MODIFIED ADENOVIRUSES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/854,865 filed May 30, 2019, which is hereby incorporated by reference in its entirety 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 7, 2022, is named GSO-033WO_SL.txt and is 820,368 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 responses[6] 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.

MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, and optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein; (ii) at least one promoter sequence operably linked to the at least one payload nucleic acid sequence, wherein the at least one promoter is a tetracycline (TET) repressor protein (TETr) controlled promoter, (iii) optionally, at least one MHC class II antigen-encoding nucleic acid sequence; (iv) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and (v) optionally, at least one polyadenylation sequence; b. providing a cell engineered to express the TETr protein; and c. contacting the viral vector with the cell under conditions sufficient for production of the virus.

[0015] In some examples, the viral vector comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector. In some examples, the production of the virus is increased using the vector comprising the TETr controlled promoter relative to production of a virus produced using a vector without the TETr controlled promoter. In some examples, the increased production is increased at least 1.5-fold, at least 2-fold, at least 2.5-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold relative to production using a vector without the TETr controlled promoter. In some examples, the increased production is increased at least 15-fold, at least 20-fold, at least 25-fold, at least 30-fold, at least 35-fold,at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, or at least 100-fold relative to production using a vector without the TETr controlled promoter. In some examples, the production of the virus is increased using the vector comprising the TETr controlled promoter relative to production of a virus produced using a cell that is not engineered to express the TETr protein. In some examples, the increased production is increased at least 1.5-fold, at least 2-fold, at least 2.5-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold relative to production using a cell that is not engineered to express the TETr protein.

[0016] Also provided herein is a viral vector comprising a cassette, the cassette comprising: (i) at least one payload nucleic acid sequence, optionally wherein the at least one payload nucleic acid sequence encodes a polypeptide, optionally wherein the polypeptide comprises an antigen, optionally wherein the antigen comprises: a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, and optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein; (ii) at

least one promoter sequence operably linked to the at least one payload nucleic acid sequence, wherein the at least one promoter is a tetracycline (TET) repressor protein (TETr) controlled promoter, (iii) optionally, at least one MHC class II antigen-encoding nucleic acid sequence; (iv) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and (v) optionally, at least one polyadenylation sequence.

[0017] In some examples, the TETr controlled promoter comprises one or more TET operator (TETo) nucleic acid sequences, optionally wherein the one or more TETo nucleic acid sequences comprises the nucleotide sequence shown in SEQ ID NO:60. In some examples, the one or more TETo nucleic acid sequences comprises 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more TETo nucleic acid sequences, optionally wherein each of TETo nucleic acid sequences comprises the nucleotide sequence shown in SEQ ID NO:60. In some examples, the 2 or more TETo nucleic acid sequences are linked together. In some examples, the 2 or more TETo nucleic acid sequences are directly linked together. In some examples, the 2 or more TETo nucleic acid sequences are linked together with a linker sequence, wherein the linker comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more nucleotides, and optionally wherein the linker sequence comprises the linker nucleotide sequence shown in SEQ ID NO:61. In some examples, the one or more TETo nucleic acid sequences are 5' of a RNA polymerase binding sequence of the promoter sequence. In some examples, the one or more TETo nucleic acid sequences are 3' of a RNA polymerase binding sequence of the promoter sequence. In some examples, the at least one promoter sequence comprises a CMV, SV40, EF-1, RSV, PGK, HSA, MCK or EBV promoter sequence. In some examples, the at least one promoter sequence is a CMV-derived promoter sequence, optionally wherein the CMV promoter sequence comprises the CMV promoter nucleotide sequence shown in SEQ ID NO:64. In some examples, the CMV-derived promoter sequence is a minimal CMV promoter sequence, optionally wherein the minimal CMV promoter sequence comprises the minimal CMV promoter nucleotide sequence as shown in SEQ ID NO:61.

[0018] In some examples, the TETr controlled promoter operably linked to the at least one payload nucleic acid sequence comprises an ordered sequence described in the formula, from 5' to 3', comprising: **(T-L_Y)$_X$-P-N** wherein, N comprises one of the at least one payload nucleic acid sequences, optionally wherein each N encodes a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, optionally with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence P a RNA

polymerase binding sequence of the promoter sequence operably linked to at least one of the at least one payload nucleic acid sequences, T comprises a TETo nucleic acid sequences comprising the nucleotide sequence shown in SEQ ID NO:60, L comprises a linker sequence, where Y = 0 or 1 for each X, and wherein X = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In some examples, the TETr controlled promoter operably linked to the at least one payload nucleic acid sequence comprises an ordered sequence described in the formula, from 5' to 3', comprising: **P-(T-L$_Y$)$_X$-N** wherein, N comprises one of the at least one payload nucleic acid sequences, optionally wherein each N encodes a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, optionally with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence P a RNA polymerase binding sequence of the promoter sequence operably linked to at least one of the at least one payload nucleic acid sequences, T comprises a TETo nucleic acid sequences comprising the nucleotide sequence shown in SEQ ID NO:60, L comprises a linker sequence, where Y = 0 or 1 for each X, and wherein X = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

[0019] In some examples, the TETr controlled promoter comprises: (1) a minimal CMV promoter sequence; (2) 7 TETo nucleic acid sequences, wherein each of TETo nucleic acid sequences comprises the nucleotide sequence shown in SEQ ID NO:60, and wherein each of the TETo nucleic acid sequences are linked together with a linker sequence, the 7 TETo nucleic acid sequences are 5' of the minimal CMV promoter sequence, and optionally wherein the TETr controlled promoter comprises the nucleotide sequence as shown in SEQ ID NO:61. In some examples, the TETr controlled promoter comprises: (1) a CMV promoter sequence; (2) 2 TETo nucleic acid sequences, wherein each of the TETo nucleic acid sequences comprises the nucleotide sequence shown in SEQ ID NO:60, and wherein each of the TETo nucleic acid sequences are directly linked together, the 2 TETo nucleic acid sequences are 3' of the CMV promoter sequence, and optionally wherein the TETr controlled promoter comprises the nucleotide sequence as shown in SEQ ID NO:64.

[0020] In some examples, the viral vector comprises a vector backbone, wherein the vector backbone comprises a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector.

[0021] In some examples, the cassette comprises an ordered sequence described in the formula, from 5' to 3', comprising: **P$_a$-(L5$_b$-N$_c$-L3$_d$)$_X$-(G5$_e$-U$_f$)$_Y$-G3$_g$-A$_h$** wherein, N comprises one of the at least one payload nucleic acid sequences, optionally wherein each N

encodes a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, optionally with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, where c = 1, P comprises the at least one promoter sequence operably linked to at least one of the at least one payload nucleic acid sequences, where a = 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 (SEQ ID NO: 56), where e = 0 or 1, G3 comprises one of the at least one nucleic acid sequences encoding a GPGPG amino acid linker (SEQ ID NO: 56), where g = 0 or 1, U comprises one of the at least one universal MHC class II antigen-encoding nucleic acid sequence, where f = 1, A comprises the at least one polyadenylation sequence, where h = 0 or 1, X = 2 to 400, where for each X the corresponding N_c is a payload nucleic acid sequence, optionally wherein for each X the corresponding N_c is a distinct payload nucleic acid sequence, and Y = 0-2, where for each Y the corresponding U_f is a universal MHC class II antigen-encoding nucleic acid sequence, optionally wherein for each Y the corresponding U_f is a distinct universal MHC class II antigen-encoding nucleic acid sequence.

[0022] In some examples, the cassette further comprises at least one additional payload nucleic acid sequence not encoded in the ordered sequence. In some examples, b = 1, d = 1, e = 1, g = 1, h = 1, X = 10, Y = 2, P is a CMV-derived promoter sequence, each N encodes a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof, L5 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 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, and the vector comprises a modified ChAdV68 sequence, wherein the modified ChAdV68 sequence comprises: (a) a partially deleted E4 gene of the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1; (b) 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; and (c) 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.

[0023] In some examples, the vector is a chimpanzee adenovirus vector, optionally wherein the chimpanzee adenovirus vector is a ChAdV68 vector.

[0024] In some examples, the partially deleted E4 gene comprises: A. the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1, B. 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, 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 shown in SEQ ID NO:1, C. 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, and wherein the vector comprises at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, D. 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, and wherein the vector comprises at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, E. an E4 deletion of at least a partial deletion of E4Orf2, a fully deleted E4Orf3, and at least a partial deletion of E4Orf4, F. 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, G. an E4 deletion of at least a partial deletion of E4Orf1, a fully deleted E4Orf2, and at least a partial deletion of E4Orf3, or H. an E4 deletion of at least a partial deletion of E4Orf2 and at least a partial deletion of E4Orf3.

[0025] In some examples, the vector comprises 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 are selected from the group consisting of the chimpanzee adenovirus inverted terminal repeat (ITR), E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence shown in SEQ ID NO: 1. In some examples, the adenoviral backbone or modified ChAdV68 sequence further comprises a functional deletion in at least one gene selected from the group consisting of an adenovirus E1A, E1B, E2A, E2B, E3, L1, L2, L3, L4, and L5 gene with reference to the adenovirus genome or with reference to the sequence shown in SEQ ID NO: 1, optionally wherein the adenoviral backbone or modified ChAdV68 sequence is fully deleted or functionally deleted in: (1) E1A and E1B; or (2) E1A, E1B, and E3 with reference to the adenovirus genome or with reference to the sequence shown in SEQ ID NO: 1, optionally wherein the E1 gene is functionally deleted through an E1 deletion of at least nucleotides 577 to 3403 with reference to the sequence shown in SEQ ID NO: 1 and

optionally wherein the E3 gene is functionally deleted through an E3 deletion of at least nucleotides 27,125 to 31,825 with reference to the sequence shown in SEQ ID NO: 1.

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

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

[0028] In some examples, the modified ChAdV68 sequence comprises 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. In some examples, the nucleotides 2 to 34,916 lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion. In some examples, the nucleotides 2 to 34,916 lack nucleotides 456-3014 with reference to the sequence shown in SEQ ID NO: 1. In some examples, the nucleotides 2 to 34,916 lack nucleotides 27,125-31,825 with reference to the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion. In some examples, the nucleotides 2 to 34,916 lack nucleotides 27,816- 31,333 with reference to the sequence shown in SEQ ID NO:1. In some examples, the nucleotides 2 to 34,916 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. In some examples, the nucleotides 2 to 34,916 further lack nucleotides 3957-10346, nucleotides 21787-23370, nucleotides 33486-36193, or a combination thereof with reference to the sequence shown in SEQ ID NO: 1.

[0029] In some examples, at least one of the at least one payload nucleic acid sequences encodes an antigen, wherein the antigen comprises: a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof. In some examples, at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence capable of undergoing antigen processing into an epitope, optionally wherein the epitope is 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.

