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(71) Applicant: **EPICENTRX, INC.** [US/US]; 4445 Eastgate Mall, Suite 200, San Diego, CA 92121 (US).

(72) Inventors: **REID, Tony, R.**; 5517 Carriage Court, San Diego, CA 92130 (US). **LARSON, Christopher**; 3815 Lebon Dr. Apt. 335, San Diego, CA 92122 (US). **ORONSKY, Bryan, T.**; 28540 Matadero Creek Lane, Los Altos Hills, CA 94022 (US).

(74) Agent: **DAVENPORT, Andrew, M.** et al.; Goodwin Procter LLP, 100 Northern Avenue, Boston, MA 02210 (US).

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(54) Title: MULTIPLE TRANSGENE RECOMBINANT ADENOVIRUS

(57) Abstract: The invention relates to a recombinant adenovirus comprising two or more therapeutic transgenes, e.g., two components of a heterodimeric cytokine, separated by a cleavable linker.

MULTIPLE TRANSGENE RECOMBINANT ADENOVIRUS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and priority to, U.S. Provisional Patent Application serial number 62/484,841 filed April 12, 2017, which is hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The field of the invention is molecular biology and virology, specifically recombinant viruses that express two or more therapeutic transgenes.

BACKGROUND

[0003] Despite extensive knowledge of the underlying molecular mechanisms that cause cancer, most advanced cancers remain incurable with current chemotherapy and radiation protocols. Oncolytic viruses have emerged as a platform technology that has the potential to significantly augment current standard treatment for a variety of malignancies (Kumar, S. et al. (2008) CURRENT OPINION IN MOLECULAR THERAPEUTICS 10(4):371-379; Kim, D. (2001) EXPERT OPINION ON BIOLOGICAL THERAPY 1(3):525-538; Kim D. (2000) ONCOGENE 19(56):6660-6669). These viruses have shown promise as oncolytic agents that not only directly destroy malignant cells via an infection-to-reproduction-to-lysis chain reaction but also indirectly induce anti-tumor immunity. These immune stimulatory properties have been augmented with the insertion of therapeutic transgenes that are copied and expressed each time the virus replicates.

[0004] Previously developed oncolytic viruses include the oncolytic serotype 5 adenovirus (Ad5) referred to as TAV-255 that is transcriptionally attenuated in normal cells but transcriptionally active in cancer cells (see, PCT Publication No. WO2010101921). It is believed that the mechanism by which the TAV-255 vector achieves this tumor selectivity is through targeted deletion of three transcriptional factor (TF) binding sites for the transcription factors Pea3 and E2F, proteins that regulate adenovirus expression of E1a, the earliest gene to be transcribed after virus entry into the host cell, through binding to specific DNA sequences.

[0005] Despite the efforts to date, there is a need for improved recombinant viruses, *e.g.,* recombinant oncolytic viruses, for treating cancers and hyperproliferative disorders in human patients.

SUMMARY OF THE INVENTION

[0006] The invention is based, in part, upon the discovery that for certain recombinant adenoviruses, such as recombinant oncolytic adenoviruses, that express two therapeutic transgenes, the expression of each therapeutic transgene can be greatly enhanced when the two therapeutic transgenes are expressed as a single polypeptide chain with an intervening cleavage site, for example, a proteolytic cleavage site. The cleavage site can then be cleaved posttranslationally by one or more cleavage agents, for example, endogenous or exogenous cleavage agents, to produce the mature protein products encoded by each therapeutic transgene. Such an approach has the additional benefit of ensuring stoichiometric expression and simultaneous delivery of each therapeutic transgene.

[0007] In one aspect, the invention provides a recombinant adenovirus comprising a first nucleotide sequence encoding a first therapeutic transgene, a second nucleotide sequence encoding a second therapeutic transgene, and a third nucleotide sequence encoding a cleavage site disposed between the first nucleotide sequence and the second nucleotide sequence. In certain embodiments, the recombinant adenovirus comprises a recombinant polynucleotide sequence comprising, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence. In certain embodiments, the recombinant adenovirus comprises a recombinant polynucleotide sequence comprising, consecutively, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence, *e.g.* there are no intervening nucleotide sequences (for example, sequences containing another transgene or regulatory sequence) disposed between the first nucleotide sequence and the third nucleotide sequence and/or between the third nucleotide sequence and the second nucleotide sequence. In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are operably linked to a single promoter (optionally positioned 5' to the first nucleotide of the first nucleotide sequence) and expressed as a single polypeptide chain.

[0008] The cleavage site may be a proteolytic cleavage site, *e.g.,* a proteolytic cleavage site that is cleaved by a protease present in an endoplasmic reticulum or golgi of a eukaryotic cell.

In certain embodiments, the proteolytic cleavage site is a furin cleavage site, *e.g.,* a furin cleavage site comprising the sequence RX_1X_2R (SEQ ID NO: 6), wherein X_1 is any amino acid, and X_2 is Lys or Arg, *e.g.,* a furin cleavage site comprising the sequence RAKR (SEQ ID NO: 7).

[0009] In certain embodiments, the recombinant adenovirus is a type 5 adenovirus (Ad5) or a type 2 adenovirus (Ad2).

[0010] In certain embodiments, the first, second, and third nucleotide sequences are inserted into an E1b-19k insertion site located between the start site of E1b-19K and the stop site of E1b-19K. In certain embodiments, the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K. In certain embodiments, the E1b-19K insertion site comprises a deletion of from about 100 to about 305, about 100 to about 300, about 100 to about 250, about 100 to about 200, about 100 to about 150, about 150 to about 305, about 150 to about 300, about 150 to about 250, or about 150 to about 200 nucleotides adjacent the start site of E1b-19K. In certain embodiments, the E1b-19K insertion site comprises a deletion of about 200 nucleotides, *e.g.,* 202 or 203 nucleotides adjacent the start site of E1b-19K. In certain embodiments, the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 or 1714-1916 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first, second, and third nucleotide sequences are inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, first, second, and third nucleotide sequences are inserted between CTGACCTC (SEQ ID NO: 2) and TCACCAGG (SEQ ID NO: 3), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 2), the first nucleotide sequence, the third nucleotide sequence, the second nucleotide sequence, and TCACCAGG (SEQ ID NO: 3).

[0011] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional Pea3 binding site. For example, the virus may comprise a deletion of nucleotides corresponding to about -300 to about -250 upstream of the initiation site of E1a, *e.g.,* a deletion of nucleotides corresponding to -305 to -255 or -304 to -255 upstream of the initiation site of E1a. In certain embodiments, the deletion comprises a deletion of nucleotides corresponding to 195-244 of the Ad5 genome (SEQ ID NO: 1), and/or the E1a promoter comprises the sequence GGTGTTTTGG (SEQ ID NO: 4).

[0012] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional TATA box, *e.g.,* the deletion of an entire TATA box. For example, in certain embodiments, the virus comprises a deletion of nucleotides corresponding to -27 to -24, -31 to -24, -44 to +54, or -146 to +54 of the adenovirus type 5 E1a promoter, which correspond, respectively, to nucleotides 472 to 475, 468 to 475, 455 to 552, and 353 to 552 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5), AGTGCCCG (SEQ ID NO: 16), or TATTCCCG (SEQ ID NO: 17), which result from joining the two polynucleotide sequences that would otherwise flank the deleted polynucleotide sequence.

[0013] In certain embodiments, the recombinant adenovirus comprises a deletion of nucleotides corresponding to -29 to -26, -33 to -26, -44 to +52, or -148 to +52 of the adenovirus type 5 E1a promoter. In certain embodiments, the virus comprises a deletion of nucleotides corresponding to 353 to 552 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5), which results from joining the two polynucleotide sequences that would otherwise flank the deleted polynucleotide sequence.

[0014] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional CAAT box, *e.g.,* the deletion of an entire CAAT box. For example, in certain embodiments, the virus comprises a deletion of nucleotides corresponding to -76 to -68 of the adenovirus type 5 E1a promoter, which corresponds to nucleotides 423 to 431 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence TTCCGTGGCG (SEQ ID NO: 18), which results from joining the two polynucleotide sequences that would otherwise flank the deleted polynucleotide sequence.

[0015] In certain embodiments the recombinant adenovirus comprises an E3 deletion. In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to

about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides. In certain embodiments, the E3 deletion site is located between the stop site of pVIII and the start site of Fiber. In certain embodiments, the E3 deletion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K. In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K, *e.g.,* the E3 deletion comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain embodiments, the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 1).

