at least two distinct epitope-encoding nucleic acid sequences linearly linked to each other, each epitope-encoding nucleic acid sequence optionally comprising:

(A) a MHC class I epitope encoding nucleic acid sequence, wherein the MHC class 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 class 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 class I epitope, and wherein the 3' linker sequence encodes a peptide that is at least 3 amino acids in length, and

wherein the cassette is operably linked to the native promoter nucleotide sequence, wherein each of the epitope-encoding nucleic acid sequences encodes a polypeptide that is between 13 and 25 amino acids in length, and wherein each 3' end of each epitope-encoding nucleic acid sequence is linked to the 5' end of the following epitope-encoding nucleic acid sequence with the exception of the final epitope-encoding nucleic acid sequence in the cassette; and

(ii) at least two MHC class II epitope-encoding nucleic acid sequences comprising:

- (I) a PADRE MHC class II sequence (SEQ ID NO:48),
- (II) a Tetanus toxoid MHC class II sequence (SEQ ID NO:46),
- (III) 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,
- (IV) 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 epitope-encoding nucleic acid sequences to the epitope-encoding nucleic acid sequences,
- (V) 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 epitope-encoding nucleic acid sequences, and

(iii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the native promoter nucleotide sequence, and

wherein the cassette is 700 nucleotides in length or less.

53. The composition of any one of claims 1-52, wherein the cassette is between 375-700 nucleotides in length.
54. The composition of any one of claims 1-52, wherein the cassette is 600, 500, 400, 300, 200, or 100 nucleotides in length or less.
55. The composition of any one of claims 1-52, wherein the cassette is between 375-600, between 375-500, or between 375-400 nucleotides in length.
56. The composition of any one of claims 1-55, wherein one or more of the epitope-encoding nucleic acid sequences are derived from a tumor, an infection, or an infected cell of a subject.
57. The composition of any one of claims 1-55, wherein each of the epitope-encoding nucleic acid sequences are derived from a tumor, an infection, or an infected cell of a subject.

58. The composition of any one of claims 1-55, wherein one or more of the epitope-encoding nucleic acid sequences are not derived from a tumor, an infection, or an infected cell of a subject.
59. The composition of any one of claims 1-55, wherein each of the epitope-encoding nucleic acid sequences are not derived from a tumor, an infection, or an infected cell of a subject.
60. The composition of any one of claims 1-59, wherein the epitope-encoding nucleic acid sequence encodes an epitope known or suspected to be presented by MHC class I on a surface of a cell, optionally wherein the surface of the cell is a tumor cell surface or an infected cell surface, and optionally wherein the cell is a subject's cell.
61. The composition of claim 60, wherein the cell is a tumor cell 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, or wherein the cell is an infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, a fungally infected cell, and a parasitically infected cell.
62. The composition of claim 61, wherein the virally infected cell is selected from the group consisting of: an HIV infected cell, a Severe acute respiratory syndrome-related coronavirus (SARS) infected cell, a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected cell, a Ebola infected cell, a Hepatitis B virus (HBV) infected cell, an influenza infected cell, a Hepatitis C virus (HCV) infected cell, a Human papillomavirus (HPV) infected cell, a Cytomegalovirus (CMV) infected cell, a Chikungunya virus infected cell, a Respiratory syncytial virus (RSV) infected cell, a Dengue virus infected cell, and an orthomyxoviridae family virus infected cell.
63. The composition of any one of the above claims, wherein the composition further comprises a nanoparticulate delivery vehicle.
64. The composition of claim 63, wherein the nanoparticulate delivery vehicle is a lipid nanoparticle (LNP).
65. The composition of claim 64, wherein the LNP comprises ionizable amino lipids.

66. The composition of claim 65, wherein the ionizable amino lipids comprise MC3-like (dilinoleylmethyl-4-dimethylaminobutyrate) molecules.
67. The composition of any of claims claim 24-66, wherein the nanoparticulate delivery vehicle encapsulates the antigen expression system.
68. The composition of any one of claims 16-18, 20-22, or 24-67, wherein the cassette is integrated between the at least one promoter nucleotide sequence and the at least one poly(A) sequence.
69. The composition of any one of claims 16-18, 20-22, or 24-68, wherein the second promoter is absent and the at least one promoter nucleotide sequence is operably linked to the antigen-encoding nucleic acid sequence.
70. The composition of any one of claims 16-18, 20-22, or 24-69, wherein the one or more vectors comprise one or more +-stranded RNA vectors.
71. The composition of claim 70 wherein the one or more +-stranded RNA vectors comprise a 5' 7-methylguanosine (m7g) cap.
72. The composition of claim 70 or 71, wherein the one or more +-stranded RNA vectors are produced by *in vitro* transcription.
73. The composition of any one of claims 16-18, 20-22, or 24-72, wherein the one or more vectors are self-replicating within a mammalian cell.
74. The composition of any one of claims 16-18, 20-22, or 24-73, wherein 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.
75. The composition of any one of claims 16-18, 20-22, or 24-73, wherein the backbone comprises at least one nucleotide sequence of a Venezuelan equine encephalitis virus.
76. The composition of claim 74 or 75, wherein the backbone comprises at least sequences for nonstructural protein-mediated amplification, a subgenomic 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.
77. The composition of claim 74 or 75, wherein the backbone comprises at least sequences for nonstructural protein-mediated amplification, a subgenomic 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.

78. The composition of claim 76 or 77, wherein 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.
79. The composition of any one of claims 76-78, wherein the backbone does not encode structural virion proteins capsid, E2 and E1.
80. The composition of claim 79, wherein the cassette is inserted in place of 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.
81. The composition of claim 74 or 75, wherein the Venezuelan equine encephalitis virus comprises the sequence of SEQ ID NO:3 or SEQ ID NO:5.
82. The composition of claim 74 or 75, wherein 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.
83. The composition of claim 82, wherein the backbone comprises the sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.
84. The composition of claim 82 or 83, wherein the cassette is inserted at position 7544 to replace the deletion between base pairs 7544 and 11175 as set forth in the sequence of SEQ ID NO:3 or SEQ ID NO:5.
85. The composition of claim 80-84, wherein the insertion of the cassette provides for transcription of a polycistronic RNA comprising the nsP1-4 genes and the at least one antigen-encoding nucleic acid sequence, wherein the nsP1-4 genes and the at least one antigen-encoding nucleic acid sequence are in separate open reading frames.
86. The composition of any one of claims 16-18, 20-22, or 24-73, wherein the backbone comprises at least one nucleotide sequence of a chimpanzee adenovirus vector.
87. The composition of claim 86, wherein the chimpanzee adenovirus vector is a ChAdV68 vector, optionally wherein the ChAdV vector comprises:
 - (a) an ChAdV backbone, wherein the ChAdV backbone comprises:

- (i) a modified ChAdV68 sequence comprising at least nucleotides 2 to 36,518 of the sequence set forth in SEQ ID NO:1, wherein the nucleotides 2 to 36,518 lack: (1) nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion; (2) nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion; and optionally (3) nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1 corresponding to a partial E4 deletion ChAdV68;
 - (ii) optionally, a CMV promoter nucleotide sequence; and
 - (iii) optionally, an SV40 polyadenylation (poly(A)) sequence; and
- (b) the antigen-encoding cassette of any one of the above claims, optionally wherein the antigen-encoding cassette is inserted within the E1 deletion and the cassette is operably linked to the CMV promoter nucleotide sequence and the SV40 poly(A) sequence.
88. The composition of any one of claims 16-18, 20-22, or 24-87, wherein the at least one promoter nucleotide sequence is the native subgenomic promoter nucleotide sequence encoded by the backbone.
89. The composition of any one of claims 16-18, 20-22, or 24-87, wherein the at least one promoter nucleotide sequence is an exogenous RNA promoter.
90. The composition of any one of claims 16-18, 20-22, or 24-89, wherein the second promoter nucleotide sequence is a subgenomic promoter nucleotide sequence.
91. The composition of any one of claims 16-18, 20-22, or 24-89, wherein the second promoter nucleotide sequence comprises multiple subgenomic promoter nucleotide sequences, wherein each subgenomic promoter nucleotide sequence provides for transcription of one or more of the separate open reading frames.
92. The composition of any one of the above claims, wherein the one or more vectors are each at least 300nt in size.
93. The composition of any one of the above claims, wherein the one or more vectors are each at least 1kb in size.
94. The composition of any one of the above claims, wherein the one or more vectors are each 2kb in size.