[0030] In some examples, at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I and/or MHC class II on a surface of a cell, optionally wherein the surface of the cell is a tumor cell surface or an infected cell surface. In some examples, the 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 the infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, an fungally infected cell, and a parasitically infected cell, optionally 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, and a Hepatitis C virus (HCV) infected cell.

[0031] In some examples, at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence or portion thereof comprising an epitope capable of stimulating a B cell response, optionally wherein the polypeptide sequence or portion thereof comprises a full-length protein, a protein domain, a protein subunit, or an antigenic fragment predicted or known to be capable of being bound by an antibody.

[0032] In some examples, at least one of the at least one payload nucleic acid sequences encodes 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. In some examples, at least one of the at least one payload nucleic acid sequences encodes an epitope with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence. In some examples, at least one of the at least one payload nucleic acid sequences encodes a MHC class I epitope or MHC class II epitope with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, optionally wherein the encoded polypeptide sequence or portion thereof has increased binding affinity to, increased binding stability to, and/or an increased likelihood of presentation on its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence. In some examples, 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.

[0033] In some examples, at least one of the at least one payload nucleic acid sequences encodes a full-length protein, a protein domain, or a protein subunit. In some examples, at least

one of the at least one payload nucleic acid sequences encodes an antibody, a cytokine, a chimeric antigen receptor (CAR), a T-cell receptor, and a genome-editing system nuclease.

[0034] In some examples, at least one of the at least one payload nucleic acid sequences comprises a non-coding nucleic acid sequence. In some examples, the non-coding nucleic acid sequence comprises an RNA interference (RNAi) polynucleotide or genome-editing system polynucleotide.

[0035] In some examples, each of the at least one payload nucleic acid sequences is linked directly to one another. In some examples, at least one of the at least one payload nucleic acid sequences is linked to a distinct payload nucleic acid sequence with a nucleic acid sequence encoding a linker. In some examples, the linker links two payload nucleic acid sequences encoding MHC class I epitopes or links a first payload nucleic acid sequence encoding an MHC class I epitope to a second payload nucleic acid sequence encoding an MHC class II epitope or encoding an epitope sequence capable of stimulating a B cell response. In some examples, 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 examples, the linker links two payload nucleic acid sequences encoding MHC class II epitopes or links a first payload nucleic acid sequence encoding an MHC class II epitope to a second payload nucleic acid sequence encoding an MHC class I epitope or encoding an epitope sequence capable of stimulating a B cell response. In some examples, the linker comprises the sequence GPGPG (SEQ ID NO: 56).

[0036] In some examples, at least one of the at least one payload 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 payload nucleic acid sequence, and optionally the expression, stability, cell trafficking, processing and presentation, and/or immunogenicity of the polypeptide encoded by the at least one payload nucleic acid sequence. In some examples, the separate or contiguous sequence comprises at least one of: a ubiquitin sequence, a ubiquitin sequence modified to increase proteasome targeting, optionally wherein the ubiquitin sequence contains a Gly to Ala substitution at position 76, an immunoglobulin signal sequence, optionally wherein the

immunoglobulin signal sequence comprises 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.

[0037] In some examples, the expression of each of the at least one payload nucleic acid sequences is driven by the at least one promoter.

[0038] In some examples, the at least one payload nucleic acid sequence comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 payload nucleic acid sequences. In some examples, the at least one payload nucleic acid sequence comprises at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or up to 400 payload nucleic acid sequences. In some examples, the at least one payload nucleic acid sequence comprises at least 2-400 payload nucleic acid sequences and wherein at least one of the at least one payload nucleic acid sequences encodes a MHC class I epitope, a MHC class II epitope, an epitope capable of stimulating a B cell response, or a combination thereof. In some examples, the at least one payload nucleic acid sequence comprises at least 2-400 payload nucleic acid sequences and wherein, when administered to the subject and translated, at least one of the at least one payload nucleic acid sequences encodes an antigen presented on antigen presenting cells resulting in an immune response targeting the antigen. In some examples, the at least one payload nucleic acid sequence comprises at least 2-400 MHC class I and/or class II antigen-encoding nucleic acid sequences, wherein, when administered to the subject and translated, at least one of the MHC class I or class II antigens are presented on antigen presenting cells resulting in an immune response targeting at least one of the antigens on a cell surface, and optionally wherein the expression of each of the at least 2-400 MHC class I or class II antigen-encoding nucleic acid sequences is driven by the at least one promoter.

[0039] In some examples, each MHC class I epitope is independently between 8 and 35 amino acids in length, optionally 7-15, 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. In some examples, the at least one universal MHC class II antigen-encoding nucleic acid sequence is present. In some examples, the at least one universal MHC class II antigen-encoding nucleic acid sequence is present and comprises at least one universal MHC class II antigen-encoding nucleic acid sequence that comprises at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence. In some examples, the at least one universal MHC class II antigen-encoding nucleic acid sequence is 12-20, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 20-40 amino acids in

length. In some examples, the at least one universal MHC class II antigen-encoding nucleic acid sequence is present and wherein the at least one universal sequence comprises at least one of Tetanus toxoid and PADRE.

[0040] In some examples, the at least one promoter sequence is a regulatable promoter, optionally wherein the regulatable promoter is a tetracycline (TET) repressor protein (TETr) controlled promoter, optionally wherein the regulatable promoter comprises multiple TET operator (TETo) sequences 5' or 3'of a RNA polymerase binding sequence of the promoter. multiple TET operator (TETo) sequences are 5' or 3'of a RNA the at least one promoter sequence is constitutive. multiple TET operator (TETo) sequences are 5' or 3'of a RNA the at least one promoter sequence is a CMV, SV40, EF-1, RSV, PGK, HSA, MCK or EBV promoter sequence.

[0041] In some examples, the cassette further comprises at least one poly-adenylation (polyA) sequence operably linked to at least one of the at least one payload nucleic acid sequences, optionally wherein the polyA sequence is located 3' of the at least one payload nucleic acid sequence. In some examples, the polyA sequence comprises an SV40 or Bovine Growth Hormone (BGH) polyA sequence.

[0042] In some examples, 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, a nucleotide sequence encoding a TEV 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 payload nucleic acid sequences.

[0043] In some examples, the cassette comprises a reporter gene, including but not limited to, green fluorescent protein (GFP), a GFP variant, secreted alkaline phosphatase, luciferase, or a luciferase variant.

[0044] In some examples, the vector further comprises one or more payload nucleic acid sequences encoding at least one immune modulator, optionally wherein the at least one immune modulator inhibits an immune checkpoint molecule. In some examples, 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 examples, 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 examples, the heavy and light chain sequences of the antibody are a contiguous sequence separated by either a self-cleaving sequence such as 2A, optionally wherein the self-cleaving sequence has a Furin cleavage site sequence 5' of the self-cleaving sequence, or an IRES sequence; or the heavy and light chain sequences of the antibody are linked by a flexible linker such as consecutive glycine residues. In some examples, the immune modulator is a cytokine. In some examples, the cytokine is at least one of IL-2, IL-7, IL-12, IL-15, or IL-21 or variants thereof of each.

[0045] In some examples, at least one of the at least one payload nucleic acid sequences are selected by performing the steps of: (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor cell, 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 payload nucleic acid sequence.

[0046] In some examples, each of the at least one payload nucleic acid sequences are selected by performing the steps of: (a) obtaining at least one of exome, transcriptome, or whole genome nucleotide sequencing data from a tumor cell, 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 each of the at least one payload nucleic acid sequences. In some examples, a number of the set of selected antigens is 2-20. In some examples, 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 examples, 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. In some examples, selecting the set of selected antigens comprises selecting antigens that have an increased likelihood of being capable of inducing a cell-specific immune response in the subject relative to unselected antigens based on the presentation model. In some examples, 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 examples, 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 examples, selecting the set of selected antigens comprises selecting antigens that have a decreased likelihood of being capable of inducing an autoimmune response to normal tissue in the subject relative to unselected antigens based on the presentation model. In some examples, 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 examples, the sequencing is next generation sequencing (NGS) or any massively parallel sequencing approach.

[0047] In some examples, the cassette comprises junctional epitope sequences formed by adjacent sequences in the cassette. In some examples, at least one or each junctional epitope sequence has an affinity of greater than 500 nM for MHC. In some examples, each junctional epitope sequence is non-self. In some examples, the cassette does not encode a non-therapeutic MHC class I or class II epitope, wherein the non-therapeutic epitope is predicted to be displayed on an MHC allele of a subject. In some examples, the non-therapeutic predicted MHC class I or class II epitope sequence is a junctional epitope sequence formed by adjacent sequences in the cassette. In some examples, the prediction in based on presentation likelihoods generated by inputting sequences of the non-therapeutic epitopes into a presentation model. In some examples, an order of the at least one payload nucleic acid sequences in the cassette is determined by a series of steps comprising: i. generating a set of candidate cassette sequences corresponding to different orders of the at least one payload nucleic acid sequences; ii.

determining, for each candidate cassette sequence, a presentation score based on presentation of non-therapeutic epitopes in the candidate cassette sequence; and iii. selecting a candidate cassette sequence associated with a presentation score below a predetermined threshold as the cassette sequence.

[0048] In some examples, each of the MHC class I and/or class II epitopes is predicted or validated to be capable of presentation by at least one HLA allele present in at least 5% of a human population. In some examples, each of the MHC class I and/or class II 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 human population. In some examples, each of the MHC class I and/or class II 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 human population. In some examples, the at least one payload nucleic acid sequence encoding the polypeptide is codon optimized relative to a native nucleic acid sequence directly extracted from a subject tissue or sample.

[0049] Also disclosed herein is a pharmaceutical composition comprising any of the vectors described herein and a pharmaceutically acceptable carrier. In some examples, the composition further comprises an adjuvant. In some examples, the composition further comprises an immune modulator. In some examples, 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.

[0050] Also disclosed herein is an isolated nucleotide sequence comprising the cassette of any of the vectors described herein and a gene of the sequence of SEQ ID NO: 1, optionally wherein the gene is selected from the group consisting of the chimpanzee adenovirus ITR, E1A, E1B, E2A, E2B, E3, E4, L1, L2, L3, L4, and L5 genes of the sequence shown in SEQ ID NO: 1, and optionally wherein the nucleotide sequence is cDNA.

[0051] Also disclosed herein is an isolated cell comprising any of the isolated nucleotide sequences described herein, optionally wherein the cell is a CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.

[0052] Also disclosed is vector comprising any of the isolated nucleotide sequences described herein.

[0053] Also disclosed herein is a kit comprising any of the vectors or compositions described herein and instructions for use.