[0016] In certain embodiments, the E3 deletion is located between stop site of E3-gp19K and the stop site of E3-14.7K. In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 1824, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 deletion comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K, *e.g.,* the E3 deletion comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 1).

[0017] In certain embodiments, the recombinant adenovirus further comprises an E4 deletion. In certain embodiments, the E4 deletion is located between the start site of E4-ORF6/7 and the right inverted terminal repeat (ITR). In certain embodiments, the E4 deletion is located between the start site of E4-ORF6/7 and the start site of E4-ORF1. In certain embodiments, the E4 deletion comprises a deletion of from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 2500, from about 1500 to about 2000, or from about 2000 to about 2500 nucleotides. In certain embodiments, the E4 deletion comprises a deletion of from about

250 to about 1500, from about 250 to about 1250, from about 250 to about 1000, from about 250 to about 750, from about 250 to about 500, from 500 to about 1500, from about 500 to about 1250, from about 500 to about 1000, from about 500 to about 750, from 750 to about 1500, from about 750 to about 1250, from about 750 to about 1000, from about 1000 to about 1500, or from about 1000 to about 1250 nucleotides adjacent the start site of E4-ORF6/7. In certain embodiments, the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7, *e.g.,* the E4 deletion comprises a deletion of about 1449 nucleotides adjacent the start site of E4-ORF6/7. In certain embodiments, the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 1).

[0018] In certain embodiments, the first and/or second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-CTLA-4 antibody heavy chain or light chain, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD40L, CD70, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, GITRL, GM-CSF, ICAM, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, an IL-10 trap, IL-15, an IL-15 IL-15 receptor fusion protein, IL-17, IL-23, IL-23A/p19, IL-12B/p40, IL-24, IL-27, IL-27A/p28, IL-27B/EBI3, IL-35, interferon-gamma, MAGE, NY-ESO-1, Ox40L, p53, secreted flagellin, TGF-β, a TGF-β trap, thymidine kinase, and TNF-alpha.

[0019] In certain embodiments, the first and/or second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-CTLA-4 antibody heavy chain or light chain, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD40L, CD70, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, GITRL, GM-CSF, ICAM, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, an IL-10 trap, IL-12, IL-12A/p35, IL-12B/p40, IL-15, an IL-15 IL-15 receptor fusion protein, IL-23A/p19, interferon-gamma, MAGE, NY-ESO-1, Ox40L, p53, secreted flagellin, TGF-β, a TGF-β trap, thymidine kinase, and TNF-alpha.

[0020] In certain embodiments, the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric protein, *e.g.,* a heterodimeric cytokine. For example, in certain embodiments, in any of the foregoing recombinant adenoviruses, the first and/or second therapeutic transgenes are selected from IL-12A/p35 and IL-12B/p40, *e.g.,* the

first therapeutic transgene encodes IL-12B/p40 and the second therapeutic transgene encodes IL-12A/p35. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1000 of SEQ ID NO: 8, nucleotides 1013-1606 of SEQ ID NO: 8, and/or nucleotides 17-1606 of SEQ ID NO: 8. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 8, or comprises a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 8.

[0021] In certain embodiments, in any of the foregoing recombinant adenoviruses, the first and/or second therapeutic transgenes are selected from IL-23A/p19 and IL-12B/p40, *e.g.,* the first therapeutic transgene encodes IL-12B/p40 and the second therapeutic transgene encodes IL-23A/p19. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1000 of SEQ ID NO: 9, nucleotides 1013-1582 of SEQ ID NO: 9, and/or nucleotides 17-1582 of SEQ ID NO: 9. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 9, or comprises a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 9.

[0022] In certain embodiments, any of the foregoing recombinant viruses may selectively replicate in a hyperproliferative cell. In certain embodiments, any of the foregoing recombinant viruses may selectively express two or more therapeutic transgenes in a hyperproliferative cell. The hyperproliferative cell may be a cancer cell, *e.g.*, a lung cancer cell, a colon cancer cell, and a pancreatic cancer cell. In certain embodiments, any of the foregoing recombinant viruses may be an oncolytic virus.

[0023] In certain embodiments, in any of the foregoing recombinant adenoviruses, the first and/or second therapeutic transgenes are not operably linked to an exogenous promoter sequence. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides. In certain embodiments, the size of the first and

second therapeutic transgenes when combined comprises from about 500 to about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

[0024] In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least from about 500 to about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

[0025] In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic

transgenes when combined comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000 nucleotides, about 6000, or about 7000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises about 1600 nucleotides, about 1650 nucleotides, or about 3100 nucleotides.

[0026] In another aspect, the invention provides a pharmaceutical composition comprising each of the foregoing recombinant adenoviruses and at least one pharmaceutically acceptable carrier or diluent.

[0027] In another aspect, the invention provides a method of treating cancer in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to treat the cancer disease in the subject. In certain embodiments, the cancer is selected from anal cancer, basal cell carcinoma, bladder cancer, bone cancer, brain cancer, breast cancer, carcinoma, cholangiocarcinoma, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, gastroesophageal cancer, gastrointestinal (GI) cancer, gastrointestinal stromal tumor, hepatocellular carcinoma, gynecologic cancer, head and neck cancer, hematologic cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, merkel cell carcinoma, mesothelioma, neuroendocrine cancer, non-small cell lung cancer, ovarian cancer, pancreatic cancer, pediatric cancer, prostate cancer, renal cell carcinoma, sarcoma, skin cancer, small cell lung cancer, squamous cell carcinoma of the skin, stomach cancer, testicular cancer and thyroid cancer.

[0028] In another aspect, the invention provides a method of inhibiting proliferation of a tumor cell in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to inhibit proliferation of the tumor cell.

[0029] In another aspect, the invention provides a method of inhibiting tumor growth in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to inhibit proliferation of the tumor cell.

[0030] In each of the foregoing methods, the recombinant adenovirus can, *e.g.*, be administered in combination with one or more therapies selected from surgery, radiation, chemotherapy, immunotherapy, hormone therapy, and virotherapy. In each of the foregoing methods, the effective amount of the recombinant adenovirus can be, *e.g.*, 10^2-10^{15} plaque forming units (pfus). In certain embodiments of any of the foregoing methods, the subject can be a human, *e.g.,* a pediatric human.

[0031] In another aspect, the invention provides a method of expressing two or more therapeutic transgenes in a target cell. The method comprises delivering to the cell, *e.g.*, exposing the cell, to an effective amount of a recombinant adenovirus described herein to express the target transgenes. In certain embodiments, the two therapeutic transgenes, when expressed, produce a single polypeptide chain, which may be cleaved posttranslationally into two polypeptide chains.

[0032] These and other aspects and advantages of the invention are illustrated by the following figures, detailed description and claims.

DESCRIPTION OF THE DRAWINGS

[0033] The invention can be more completely understood with reference to the following drawings.

[0034] **FIGURE 1** is a graph depicting IL-12 expression by HEK-293 cells transiently transfected with plasmids encoding IL-12 with different linkers, as determined by ELISA. Dots represent concentrations measured in triplicates, thick bars represent mean, and error bars represent standard deviation.

[0035] **FIGURE 2** is a gel depicting PCR products of DNA from the TAV-hIL-12-furin adenovirus (indicated as "furin"), the TAV-hIL-12-Separate adenovirus (indicated as "separate") and the control dl309 adenovirus, demonstrating the anticipated size products.

[0036] **FIGURE 3** depicts crystal violet staining of ADS-12 cells at the indicated time points following infection with the indicated virus. Crystal violet stains viable cells purple.

[0037] **FIGURE 4** is a bar graph depicting IL-12 expression from ADS-12 cells infected with TAV-Δ19k and TAV-mIL12-IRES, as determined by ELISA. Error bars represent standard deviation.

[0038] **FIGURE 5** is a bar graph depicting viral replication of TAV-Δ19k and TAV-mIL-12-IRES in ADS-12 cells. Error bars represent standard deviation.

[0039] **FIGURE 6** is a bar graph depicting IL-12 expression from A549 cells infected with TAV-Δ19k, TAV-hIL-12-IRES, TAV-hIL-12-furin, or no virus, as determined by ELISA.

[0040] **FIGURE 7** depicts crystal violet staining of A549 cells at the indicated time points following infection with the indicated virus. Crystal violet stains viable cells purple.

[0041] **FIGURE 8** is bar graph a depicting viral replication of TAV-Δ19k, TAV-hIL-12-IRES, and TAV-hIL-12-furin in A549 cells.