95. The composition of any one of the above claims, wherein the one or more vectors are each less than 5kb in size.
96. The composition of any one of the above claims, wherein at least one of the at least one antigen-encoding nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface.
97. The composition of any one of claims 1-18, 20-22, or 24-96, wherein each epitope-encoding nucleic acid sequence is linked directly to one another.
98. The composition of any one of claims 1-18, 20-22, or 24-97, wherein at least one of the at least one epitope-encoding nucleic acid sequences is linked to a distinct epitope-encoding nucleic acid sequence with a nucleic acid sequence encoding a linker.
99. The composition of claim 98, wherein the linker links two MHC class I sequences or an MHC class I sequence to an MHC class II sequence.
100. The composition of claim 99, 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.
101. The composition of claim 98, wherein the linker links two MHC class II sequences or an MHC class II sequence to an MHC class I sequence.
102. The composition of claim 101, wherein the linker comprises the sequence GPGPG.
103. The composition of any one of claims 1-18, 20-22, or 24-102, wherein at least one sequence of the at least one epitope-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 epitope-encoding nucleic acid sequences of epitope encoded therefrom.
104. The composition of claim 103, 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.

105. The composition of any one of the above claims, wherein at least one of the at least one epitope-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.
106. The composition of any one of the above claims, wherein at least one of the at least one epitope-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.
107. The composition of any one of the above claims, wherein at least one of the at least one epitope-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.
108. The composition of any one 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.
109. The composition of any one 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, bladder cancer, brain cancer, B-cell lymphoma, acute myelogenous leukemia, adult acute lymphoblastic leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, T cell lymphocytic leukemia, non-small cell lung cancer, and small cell lung cancer
or the infectious disease organism is selected from the group consisting of: Severe acute respiratory syndrome-related coronavirus (SARS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Ebola, HIV, Hepatitis B virus (HBV), influenza, Hepatitis C virus (HCV), Human papillomavirus (HPV), Cytomegalovirus (CMV),

Chikungunya virus, Respiratory syncytial virus (RSV), Dengue virus, an orthomyxoviridae family virus, and tuberculosis.

110. The composition of any one claims 1-18, 20-22, or 24-109, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-10, 2, 3, 4, 5, 6, 7, 8, 9, or 10 epitope-encoding nucleic acid sequences.
111. The composition of any one of claims 1-18, 20-22, or 24-109, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 11-20, 15-20, 11-100, 11-200, 11-300, 11-400, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 epitope-encoding nucleic acid sequences.
112. The composition of any one of claims 1-18, 20-22, or 24-109, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 epitope-encoding nucleic acid sequences and wherein at least two of the epitope-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface.
113. The composition of any one claims 1-18, 20-22, or 24-112, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-10, 2, 3, 4, 5, 6, 7, 8, 9, or 10 antigen-encoding nucleic acid sequences.
114. The composition of any one of claims 1-18, 20-22, or 24-112, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 11-20, 15-20, 11-100, 11-200, 11-300, 11-400, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 antigen-encoding nucleic acid sequences.
115. The composition of any one of claims 1-18, 20-22, or 24-112, wherein the at least one antigen-encoding nucleic acid sequence comprises at least 2-400 antigen-encoding 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 a cell surface, optionally a tumor cell surface or an infected cell surface.
116. The composition of claim 19 or 23, wherein at least two of the epitope-encoding nucleic acid sequences encode polypeptide sequences or portions thereof that are presented by MHC class I on a cell surface, optionally a tumor cell surface or an infected cell surface.
117. The composition of any one of the above claims, wherein when administered to the subject and translated, at least one of the epitopes encoded by the at least one epitope-encoding nucleic acid sequence are presented on antigen presenting cells resulting in an

immune response targeting at least one of the antigens on the tumor cell surface or the infected cell surface.

118. The composition of any one of the above claims, wherein the at least one antigen-encoding nucleic acid sequences when administered to the subject and translated, at least one of the MHC class I or class II epitopes are presented on antigen presenting cells resulting in an immune response targeting at least one of the epitopes on a tumor cell surface or the infected cell surface, and optionally wherein the expression of each of the at least one antigen-encoding nucleic acid sequences is driven by the at least one promoter nucleotide sequence.
119. The composition of any one of claims 1-18, 20-22, or 24-118, wherein each epitope-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.
120. The composition of any one of claims 1-18, 20-22, or 24-119, wherein the at least one MHC class II epitope-encoding nucleic acid sequence is present.
121. The composition of any one of claims 1-18, 20-22, or 24-119, wherein the at least one MHC class II epitope-encoding nucleic acid sequence is present and comprises at least one MHC class II epitope-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.
122. The composition of any one of claims 1-18, 20-22, or 24-121, wherein the at least one MHC class II epitope-encoding nucleic acid sequence is 12-20, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 20-40 amino acids in length.
123. The composition of any one of claims 1-18, 20-22, or 24-122, wherein the at least one MHC class II epitope-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.
124. The composition of any one of claims 16-18, 20-22, or 24-123, wherein the at least one promoter nucleotide sequence or the second promoter nucleotide sequence is inducible.
125. The composition of any one of claims 16-18, 20-22, or 24-123, wherein the at least one promoter nucleotide sequence or the second promoter nucleotide sequence is non-inducible.

126. The composition of any one of claims 16-18, 20-22, or 24-125, wherein the at least one poly(A) sequence comprises a poly(A) sequence native to the backbone.
127. The composition of any one of claims 16-18, 20-22, or 24-125, wherein the at least one poly(A) sequence comprises a poly(A) sequence exogenous to the backbone.
128. The composition of any one claims 16-18, 20-22, or 24-127, wherein the at least one poly(A) sequence is operably linked to at least one of the at least one antigen-encoding nucleic acid sequences.
129. The composition of any one of claims 16-18, 20-22, or 24-128, wherein the at least one poly(A) sequence is at least 20 , at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 consecutive A nucleotides.
130. The composition of any one of claims 16-18, 20-22, or 24-128, wherein the at least one poly(A) sequence is at least 80 consecutive A nucleotides.
131. The composition of any one of the above claims, wherein the 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.
132. The composition of any one of the above claims, wherein the cassette further comprises a reporter gene, including but not limited to, green fluorescent protein (GFP), a GFP variant, secreted alkaline phosphatase, luciferase, a luciferase variant, or a detectable peptide or epitope.
133. The composition of claim 132, wherein the detectable peptide or epitope is selected from the group consisting of an HA tag, a Flag tag, a His-tag, or a V5 tag.
134. The composition of any one of the above claims, wherein the one or more vectors further comprises one or more nucleic acid sequences encoding at least one immune modulator.
135. The composition of claim 134, 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.
136. The composition of claim 135, 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).
137. The composition of claim 135 or 136, wherein the heavy and light chain sequences of the antibody are a contiguous sequence separated by either a self-cleaving sequence such as 2A or IRES; or the heavy and light chain sequences of the antibody are linked by a flexible linker such as consecutive glycine residues.
138. The composition of claim 134, wherein the immune modulator is a cytokine.
139. The composition of claim 138, wherein the cytokine is at least one of IL-2, IL-7, IL-12, IL-15, or IL-21 or variants thereof of each.
140. The composition of any one of claims 1-18, 20-22, or 24-139, wherein at least one epitope-encoding nucleic acid sequence is selected by performing the steps of:
- (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor, an infected cell, or an infectious disease organism, wherein the nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens;
 - (b) 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 a cell surface, optionally a tumor cell surface or an infected cell surface, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and
 - (c) 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 epitope-encoding nucleic acid sequence.
141. The composition of claim 19 or 23, wherein each of the epitope-encoding nucleic acid sequences is selected by performing the steps of:
- (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor, an infected cell, or an infectious disease organism,

wherein the nucleotide sequencing data is used to obtain data representing peptide sequences of each of a set of antigens;

(b) 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 a cell surface, optionally a tumor cell surface or an infected cell surface, the set of numerical likelihoods having been identified at least based on received mass spectrometry data; and

(c) 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 20 epitope-encoding nucleic acid sequences.

142. The composition of claim 140, wherein a number of the set of selected antigens is 2-20.

143. The composition of claim 140-142, wherein the presentation model represents dependence between:

(a) 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

(b) likelihood of presentation on a cell surface, optionally a tumor cell surface or an infected 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.

144. The composition of claim 140-143, wherein selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being presented on the cell surface relative to unselected antigens based on the presentation model, optionally wherein the selected antigens have been validated as being presented by one or more specific HLA alleles.

145. The composition of claim 140-144, wherein selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of stimulating a tumor-specific or infectious disease-specific immune response in the subject relative to unselected antigens based on the presentation model.