[0054] Also disclosed herein is amethod for stimulating an immune response in a subject, the method comprising administering to the subject any of the vectors or compositions described herein. In some examples, the vector or composition is administered intramuscularly (IM), intradermally (ID), or subcutaneously (SC). In some examples, the method further comprises administering to the subject an immune modulator, optionally wherein the immune modulator is administered before, concurrently with, or after administration of the vector or pharmaceutical composition. In some examples, 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 examples, the immune modulator is administered intravenously (IV), intramuscularly (IM), intradermally (ID), or subcutaneously (SC). In some examples, the subcutaneous administration is near the site of the vector or composition administration or in close proximity to one or more vector or composition draining lymph nodes.

[0055] In some examples, the method further comprises administering to the subject a second vaccine composition. In some examples, the second vaccine composition is administered prior to the administration of any of the vectors or compositions described herein. In some examples, the second vaccine composition is administered subsequent to the administration of any of the vectors or compositions described herein. In some examples, the second vaccine composition is the same as any of the vectors or compositions described herein. In some examples, the second vaccine composition is different from any of the vectors or compositions described herein. In some examples, the second vaccine composition comprises a self-amplifying RNA (samRNA) vector encoding at least one payload nucleic acid sequence. In some examples, the at least one payload nucleic acid sequence encoded by the samRNA vector is the same as at least one of the at least one payload nucleic acid sequence of any of the above vector claims.

[0056] Also disclosed herein is a method of manufacturing the vector of any of the above vector claims, the method comprising: obtaining a plasmid sequence comprising the adenovirus vector or chimpanzee adenovirus vector; transfecting the plasmid sequence into one or more host cells; and isolating the vector from the one or more host cells. In some examples, the isolating comprises: lysing the one or more host cells to obtain a cell lysate comprising the vector; and purifying the vector from the cell lysate and optionally also from media used to culture the one or more host cells. In some examples, the plasmid sequence is

generated using one of the following; DNA recombination or bacterial recombination or full genome DNA synthesis or full genome DNA synthesis with amplification of synthesized DNA in bacterial cells. In some examples, 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 examples, the purifying the vector from the cell lysate involves one or more of chromatographic separation, centrifugation, virus precipitation, and filtration.

[0057] Also provided herein is a method of producing a virus, wherein the virus is produced using any of the vectors described herein. In some examples, the production of the virus is increased using the vector comprising the partially deleted E4 gene relative to production of a virus produced using a vector without the partially deleted E4 gene. In some examples, the infectious unit titer of the virus is increased using the vector comprising the partially deleted E4 gene relative to the infectious unit titer of a virus produced using a vector without the partially deleted E4 gene. In some examples, the increased production is increased at least 1.5-fold, at least 2-fold, at least 2.5-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, or at least 9-fold relative to production using a vector without the partially deleted E4 gene. In some examples, the increased production is increased at least 10-fold, at least 18-fold, at least 20-fold, at least 25-fold, or at least 27-fold, relative to production using a vector without the partially deleted E4 gene.

[0057a] Also provided herein is an isolated nucleotide sequence encoding the vector as described herein, and optionally wherein the nucleotide sequence is cDNA.

[0057b] Also provided herein is a use of the vector as described herein in the manufacture of a medicament for stimulating an immune response in a subject.

[00057c] Also provided herein is a use of the vector as described herein in the manufacture of a medicament for treating a subject with a disease, optionally wherein the disease is cancer or an infection.

[0057d] Also provided herein is a method of producing a virus, wherein the virus is produced using the vector as described herein, wherein the production and/or infectious unit titer of the virus is increased using the vector comprising the partially deleted E4 gene relative to production of a virus produced using a vector without the partially deleted E4 gene.

[0057e] Also provided herein is a use of an immunotherapy in the manufacture of a medicament for treating a subject with cancer, wherein the immunotherapy is to be administered to the subject, and wherein the the immunotherapy comprises an adenovirus

vector comprising: an adenoviral backbone comprising one or more genes or regulatory sequences of an adenovirus genome, wherein the adenoviral backbone comprises a partially deleted E4 gene with reference to the adenovirus genome, wherein the partially deleted E4 gene comprises a deleted or partially-deleted E4orf2 region and a deleted or partially-deleted E4orf3 region, and optionally a deleted or partially-deleted E4orf4 region, and wherein the adenovirus vector further comprises a cassette, the cassette comprising: (1) at least one payload nucleic acid sequence, optionally wherein the at least one payload nucleic acid sequence encodes a polypeptide, optionally wherein the polypeptide comprises an antigen, optionally wherein the antigen comprises: - a MHC class I epitope, - a MHC class II epitope, - an epitope capable of stimulating a B cell response, or - a combination thereof, and optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein; (2) at least one promoter sequence operably linked to the at least one payload nucleic acid sequence, (3) optionally, at least one universal MHC class II antigen-encoding nucleic acid sequence; (4) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and (5) optionally, at least one polyadenylation sequence.

[0057f] Also provided herein is the vector as described herein for use in therapy.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0058] 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:

[0059] **Figure (Fig.) 1** illustrates development of an *in vitro* T cell activation assay. Schematic of the assay in which the delivery of a vaccine cassette to antigen presenting cells, leads to expression, processing and MHC-restricted presentation of distinct peptide antigens. Reporter T cells engineered with T cell receptors that match the specific peptide-MHC combination become activated resulting in luciferase expression.

[0060] **Fig. 2A** illustrates evaluation of linker sequences in short cassettes and shows five class I MHC restricted epitopes (epitopes 1 through 5) concatenated in the same position relative to each other followed by two universal class II MHC epitopes (MHC-II). Various iterations were generated using different linkers. In some cases the T cell epitopes are directly linked to each other. In others, the T cell epitopes are flanked on one or both sides by its

natural sequence. In other iterations, the T cell epitopes are linked by the non-natural sequences AAY, RR, and DPP.

[0061] **Fig. 2B** illustrates evaluation of linker sequences in short cassettes and shows sequence information on the T cell epitopes embedded in the short cassettes. Fig. 2B discloses SEQ ID NOS 78-79, 82, 81, 80, 49 and 194, respectively, in order of appearance.

[0062] **Fig. 3** illustrates evaluation of cellular targeting sequences added to model vaccine cassettes. The targeting cassettes extend the short cassette designs with ubiquitin (Ub), signal peptides (SP) and/or transmembrane (TM) domains, feature next to the five marker human T cell epitopes (epitopes 1 through 5) also two mouse T cell epitopes SIINFEKL (SII) (SEQ ID NO: 72) and SPSYAYHQF (A5) (SEQ ID NO: 73), and use either the non-natural linker AAY- or natural linkers flanking the T cell epitopes on both sides (25mer) .

[0063] **Fig. 4** illustrates *in vivo* evaluation of linker sequences in short cassettes. A) Experimental design of the *in vivo* evaluation of vaccine cassettes using HLA-A2 transgenic mice.

[0064] **Fig. 5A** illustrates *in vivo* evaluation of the impact of epitope position in long 21-mer cassettes and shows the design of long cassettes entails five marker class I epitopes (epitopes 1 through 5) contained in their 25-mer natural sequence (linker = natural flanking sequences), spaced with additional well-known T cell class I epitopes (epitopes 6 through 21) contained in their 25-mer natural sequence, and two universal class II epitopes (MHC-II0, with only the relative position of the class I epitopes varied.

[0065] **Fig. 5B** illustrates *in vivo* evaluation of the impact of epitope position in long 21-mer cassettes and shows the sequence information on the T cell epitopes used. Fig. 5B discloses SEQ ID NOS 78-79, 82, 81, 80, 195-197, 83 and 198-209, respectively, in order of appearance.

[0066] **Fig. 6A** illustrates final cassette design for preclinical IND-enabling studies and shows the design of the final cassettes comprises 20 MHC I epitopes contained in their 25-mer natural sequence (linker = natural flanking sequences), composed of 6 non-human primate (NHP) epitopes, 5 human epitopes, 9 murine epitopes, as well as 2 universal MHC class II epitopes.

[0067] **Fig. 6B** illustrates final cassette design for preclinical IND-enabling studies and shows the sequence information for the T cell epitopes used that are presented on class I MHC of non-human primate, mouse and human origin, as well as sequences of 2 universal MHC class II epitopes PADRE and Tetanus toxoid. Fig. 6B discloses SEQ ID NOS 112-117, 80-82, 78-79, 72-73, 142, 210, 146-148, 144-145, 49 and 47, respectively, in order of columns.

[0068] **Fig. 7A** illustrates ChAdV68.4WTnt.GFP virus production after transfection. HEK293A cells were transfected with ChAdV68.4WTnt.GFP DNA using the calcium phosphate protocol. Viral replication was observed 10 days after transfection and

immunization of C57BL/6J mice with VEE-Luciferase srRNA (10 ug per mouse, bilateral intramuscular injection, MC3 encapsulated) at various timepoints.

[0077] **Fig. 12A** illustrates T-cell responses measured 14 days after immunization with VEE srRNA formulated with MC3 LNP in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with 10 ug of VEE-Luciferase srRNA (control), VEE-UbAAY srRNA (Vax), VEE-Luciferase srRNA and anti-CTLA-4 (aCTLA-4) or VEE-UbAAY srRNA and anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD1 mAb starting at day 7. Each group consisted of 8 mice. Mice were sacrificed and spleens and lymph nodes were collected 14 days after immunization. SIINFEKL-specific T-cell ("SIINFEKL" disclosed as SEQ ID NO: 72) responses were assessed by IFN-gamma ELISPOT and are reported as spot-forming cells (SFC) per 106 splenocytes. Lines represent medians.

[0078] **Fig. 12B** illustrates T-cell responses measured 14 days after immunization with VEE srRNA formulated with MC3 LNP in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with 10 ug of VEE-Luciferase srRNA (control), VEE-UbAAY srRNA (Vax), VEE-Luciferase srRNA and anti-CTLA-4 (aCTLA-4) or VEE-UbAAY srRNA and anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD1 mAb starting at day 7. Each group consisted of 8 mice. Mice were sacrificed and spleens and lymph nodes were collected 14 days after immunization. SIINFEKL-specific T-cell ("SIINFEKL" disclosed as SEQ ID NO: 72) responses were assessed by MHCI-pentamer staining, reported as pentamer positive cells as a percent of CD8 positive cells. Lines represent medians.

[0079] **Fig. 13A** illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A third group was treated with the Ad5-GFP prime/VEE-Luciferase srRNA boost in combination with anti-CTLA-4 (aCTLA-4), while the fourth group was treated with the Ad5-UbAAY prime/VEE-UbAAY boost in combination with anti-CTLA-4 (Vax + aCTLA-4). In addition, all mice were treated with anti-PD-1 mAb starting at day 21. T-cell responses were measured by IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 14 days post immunization with adenovirus.