[0042] **FIGURE 9** is a line graph depicting expression of IL-12 from the virus TAV-mIL-12-furin. A549 cells were infected at the indicated MOI, and IL-12 concentration in conditioned media was measured at the indicated timepoints by ELISA. CT indicates control non-infected cells.

[0043] **FIGURE 10** is a bar graph depicting expression of IL-23 from the virus TAV-mIL-23-furin and IL-27 from the virus TAV-mIL-27-furin. A549 cells were infected at an MOI of 5 and the concentration of the corresponding cytokine for each virus in conditioned media was measured four days after infection by ELISA.

DETAILED DESCRIPTION

[0044] The invention is based, in part, upon the discovery that for certain recombinant adenoviruses, such as recombinant oncolytic adenoviruses, that express two therapeutic transgenes, the expression of each therapeutic transgene can be greatly enhanced when the two therapeutic transgenes are expressed as a single polypeptide chain with an intervening cleavage site, for example, a proteolytic cleavage site. The cleavage site can then be cleaved posttranslationally by one or more cleavage agents, for example, endogenous or exogenous cleavage agents, to produce the mature protein products encoded by each therapeutic transgene. Such an approach has the additional benefit of ensuring stoichiometric expression and simultaneous delivery of each therapeutic transgene.

[0045] Accordingly, in one aspect, the invention provides a recombinant adenovirus comprising a first nucleotide sequence encoding a first therapeutic transgene, a second nucleotide sequence encoding a second therapeutic transgene, and a third nucleotide sequence encoding a cleavage site disposed between the first nucleotide sequence and the second nucleotide sequence. In certain embodiments, the recombinant adenovirus comprises a recombinant polynucleotide sequence comprising, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence. In certain embodiments, the recombinant adenovirus comprises a recombinant polynucleotide sequence comprising, consecutively, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence, *e.g.* there are no intervening nucleotide sequences (for example, sequences containing another transgene or regulatory

sequence) disposed between the first nucleotide sequence and the third nucleotide sequence and/or between the third nucleotide sequence and the second nucleotide sequence. In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are operably linked to a single promoter (optionally positioned 5' to the first nucleotide of the first nucleotide sequence) and expressed as a single polypeptide chain.

[0046] In certain embodiments, the recombinant adenovirus is an oncolytic virus, *e.g.*, a virus that exhibits tumor-selective replication and/or viral mediated lysis. In certain embodiments, the oncolytic virus allows for selective expression of a therapeutic transgene, *e.g.*, the virus permits expression of the therapeutic transgene in neoplastic cells, but attenuates expression in normal cells. In certain embodiments, the expression of the therapeutic transgene in a non-hyperproliferative cell is about 90%, about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20%, about 10% , or about 5% of the expression in a hyperproliferative cell. In certain embodiments, the virus exhibits no detectable expression of the therapeutic transgene in a non-hyperproliferative cell. Therapeutic transgene expression may be determined by any appropriate method known in the art, *e.g.,* Western blot or ELISA. The hyperproliferative cell may be a cancer cell, *e.g.,* a carcinoma, sarcoma, leukemia, lymphoma, prostate cancer, lung cancer, gastrointestinal tract cancer, colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, cervical cancer, stomach cancer, thyroid cancer, mesothelioma, liver cancer, kidney cancer, skin cancer, head and neck cancer, or brain cancer cell.

I. Viruses

[0047] The term "virus" is used herein to refer any of the obligate intracellular parasites having no protein-synthesizing or energy-generating mechanism. The viral genome may be RNA or DNA. The viruses useful in the practice of the present invention include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridiae, parvoviridiae, picornoviridiae, herpesviridiae, poxyiridae, or adenoviridiae. A recombinantly modified virus is referred to herein as a "recombinant virus." A recombinant virus may, *e.g.,* be modified by recombinant DNA techniques to be replication deficient, conditionally replicating, or replication competent, and/or be modified by recombinant DNA techniques to include expression of exogenous transgenes. Chimeric viral vectors which exploit advantageous elements of each of the parent vector properties (See, *e.g.*,

Feng *et al.* (1997) NATURE BIOTECHNOLOGY 15:866-870) may also be useful in the practice of the present invention. Although it is generally favored to employ a virus from the species to be treated, in some instances it may be advantageous to use vectors derived from different species that possess favorable pathogenic features. For example, equine herpes virus vectors for human gene therapy are described in PCT Publication No. WO 98/27216. The vectors are described as useful for the treatment of humans as the equine virus is not pathogenic to humans. Similarly, ovine adenoviral vectors may be used in human gene therapy as they are claimed to avoid the antibodies against the human adenoviral vectors. Such vectors are described in PCT Publication No. WO 97/06826.

[0048] Preferably, the recombinant virus is an adenovirus. Adenoviruses are medium-sized (90-100 nm), non-enveloped (naked), icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome. Adenoviruses replicate in the nucleus of mammalian cells using the host's replication machinery. The term "adenovirus" refers to any virus in the genus Adenoviridiae including, but not limited to, human, bovine, ovine, equine, canine, porcine, murine, and simian adenovirus subgenera. In particular, human adenoviruses includes the A-F subgenera as well as the individual serotypes thereof, the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11a and Ad11p), 12, 13, 14, 15, 16, 17, 18, 19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. Preferred are recombinant viruses derived from human adenovirus types 2 and 5. Unless stated otherwise, all adenovirus type 5 nucleotide numbers are relative to the NCBI reference sequence AC_000008.1, which is depicted herein in SEQ ID NO: 1.

[0049] The adenovirus replication cycle has two phases: an early phase, during which 4 transcription units E1, E2, E3, and E4 are expressed, and a late phase which occurs after the onset of viral DNA synthesis when late transcripts are expressed primarily from the major late promoter (MLP). The late messages encode most of the virus's structural proteins. The gene products of E1, E2 and E4 are responsible for transcriptional activation, cell transformation, viral DNA replication, as well as other viral functions, and are necessary for viral growth.

[0050] The term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a gene if it affects the transcription of the gene. Operably linked

nucleotide sequences are typically contiguous. However, as enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not directly flanked and may even function *in trans* from a different allele or chromosome.

[0051] In certain embodiments, the recombinant adenovirus has one or more modifications to a regulatory sequence or promoter. A modification to a regulatory sequence or promoter comprises a deletion, substitution, or addition of one or more nucleotides compared to the wild-type sequence of the regulatory sequence or promoter.

[0052] In certain embodiments, the modification of a regulatory sequence or promoter comprises a modification of the sequence of a transcription factor binding site to reduce affinity for the transcription factor, for example, by deleting a portion thereof, or by inserting a single point mutation into the binding site. In certain embodiments, the modified regulatory sequence enhances expression in neoplastic cells and/or attenuates expression in normal cells.

[0053] In certain embodiments, the modified regulatory sequence is operably linked to a sequence encoding a protein. In certain embodiments, at least one of the adenoviral E1a and E1b genes (coding regions) is operably linked to a modified regulatory sequence. In certain embodiments, the E1a gene is operably linked to a modified regulatory sequence.

[0054] The E1a regulatory sequence contains five binding sites for the transcription factor Pea3, designated Pea3 I, Pea3 II, Pea3 III, Pea3 IV, and Pea3 V, where Pea3 I is the Pea3 binding site most proximal to the E1a start site, and Pea3 V is most distal. The E1a regulatory sequence also contains binding sites for the transcription factor E2F, hereby designated E2F I and E2F II, where E2F I is the E2F binding site most proximal to the E1a start site, and E2F II is more distal. From the E1a start site, the binding sites are arranged: Pea3 I, E2F I, Pea3 II, E2F II, Pea3 III, Pea3 IV, and Pea3 V.

[0055] In certain embodiments, at least one of these seven binding sites, or at least one of seven functional binding sites, is deleted. As used herein, a "functional binding site" refers to a binding site that is capable of binding to a respective binding partner, *e.g.,* a transcription factor, *e.g.,* a binding site that has at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, or at least 40%, of the binding activity of a corresponding wild-type binding site sequence. As used herein, a "non-functional binding site" refers to a binding site

that, *e.g.*, has less than 30%, less than 20%, less than 10%, or 0% of the binding activity of a corresponding wild-type binding site sequence.

[0056] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional Pea3 binding site, *e.g.,* the deletion of an entire Pea3 binding site. As used herein, a "functional Pea3 binding site" refers to a Pea3 binding site that is capable of binding to its respective transcription factor (*e.g.,* Pea3), *e.g.,* a Pea3 binding site that has at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, or at least 40%, of the Pea3 binding activity of a corresponding wild-type Pea3 binding site sequence. As used herein, a "non-functional Pea3 binding site" refers to a Pea3 binding site that, *e.g.*, has less than 30%, less than 20%, less than 10%, or 0% of the Pea3 binding activity of a corresponding wild-type Pea3 binding site sequence. Assays for determining whether a Pea3 binding site binds to Pea3 are known in the art. Exemplary binding assays include electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and DNAse footprinting assays.