146. The composition of claim 140-145, 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).

147. The composition of claim 140-146, 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.
148. The composition of claim 140-147, wherein selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being capable of stimulating an autoimmune response to normal tissue in the subject relative to unselected antigens based on the presentation model.
149. The composition of claim 140-148, wherein exome or transcriptome nucleotide sequencing data is obtained by performing sequencing on a tumor cell or tissue, an infected cell, or an infectious disease organism.
150. The composition of claim 149, wherein the sequencing is next generation sequencing (NGS) or any massively parallel sequencing approach.
151. The composition of any one of the above claims, wherein the cassette comprises junctional epitope sequences formed by adjacent sequences in the cassette.
152. The composition of claim 151, wherein at least one or each junctional epitope sequence has an affinity of greater than 500 nM for MHC.
153. The composition of claims 151 or 152, wherein each junctional epitope sequence is non-self.
154. The composition of any one of the above claims, wherein each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele present in at least 5% of a population.
155. The composition of any one of the above claims, wherein each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele, wherein each antigen/HLA pair has an antigen/HLA prevalence of at least 0.01% in a population.
156. The composition of any one of the above claims, wherein each of the MHC class I epitopes is predicted or validated to be capable of presentation by at least one HLA allele, wherein each antigen/HLA pair has an antigen/HLA prevalence of at least 0.1% in a population.
157. The composition of any one of the above claims, wherein the 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.
158. The composition of claim 157, wherein the non-therapeutic predicted MHC class I or class II epitope sequence is a junctional epitope sequence formed by adjacent sequences in the cassette.
159. The composition of claims 151-158, wherein the prediction is based on presentation likelihoods generated by inputting sequences of the non-therapeutic epitopes into a presentation model.
160. The composition of any one of claims 151-159, wherein an order of the at least one antigen-encoding nucleic acid sequences in the cassette is determined by a series of steps comprising:
- (a) generating a set of candidate cassette sequences corresponding to different orders of the at least one antigen-encoding nucleic acid sequences;
 - (b) determining, for each candidate cassette sequence, a presentation score based on presentation of non-therapeutic epitopes in the candidate cassette sequence; and
 - (c) selecting a candidate cassette sequence associated with a presentation score below a predetermined threshold as the cassette sequence for an antigen vaccine.
161. A pharmaceutical composition comprising the composition of any one of the above claims and a pharmaceutically acceptable carrier.
162. The composition of claim 161, wherein the composition further comprises an adjuvant.
163. The pharmaceutical composition of claim 161 or 162, wherein the composition further comprises an immune modulator.
164. The pharmaceutical composition of claim 163, 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.
165. An isolated nucleotide sequence or set of isolated nucleotide sequences comprising the cassette of any of the above composition claims and one or more elements obtained from the sequence of SEQ ID NO:3 or SEQ ID NO:5, optionally wherein the one or more elements are selected from the group consisting of the sequences necessary for nonstructural protein-mediated amplification, the subgenomic promoter nucleotide

- sequence, the poly(A) sequence, and the nsP1-4 genes of the sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, and optionally wherein the nucleotide sequence is cDNA.
166. The isolated nucleotide sequence of claim 165, wherein the sequence or set of isolated nucleotide sequences comprises the cassette of any of the above composition claims inserted at position 7544 of the sequence set forth in SEQ ID NO:6 or SEQ ID NO:7.
167. The isolated nucleotide sequence of claim 165 or 166, further comprising:
- a) a T7 or SP6 RNA polymerase promoter nucleotide sequence 5' of the one or more elements obtained from the sequence of SEQ ID NO:3 or SEQ ID NO:5; and
 - b) optionally, one or more restriction sites 3' of the poly(A) sequence.
168. The isolated nucleotide sequence of claim 165, wherein the cassette of any of the above composition claims is inserted at position 7563 of SEQ ID NO:8 or SEQ ID NO:9.
169. A vector or set of vectors comprising the nucleotide sequence of claims 165-168.
170. An isolated cell comprising the nucleotide sequence or set of isolated nucleotide sequences of claims 165-169, optionally wherein the cell is a BHK-21, CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.
171. A kit comprising the composition of any of the above composition claims and instructions for use.
172. A method for treating a subject with cancer, the method comprising administering to the subject the composition of any of the above composition claims or the pharmaceutical composition of any of claims 161-164.
173. The method of claim 172, wherein the at least one epitope-encoding nucleic acid sequence is derived from the tumor of the subject with cancer or from a cell or sample of the infected subject.
174. The method of claim 172, wherein the at least one epitope-encoding nucleic acid sequence are not derived from the tumor of the subject with cancer or from a cell or sample of the infected subject.
175. A method for stimulating an immune response in a subject, the method comprising administering to the subject the composition of any of the above composition claims or the pharmaceutical composition of any of claims 161-164.
176. The method any of claims 172-175, wherein the subject expresses at least one HLA allele predicted or known to present the MHC class I epitope.
177. The method of any of claims 172-176, wherein the composition is administered intramuscularly (IM), intradermally (ID), subcutaneously (SC), or intravenously (IV).

178. The method of any of claims 172-176, wherein the composition is administered intramuscularly.
179. The method of any of claims 172-178, the method further comprising administration of one or more immune modulators, optionally wherein the immune modulator is administered before, concurrently with, or after administration of the composition or pharmaceutical composition.
180. The method of claim 179, wherein the one or more immune modulators are selected from the group consisting of: 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.
181. The method of claim 179 or 180, wherein the immune modulator is administered intravenously (IV), intramuscularly (IM), intradermally (ID), or subcutaneously (SC).
182. The method of claim 181, wherein the subcutaneous administration is near the site of the composition or pharmaceutical composition administration or in close proximity to one or more vector or composition draining lymph nodes.
183. The method of any one of claims 172-182, further comprising administering to the subject a second vaccine composition.
184. The method of claim 183, wherein the second vaccine composition is administered prior to the administration of the composition or the pharmaceutical composition of any one of claims 172-182.
185. The method of claim 183, wherein the second vaccine composition is administered subsequent to the administration of the composition or the pharmaceutical composition of any one of claims 172-182.
186. The method of claim 184 or 185, wherein the second vaccine composition is the same as the composition or the pharmaceutical composition of any one of claims 172-182.
187. The method of claim 184 or 185, wherein the second vaccine composition is different from the composition or the pharmaceutical composition of any one of claims 172-182.
188. The method of claim 187, wherein the second vaccine composition comprises a chimpanzee adenovirus vector encoding at least one antigen-encoding nucleic acid sequence.

189. The method of claim 188, wherein the at least one antigen-encoding nucleic acid sequence encoded by the chimpanzee adenovirus vector is the same as the at least one antigen-encoding nucleic acid sequence of any of the above composition claims.
190. A method of manufacturing the one or more vectors of any of the above composition claims, the method comprising:
- (a) obtaining a linearized DNA sequence comprising the backbone and the cassette;
 - (b) *in vitro* transcribing the linearized DNA sequence by addition of the linearized DNA sequence to an *in vitro* transcription reaction containing all the necessary components to transcribe the linearized DNA sequence into RNA, optionally further comprising *in vitro* addition of the m7g cap to the resulting RNA; and
 - (c) isolating the one or more vectors from the *in vitro* transcription reaction.
191. The method of manufacturing of claim 190, wherein the linearized DNA sequence is generated by linearizing a DNA plasmid sequence or by amplification using PCR.
192. The method of manufacturing of claim 191, wherein the DNA plasmid sequence is generated using one of bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells.
193. The method of manufacturing of claim 190, wherein isolating the one or more vectors from the *in vitro* transcription reaction involves one or more of phenol chloroform extraction, silica column based purification, or similar RNA purification methods.
194. A method of manufacturing the composition of any of the above composition claims for delivery of the antigen expression system, the method comprising:
- (a) providing components for the nanoparticulate delivery vehicle;
 - (b) providing the antigen expression system; and
 - (c) providing conditions sufficient for the nanoparticulate delivery vehicle and the antigen expression system to produce the composition for delivery of the antigen expression system.
195. The method of manufacturing of claim 194, wherein the conditions are provided by microfluidic mixing.
196. A method for treating a subject with a disease, optionally wherein the disease is cancer or an infection, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-

encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$

wherein,

E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,

n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,

E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,

for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and

z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

197. A method for treating a subject with a disease, optionally wherein the disease is cancer, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising:

the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

(ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least one epitope-encoding nucleic acid sequence, optionally

comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group

consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences.