[0080] **Fig. 13B** illustrates antigen-specific T-cell responses following heterologous prime/boost in B16-OVA tumor bearing mice. B16-OVA tumor bearing C57BL/6J mice were injected with adenovirus expressing GFP (Ad5-GFP) and boosted with VEE-Luciferase

boosted 15 days after the adenovirus prime with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or primed with Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A separate group was administered the Ad5-GFP/VEE-Luciferase srRNA prime/boost in combination with anti-PD-1 (aPD1), while a fourth group received the Ad5-UbAAY/VEE-UbAAY srRNA prime/boost in combination with an anti-PD-1 mAb (Vax + aPD1). T-cell responses to the AH1 peptide were measured using IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 12 days post immunization with adenovirus.

[0084] **Fig. 14B** illustrates antigen-specific T-cell responses following heterologous prime/boost in CT26 (Balb/c) tumor bearing mice. Mice were immunized with Ad5-GFP and boosted 15 days after the adenovirus prime with VEE-Luciferase srRNA formulated with MC3 LNP (Control) or primed with Ad5-UbAAY and boosted with VEE-UbAAY srRNA (Vax). Both the Control and Vax groups were also treated with an IgG control mAb. A separate group was administered the Ad5-GFP/VEE-Luciferase srRNA prime/boost in combination with anti-PD-1 (aPD1), while a fourth group received the Ad5-UbAAY/VEE-UbAAY srRNA prime/boost in combination with an anti-PD-1 mAb (Vax + aPD1). T-cell responses to the AH1 peptide were measured using IFN-gamma ELISPOT. Mice were sacrificed and spleens and lymph nodes collected at 12 days post immunization with adenovirus and 6 days post boost with srRNA (day 21 after prime).

[0085] **Fig. 15** illustrates ChAdV68 eliciting T-Cell responses to mouse tumor antigens in mice. Mice were immunized with ChAdV68.5WTnt.MAG25mer, and T-cell responses to the MHC class I epitope SIINFEKL (SEQ ID NO: 72) (OVA) were measured in C57BL/6J female mice and the MHC class I epitope AH1-A5 measured in Balb/c mice. Mean spot forming cells (SFCs) per 10^6 splenocytes measured in ELISpot assays presented. Error bars represent standard deviation.

[0086] **Fig. 16** illustrates cellular immune responses in a CT26 tumor model following a single immunization with either ChAdV6, ChAdV + anti-PD-1, srRNA, srRNA + anti-PD-1, or anti-PD-1 alone. Antigen-specific IFN-gamma production was measured in splenocytes for 6 mice from each group using ELISpot. Results are presented as spot forming cells (SFC) per 10^6 splenocytes. Median for each group indicated by horizontal line. P values determined using the Dunnett's multiple comparison test; *** P<0.0001, **P<0.001, *P<0.05. ChAdV= ChAdV68.5WTnt.MAG25mer; srRNA= VEE-MAG25mer srRNA.

LNP1 homologous prime/boost regimen using ELISpot prior to immunization and 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, or 15 weeks after the initial immunization. Results are presented as mean spot forming cells (SFC) per 10^6 PBMCs for each epitope (6 rhesus macaques per group) in a stacked bar graph format.

[0097] **Fig. 24A** shows an example peptide spectrum generated from Promega's dynamic range standard. Fig. 24A discloses SEQ ID NO: 77.

[0098] **Fig. 24B** shows an example peptide spectrum generated from Promega's dynamic range standard.

[0099] **Fig. 25** shows productivity, as assessed by IU titers, of the eight selected ChAdV68-MAG rapidly growing plaques compared to the non-purified pooled virus. Numbers above the columns on the graph indicate fold improvement over the pooled virus in a controlled infection at an MOI of 0.1.

[00100] **Fig. 26** shows a schematic of the E4 locus and the 727 bp deletion between E4orf2-E4orf4 identified in Clone 1A.

[00101] **Fig. 27** shows virus productivity with viruses plus and minus the E4 deletion. Numbers above the bar indicate fold improvement over non-E4 deleted virus. The ChAdV68-MAG comparison to ChAdV68-MAG-E4 virus was performed on 3 separate occasions. In each case a 400 mL production run with both viruses was performed at an MOI of 1.0. Shown are viral particle (VP) titers (left panel) and infectious unit (IU) titers (right panel).

[00102] **Fig. 28** shows a Western blot analysis of MAG expression using rabbit anti-class II epitope antibody expression in cells infected with ChAdV68.5WTnt.MAG25mer ("MAG") and ChAdV68-MAG-E4deleted ("MAG-E4") viruses. Samples were treated with and without the proteasome inhibitor, MG-132, as indicated by plus and minus signs.

[00103] **Fig. 29** illustrates the general organization of the model epitopes from the various species for large antigen cassettes that had either 30 (L), 40 (XL) or 50 (XXL) epitopes.

[00104] **Fig. 30** shows ChAd vectors express long cassettes as indicated by the above Western blot using an anti-class II (PADRE) antibody that recognizes a sequence common to all cassettes. HEK293 cells were infected with ChAdV68 vectors expressing large cassettes (ChAdV68-50XXL, ChAdV68-40XL & ChAdV68-30L) of variable size. Infections were set up at a MOI of 0.2. Twenty-four hours post infection MG132 a proteasome inhibitor was added to a set of the infected wells (indicated by the plus sign). Another set of virus treated wells were not treated with MG132 (indicated by minus sign). Uninfected HEK293 cells (293F) were used as a negative control. Forty-eight hours post infection cell pellets were harvested and analyzed by SDS/PAGE electrophoresis, and immunoblotting using a rabbit

anti-Class II PADRE antibody. A HRP anti-rabbit antibody and ECL chemiluminescent substrate was used for detection.

[00105] **Fig. 31** shows CD8+ immune responses in ChAdV68 large cassette immunized mice, detected against AH1 (top) and SIINFEKL (SEQ ID NO: 72) (bottom) by ICS. Data is presented as IFNg+ cells against the model epitope as % of total CD8 cells

[00106] **Fig. 32** shows CD8+ responses to LD-AH1+ (top) and Kb-SIINFEKL+ (bottom) ("SIINFEKL" disclosed as SEQ ID NO: 72) Tetramers post ChAdV68 large cassette vaccination. Data is presented as % of total CD8 cells reactive against the model Tetramer peptide complex. *p<0.05, **p<0.01 by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

[00107] **Fig. 33** shows CD8+ immune responses in alphavirus large cassette treated mice, detected against AH1 (top) and SIINFEKL (SEQ ID NO: 72) (bottom) by ICS. Data is presented as IFNg+ cells against the model epitope as % of total CD8 cells. *p<0.05, **p<0.01, ***p<0.001 by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

[00108] **Fig. 34** illustrates the vaccination strategy used to evaluate immunogenicity of the antigen-cassette containing vectors in rhesus macaques. Triangles indicate ChAdV68 vaccination (1e12 vp/animal) at weeks 0 & 32. Circles represent alphavirus vaccination at weeks 0, 4, 12,20, 28 & 32. Squares represent administration of an anti-CTLA4 antibody.

[00109] **Fig. 35** shows a time course of CD8+ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG alone (Group 4). Mean SFC/1e6 splenocytes is shown.

[00110] **Fig. 36** shows a time course of CD8+ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered IV (Group 5). Mean SFC/1e6 splenocytes is shown.

[00111] **Fig. 37** shows a time course of CD8+ anti-epitope responses in Rhesus Macaques dosed with chAd-MAG plus anti-CTLA4 antibody (Ipilimumab) delivered SC (Group 6). Mean SFC/1e6 splenocytes is shown.

[00112] **Fig. 38** shows antigen-specific memory responses generated by ChAdV68/samRNA vaccine protocol measured by ELISpot. Results are presented as individual dot plots, with each dot representing a single animal. Pre-immunization baseline (left panel) and memory response at 18 months post-prime (right panel) are shown.

[00113] **Fig. 39** shows memory cell phenotyping of antigen-specific CD8+ T-cells by flow cytometry using combinatorial tetramer staining and CD45RA/CCR7 co-staining.

[00114] **Fig. 40** shows the distribution of memory cell types within the sum of the four Mamu-A*01 tetramer+ CD8+ T-cell populations at study month 18. Memory cells were

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, *e.g.*, *see* "V.A. Cassettes" section.

[00277] 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 nucleic acid 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 (SEQ ID NO: 74) encoding repeats of 25mer antigens Trp1 (VTNTEMFVTAPDNLGYMYEVQWPGQ (SEQ ID NO: 75)) and Trp2 (TQPQIANCSVYDFFVWLHYYSVRDT (SEQ ID NO: 76)), 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.

[00278] In one embodiment, different peptides and/or polypeptides or nucleic acid 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

to dryness by SpeedVac evaporation and in some instances are stored at -20C prior to MS analysis.

[00421] 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.

[00422] 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.

[00423] Using the peptide YVYVADVAAK (SEQ ID NO: 77) 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}).

XIV.B. Antigen Cassette Design Evaluation

XIV.B.1. Methods and Materials

TCR and cassette design and cloning

[00446] The selected TCRs recognize peptides NLVPMVATV (SEQ ID NO: 78) (PDB# 5D2N), CLGGLLTMV (SEQ ID NO: 79) (PDB#3REV), GILGFVFTL (SEQ ID NO: 80) (PDB#1OGA) LLFGYPVYV (SEQ ID NO: 81) (PDB#1AO7) when presented by A*0201. Transfer vectors were constructed that contain 2A peptide-linked TCR subunits (beta followed by alpha), the EMCV IRES, and 2A-linked CD8 subunits (beta followed by alpha and by the puromycin resistance gene). Open reading frame sequences were codon-optimized and synthesized by GeneArt.

Cell line generation for in vitro epitope processing and presentation studies

[00447] Peptides were purchased from ProImmune or Genscript diluted to 10mg/mL with 10mM tris(2-carboxyethyl)phosphine (TCEP) in water/DMSO (2:8, v/v). Cell culture medium and supplements, unless otherwise noted, were from Gibco. Heat inactivated fetal bovine serum (FBShi) was from Seradigm. QUANTI-Luc Substrate, Zeocin, and Puromycin were from InvivoGen. Jurkat-Lucia NFAT Cells (InvivoGen) were maintained in RPMI 1640 supplemented with 10% FBShi, Sodium Pyruvate, and 100µg/mL Zeocin. Once transduced, these cells additionally received 0.3 µg/mL Puromycin. T2 cells (ATCC CRL-1992) were cultured in Iscove's Medium (IMDM) plus 20% FBShi. U-87 MG (ATCC HTB-14) cells were maintained in MEM Eagles Medium supplemented with 10% FBShi.