[0057] In certain embodiments, at least one Pea3 binding site, or a functional Pea3 binding site, is deleted. The deleted Pea3 binding site can be Pea3 I, Pea3 II, Pea3 III, Pea3 IV, and/or Pea3 V. In certain embodiments, the deleted Pea3 binding site is Pea3 II, Pea3 III, Pea3 IV, and/or Pea3 V. In certain embodiments, the deleted Pea3 binding site is Pea3 IV and/or Pea3 V. In certain embodiments, the deleted Pea3 binding site is Pea3 II and/or Pea3 III. In certain embodiments, the deleted Pea3 binding site is both Pea3 II and Pea3 III. In certain embodiments, the Pea3 I binding site, or a functional Pea3 I binding site, is retained.

[0058] In certain embodiments, at least one E2F binding site, or a functional E2F binding site, is deleted. In certain embodiments, at least one E2F binding site, or a functional E2F binding site, is retained. In certain embodiments, the retained E2F binding site is E2F I and/or E2F II. In certain embodiments, the retained E2F binding site is E2F II. In certain embodiments, the total deletion consists essentially of one or more of Pea3 II, Pea3 III, Pea3 IV, and/or Pea3 V.

[0059] In certain embodiments, the recombinant adenovirus comprises a deletion of at least one E2F binding site, or a functional portion thereof. In certain embodiments, the recombinant adenovirus comprises a deletion of at least one E2F binding site, or a functional portion thereof, and does not comprise a deletion of a Pea3 binding site.

[0060] In certain embodiments, the virus has a deletion of a 50 base pair region located from -304 to -255 upstream of the E1a initiation site, *e.g.,* corresponding to 195-244 of the Ad5 genome (SEQ ID NO: 1), hereafter referred to as the TAV-255 deletion. In certain embodiments, the TAV-255 deletion results in an E1a promoter that comprises the sequence GGTGTTTTGG (SEQ ID NO: 4).

[0061] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional TATA box, *e.g.,* the deletion of an entire TATA box. As used herein, a "functional TATA box" refers to a TATA box that is capable of binding to a TATA box binding protein (TBP), *e.g.,* a TATA box that has at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, or at least 40%, of the TBP binding activity of a corresponding wild-type TATA box sequence. As used herein, a "non-functional TATA box" refers to a TATA box that, *e.g.*, has less than 30%, less than 20%, less than 10%, or 0% of the TBP binding activity of a corresponding wild-type TATA box sequence. Assays for determining whether a TBP binds to a TATA box are known in the art. Exemplary binding assays include electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and DNAse footprinting assays.

[0062] For example, in certain embodiments, the recombinant adenovirus comprises a deletion of nucleotides corresponding to -27 to -24, -31 to -24, -44 to +54, or -146 to +54 of the adenovirus type 5 E1a promoter, which correspond, respectively, to nucleotides 472 to 475, 468 to 475, 455 to 552, and 353 to 552 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a deletion of nucleotides corresponding to -29 to -26, -33 to -26, -44 to +52, or -148 to +52 of the adenovirus type 5 E1a promoter. In certain embodiments, the virus comprises a deletion of nucleotides corresponding to 353 to 552 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5), AGTGCCCG (SEQ ID NO: 16), or TATTCCCG (SEQ ID NO: 17), which result from joining the two polynucleotide sequences that would otherwise flank the deleted polynucleotide sequence. In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5).

[0063] In certain embodiments, the recombinant adenovirus comprises an E1a promoter having a deletion of a functional CAAT box, *e.g.,* the deletion of an entire CAAT box. As used

herein, a "functional CAAT box" refers to a CAAT box that is capable of binding to a C/EBP or NF-Y protein, *e.g.,* a CAAT box that has at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, or at least 40%, of the a C/EBP or NF-Y binding activity of a corresponding wild-type CAAT box sequence. As used herein, a "non-functional CAAT box" refers to a CAAT box that, *e.g.*, has less than 30%, less than 20%, less than 10%, or 0% of the a C/EBP or NF-Y binding activity of a corresponding wild-type CAAT box sequence. Assays for determining whether a C/EBP or NF-Y protein binds to a CAAT box are known in the art. Exemplary binding assays include electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and DNAse footprinting assays.

[0064] For example, in certain embodiments, a recombinant adenovirus comprises a deletion of nucleotides corresponding to -76 to -68 of the adenovirus type 5 E1a promoter, which corresponds to nucleotides 423 to 431 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the virus comprises a polynucleotide deletion that results in a virus comprising the sequence TTCCGTGGCG (SEQ ID NO: 18), which results from joining the two polynucleotide sequences that would otherwise flank the deleted polynucleotide sequence.

[0065] The adenoviral E1b-19k gene functions primarily as an anti-apoptotic gene and is a homolog of the cellular anti-apoptotic gene, BCL-2. Since host cell death prior to maturation of the progeny viral particles would restrict viral replication, E1b-19k is expressed as part of the El cassette to prevent premature cell death thereby allowing the infection to proceed and yield mature virions. Accordingly, in certain embodiments, a recombinant adenovirus is provided that includes an E1b-19K insertion site, *e.g.*, the recombinant adenovirus has a nucleotide sequence encoding a transgene inserted into an E1b-19K insertion site. In certain embodiments, the insertion site is located between the start site of E1b-19K (*i.e.,* the nucleotide sequence encoding the start codon of E1b-19k, *e.g.,* corresponding to nucleotides 1714-1716 of SEQ ID NO: 1) and the start site of E1b-55K (*i.e.,* the nucleotide sequence encoding the start codon of E1b-55k, *e.g.,* corresponding to nucleotides 2019-2021 of SEQ ID NO: 1). In certain embodiments, the E1b-19K insertion site is located between the start site of E1b-19K (*i.e.,* the nucleotide sequence encoding the start codon of E1b-19k, *e.g.,* corresponding to nucleotides 1714-1716 of SEQ ID NO: 1) and the stop site of E1b-19K (*i.e.,* the nucleotide sequence encoding the stop codon of E1b-19k, *e.g.,* corresponding to nucleotides 2242-2244 of SEQ ID NO: 1).

[0066] Throughout the description and claims, an insertion between two sites, for example, an insertion between (i) a start site of a first gene (*e.g.,* E1b-19k) and a start site of a second gene, (*e.g.,* E1b-55K), (ii) a start site of a first gene and a stop site of a second gene, (iii) a stop site of a first gene and start site of a second gene, or (iv) a stop site of first gene and a stop site of a second gene, is understood to mean that all or a portion of the nucleotides constituting a given start site or a stop site surrounding the insertion may be present or absent in the final virus. Similarly, an insertion between two nucleotides is understood to mean that the nucleotides surrounding the insertion may be present or absent in the final virus.

[0067] In certain embodiments, the E1b-19K insertion site comprises a deletion of from about 100 to about 305, about 100 to about 300, about 100 to about 250, about 100 to about 200, about 100 to about 150, about 150 to about 305, about 150 to about 300, about 150 to about 250, or about 150 to about 200 nucleotides adjacent the start site of E1b-19K. In certain embodiments, the E1b-19K insertion site comprises a deletion of about 200 nucleotides, *e.g.,* 202 or 203 nucleotides adjacent the start site of E1b-19K. In certain embodiments, the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 or 1714-1916 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence is inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence is inserted between CTGACCTC (SEQ ID NO: 2) and TCACCAGG (SEQ ID NO: 3), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 2), the first nucleotide sequence, the third nucleotide sequence, the second nucleotide sequence, and TCACCAGG (SEQ ID NO: 3). CTGACCTC (SEQ ID NO: 2) and TCACCAGG (SEQ ID NO: 3) define unique boundary sequences for an E1b-19K insertion site within the Ad5 genome (SEQ ID NO: 1). Throughout the description and claims, a deletion adjacent a site, for example, a deletion adjacent a start site of a gene or a deletion adjacent a stop site of a gene, is understood to mean that the deletion may include a deletion of all, a portion, or none of the nucleotides constituting a given start site or a stop site.