198. A method for treating a subject with a disease, optionally wherein the disease is cancer, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising:

the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

- (ii) optionally, at least one polyadenylation (poly(A)) sequence; and
 - (b) a cassette, wherein the cassette comprises:
 - (i) at least one antigen-encoding nucleic acid sequence, comprising:
 - (I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and
 - wherein each of the epitope-encoding nucleic acid sequences comprises;
 - (A) optionally, a 5' linker sequence, and
 - (B) optionally, a 3' linker sequence;
 - (ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and
 - (iii) optionally, at least one MHC class II epitope-encoding nucleic acid sequence;
 - (iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and
 - (v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,
 - wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and
 - wherein the cassette is 700 nucleotides in length or less.
199. The method of any one of claims 196-198, wherein the at least one epitope-encoding nucleic acid sequence is derived from a tumor of the subject with cancer or from a cell or sample of the infected subject.

200. The method any one of claims 196-198, wherein the at least one epitope-encoding nucleic acid sequence are not derived from a tumor of the subject with cancer or from a cell or sample of the infected subject.
201. A method for stimulating an immune response in a subject, the method comprising the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-encoding cassette, wherein the antigen-encoding cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$
 wherein,
 E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,
 n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,
 E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,
 for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and
 z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.
202. A method for stimulating an immune response in a subject, the method comprising the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises:
 an antigen expression system, comprising:
 the antigen expression system,
 wherein the antigen expression system comprises one or more vectors,
 the one or more vectors comprising:
 (a) a vector backbone, wherein the backbone comprises:
 (i) at least one promoter nucleotide sequence, and
 (ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least one epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences.

203. A method for treating a subject with a disease, optionally wherein the disease is cancer of an infection, the method comprising administering to the subject an antigen-based

vaccine to the subject, wherein the antigen-based vaccine comprises an antigen expression system, comprising:

the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

(ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least two distinct epitope-encoding nucleic acid sequences, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the cassette is 700 nucleotides in length or less.

204. The method any of claims 196-203, wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence.
205. The method any of claims 196-203, wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence, and wherein the at least one epitope sequence comprises a an epitope known or suspected to be presented by MHC class I on a surface of a cell.
206. The method of claim 205, wherein the surface of the cell is a tumor cell surface.
207. The method of claim 206, wherein the cell is a tumor cell 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.
208. The method of claim 205, wherein the surface of the cell is an infected cell surface.
209. The method of claim 208, wherein the cell is an infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, a fungally infected cell, and a parasitically infected cell.
210. The method of claim 209, wherein the virally infected cell is selected from the group consisting of: an HIV infected cell, a Severe acute respiratory syndrome-related coronavirus (SARS) infected cell, a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected cell, an Ebola infected cell, a Hepatitis B virus (HBV) infected cell, an influenza infected cell, a Hepatitis C virus (HCV) infected cell, a Human papillomavirus (HPV) infected cell, a Cytomegalovirus (CMV) infected cell, a Chikungunya virus infected cell, a Respiratory syncytial virus (RSV) infected cell, a Dengue virus infected cell, and an orthomyxoviridae family virus infected cell.
211. A method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises an antigen-encoding cassette, wherein the antigen-encoding

cassette comprises at least one antigen-encoding nucleic acid sequence described, from 5' to 3', by the formula:

$$(E_x-(E^N_n)_y)_z$$

wherein,

E represents a nucleotide sequence comprising at least one of the at least one distinct epitope-encoding nucleic acid sequences,

n represents the number of separate distinct epitope-encoding nucleic acid sequences and is any integer including 0,

E^N represents a nucleotide sequence comprising the separate distinct epitope-encoding nucleic acid sequence for each corresponding n,

for each iteration of z: x = 0 or 1, y = 0 or 1 for each n, and at least one of x or y = 1, and

z = 2 or greater, wherein the antigen-encoding nucleic acid sequence comprises at least two iterations of E, a given E^N , or a combination thereof.

212. A method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises:

an antigen expression system, comprising:

the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

(ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least one epitope-encoding nucleic acid sequence, optionally

comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding

an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope, and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GP GPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence,

wherein the at least one antigen-encoding nucleic acid sequence comprises at least two repeats of at least one of the at least one epitope-encoding nucleic acid sequences, and

wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence.

213. A method for stimulating an immune response in a subject, the method comprising administering to the subject an antigen-based vaccine to the subject, wherein the antigen-based vaccine comprises:

an antigen expression system, comprising:

the antigen expression system,

wherein the antigen expression system comprises one or more vectors,

the one or more vectors comprising:

(a) a vector backbone, wherein the backbone comprises:

(i) at least one promoter nucleotide sequence, and

(ii) optionally, at least one polyadenylation (poly(A)) sequence; and

(b) a cassette, wherein the cassette comprises:

(i) at least one antigen-encoding nucleic acid sequence, comprising:

(I) at least two epitope-encoding nucleic acid sequence, optionally comprising: (1) at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, or (2) a nucleic acid sequence encoding an infectious disease organism peptide selected from the group consisting of: a pathogen-derived peptide, a virus-derived peptide, a bacteria-derived peptide, a fungus-derived peptide, and a parasite-derived peptide, and optionally wherein the epitope-encoding nucleic acid sequence encodes a MHC class I epitope , and

wherein each of the epitope-encoding nucleic acid sequences comprises;

(A) optionally, a 5' linker sequence, and

(B) optionally, a 3' linker sequence;

(ii) optionally, a second promoter nucleotide sequence operably linked to the antigen-encoding nucleic acid sequence; and

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

(iv) optionally, at least one nucleic acid sequence encoding a GPGPG amino acid linker sequence (SEQ ID NO:56); and

(v) optionally, at least one second poly(A) sequence, wherein the second poly(A) sequence is a native poly(A) sequence or an exogenous poly(A) sequence to the vector backbone,

wherein if the second promoter nucleotide sequence is absent, the antigen-encoding nucleic acid sequence is operably linked to the at least one promoter nucleotide sequence, and

wherein the cassette is 700 nucleotides in length or less, and

- wherein the subject expresses at least one HLA allele predicted or known to present the at least one epitope sequence.
214. The method of any of claims 196-213, wherein the antigen expression system comprises any one of the antigen expression systems in any one of claims 1-160.
215. The method of any of claims 196-213, wherein the antigen-based vaccine comprises any one of the pharmaceutical compositions in any one of claims 161-164.
216. The method of any of claims 196-215, wherein the antigen-based vaccine is administered as a priming dose.
217. The method of any of claims 196-216, wherein the antigen-based vaccine is administered as one or more boosting doses.
218. The method of claim 217, wherein the boosting dose is different than the priming dose.
219. The method of claim 218, wherein:
- a) the priming dose comprises a chimpanzee adenovirus vector and the boosting dose comprises an alphavirus vector; or
 - b) the priming dose comprises an alphavirus vector and the boosting dose comprises a chimpanzee adenovirus vector.
220. The method of claim 217, wherein the boosting dose is the same as the priming dose.
221. The method of any one of claims 217-220, wherein the injection site of the one or more boosting doses is as close as possible to the injection site of the priming dose.
222. The method of any one of the above method claims, further comprising determining or having determined the HLA-haplotype of the subject.
223. The method of any one of the above method claims, wherein the antigen-based vaccine is administered intramuscularly (IM), intradermally (ID), subcutaneously (SC), or intravenously (IV).
224. The method of any one of the above method claims, wherein the antigen-based vaccine is administered intramuscularly (IM).
225. The method of claim 224, wherein the IM administration is administered at separate injection sites.
226. The method of claim 225, wherein the separate injection sites are in opposing deltoid muscles.