[00448] Jurkat-Lucia NFAT cells contain an NFAT-inducible Lucia reporter construct. The Lucia gene, when activated by the engagement of the T cell receptor (TCR), causes secretion of a coelenterazine-utilizing luciferase into the culture medium. This luciferase can be measured using the QUANTI-Luc luciferase detection reagent. Jurkat-Lucia cells were transduced with lentivirus to express antigen-specific TCRs. The HIV-derived lentivirus transfer vector was obtained from GeneCopoeia, and lentivirus support plasmids expressing VSV-G (pCMV-VsvG), Rev (pRSV-Rev) and Gag-pol (pCgpV) were obtained from Cell Design Labs.

[00449] Lentivirus was prepared by transfection of 50-80% confluent T75 flasks of HEK293 cells with Lipofectamine 2000 (Thermo Fisher), using 40 µl of lipofectamine and 20 µg of the DNA mixture (4:2:1:1 by weight of the transfer plasmid:pCgpV:pRSV-Rev:pCMV-VsvG). 8-10 mL of the virus-containing media were concentrated using the Lenti-X system (Clontech), and the virus resuspended in 100-200 µl of fresh medium. This volume was used to overlay an equal volume of Jurkat-Lucia cells (5x10E4-1x10E6 cells

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

In vitro epitope processing and presentation assay

[00450] T2 cells are routinely used to examine antigen recognition by TCRs. T2 cells lack a peptide transporter for antigen processing (TAP deficient) and cannot load endogenous peptides in the endoplasmic reticulum for presentation on the MHC. However, the T2 cells can easily be loaded with exogenous peptides. The five marker peptides (NLVPMVATV (SEQ ID NO: 78), CLGGLLTMV (SEQ ID NO: 79), GLCTLVAML (SEQ ID NO: 82), LLFGYPVYV (SEQ ID NO: 81), GILGFVFTL (SEQ ID NO: 80)) and two irrelevant peptides (WLSLLVPFV (SEQ ID NO: 83), FLLTRICT (SEQ ID NO: 84)) were loaded onto T2 cells. Briefly, T2 cells were counted and diluted to 1x106 cells/mL with IMDM plus 1% FBShi. Peptides were added to result in 10 µg peptide/1x106 cells. Cells were then incubated at 37oC for 90 minutes. Cells were washed twice with IMDM plus 20% FBShi, diluted to 5x10E5 cells/mL and 100 µL plated into a 96-well Costar tissue culture plate. Jurkat-Lucia TCR clones were counted and diluted to 5x10E5 cells/mL in RPMI 1640 plus 10% FBShi and 100 µL added to the T2 cells. Plates were incubated overnight at 37ºC, 5% CO2. Plates were then centrifuged at 400g for 3 minutes and 20 µL supernatant removed to a white flat bottom Greiner plate. QUANTI-Luc substrate was prepared according to instructions and 50 µL/well added. Luciferase expression was read on a Molecular Devices SpectraMax iE3x.

[00451] To test marker epitope presentation by the adenoviral cassettes, U-87 MG cells were used as surrogate antigen presenting cells (APCs) and were transduced with the adenoviral vectors. U-87 MG cells were harvested and plated in culture media as 5x10E5 cells/100 µl in a 96-well Costar tissue culture plate. Plates were incubated for approximately 2 hours at 37ºC. Adenoviral cassettes were diluted with MEM plus 10% FBShi to an MOI of 100, 50, 10, 5, 1 and 0 and added to the U-87 MG cells as 5µl/well. Plates were again incubated for approximately 2 hours at 37ºC. Jurkat-Lucia TCR clones were counted and diluted to 5x10E5 cells/mL in RPMI plus 10% FBShi and added to the U-87 MG cells as 100 µL/well. Plates were then incubated for approximately 24 hours at 37ºC, 5% CO2. Plates were centrifuged at 400g for 3 minutes and 20 µL supernatant removed to a white flat bottom Greiner plate. QUANTI-Luc substrate was prepared according to instructions and 50 µL/well added. Luciferase expression was read on a Molecular Devices SpectraMax iE3x.

Mouse strains for immunogenicity studies

[00452] Transgenic HLA-A2.1 (HLA-A2 Tg) mice were obtained from Taconic Labs, Inc. These mice carry a transgene consisting of a chimeric class I molecule comprised of the

CD8 T cell responses were comparable in magnitude and breadth for both long and short vaccine cassettes, demonstrating that (a) the addition of more epitopes did not substantially impact the magnitude of immune response to the original set of epitopes, and (b) the position of an epitope in a cassette did not substantially influence the ensuing T cell response to it (**Table 6**).

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

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

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

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

[00465] In summary, the findings of the model cassette evaluations (**Fig. 2-5, Tables 2-6**) demonstrated that, for model vaccine cassettes, robust immunogenicity was achieved when a "string of beads" approach was employed that encodes around 20 epitopes in the context of an adenovirus-based vector. The epitopes were assembled by concatenating 25mer sequences, each embedding a minimal CD8 T cell epitope (e.g. 9 amino acid residues) that were flanked on both sides by its natural, surrounding peptide sequence (e.g. 8 amino acid residues on each side). As used herein, a "natural" or "native" flanking sequence refers to the N- and/or C-terminal flanking sequence of a given epitope in the naturally occurring context of that epitope within its source protein. For example, the HCMV pp65 MHC I epitope NLVPMVATV (SEQ ID NO: 78) is flanked on its 5' end by the native 5' sequence WQAGILAR (SEQ ID NO: 85) and on its 3' end by the native 3' sequence QGQNLKYQ (SEQ ID NO: 86), thus generating the WQAGILARNLVPMVATVQGQNLKYQ (SEQ ID NO: 87) 25mer peptide found within the HCMV pp65 source protein. The natural or native sequence can also refer to a nucleic acid sequence that encodes an epitope flanked by native flanking sequence(s). Each 25mer sequence is directly

connected to the following 25mer sequence. In instances where the minimal CD8 T cell epitope is greater than or less than 9 amino acids, the flanking peptide length can be adjusted such that the total length is still a 25mer peptide sequence. For example, a 10 amino acid CD8 T cell epitope can be flanked by an 8 amino acid sequence and a 7 amino acid. The concatamer was followed by two universal class II MHC epitopes that were included to stimulate CD4 T helper cells and improve overall *in vivo* immunogenicity of the vaccine cassette antigens. (Alexander et al., 1994; Panina-Bordignon et al., 1989) The class II epitopes were linked to the final class I epitope by a GPGPG amino acid linker (SEQ ID NO:56). The two class II epitopes were also linked to each other by a GPGPG amino acid linker (SEQ ID NO: 56), as a well as flanked on the C-terminus by a GPGPG amino acid linker (SEQ ID NO: 56). Neither the position nor the number of epitopes appeared to substantially impact T cell recognition or response. Targeting sequences also did not appear to substantially impact the immunogenicity of cassette-derived antigens.

[00466] As a further example, based on the in vitro and in vivo data obtained with model cassettes (**Fig. 2-5, Tables 2-6**), a cassette design was generated that alternates well-characterized T cell epitopes known to be immunogenic in nonhuman primates (NHPs), mice and humans. The 20 epitopes, all embedded in their natural 25mer sequences, are followed by the two universal class II MHC epitopes that were present in all model cassettes evaluated (**Fig. 6**). This cassette design was used to study immunogenicity as well as pharmacology and toxicology studies in multiple species.

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

[00467] Large antigen cassettes were designed that had either 30 (L), 40 (XL) or 50 (XXL) epitopes, each 25 amino acids in length. The epitopes were a mix of human, NHP and mouse epitopes to model disease antigens including tumor antigens. **Fig. 29** illustrates the general organization of the epitopes from the various species. The model antigens used are described in **Tables 32, 33 and 34** for human, primate, and mouse model epitopes, respectively. Each of **Tables 32, 33 and 34** described the epitope position, name, minimal epitope description, and MHC class. Table 32 discloses SEQ ID NOS 80-82, 78-79, 88-100, 87 and 101-111, respectively, in order of columns. Table 33 discloses SEQ ID NOS 112-141, respectively, in order of columns. Table 34 discloses SEQ ID NOS 72, 142-144, 73, 145-161, 75-76 and 162-177, respectively, in order of columns

[00468] These cassettes were cloned into the ChAdV68 and alphavirus vaccine vectors as described to evaluate the efficacy of longer multiple-epitope cassettes. **Fig. 30** shows that each of the large antigen cassettes were expressed from a ChAdV vector as indicated by at least one major band of the expected size by Western blot.

[00469] Mice were immunized as described to evaluate the efficacy of the large cassettes. T cell responses were analyzed by ICS and tetramer staining following immunization with a ChAdV68 vector (**Fig. 31/Table 35** and **Fig. 32/Table 36**, respectively) and by ICS following immunization with a srRNA vector (**Fig. 33/Table 37**) for epitopes AH1 (top panels) and SIINFEKL (SEQ ID NO: 72) (bottom panels). Immunizations using ChAdV68 and srRNA vaccine vectors expressing either 30 (L), 40 (XL) or 50 (XXL) epitopes induced CD8+ immune responses to model disease epitopes.

Table 35: Average IFNg+ cells in response to AH1 and SIINFEKL (SEQ ID NO: 72) peptides in ChAd large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL (SEQ ID NO: 72)	5.308	0.660	n/a	8
30	SIINFEKL (SEQ ID NO: 72)	4.119	1.019	0.978	8
40	SIINFEKL (SEQ ID NO: 72)	6.324	0.954	0.986	8
50	SIINFEKL (SEQ ID NO: 72)	8.169	1.469	0.751	8
20	AH1	6.405	2.664	n/a	8
30	AH1	4.373	1.442	0.093	8
40	AH1	4.126	1.135	0.050	8
50	AH1	4.216	0.808	0.063	8

Table 36: Average tetramer+ cells for AH1 and SIINFEKL (SEQ ID NO: 72) antigens in ChAd large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL (SEQ ID NO: 72)	10.314	2.384	n/a	8
30	SIINFEKL (SEQ ID NO: 72)	4.551	2.370	0.003	8
40	SIINFEKL (SEQ ID NO: 72)	5.186	3.254	0.009	8
50	SIINFEKL (SEQ ID NO: 72)	14.113	3.660	0.072	8
20	AH1	6.864	2.207	n/a	8
30	AH1	4.713	0.922	0.036	8
40	AH1	5.393	1.452	0.223	8
50	AH1	5.860	1.041	0.543	8

Table 37: Average IFNg+ cells in response to AH1 and SIINFEKL (SEQ ID NO: 72) peptides in SAM large cassette treated mice. Data is presented as % of total CD8 cells. Shown is average and standard deviation per group and p-value by ANOVA with Tukey's test. All p-values compared to MAG 20-antigen cassette.