[0068] In certain embodiments the recombinant adenovirus comprises an E3 deletion. In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000,

from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides. In certain embodiments the E3 deletion is located between the stop site of pVIII (*i.e.,* the nucleotide sequence encoding the stop codon of pVIII, *e.g.,* corresponding to nucleotides 27855-27857 of SEQ ID NO: 1) and the start site of Fiber (*i.e.,* the nucleotide sequence encoding the start codon of Fiber, *e.g.,* corresponding to nucleotides 31042-31044 of SEQ ID NO: 1). In certain embodiments, the E3 deletion is located between the stop site of E3-10.5K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-10.5K, *e.g.,* corresponding to nucleotides 29770-29772 of SEQ ID NO: 1) and the stop site of E3-14.7K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-14.7K, *e.g.,* corresponding to nucleotides 30837-30839 of SEQ ID NO: 1). In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K , *e.g.,* the E3 deletion comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain embodiments, the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 1).

[0069] In certain embodiments, the E3 deletion is located between stop site of E3-gp19K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-gp19K, *e.g.,* corresponding to nucleotides 29215-29217 of SEQ ID NO: 1) and the stop site of E3-14.7K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-14.7K, *e.g.,* corresponding to nucleotides 30837-30839 of SEQ ID NO: 1). In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 1824, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 deletion comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K. *e.g.,* the E3

deletion comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 1).

[0070] In certain embodiments, a recombinant adenovirus is provided that includes an E3 insertion site, *e.g.*, the recombinant adenovirus has a nucleotide sequence encoding a therapeutic transgene inserted into an E3 insertion site. In certain embodiments the recombinant adenovirus comprises an E3 deletion. In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides. In certain embodiments the E3 deletion is located between the stop site of pVIII (*i.e.,* the nucleotide sequence encoding the stop codon of pVIII, *e.g.,* corresponding to nucleotides 27855-27857 of SEQ ID NO: 1) and the start site of Fiber (*i.e.,* the nucleotide sequence encoding the start codon of Fiber, *e.g.,* corresponding to nucleotides 31042-31044 of SEQ ID NO: 1). In certain embodiments, the E3 deletion is located between the stop site of E3-10.5K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-10.5K, *e.g.,* corresponding to nucleotides 29770-29772 of SEQ ID NO: 1) and the stop site of E3-14.7K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-14.7K, *e.g.,* corresponding to nucleotides 30837-30839 of SEQ ID NO: 1). In certain embodiments, the E3 deletion comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K , *e.g.,* the E3 deletion comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain embodiments, the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence,

and the second nucleotide sequence are inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are inserted between CAGTATGA (SEQ ID NO: 19) and TAATAAAAAA (SEQ ID NO: 20), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 19), the first nucleotide sequence, the third nucleotide sequence, the second nucleotide sequence, and TAATAAAAAA (SEQ ID NO: 20). CAGTATGA (SEQ ID NO: 19) and TAATAAAAAA (SEQ ID NO: 20) define unique boundary sequences for an E3 insertion site within the Ad5 genome (SEQ ID NO: 1).

[0071] In certain embodiments, the E3 insertion site is located between stop site of E3-gp19K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-gp19K, *e.g.,* corresponding to nucleotides 29215-29217 of SEQ ID NO: 1) and the stop site of E3-14.7K (*i.e.,* the nucleotide sequence encoding the stop codon of E3-14.7K, *e.g.,* corresponding to nucleotides 30837-30839 of SEQ ID NO: 1). In certain embodiments, the E3 insertion site comprises a deletion of from about 500 to about 1824, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K. *e.g.,* the E3 insertion site comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K. In certain embodiments, the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 1). In certain embodiments, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are inserted between TGCCTTAA (SEQ ID NO: 21) and TAAAAAAAAAT (SEQ ID NO: 22), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 21), the first nucleotide sequence, the third nucleotide sequence, the second nucleotide sequence, and TAAAAAAAAAT (SEQ ID NO: 22). TGCCTTAA (SEQ ID NO: 21) and TAAAAAAAAAT (SEQ ID NO: 22) define unique boundary sequences for an E3 insertion site within the Ad5 genome (SEQ ID NO: 1).

[0072] In certain embodiments, a recombinant adenovirus comprises an E4 deletion. In certain embodiments, the E4 deletion is located between the start site of E4-ORF6/7 (*i.e.,* the

nucleotide sequence encoding the start codon of E4-ORF6/7, *e.g.,* corresponding to nucleotides 34075-34077 of SEQ ID NO: 1) and the right inverted terminal repeat (ITR; *e.g.,* corresponding to nucleotides 35836-35938 of SEQ ID NO: 1). In certain embodiments, the E4 deletion is located between the start site of E4-ORF6/7 and the start site of E4-ORF1 (*i.e.,* the nucleotide sequence encoding the start codon of E4-ORF1, *e.g.,* corresponding to nucleotides 35524-35526 of SEQ ID NO: 1). In certain embodiments, the E4 deletion comprises a deletion of a nucleotide sequence between the start site of E4-ORF6/7 and the start site of E4-ORF1. In certain embodiments, the E4 deletion comprises a deletion of from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 2500, from about 1500 to about 2000, or from about 2000 to about 2500 nucleotides. In certain embodiments, the E4 deletion comprises a deletion of from about 250 to about 1500, from about 250 to about 1250, from about 250 to about 1000, from about 250 to about 750, from about 250 to about 500, from 500 to about 1500, from about 500 to about 1250, from about 500 to about 1000, from about 500 to about 750, from 750 to about 1500, from about 750 to about 1250, from about 750 to about 1000, from about 1000 to about 1500, or from about 1000 to about 1250 nucleotides adjacent the start site of E4-ORF6/7. In certain embodiments, the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7, e.g., the E4 deletion comprises a deletion of about 1449 nucleotides adjacent the start site of E4-ORF6/7. In certain embodiments, the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 1).

II. Methods of Viral Production

[0073] Methods for producing recombinant viruses of the invention are known in the art. Typically, a disclosed virus is produced in a suitable host cell line using conventional techniques including culturing a transfected or infected host cell under suitable conditions so as to allow the production of infectious viral particles. Nucleic acids encoding viral genes can be incorporated into plasmids and introduced into host cells through conventional transfection or transformation techniques. Exemplary suitable host cells for production of disclosed viruses include human cell lines such as HeLa, Hela-S3, HEK293, 911, A549, HER96, or PER-C6 cells. Specific production and purification conditions will vary depending upon the virus and the production system employed. For adenovirus, the traditional method for the generation of

viral particles is co-transfection followed by subsequent *in vivo* recombination of a shuttle plasmid (usually containing a small subset of the adenoviral genome and optionally containing a potential transgene an expression cassette) and an adenoviral helper plasmid (containing most of the entire adenoviral genome).

[0074] Alternative technologies for the generation of adenovirus include utilization of the bacterial artificial chromosome (BAC) system, *in vivo* bacterial recombination in a recA+ bacterial strain utilizing two plasmids containing complementary adenoviral sequences, and the yeast artificial chromosome (YAC) system.

[0075] Following production, infectious viral particles are recovered from the culture and optionally purified. Typical purification steps may include plaque purification, centrifugation, *e.g.,* cesium chloride gradient centrifugation, clarification, enzymatic treatment, *e.g.*, benzonase or protease treatment, chromatographic steps, *e.g.,* ion exchange chromatography or filtration steps.

III. Therapeutic Transgenes

[0076] A disclosed recombinant adenovirus may comprise a nucleotide sequence that encodes for a therapeutic transgene. In certain embodiments, a disclosed recombinant adenovirus may comprise a first nucleotide sequence and a second nucleotide sequence that encode for a first and a second therapeutic transgene, respectively.

[0077] A therapeutic transgene may encode a therapeutic nucleic acid, *e.g.,* an antisense RNA or ribozyme RNA. The therapeutic transgene may encode a therapeutic peptide or polypeptide, *e.g.,* an apoptotic agent, antibody, CTL responsive peptide, cytokine, cytolytic agent, cytotoxic agent, enzyme, heterologous antigen expressed on the surface of a tumor cell to elicit an immune response, immunostimulatory or immunomodulatory agent, interferon, lytic peptide, oncoprotein, polypeptide which catalyzes processes leading to cell death, polypeptide which complements genetic defects in somatic cells, tumor suppressor protein, vaccine antigen, or any combination thereof.

[0078] In certain embodiments, the first and/or second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-CTLA-4 antibody heavy chain or light chain, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD40L, CD70, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, GITRL, GM-CSF, ICAM, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, an IL-10 trap, IL-15, an IL-15 IL-15 receptor fusion protein, IL-17, IL-23, IL-23A/p19, IL-

12B/p40, IL-24, IL-27, IL-27A/p28, IL-27B/EBI3, IL-35, interferon-gamma, MAGE, NY-ESO-1, Ox40L, p53, secreted flagellin, TGF-β, a TGF-β trap, thymidine kinase, and TNF-alpha.