227. The method of claim 226, wherein the separate injection sites are in gluteus or rectus femoris sites on each side.

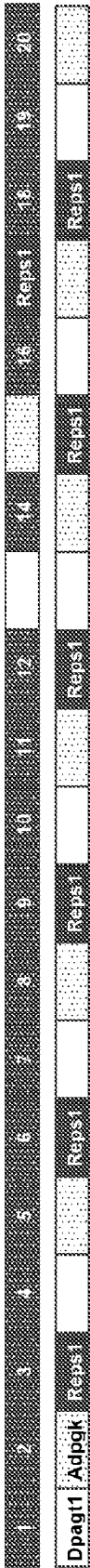


FIG. 1A

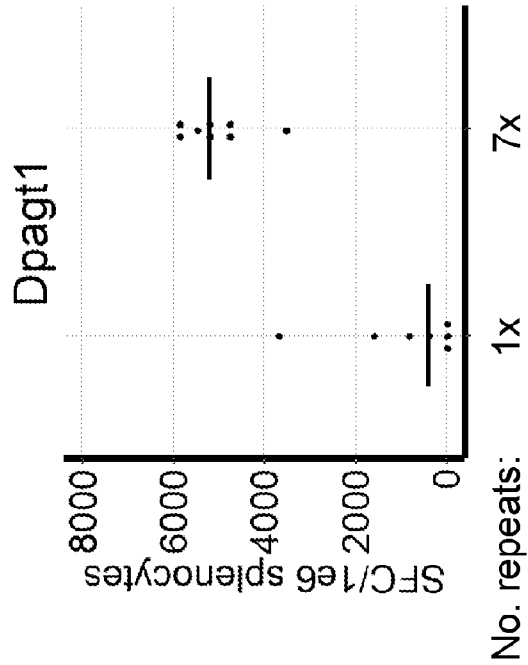


FIG. 1B

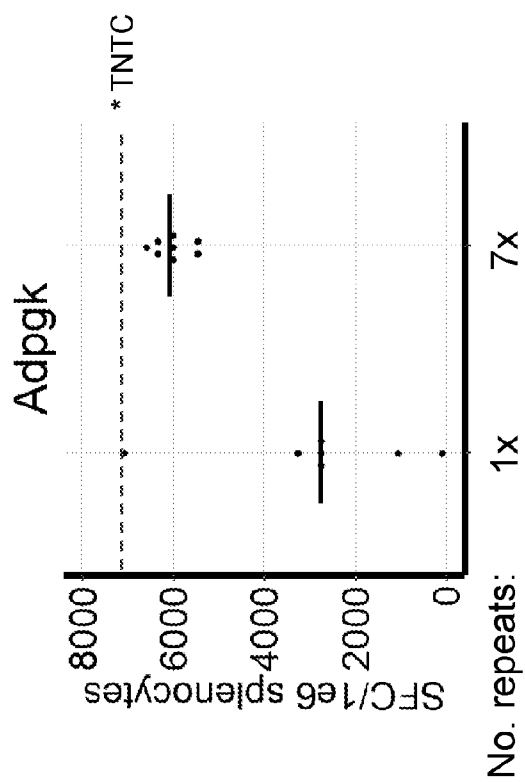


FIG. 1C