# antigens	Antigen	Average	Standard deviation	p-value	N
20	SIINFEKL (SEQ ID NO: 72)	1.843	0.422	n/a	8
30	SIINFEKL (SEQ ID NO: 72)	2.112	0.522	0.879	7
40	SIINFEKL (SEQ ID NO: 72)	1.754	0.978	0.995	7
50	SIINFEKL (SEQ ID NO: 72)	1.409	0.766	0.606	8
20	AH1	3.050	0.909	n/a	8
30	AH1	0.618	0.427	1.91E-05	7
40	AH1	1.286	0.284	0.001	7
50	AH1	1.309	1.149	0.001	8

XV. ChAd Antigen Cassette Delivery Vector

XV.A. ChAd Antigen Cassette Delivery Vector Construction

[00470] In one example, Chimpanzee adenovirus (ChAd) was engineered to be a delivery vector for antigen cassettes. In a further example, a full-length ChAdV68 vector was synthesized based on AC_000011.1 (sequence 2 from Patent US 6083716) with E1 (nt 457 to 3014) and E3 (nt 27,816- 31,332) sequences deleted. Reporter genes under the control of the CMV promoter/enhancer were inserted in place of the deleted E1 sequences. Transfection of this clone into HEK293 cells did not yield infectious virus. To confirm the sequence of the wild-type C68 virus, isolate VR-594 was obtained from the ATCC, passaged, and then independently sequenced (SEQ ID NO:10). When comparing the AC_000011.1 sequence to the ATCC VR-594 sequence (SEQ ID NO:10) of wild-type ChAdV68 virus , 6 nucleotide differences were identified. In one example, a modified ChAdV68 vector was generated based on AC_000011.1, with the corresponding ATCC VR-594 nucleotides substituted at five positions (ChAdV68.5WTnt SEQ ID NO:1).

[00471] In another example, a modified ChAdV68 vector was generated based on AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,816- 31,332) sequences deleted and the corresponding ATCC VR-594 nucleotides substituted at four positions. A GFP reporter (ChAdV68.4WTnt.GFP; SEQ ID NO:11) or model neoantigen cassette (ChAdV68.4WTnt.MAG25mer; SEQ ID NO:12) under the control of the CMV promoter/enhancer was inserted in place of deleted E1 sequences.

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

[00489] In one example, ChAdV68.4WTnt.GFP (**Fig. 7**) and ChAdV68.5WTnt.GFP (**Fig. 8**) DNA was transfected into HEK293A cells and virus replication (viral plaques) was observed 7-10 days after transfection. ChAdV68 viral plaques were visualized using light (**Fig. 7A** and **8A**) and fluorescent microscopy (**Fig. 7B-C** and **Fig. 8B-C**). GFP denotes productive ChAdV68 viral delivery particle production.

XV.B.3. ChAdV68 Viral Delivery Particles Expansion

[00490] In one example, ChAdV68.4WTnt.GFP, ChAdV68.5WTnt.GFP, and ChAdV68.5WTnt.MAG25mer viruses were expanded in HEK293F cells and a purified virus stock produced 18 days after transfection (**Fig. 9**). Viral particles were quantified in the purified ChAdV68 virus stocks and compared to adenovirus type 5 (Ad5) and ChAdVY25 (a closely related ChAdV; Dicks, 2012, PloS ONE 7, e40385) viral stocks produced using the same protocol. ChAdV68 viral titers were comparable to Ad5 and ChAdVY25 (**Table 7**).

Table 7. Adenoviral vector production in 293F suspension cells

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

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

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

[00491] C68 vector expressing mouse tumor antigens were evaluated in mouse immunogenicity studies to demonstrate the C68 vector elicits T-cell responses. T-cell responses to the MHC class I epitope SIINFEKL (SEQ ID NO: 72) were measured in C57BL/6J female mice and the MHC class I epitope AH1-A5 (Slansky et al., 2000, Immunity13:529-538) measured in Balb/c mice. As shown in **Fig. 15**, strong T-cell responses relative to control were measured after immunization of mice with ChAdV68.5WTnt.MAG25mer. Mean cellular immune responses of 8957 or 4019 spot forming cells (SFCs) per 10^6 splenocytes were observed in ELISpot assays when C57BL/6J or Balb/c mice were immunized with ChAdV68.5WTnt.MAG25mer, respectively, 10 days after immunization.

Table 8: qPCR primers/probes

Target			SEQ ID NO:
Luci	Primer1	GTGGTGTGCAGCGAGAATAG	178
	Primer2	CGCTCGTTGTAGATGTCGTTAG	179
	Probe	/56-FAM/TTGCAGTTC/ZEN/TTCATGCCCGTGTTG/3IABkFQ/	180
GusB	Primer1	GTTTTTGATCCAGACCCAGATG	181
	Primer2	GCCCATTATTCAGAGCGAGTA	182
	Probe	/56-FAM/TGCAGGGTT/ZEN/TCACCAGGATCCAC/3IABkFQ/	183
ActB	Primer1	CCTTGCACATGCCGGAG	184
	Primer2	ACAGAGCCTCGCCTTTG	185
	Probe	/56-FAM/TCATCCATG/ZEN/GTGAGCTGGCGG/3IABkFQ/	186
MAG-25mer Set1	Primer1	CTGAAAGCTCGGTTTGCTAATG	187
	Primer2	CCATGCTGGAAGAGACAATCT	188
	Probe	/56-FAM/CGTTTCTGA/ZEN/TGGCGCTGACCGATA/3IABkFQ/	189
MAG-25mer Set2	Primer1	TATGCCTATCCTGTCTCCTCTG	190
	Primer2	GCTAATGCAGCTAAGTCCTCTC	191
	Probe	/56-FAM/TGTTTACCC/ZEN/TGACCGTGCCTTCTG/3IABkFQ/	192

B16-OVA tumor model

[00499] C57BL/6J mice were injected in the lower left abdominal flank with 10^5 B16-OVA cells/animal. Tumors were allowed to grow for 3 days prior to immunization.

CT26 tumor model

[00500] Balb/c mice were injected in the lower left abdominal flank with 10^6 CT26 cells/animal. Tumors were allowed to grow for 7 days prior to immunization.

Immunizations

[00501] For srRNA vaccine, mice were injected with 10 ug of RNA in 100 uL volume, bilateral intramuscular injection (50 uL per leg). For Ad5 vaccine, mice were injected with $5x10^{10}$ viral particles (VP) in 100 uL volume, bilateral intramuscular injection (50 uL per leg). Animals were injected with anti-CTLA-4 (clone 9D9, BioXcell), anti-PD-1 (clone RMP1-14, BioXcell) or anti-IgG (clone MPC-11, BioXcell), 250 ug dose, 2 times per week, via intraperitoneal injection.

In vivo bioluminescent imaging

[00502] At each timepoint mice were injected with 150 mg/kg luciferin substrate via intraperitoneal injection and bioluminescence was measured using the IVIS In vivo imaging system (PerkinElmer) 10-15 minutes after injection.

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

XVI.B.3. Alphavirus Vector Tumor Model Evaluation

[00508] In one implementation, to determine if the VEE srRNA vector directs antigen-specific immune responses *in vivo*, a VEE srRNA vector was generated (VEE-UbAAY, SEQ ID NO:14) that expresses 2 different MHC class I mouse tumor epitopes, SIINFEKL (SEQ ID NO: 72) and AH1-A5 (Slansky et al., 2000, Immunity 13:529-538). The SFL (SIINFEKL (SEQ ID NO: 72)) epitope is expressed by the B16-OVA melanoma cell line, and the AH1-A5 (SPSYAYHQF (SEQ ID NO: 73); Slansky et al., 2000, Immunity) epitope induces T cells targeting a related epitope (AH1/ SPSYVYHQF (SEQ ID NO: 193); Huang et al., 1996, Proc Natl Acad Sci USA 93:9730-9735) that is expressed by the CT26 colon carcinoma cell line. In one example, for *in vivo* studies, VEE-UbAAY srRNA was generated by *in vitro* transcription using T7 polymerase (TriLink Biotechnologies) and encapsulated in a lipid nanoparticle (MC3).

[00509] A strong antigen-specific T-cell response targeting SFL, relative to control, was observed two weeks after immunization of B16-OVA tumor bearing mice with MC3 formulated VEE-UbAAY srRNA. In one example, a median of 3835 spot forming cells (SFC) per 10^6 splenocytes was measured after stimulation with the SFL peptide in ELISpot assays (**Fig. 12A**, **Table 12**) and 1.8% (median) of CD8 T-cells were SFL antigen-specific as measured by pentamer staining (**Fig. 12B**, **Table 12**). In another example, co-administration of an anti-CTLA-4 monoclonal antibody (mAb) with the VEE srRNA vaccine resulted in a moderate increase in overall T-cell responses with a median of 4794.5 SFCs per 10^6 splenocytes measured in the ELISpot assay (**Fig. 12A**, **Table 12**).

cat. No. A14528) suspension cultures. The virus was purified by 2X CsCl gradient purification and formulated into ARM buffer (25 mM NaCl, 20mm Tris pH 8.0, 2.5% Glycerol) by 3 rounds of dialysis. Viral particle titers were determined by Absorbance at 260 nm measurement post 0.1% SDS lysis at 56 C. Infectious titers were determined using an anti-capsid immunostaining assay.

Next Generation Sequencing

[00551] DNA was purified from the purified virus using the QiAmp viral DNA kit (Qiagen) and subjected to NGS using the Illumina platform.

MOI Evaluation of Clone Productivity

[00552] Controlled infections were set up using the purified virus at an MOI of 0.1 IU and incubated for 96 h. Infectious units were measured in cell lysates. Production was compared to a non-plaque selected virus (pool).

Immunizations

[00553] Balb/c female mice were injected with $1x10^9$ or $1x10^{10}$ viral particles (VP) of ChAdV68.5WTnt.MAG25mer (SEQ ID NO:2) or ChAdV68-MAG-E4deleted (SEQ ID NO: 57; "MAG E4 Delta" and "ChAdV68-MAG-E4") in 100 uL volume, bilateral intramuscular injection (50 uL per leg).

[00554] Mamu-A*01 Indian rhesus macaques were immunized as bilateral intramuscular injections into the quadriceps muscle with $1x10^{12}$ viral particles ($5x10^{11}$ viral particles per injection) of ChAdV68.5WTnt.MAG25mer ("ChAdV68-CMV-MAG"; SEQ ID NO:2; no E4 deletion or TET response element) or ChAdV68-E4d-CMT-MAG (SEQ ID NO:71; E4 deletion and CMT TET response element [see below]). Macaques were also administered 50 mg of an anti-CTLA4 antibody (Ipilimumab) SC on the day of injection.