[0079] In certain embodiments, the first and/or second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-CTLA-4 antibody heavy chain or light chain, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD40L, CD70, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, GITRL, GM-CSF, ICAM, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, an IL-10 trap, IL-12, IL-12A/p35, IL-12B/p40, IL-15, an IL-15 IL-15 receptor fusion protein, IL-23A/p19, interferon-gamma, MAGE, NY-ESO-1, Ox40L, p53, secreted flagellin, TGF-β, a TGF-β trap, thymidine kinase, and TNF-alpha.

[0080] In certain embodiments, the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric protein, *e.g.,* a heterodimeric cytokine. For example, in certain embodiments the first and/or second therapeutic transgenes are selected from IL-12A/p35 and IL-12B/p40, which make up the heterodimeric cytokine IL-12. For example the first therapeutic transgene may encode IL-12B/p40 and the second therapeutic transgene may encode IL-12A/p35. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1000 of SEQ ID NO: 8, nucleotides 1013-1606 of SEQ ID NO: 8, and/or nucleotides 17-1606 of SEQ ID NO: 8. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 8, or comprises a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 8.

[0081] Additionally, in certain embodiments, the first and/or second therapeutic transgenes are selected from IL-23A/p19 and IL-12B/p40, which make up the heterodimeric cytokine IL-23. For example the first therapeutic transgene may encode IL-12B/p40 and the second therapeutic transgene may encode IL-23A/p19. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1000 of SEQ ID NO: 9, nucleotides 1013-1582 of SEQ ID NO: 9, and/or nucleotides 17-1582 of SEQ ID NO: 9. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 9, or comprises a sequence having 80%,

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 9.

[0082] Additionally, in certain embodiments, the first and/or second therapeutic transgenes are selected from IL-27A/p28 and IL-27B/EBI3, which make up the heterodimeric cytokine IL-23.

[0083] Sequence identity may be determined in various ways that are within the skill in the art, e.g., using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin *et al.*, (1990) PROC. NATL. ACAD. SCI. USA 87:2264-2268; Altschul, (1993) J. MOL. EVOL. 36, 290-300; Altschul *et al.*, (1997) NUCLEIC ACIDS RES. 25:3389-3402, incorporated by reference) are tailored for sequence similarity searching. For a discussion of basic issues in searching sequence databases see Altschul *et al.*, (1994) NATURE GENETICS 6:119-129, which is fully incorporated by reference. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.*, (1992) PROC. NATL. ACAD. SCI. USA 89:10915-10919, fully incorporated by reference). Four blastn parameters may be adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every wink.sup.th position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings may be Q=9; R=2; wink=1; and gapw=32. Searches may also be conducted using the NCBI (National Center for Biotechnology Information) BLAST Advanced Option parameter (e.g.: -G, Cost to open gap [Integer]: default = 5 for nucleotides/ 11 for proteins; -E, Cost to extend gap [Integer]: default = 2 for nucleotides/ 1 for proteins; -q, Penalty for nucleotide mismatch [Integer]: default = -3; -r, reward for nucleotide match [Integer]: default = 1; -e, expect value [Real]: default = 10; -W, wordsize [Integer]: default = 11 for nucleotides/ 28 for megablast/ 3 for proteins; -y, Dropoff (X) for blast extensions in bits: default = 20 for blastn/ 7 for others; -X, X dropoff value for gapped alignment (in bits): default = 15 for all programs, not applicable to blastn; and –Z, final X dropoff value for gapped

alignment (in bits): 50 for blastn, 25 for others). ClustalW for pairwise protein alignments may also be used (default parameters may include, *e.g.*, Blosum62 matrix and Gap Opening Penalty = 10 and Gap Extension Penalty = 0.1). A Bestfit comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

[0084] In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises from about 500 to about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

[0085] In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least from about 500 to

about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

[0086] In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000 nucleotides, about 6000, or about 7000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes when combined comprises about 1600 nucleotides, about 1650 nucleotides, or about 3100 nucleotides.

[0087] In certain embodiments the first and second therapeutic transgenes are separated by a linker. The linker may comprise a cleavage site, *e.g.,* a proteolytic or a non-proteolytic cleavage site, or a ribosome skipping sequence, *e.g.,* a T2A sequence. In certain embodiments, the first and second therapeutic transgenes are separated by a proteolytic cleavage site. In certain embodiments, the proteolytic cleavage site is cleaved by a protease present in a specific tissue, organelle or intracellular compartment. In certain embodiments, the linker comprises a proteolytic cleavage site and two cysteine residues that result in a disulfide linkage following proteolytic cleavage. In certain embodiments, the proteolytic cleavage site is cleaved by a protease selected from a matrix metalloproteinase (MMP), furin, PC1, PC2, PC3, cathepsin B, proteinase 3, and caspase 3.

[0088] In certain embodiments, the cleavage site is a proteolytic cleavage site that is cleaved by a protease that is present in an endoplasmic reticulum or golgi of a eukaryotic cell. In certain embodiments, the proteolytic cleavage site is a furin cleavage site. Furin is a protease that is ubiquitously expressed and is localized to the Golgi, where it recognizes the

consensus sequence RX_1X_2R (SEQ ID NO: 6), wherein X_1 is any amino acid, and X_2 is Lys or Arg, and cleaves after the final Arg. Furin plays a biological role in cleaving propeptides of proteins that are trafficked through the Golgi. Accordingly, in certain embodiments the proteolytic cleavage site is a furin cleavage site comprising the sequence RX_1X_2R (SEQ ID NO: 6), wherein X_1 is any amino acid, and X_2 is Lys or Arg, *e.g.,* a furin cleavage site comprising the sequence RAKR (SEQ ID NO: 7).

III. Pharmaceutical Compositions

[0089] For therapeutic use, a recombinant adenovirus disclosed herein is preferably combined with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" means buffers, carriers, and excipients suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The carrier(s) should be "acceptable" in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient. Pharmaceutically acceptable carriers include buffers, solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art.

[0090] Pharmaceutical compositions containing recombinant adenoviruses can be presented in a dosage unit form and can be prepared by any suitable method. A pharmaceutical composition should be formulated to be compatible with its intended route of administration. Examples of routes of administration are intravenous (IV), intradermal, inhalation, transdermal, topical, transmucosal, and rectal administration. Useful formulations can be prepared by methods known in the pharmaceutical art. For example, see *Remington's Pharmaceutical Sciences*, 18th ed. (Mack Publishing Company, 1990). Formulation components suitable for parenteral administration include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose.

[0091] For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline

(PBS). The carrier should be stable under the conditions of manufacture and storage, and should be preserved against microorganisms. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol), and suitable mixtures thereof.

[0092] Pharmaceutical formulations preferably are sterile. Sterilization can be accomplished by any suitable method, *e.g.*, filtration through sterile filtration membranes. Where the composition is lyophilized, filter sterilization can be conducted prior to or following lyophilization and reconstitution.

[0093] The term "effective amount" as used herein refers to the amount of an active component (*e.g.*, the amount of a recombinant adenovirus) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route.

[0094] In certain embodiments, a therapeutically effective amount of active component is in the range of 0.1 mg/kg to 100 mg/kg, *e.g.*, 1 mg/kg to 100 mg/kg, 1 mg/kg to 10 mg/kg. In certain embodiments, a therapeutically effective amount of the recombinant adenovirus is in the range of 10^2 to 10^{15} plaque forming units (pfus), *e.g.*, 10^2 to 10^{10}, 10^2 to 10^5, 10^5 to 10^{15}, 10^5 to 10^{10}, or 10^{10} to 10^{15} plaque forming units. The amount administered will depend on variables such as the type and extent of disease or indication to be treated, the overall health of the patient, the *in vivo* potency of the active component, the pharmaceutical formulation, and the route of administration. The initial dosage can be increased beyond the upper level in order to rapidly achieve the desired blood-level or tissue-level. Alternatively, the initial dosage can be smaller than the optimum, and the daily dosage may be progressively increased during the course of treatment. Human dosage can be optimized, *e.g.*, in a conventional Phase I dose escalation study designed to run from 0.5 mg/kg to 20 mg/kg. Dosing frequency can vary, depending on factors such as route of administration, dosage amount, serum half-life of the recombinant adenovirus, and the disease being treated. Exemplary dosing frequencies are once per day, once per week and once every two weeks.