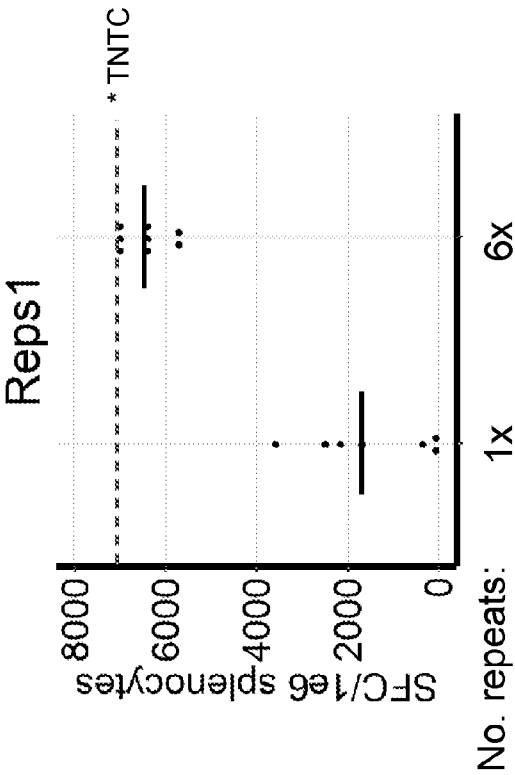


FIG. 1D



FIG. 2A

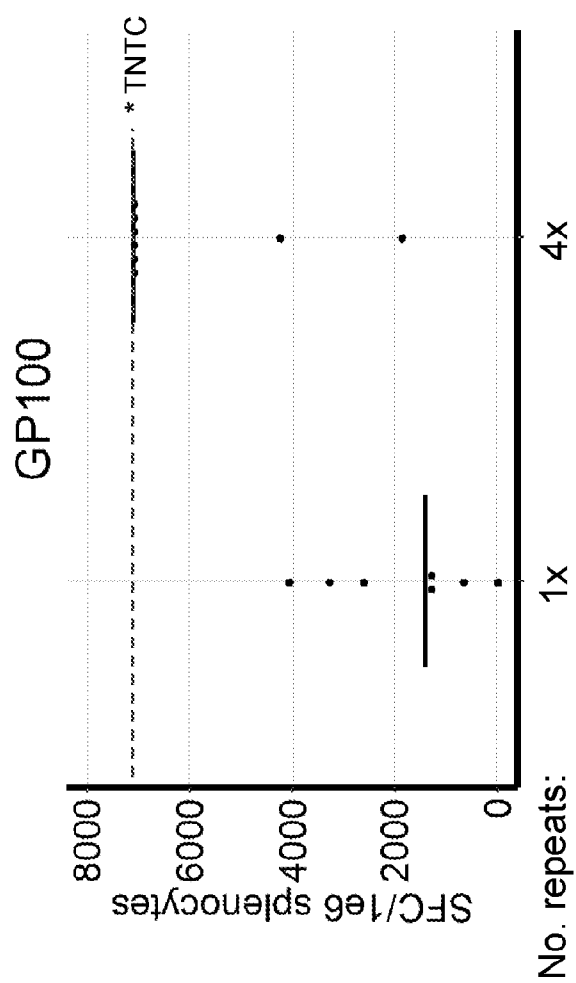


FIG. 2B

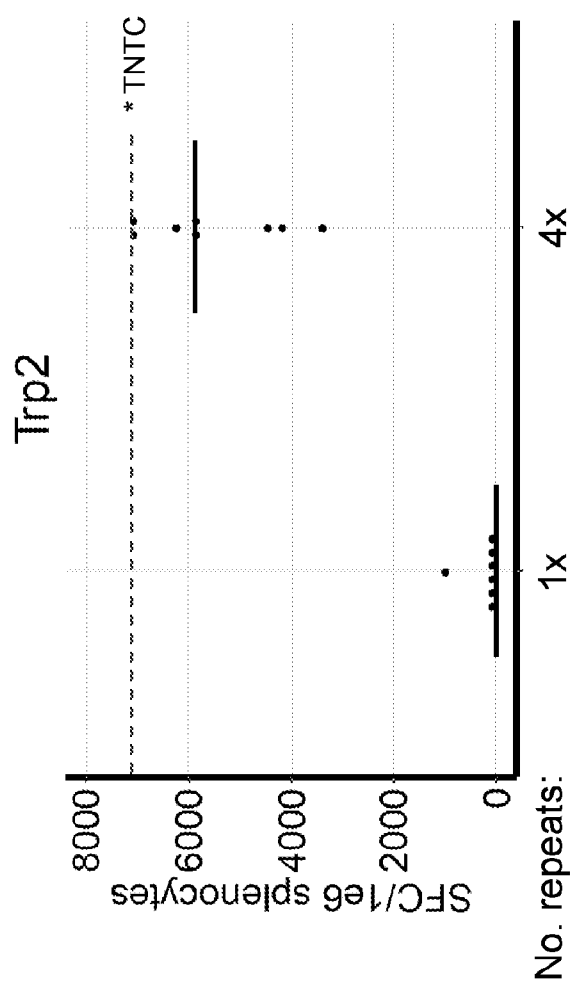


FIG. 2C

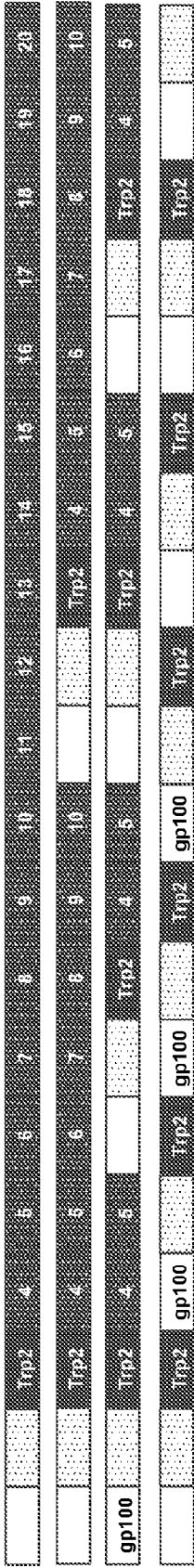


FIG. 3A

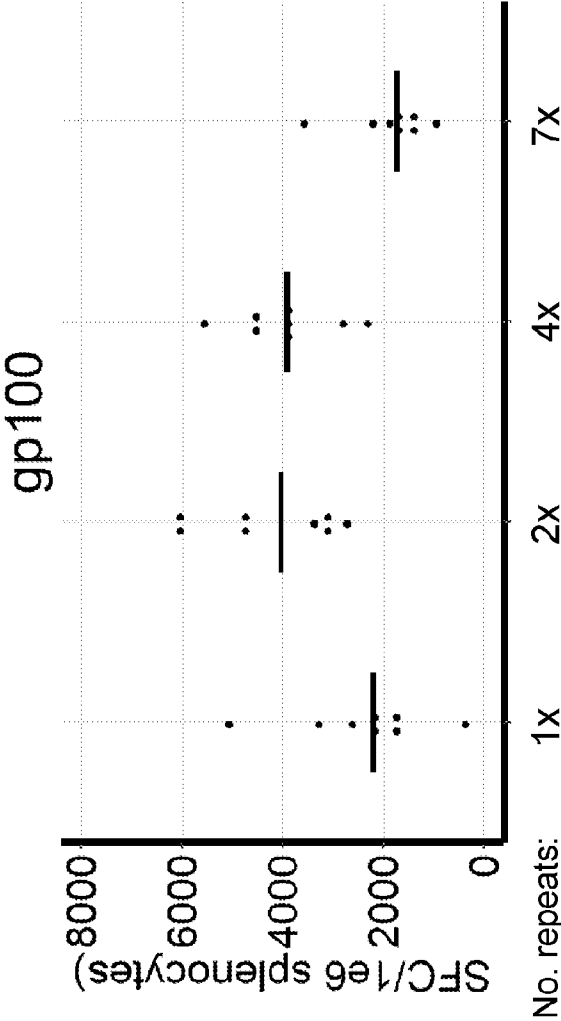


FIG. 3B