ChAdV68-E4d-CMT-MAG; SEQ ID NO: 71
CATCATCAATAATATACCTCAAACTTTTTGTGCGCGTTAATATGCAAATGAGGCGTTTGAATTTGGGGAGGA
AGGGCGGTGATTGGTCGAGGGATGAGCGACCGTTAGGGGCGGGGCGAGTGACGTTTTGATGACGTGGTTG
CGAGGAGGAGCCAGTTTGCAAGTTCTCGTGGGAAAAGTGACGTCAAACGAGGTGTGGTTTGAACACGGAA
ATACTCAATTTTCCCGCGCTCTCTGACAGGAAATGAGGTGTTTCTGGGCGGATGCAAGTGAAAACGGGCCA
TTTTCGCGCGAAAACTGAATGAGGAAGTGAAAATCTGAGTAATTTCGCGTTTATGGCAGGGAGGAGTATTT
GCCGAGGGCCGAGTAGACTTTGACCGATTACGTGGGGGTTTCGATTACCGTGTTTTTCACCTAAATTTCCGC
GTACGGTGTCAAAGTCCGGTGTTTTTACGTAGGTGTCAGCTGATCGCCAGGGTATTTAAACCTGCGCTCTCC
AGTCAAGAGGCCACTCTTGAGTGCCAGCGAGAAGAGTTTTCTCCTCCGCGCCGCGAGTCAGATCTACACTT
TGAAAGTAGGGATAACAGGGTAATGACATTGATTATTGACTAGTTGTTAATAGTAATCAATTACGGGGTCA
TTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCC
AACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTG
ACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTC
CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGAC
TTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACC
AATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTT
GTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCG
GTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGT
GATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAACGCCATCCACGCTGTTTTGAC

MEDI4736 VH CDR2 (SEQ ID NO:27)
MEDI4736 VH CDR3 (SEQ ID NO:28)
MEDI4736 VL CDR1 (SEQ ID NO:29)
MEDI4736 VL CDR2 (SEQ ID NO:30)
MEDI4736 VL CDR3 (SEQ ID NO:31)
UbA76-25merPDTT nucleotide (SEQ ID NO:32)
UbA76-25merPDTT polypeptide (SEQ ID NO:33)
MAG-25merPDTT nucleotide (SEQ ID NO:34)
MAG-25merPDTT polypeptide (SEQ ID NO:35)
Ub7625merPDTT_NoSFL nucleotide (SEQ ID NO:36)
Ub7625merPDTT_NoSFL polypeptide (SEQ ID NO:37)
ChAdV68.5WTnt.MAG25mer (SEQ ID NO:2); AC_000011.1 with E1 (nt 577 to 3403) and E3 (nt 27,125-31,825) sequences deleted; corresponding ATCC VR-594 nucleotides substituted at five positions; model neoantigen cassette under the control of the CMV promoter/enhancer inserted in place of deleted E1; SV40 polyA 3' of cassette
Venezuelan equine encephalitis virus [VEE] (SEQ ID NO:3) GenBank: L01442.2
VEE-MAG25mer (SEQ ID NO:4); contains MAG-25merPDTT nucleotide (bases 30-1755)
Venezuelan equine encephalitis virus strain TC-83 [TC-83](SEQ ID NO:5) GenBank: L01443.1
VEE Delivery Vector (SEQ ID NO:6); VEE genome with nucleotides 7544-11175 deleted [alphavirus structural proteins removed]
TC-83 Delivery Vector(SEQ ID NO:7); TC-83 genome with nucleotides 7544-11175 deleted [alphavirus structural proteins removed]
VEE Production Vector (SEQ ID NO:8); VEE genome with nucleotides 7544-11175 deleted, plus 5' T7-promoter, plus 3' restriction sites
TC-83 Production Vector(SEQ ID NO:9); TC-83 genome with nucleotides 7544-11175 deleted, plus 5' T7-promoter, plus 3' restriction sites
VEE-UbAAY (SEQ ID NO:14); VEE delivery vector with MHC class I mouse tumor epitopes SIINFEKL (SEQ ID NO: 72) and AH1-A5 inserted
VEE-Luciferase (SEQ ID NO:15); VEE delivery vector with luciferase gene inserted at 7545
ubiquitin (SEQ ID NO:38)>UbG76 0-228
Ubiquitin A76 (SEQ ID NO:39)>UbA76 0-228
HLA-A2 (MHC class I) signal peptide (SEQ ID NO:40)>MHC SignalPep 0-78
HLA-A2 (MHC class I) Trans Membrane domain (SEQ ID NO:41)>HLA A2 TM Domain 0-201
IgK Leader Seq (SEQ ID NO:42)>IgK Leader Seq 0-60
Human DC-Lamp (SEQ ID NO:43)>HumanDCLAMP 0-3178
Mouse LAMP1 (SEQ ID NO:44)>MouseLamp1 0-1858
Human Lamp1 cDNA (SEQ ID NO:45)>Human Lamp1 0-2339
Tetanus toxoid nulceic acid sequence (SEQ ID NO:46)
Tetanus toxoid amino acid sequence (SEQ ID NO:47)
PADRE nulceotide sequence (SEQ ID NO:48)
PADRE amino acid sequence (SEQ ID NO:49)
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)
ChAdV68-MAG-E4deleted (SEQ ID NO: 57)
CATCaTCAATAATATACCTCAAACTTTTTGTGCGCGTTAATATGCAAATGAGGCGTTTGAATTTGGGGAGGAA GGGCGGTGATTGGTCGAGGGATGAGCGACCGTTAGGGGCGGGGCGAGTGACGTTTTGATGACGTGGTTGCGA GGAGGAGCCAGTTTGCAAGTTCTCGTGGGAAAAGTGACGTCAAACGAGGTGTGGTTTGAACACGGAAATACT CAATTTTCCCGCGCTCTCTGACAGGAAATGAGGTGTTTCTGGGCGGATGCAAGTGAAAACGGGCCATTTTCGC GCGAAAACTGAATGAGGAAGTGAAAATCTGAGTAATTTCGCGTTTATGGCAGGGAGGAGTATTTGCCGAGGG CCGAGTAGACTTTGACCGATTACGTGGGGGTTTCGATTACCGTGTTTTTCACCTAAATTTCCGCGTACGGTGTC AAAGTCCGGTGTTTTTACGTAGGTGTCAGCTGATCGCCAGGGTATTTAAACCTGCGCTCTCCAGTCAAGAGGC CACTCTTGAGTGCCAGCGAGAAGAGTTTTCTCCTCCGCGCCGCGAGTCAGATCTACACTTTGAAAGTAGGGAT AACAGGGTAATgacattgattattgactagttGttaaTAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTT

CLAIMS

What is claimed is:

1. An adenovirus vector comprising:

an adenoviral backbone comprising one or more genes or regulatory sequences of an adenovirus genome, and

wherein the adenoviral backbone comprises a partially deleted E4 gene with reference to the adenovirus genome, wherein the partially deleted E4 gene comprises a deleted or partially-deleted E4orf2 region and a deleted or partially-deleted E4orf3 region, and optionally a deleted or partially-deleted E4orf4 region, and

optionally, wherein the adenovirus vector further comprises a cassette, the cassette comprising:

(1) at least one payload nucleic acid sequence, optionally wherein the at least one payload nucleic acid sequence encodes a polypeptide, optionally wherein the polypeptide comprises an antigen, optionally wherein the antigen comprises:

- a MHC class I epitope,

- a MHC class II epitope,

- an epitope capable of stimulating a B cell response, or

- a combination thereof, and

optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein;

(2) at least one promoter sequence operably linked to the at least one payload nucleic acid sequence,

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

(4) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and

(5) optionally, at least one polyadenylation sequence.

2. A chimpanzee adenovirus vector comprising a modified ChAdV68 sequence, wherein the modified ChAdV68 sequence comprises:

(a) a partially deleted E4 gene of the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1; and

(b) 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; and

optionally, wherein the chimpanzee adenovirus vector further comprises 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.

3. A chimpanzee adenovirus vector comprising a modified ChAdV68 sequence, wherein the modified ChAdV68 sequence comprises:

(a) a partially deleted E4 gene of the E4 gene sequence shown in SEQ ID NO:1 and that lacks at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1;

(b) 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; and

(c) 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, and

optionally, wherein the chimpanzee adenovirus vector further comprises 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.

4. The vector of any of the above claims, wherein the adenoviral backbone or modified ChAdV68 sequence further comprises a functional deletion in at least one gene selected from the group consisting of an adenovirus E1A, E1B, E2A, E2B, E3, L1, L2, L3, L4, and L5 gene with reference to the adenovirus genome or with reference to the sequence shown in SEQ ID NO:1, optionally wherein the adenoviral backbone or modified ChAdV68 sequence is fully deleted or functionally deleted in: (1) E1A and E1B; or (2) E1A, E1B, and E3 with reference to the adenovirus genome or with reference to the sequence shown in SEQ ID NO:1, optionally wherein the E1 gene is functionally deleted through an E1 deletion of at least nucleotides 577 to 3403 with reference to the sequence shown in SEQ ID NO:1 and optionally wherein the E3 gene is functionally deleted through an E3 deletion of at least nucleotides 27,125 to 31,825 with reference to the sequence shown in SEQ ID NO:1.

5. The vector of any of the above claims, wherein the cassette is present and is inserted in the vector at the E1 region, E3 region, and/or any deleted AdV region that allows incorporation of the cassette.

6. The vector of any one of claims 2-5, wherein the modified ChAdV68 sequence comprises 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.

7. The vector of claim 6, wherein the nucleotides 2 to 34,916 lack nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion.

8. The vector of any one of claims 6-7, wherein the nucleotides 2 to 34,916 lack nucleotides 27,125-31,825 with reference to the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion.

9. The vector of any one of claims 6-7, wherein the nucleotides 2 to 34,916 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.

10. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes an antigen, wherein the antigen comprises:

    - a MHC class I epitope,

    - a MHC class II epitope,

- an epitope capable of stimulating a B cell response, or

- a combination thereof.

11. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence capable of undergoing antigen processing into an epitope, optionally wherein the epitope is 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.

12. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence or portion thereof that is presented by MHC class I and/or MHC class II on a surface of a cell, optionally wherein the surface of the cell is a tumor cell surface or an infected cell surface.

13. The vector of claim 11 or 12, wherein the 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 infected cell selected from the group consisting of: a pathogen infected cell, a virally infected cell, a bacterially infected cell, an fungally infected cell, and a parasitically infected cell, optionally 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, and a Hepatitis C virus (HCV) infected cell.

14. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence or portion thereof comprising an epitope capable of stimulating a B cell response, optionally wherein the polypeptide sequence or portion thereof comprises a full-length protein, a protein domain, a protein subunit, or an antigenic fragment predicted or known to be capable of being bound by an antibody.

15. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes 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.

16. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes an epitope with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence.

17. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes a MHC class I epitope or MHC class II epitope with at least one alteration that makes the encoded peptide sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, optionally wherein the encoded polypeptide sequence or portion thereof has increased binding affinity to, increased binding stability to, and/or an increased likelihood of presentation on its corresponding MHC allele relative to the translated, corresponding wild-type nucleic acid sequence.

18. The vector of claims 4-17, 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.

19. The vector of any of the above claims, wherein at least one of the at least one payload nucleic acid sequences encodes a full-length protein, a protein domain, or a protein subunit.

20. The vector of any of the above claims, wherein the expression of each of the at least one payload nucleic acid sequences is driven by the at least one promoter.

21. The vector of any of the above claims, wherein the at least one promoter sequence is a regulatable promoter, optionally wherein the regulatable promoter is a tetracycline (TET) repressor protein (TETr) controlled promoter, optionally wherein the regulatable promoter comprises multiple TET operator (TETo) sequences 5’ or 3’of a RNA polymerase binding sequence of the promoter.

22. The vector of any of the above claims, wherein the at least one promoter sequence is constitutive, optionally wherein the at least one promoter sequence is a CMV, SV40, EF-1, RSV, PGK, HSA, MCK or EBV promoter sequence.

23. The vector of any of the above claims, wherein the cassette further comprises at least one poly-adenylation (polyA) sequence operably linked to at least one of the at least one payload nucleic acid sequences, optionally wherein the polyA sequence is located 3’ of the at least one payload nucleic acid sequence, optionally wherein the polyA sequence comprises an SV40 or Bovine Growth Hormone (BGH) polyA sequence.

24. The vector of any of the above claims, wherein the cassette comprises junctional epitope sequences formed by adjacent sequences in the cassette, optionally wherein at least one or each junctional epitope sequence has an affinity of greater than 500 nM for MHC, and optionally wherein each junctional epitope sequence is non-self.

25. A pharmaceutical composition comprising the vector of any of claims 1-24 and a pharmaceutically acceptable carrier, optionally further comprises an adjuvant.

26. An isolated nucleotide sequence encoding the vector of any of claims 1-24 , and optionally wherein the nucleotide sequence is cDNA.

27. An isolated cell comprising the nucleotide sequence of claim 26, optionally wherein the cell is a CHO, HEK293 or variants thereof, 911, HeLa, A549, LP-293, PER.C6, or AE1-2a cell.

28. A kit comprising the vector of any of claims 1-24 and instructions for use.

29. Use of the vector of any of claims 1-24 in the manufacture of a medicament for stimulating an immune response in a subject.

30. Use of the vector of any of claims 1-24 in the manufacture of a medicament for treating a subject with a disease, optionally wherein the disease is cancer or an infection.

31. A method of producing a virus, wherein the virus is produced using the vector of any of claims 1-24, wherein the production and/or infectious unit titer of the virus is increased using the vector comprising the partially deleted E4 gene relative to production of a virus produced using a vector without the partially deleted E4 gene .

32. A viral vector comprising a cassette, the cassette comprising:

(i) at least one payload nucleic acid sequence, optionally wherein the at least one payload nucleic acid sequence encodes a polypeptide, optionally wherein the polypeptide comprises an antigen, optionally wherein the antigen comprises:

- a MHC class I epitope,

- a MHC class II epitope,

- an epitope capable of stimulating a B cell response, or

- a combination thereof, and

optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein;

(ii) at least one promoter sequence operably linked to the at least one payload nucleic acid sequence, wherein the at least one promoter is a tetracycline (TET) repressor protein (TETr) controlled promoter,

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

(iv) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and

(v) optionally, at least one polyadenylation sequence.

33. The vector of any of claims 1-24, wherein the vector comprises the nucleotide sequence of any one of SEQ ID NOs: 57, 58, and 59.

34. Use of an immunotherapy in the manufacture of a medicament for treating a subject with cancer, wherein the immunotherapy is to be administered to the subject, and wherein the the immunotherapy comprises an adenovirus vector comprising:

an adenoviral backbone comprising one or more genes or regulatory sequences of an adenovirus genome,

wherein the adenoviral backbone comprises a partially deleted E4 gene with reference to the adenovirus genome,

wherein the partially deleted E4 gene comprises a deleted or partially-deleted E4orf2 region and a deleted or partially-deleted E4orf3 region, and

optionally a deleted or partially-deleted E4orf4 region, and

wherein the adenovirus vector further comprises a cassette, the cassette comprising:

(1) at least one payload nucleic acid sequence, optionally wherein the at least one payload nucleic acid sequence encodes a polypeptide, optionally wherein the polypeptide comprises an antigen, optionally wherein the antigen comprises:

- a MHC class I epitope,

- a MHC class II epitope,

- an epitope capable of stimulating a B cell response, or

- a combination thereof, and

optionally wherein the at least one payload nucleic acid sequence further comprises a 5' linker sequence and/or a 3' linker sequence, and optionally wherein;

(2) at least one promoter sequence operably linked to the at least one payload nucleic acid sequence,

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

(4) optionally, at least one GPGPG-encoding linker sequence (SEQ ID NO:56); and

(5) optionally, at least one polyadenylation sequence.

35. The use of claim 34, wherein:

(a) the partially deleted E4 gene comprises the E4 gene sequence shown in SEQ ID NO:1 except for lacking at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1; and

(b) the one or more genes or regulatory sequences of the adenovirus genome comprise 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.

36. The use of claim 35, wherein the one or more genes or regulatory sequences of the ChAdV68 sequence shown in SEQ ID NO:1 comprise:

A. nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1, or

B. nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1 except for lacking:

(i) nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion; and/or

(ii) nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion.

37. The use of claim 36, wherein the partially deleted E4 gene is 3' of the nucleotide 34,916 of the sequence shown in SEQ ID NO:1.

38. The use of claim 36, wherein the one or more genes or regulatory sequences of the ChAdV68 sequence shown in SEQ ID NO:1 further comprises 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 nucleotide 35,643 of the sequence shown in SEQ ID NO:1.

39. The use of claim 35, wherein the adenoviral backbone comprises:

A. at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, except for lacking nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1 corresponding to the partially deleted E4 gene,

B. at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, except for lacking nucleotides 34,916 to 34,942, nucleotides 34,952 to 35,305, and nucleotides 35,302 to 35,642 of the sequence shown in SEQ ID NO:1 corresponding to the partially deleted E4 gene, and optionally lacking nucleotides 577 to 3403 and/or nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1,

C. at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, except for lacking nucleotides 34,980 to 36,516 of the sequence shown in SEQ ID NO:1 corresponding to the partially deleted E4 gene, and optionally lacking nucleotides 577 to 3403 and/or nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1,

D. at least nucleotides 2 to 36,518 of the sequence shown in SEQ ID NO:1, except for lacking nucleotides 34,979 to 35,642 of the sequence shown in SEQ ID

NO:1 corresponding to the partially deleted E4 gene, and optionally lacking nucleotides 577 to 3403 and/or nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1,

E. an E4 deletion of at least a partial deletion of E4Orf2, a fully deleted E4Orf3, and at least a partial deletion of E4Orf4,

F. 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,

G. an E4 deletion of at least a partial deletion of E4Orf1, a fully deleted E4Orf2, and at least a partial deletion of E4Orf3, or

H. an E4 deletion of at least a partial deletion of E4Orf2 and at least a partial deletion of E4Orf3.

40. The use of claim 34, wherein the cassette is inserted in the vector at the E1 region, E3 region, and/or any deleted AdV region that allows incorporation of the cassette.

41. The use of claim 34, wherein at least one of the at least one payload nucleic acid sequences encodes a polypeptide sequence capable of undergoing antigen processing into an epitope, wherein the epitope is known or suspected to be presented by MHC class I on a surface of a cell, and wherein the surface of the cell is a tumor cell surface.

42. The use of claim 34, wherein at least one of the at least one payload nucleic acid sequences encodes an epitope with at least one alteration that makes the encoded epitope sequence distinct from the corresponding peptide sequence encoded by a wild-type nucleic acid sequence, optionally 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.

43. The use of claim 34, wherein one or more of the at least one payload nucleic acid sequences encode an MHC I epitope-encoding nucleic acid sequence inclusive of the optional 5’ linker sequence and the optional 3’ linker sequences that encodes a peptide 25 amino acids in length.

44. The use of claim 34, wherein at least one of the at least one payload nucleic acid sequences is linked to a distinct payload nucleic acid sequence with a nucleic acid sequence encoding a linker.

45. The use of claim 44, wherein the linker comprises 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.

46. The use of claim 34, wherein a second vaccine composition is to be further administered to the subject.

47. The use of claim 46, wherein the second vaccine composition comprises a self-amplifying RNA (samRNA) vector encoding at least one payload nucleic acid sequence.

48. The use of claim 46, wherein the at least one payload nucleic acid sequence encoded by the samRNA vector is the same as at least one of the at least one payload nucleic acid sequence of claim 34.

49. The use of claim 34, wherein the adenovirus vector comprises:

A. a modified ChAdV68 sequence, wherein the modified ChAdV68 sequence comprises:

(i) the partially deleted E4 gene comprising the E4 gene sequence shown in SEQ ID NO:1 except for lacking at least nucleotides 34,916 to 35,642 of the sequence shown in SEQ ID NO:1;

(ii) (1) nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1 or (2) nucleotides 2 to 34,916 of the sequence shown in SEQ ID NO:1 except for lacking,

a. nucleotides 577 to 3403 of the sequence shown in SEQ ID NO:1 corresponding to an E1 deletion; and/or

b. nucleotides 27,125 to 31,825 of the sequence shown in SEQ ID NO:1 corresponding to an E3 deletion

wherein the partially deleted E4 gene is 3’ of the nucleotide 34,916 of the sequence shown in SEQ ID NO:1; and

(iii) nucleotides 35,643 to 36,518 of the sequence shown in SEQ ID NO:1, wherein the partially deleted E4 gene is 5’ of the nucleotide 35,643 of the sequence shown in SEQ ID NO:1;

B. a CMV-derived promoter sequence;

C. an SV40 polyadenylation signal nucleotide sequence; and

D. a cassette, the cassette comprising at least one at least one payload nucleic acid sequence encoding:

- at least one MHC class I epitope, optionally wherein the at least one MHC class I epitope comprises at least 2 distinct MHC class I epitopes linearly linked to each other and each optionally comprising:

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

(B) 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 between 2-20 amino acids in length, and

(C) 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 between 2-20 amino acids in length, or

(D) combinations thereof,

- at least one MHC class II epitope, optionally wherein the at least one MHC class II epitope comprises at least 2 distinct MHC class II epitopes,
- at least one an epitope capable of stimulating a B cell response, or
- combinations thereof, and

wherein the cassette is inserted within a deleted region of ChAdV68 and the CMV-derived promoter sequence is operably linked to the cassette.

50. The vector of any of claims 1-24 for use in therapy.