IV. Therapeutic Uses

[0095] The recombinant adenoviruses disclosed herein can be used to treat various medical indications, for example, cancers. As used herein, "treat", "treating" and "treatment" mean the

treatment of a disease in a subject, *e.g.*, in a human. This includes: (a) inhibiting the disease, *i.e.*, arresting its development; and (b) relieving the disease, *i.e.*, causing regression of the disease state. As used herein, the terms "subject" and "patient" refer to an organism to be treated by the methods and compositions described herein. Such organisms preferably include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), and more preferably includes humans.

[0096] Examples of cancers include solid tumors, soft tissue tumors, hematopoietic tumors and metastatic lesions. Examples of hematopoietic tumors include, leukemia, acute leukemia, acute lymphoblastic leukemia (ALL), B-cell, T-cell or FAB ALL, acute myeloid leukemia (AML), chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), *e.g.*, transformed CLL, diffuse large B-cell lymphomas (DLBCL), follicular lymphoma, hairy cell leukemia, myelodyplastic syndrome (MDS), a lymphoma, Hodgkin's disease, a malignant lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, or Richter's Syndrome (Richter's Transformation). Examples of solid tumors include malignancies, *e.g.*, sarcomas, adenocarcinomas, and carcinomas, of the various organ systems, such as those affecting head and neck (including pharynx), thyroid, lung (small cell or non-small cell lung carcinoma (NSCLC)), breast, lymphoid, gastrointestinal (*e.g.*, oral, esophageal, stomach, liver, pancreas, small intestine, colon and rectum, anal canal), genitals and genitourinary tract (*e.g.*, renal, urothelial, bladder, ovarian, uterine, cervical, endometrial, prostate, testicular), CNS (*e.g.,* neural or glial cells, *e.g.*, neuroblastoma or glioma), or skin (*e.g.,* melanoma).

[0097] In certain embodiments, the cancer is selected from anal cancer, basal cell carcinoma, bladder cancer, bone cancer, brain cancer, breast cancer, carcinoma, cholangiocarcinoma, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, gastroesophageal cancer, gastrointestinal (GI) cancer, gastrointestinal stromal tumor, hepatocellular carcinoma, gynecologic cancer, head and neck cancer, hematologic cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, merkel cell carcinoma, mesothelioma, neuroendocrine cancer, non-small cell lung cancer, ovarian cancer, pancreatic cancer, pediatric cancer, prostate cancer, renal cell carcinoma, sarcoma, skin cancer, small cell lung cancer, squamous cell carcinoma of the skin, stomach cancer, testicular cancer and thyroid cancer.

[0098] In certain embodiments, the cancer is selected from nasopharyngeal cancer, basal cell carcinoma, synovial cancer, hepatocellular cancer, renal cancer, cancer of connective tissues, melanoma, lung cancer, bowel cancer, colon cancer, rectal cancer, colorectal cancer, brain cancer, throat cancer, oral cancer, liver cancer, bone cancer, pancreatic cancer, choriocarcinoma, gastrinoma, neuroendocrine, pheochromocytoma, prolactinoma, T-cell leukemia/lymphoma, neuroma, von Hippel-Lindau disease, Zollinger-Ellison syndrome, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, ureter cancer, brain cancer, oligodendroglioma, neuroblastoma, meningioma, spinal cord tumor, bone cancer, osteochondroma, chondrosarcoma, Ewing's sarcoma, cancer of unknown primary site, carcinoid, carcinoid of gastrointestinal tract, fibrosarcoma, breast cancer, Paget's disease, cervical cancer, colorectal cancer, rectal cancer, esophagus cancer, gall bladder cancer, head cancer, eye cancer, neck cancer, kidney cancer, Wilms' tumor, liver cancer, Kaposi's sarcoma, prostate cancer, lung cancer, testicular cancer, Hodgkin's disease, non-Hodgkin's lymphoma, oral cancer, skin cancer, mesothelioma, multiple myeloma, ovarian cancer, endocrine pancreatic cancer, glucagonoma, pancreatic cancer, parathyroid cancer, penis cancer, pituitary cancer, soft tissue sarcoma, retinoblastoma, small intestine cancer, stomach cancer, thymus cancer, thyroid cancer, trophoblastic cancer, hydatidiform mole, uterine cancer, endometrial cancer, vagina cancer, vulva cancer, acoustic neuroma, mycosis fungoides, insulinoma, carcinoid syndrome, somatostatinoma, gum cancer, heart cancer, lip cancer, meninges cancer, mouth cancer, nerve cancer, palate cancer, parotid gland cancer, peritoneum cancer, pharynx cancer, pleural cancer, salivary gland cancer, tongue cancer and tonsil cancer.

[0099] In certain embodiments, a recombinant adenovirus is administered to the subject in combination with one or more therapies, *e.g.*, surgery, radiation, chemotherapy, immunotherapy, hormone therapy, or virotherapy. In certain embodiments, a recombinant adenovirus is administered in combination with a tyrosine kinase inhibitor, *e.g.*, erlotinib. In certain embodiments, a recombinant adenovirus is administered in combination with a checkpoint inhibitor, *e.g.,* an anti-CTLA-4 antibody, an anti-PD-1 antibody, or an anti-PD-L1 antibody. Exemplary anti-PD-1 antibodies include, for example, nivolumab (Opdivo®, Bristol-Myers Squibb Co.), pembrolizumab (Keytruda®, Merck Sharp & Dohme Corp.), PDR001 (Novartis Pharmaceuticals), and pidilizumab (CT-011, Cure Tech). Exemplary anti-PD-L1 antibodies include, for example, atezolizumab (Tecentriq®, Genentech), duvalumab (AstraZeneca), MEDI4736, avelumab, and BMS 936559 (Bristol Myers Squibb Co.).

[00100] The term administered "in combination," as used herein, is understood to mean that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disorder, such that the effects of the treatments on the patient overlap at a point in time. In certain embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery." In other embodiments, the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, *e.g.*, an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In certain embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive. The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered.

[00101] In certain embodiments, the effective amount of the recombinant virus is identified by measuring an immune response to an antigen in the subject and/or the method of treating the subject further comprises measuring an immune response to an antigen in the subject. Cancers may be characterized by immunosuppression, and measuring an immune response to an antigen in the subject may be indicative of the level of immunosuppression in the subject. Accordingly, measuring an immune response to an antigen in the subject may be indicative of the efficacy of the treatment and/or the effective amount of the recombinant virus. The immune response to the antigen in the subject may be measured by any method known in the art. In certain embodiments, the immune response to the antigen is measured by injecting the subject with the antigen at an injection site on the skin of the subject and measuring the size of an induration or amount of inflammation at the injection site. In certain embodiments, the immune response to the antigen is measured by release of a cytokine from a cell of the subject (*e.g.,* interferon gamma, IL-4 and/or IL-5) upon exposure to the antigen.

[00102] Throughout the description, where viruses, compositions and systems are described as having, including, or comprising specific components, or where processes and methods are

described as having, including, or comprising specific steps, it is contemplated that, additionally, there are compositions, devices, and systems of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.

[00103] In the application, where an element or component is said to be included in and/or selected from a list of recited elements or components, it should be understood that the element or component can be any one of the recited elements or components, or the element or component can be selected from a group consisting of two or more of the recited elements or components.

[00104] Further, it should be understood that elements and/or features of a virus, a composition, a system, a method, or a process described herein can be combined in a variety of ways without departing from the spirit and scope of the present invention, whether explicit or implicit herein. For example, where reference is made to a particular virus, that virus can be used in various embodiments of compositions of the present invention and/or in methods of the present invention, unless otherwise understood from the context. In other words, within this application, embodiments have been described and depicted in a way that enables a clear and concise application to be written and drawn, but it is intended and will be appreciated that embodiments may be variously combined or separated without parting from the present teachings and invention(s). For example, it will be appreciated that all features described and depicted herein can be applicable to all aspects of the invention(s) described and depicted herein.

[00105] It should be understood that the expression "at least one of" includes individually each of the recited objects after the expression and the various combinations of two or more of the recited objects unless otherwise understood from the context and use. The expression "and/or" in connection with three or more recited objects should be understood to have the same meaning unless otherwise understood from the context.

[00106] The use of the term "include," "includes," "including," "have," "has," "having," "contain," "contains," or "containing," including grammatical equivalents thereof, should be understood generally as open-ended and non-limiting, for example, not excluding additional unrecited elements or steps, unless otherwise specifically stated or understood from the context.