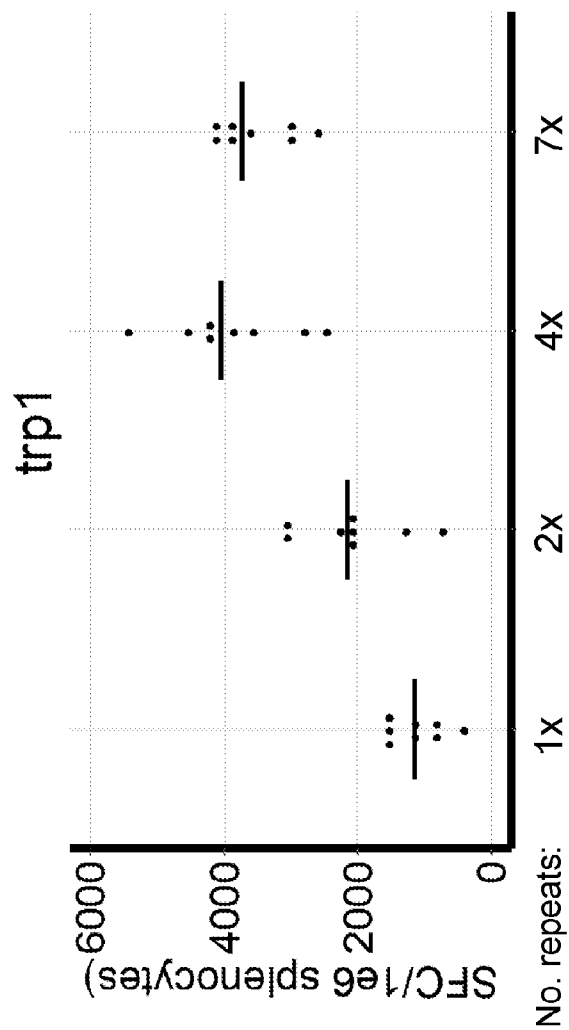


FIG. 3C

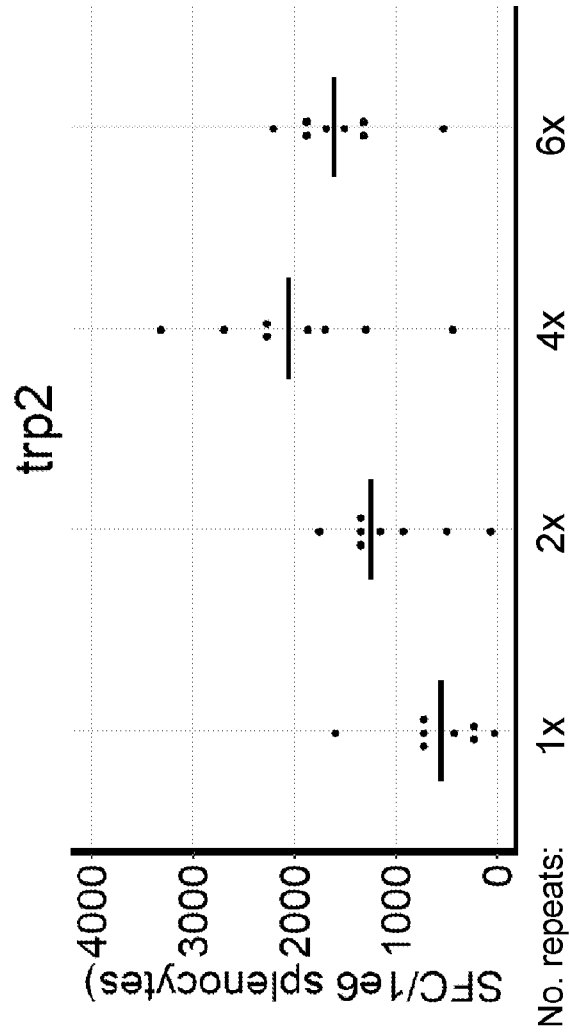


FIG. 3D

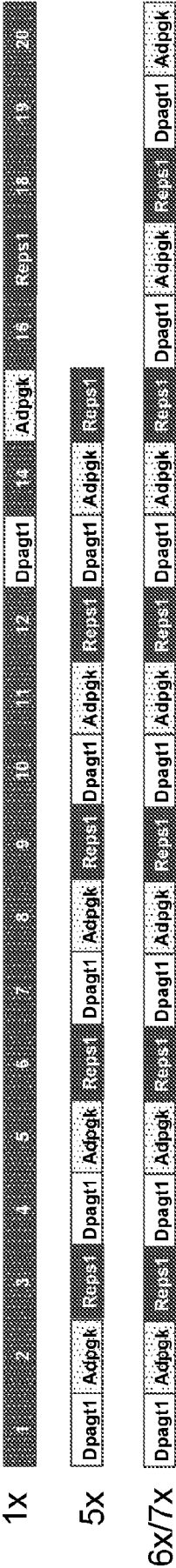


FIG. 4A

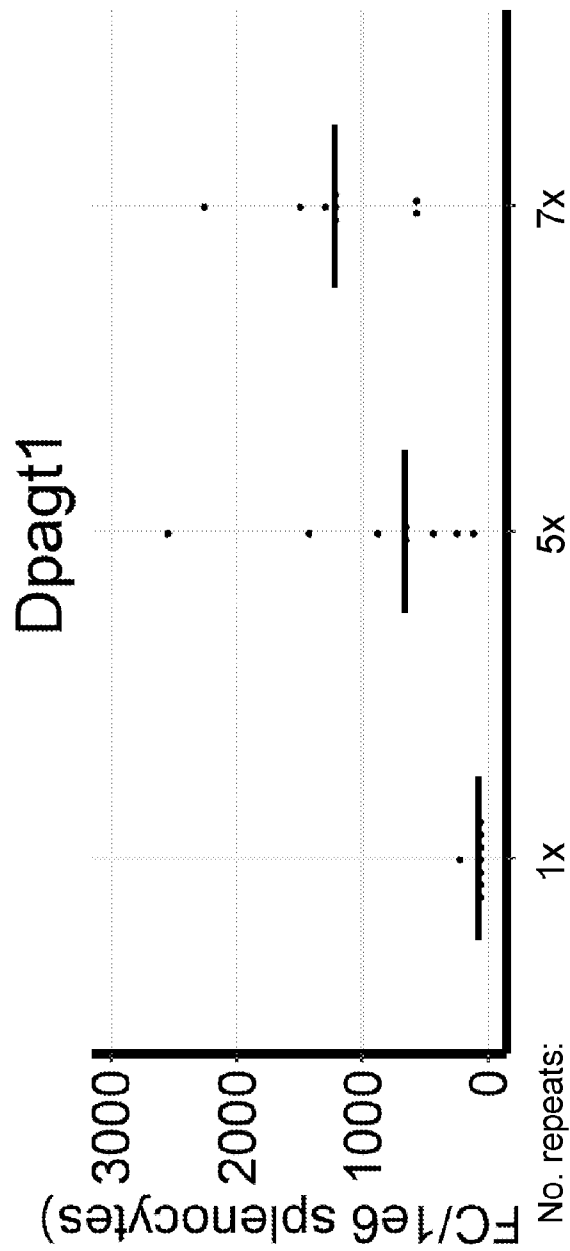


FIG. 4B

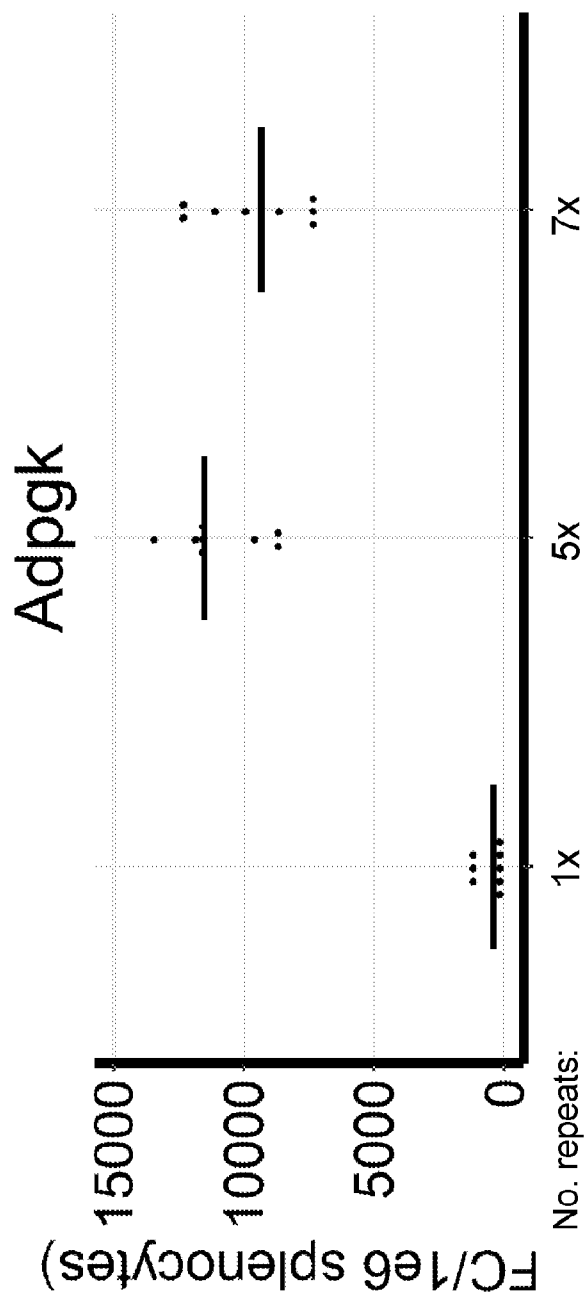


FIG. 4C

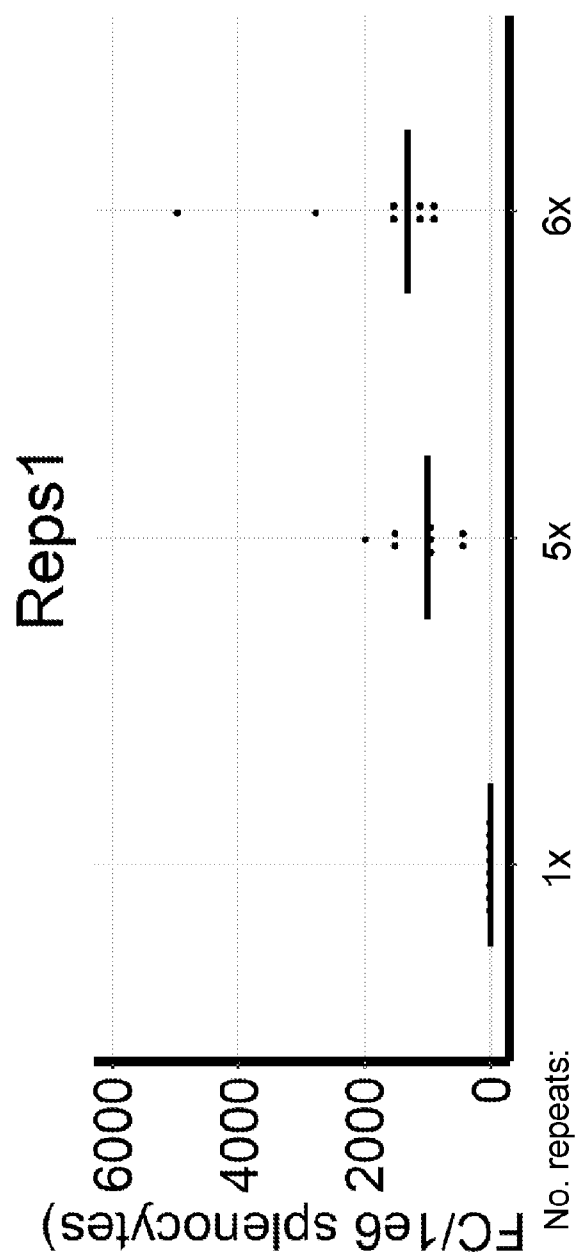


FIG. 4D

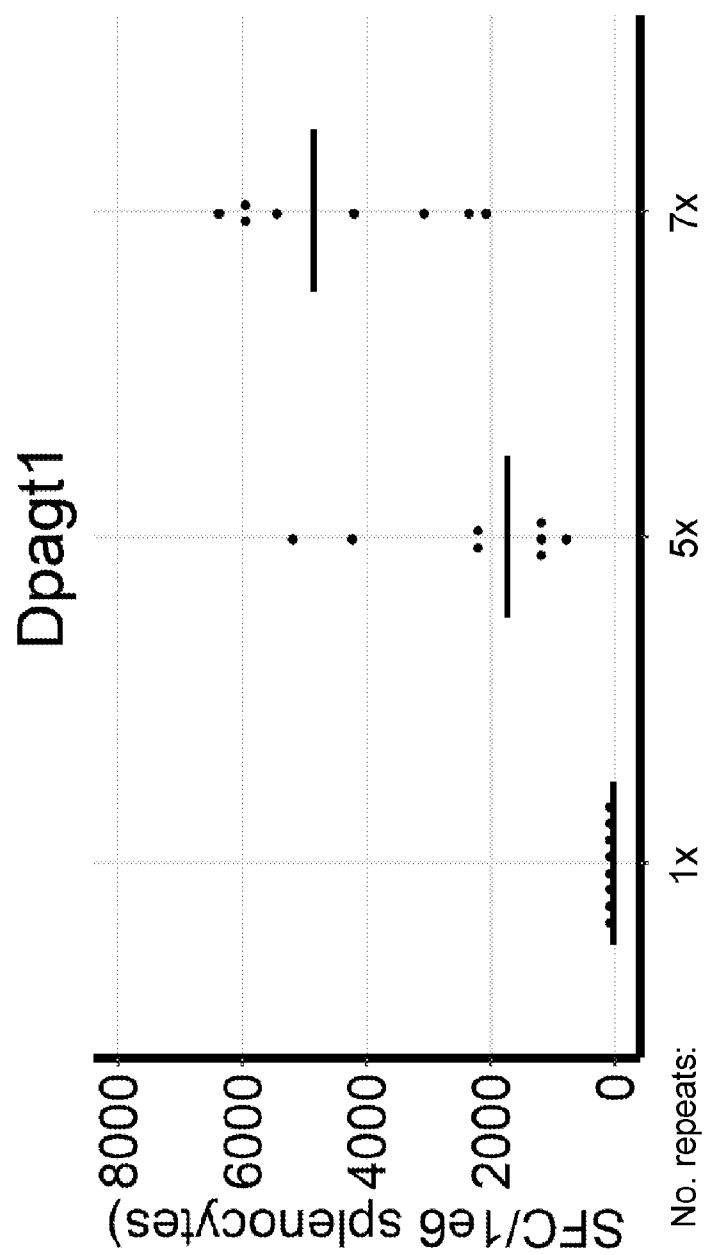


FIG. 4E

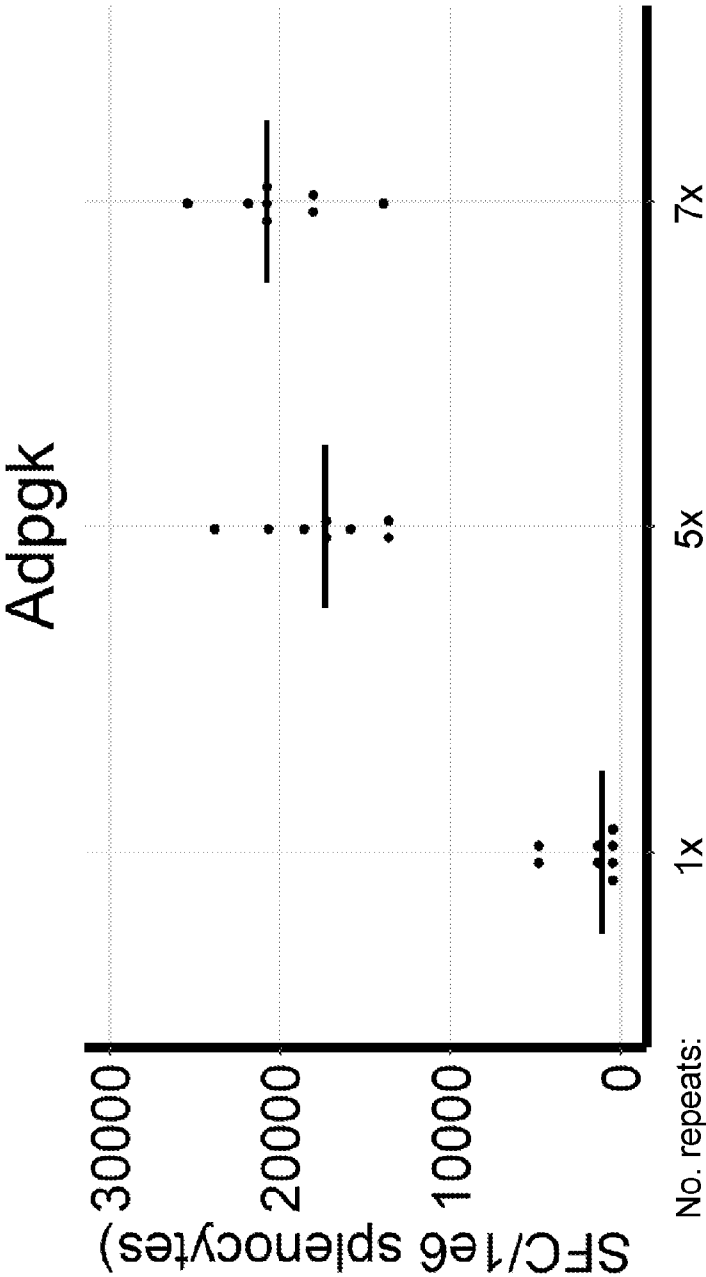


FIG. 4F

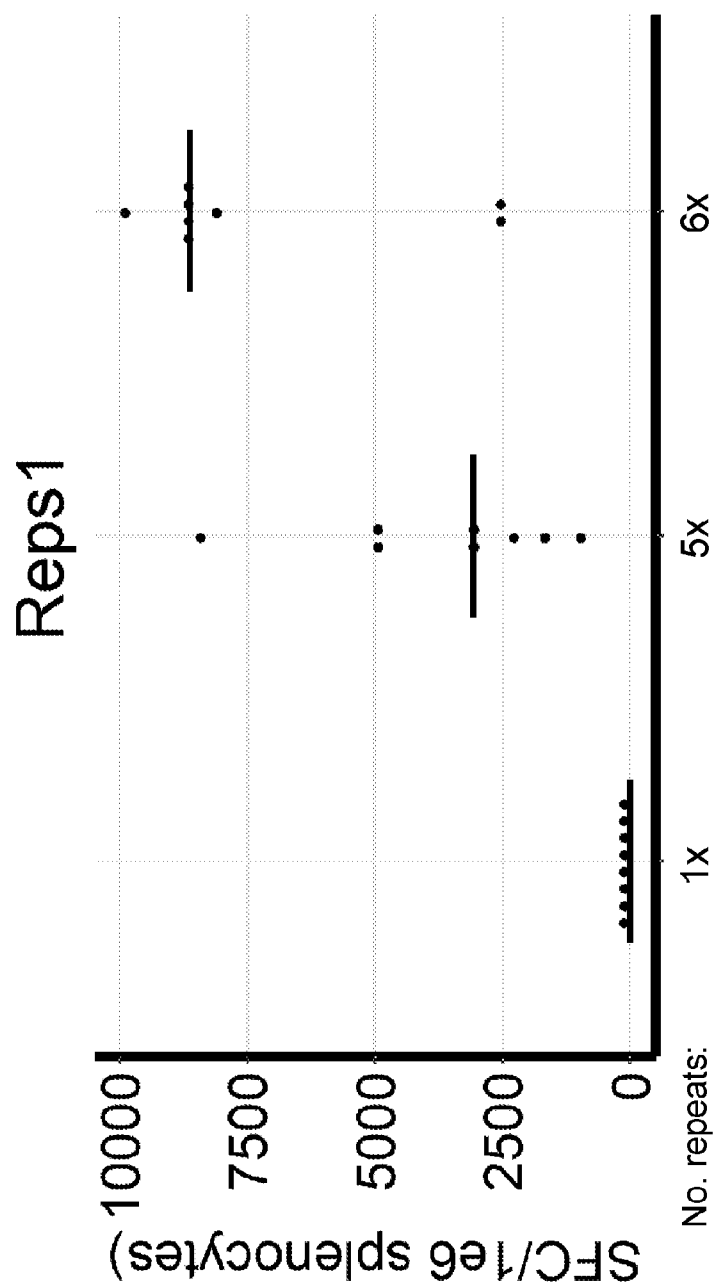


FIG. 4G



FIG. 5A

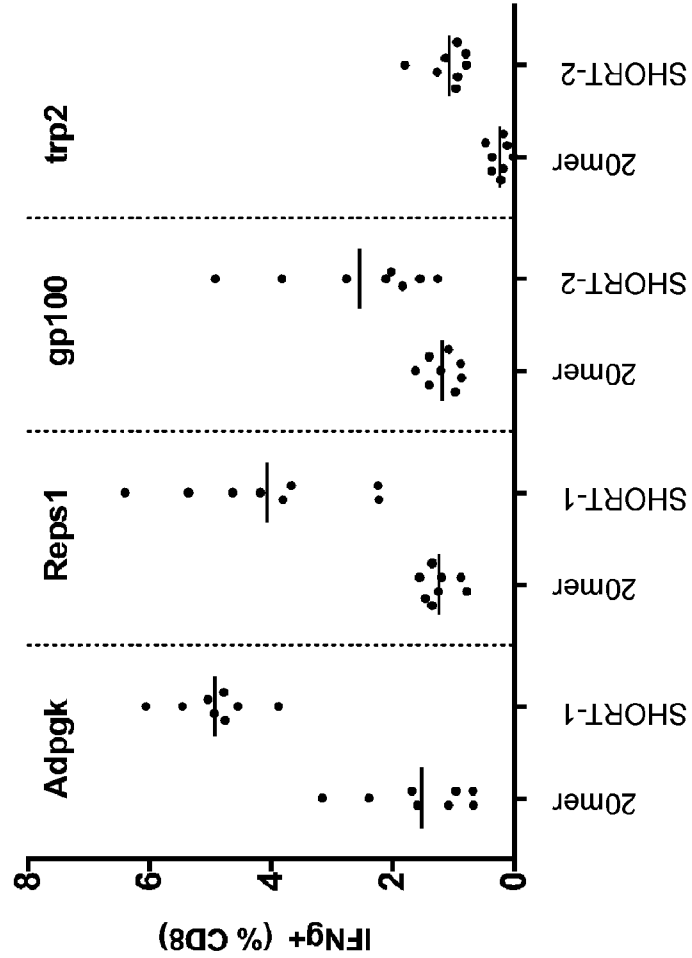


FIG. 5B

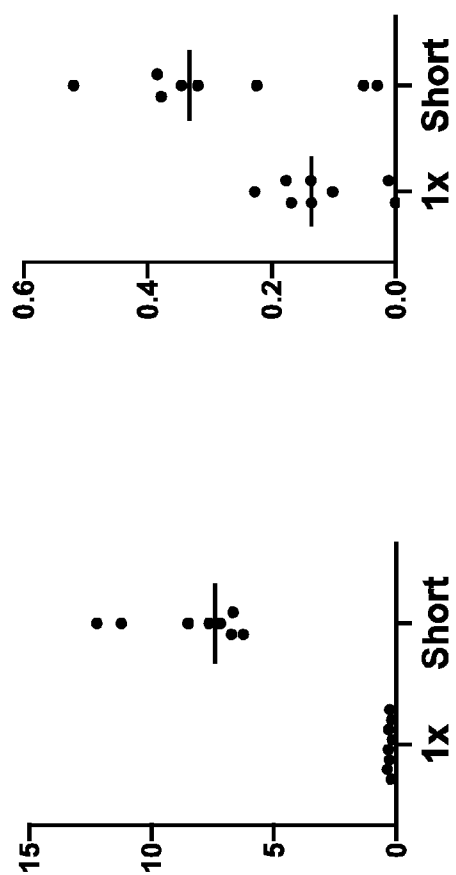


FIG. 5C