[00107] At various places in the present specification, viruses, compositions, systems, processes and methods, or features thereof, are disclosed in groups or in ranges. It is specifically intended that the description include each and every individual subcombination of the members of such groups and ranges. By way of other examples, an integer in the range of 1 to 20 is specifically intended to individually disclose 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20.

[00108] Where the use of the term "about" is before a quantitative value, the present invention also includes the specific quantitative value itself, unless specifically stated otherwise. As used herein, the term "about" refers to a ±10% variation from the nominal value unless otherwise indicated or inferred.

[00109] It should be understood that the order of steps or order for performing certain actions is immaterial so long as the present invention remain operable. Moreover, two or more steps or actions may be conducted simultaneously.

[00110] The use of any and all examples, or exemplary language herein, for example, "such as" or "including," is intended merely to illustrate better the present invention and does not pose a limitation on the scope of the invention unless claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the present invention.

EXAMPLES

[00111] The following Examples are merely illustrative and are not intended to limit the scope or content of the invention in any way.

Example 1: Expression of Heterodimeric IL-12

[00112] Interleukin 12 (IL-12) is a cytokine that promotes cell-mediated immunity. IL-12 contains two separate protein chains, IL-12A/p35 and IL-12B/p40, which combine to form the active heterodimer denoted IL-12p70. This Example describes the expression of a human IL-12 heterodimer including both of the IL-12 subunits IL-12A/p35 and IL-12B/p40.

[00113] The plasmid pAd1, which carries a portion of the adenovirus type 5 genome, was modified to carry a SalI site at the start site of the E1b-19k region and an XhoI site 200 base pairs 3' of the SalI site to facilitate insertion of therapeutic transgenes. The resulting plasmid is hereafter referred to as pAd1-Δ19k.

[00114] The nucleotide sequence of the modified E1b-19k region is as follows, with the residual bases from the fused SalI and XhoI sites underlined:

ATCTTGGTTACATCTGACCTCGTCGAGTCACCAGGCGCTTTTCCAA (SEQ ID NO: 10).

[00115] A nucleotide sequence encoding human IL-12A/p35 followed by an encephalomyocarditis virus (EMCV) IRES followed by a nucleotide sequence encoding human IL-12B/p40 was cloned into the modified E1b-19k region of pAd1-Δ19k. The resulting plasmid is hereafter referred to as pAd1-hIL-12-IRES. The nucleotide sequence encoding human IL-12A/p35 followed by the EMCV IRES followed by the nucleotide sequence encoding human IL-12B/p40 inserted into the E1b-19k region is as follows, where the IL-12A/p35 and IL-12B/p40 coding regions are capitalized, the IRES is lowercase, and the flanking E1b-19k sequences including the SalI and XhoI restriction sites are underlined:

ATCTGACCTCGTCGACATGTGGCCCCCTGGGTCAGCCTCCCAGCCACCGCCCTCACCTGCCGC
GGCCACAGGTCTGCATCCAGCGGCTCGCCCTGTGTCCCTGCAGTGCCGGCTCAGCATGTGTCC
AGCGCGCAGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAA
CCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCT
GAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCTTGCACTTC
TGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGCCTGTTTACC
ATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCATAACTAATGG
GAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGA
AGACTTGAAGATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAA
GAGGCAGATCTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAA
TTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAA
AATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGAT
GAGCTATCTGAATGCTTCCTAATAAtaacgttactggccgaagccgcttggaataaggccggt
gtgcgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagggcccgga
aacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgc
aaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgt
ctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaa
agccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttgga
tagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgccc
agaaggtaccccattgtatgggatctgatctggggcctcggtgcacatgctttacatgtgttt
agtcgaggttaaaaaacgtctaggccccccgaaccacggggacgtggttttcctttgaaaaac
acgatgataatATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCTGGCAT
CTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTGGTATC
CGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTATCACCT
GGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGTCAAAG
AGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTCGCTCC
TGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAAC
CCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTGCTGGT
GGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTCTGACC
CCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGG

AGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAGTCTGC
CCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAGCTTCT
TCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATT
CTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCT
CCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGTCTTCA
CGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGCCCAGG
ACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTTAGTAACTCGAGT
CACCAGGCG (SEQ ID NO: 11).

[00116] Additionally, a nucleotide sequence encoding human IL-12B/p40 followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding human IL-12A/p35 was cloned into the modified E1b-19k region of pAd1-Δ19k. The resulting plasmid is hereafter referred to as pAd1-hIL-12-furin. The nucleotide sequence encoding human IL-12B/p40 followed by the nucleotide sequence encoding the furin cleavage site followed by the nucleotide sequence encoding human IL-12A/p35 inserted into the E1b-19k region is as follows, where the IL-12B/p40 and IL-12A/p35 coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequences including the SalI and XhoI restriction sites are underlined:

ATCTGACCTCGTCGACATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCT
GGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTG
GTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTAT
CACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGT
CAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTC
GCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAA
AGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTG
CTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAA
CAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAG
TCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAG
CTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAA
GAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTA
CTTCTCCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGT
CTTCACGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGC
CCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTcgtgctaa
gcgaAGAAACCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCA
AAACCTGCTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCC
TTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGC
CTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCAT
AACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAG
TATTTATGAAGACTTGAAGATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGAT
GGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCA
GGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTA

TAAAACTAAAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGA
TAGAGTGATGAGCTATCTGAATGCTTCCTAACTCGAGTCACCAGGCG (SEQ ID NO: 8).

[00117] Additionally, a nucleotide sequence encoding human IL-12B/p40 followed by a nucleotide sequence encoding a 2A peptide followed by a nucleotide sequence encoding human IL-12A/p35 was cloned into the modified E1b-19k region of pAd1-Δ19k. The resulting plasmid is hereafter referred to as pAd1-hIL-12-2A. The nucleotide sequence encoding human IL-12B/p40 followed by the nucleotide sequence encoding the 2A peptide followed by the nucleotide sequence encoding human IL-12A/p35 inserted into the E1b-19k region is as follows, where the IL-12B/p40 and IL-12A/p35 coding regions are capitalized, the 2A coding region is lowercase, and the flanking E1b-19k sequences including the SalI and XhoI restriction sites are underlined:

ATCTGACCTCGTCGACATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCT
GGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTG
GTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTAT
CACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGT
CAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTC
GCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAA
AGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTG
CTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAA
CAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAG
TCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAG
CTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAA
GAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTA
CTTCTCCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGT
CTTCACGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGC
CCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTcttctgaa
cttcgacctcctcaagttggcgggagacgttgagtccaaccccgggcccAGAAACCTCCCCGT
GGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCTGAGGGCCGT
CAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCTTGCACTTCTGAAGAGAT
TGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGCCTGTTTACCATTGGAATT
AACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCATAACTAATGGGAGTTGCCT
GGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAA
GATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAGAGGCAGAT
CTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAG
TGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAAAATCAAGCT
CTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGATGAGCTATCT
GAATGCTTCCTAACTCGAGTCACCAGGCG (SEQ ID NO: 12).

[00118] Details of the plasmids tested are shown in **TABLE 1.**

TABLE 1

Plasmid	E1b-19k
pAd1-Δ19k	Deleted
pAd1-hIL-12-IRES	Deleted and Replaced with IL-12A/p35-IRES-IL-12B/p40
pAd1-hIL-12-furin	Deleted and Replaced with IL-12B/p40-furin-IL-12A/p35
pAd1-hIL-12-2A	Deleted and Replaced with IL-12B/p40-2A-IL-12A/p35

[00119] Plasmids were transiently transfected into HEK-293 cells. Conditioned media was collected four days after transfection, and IL-12 concentration was measured in an ELISA (Biolegend 431704) that specifically detects the heterodimer of the IL-12A/p35 and IL-12B/p40 subunits. As shown in **FIGURE 1**, the plasmid carrying the furin cleavage site (pAd1-hIL-12-furin) elicited significantly higher heterodimeric IL-12 expression than either the plasmid utilizing the IRES (pAd1-hIL-12-IRES) or the 2A peptide (pAd1-hIL-12-2A).

Example 2: IL-12 Expressing Adenoviruses

[00120] This Example describes recombinant type 5 (Ad5) adenoviruses that express the human IL-12 subunits IL-12A/p35 and IL-12B/p40.

[00121] An adenovirus type 5 virus was constructed that carried the deletion of a nucleotide region located from -304 to -255 upstream of the E1a initiation, which renders E1a expression cancer-selective (as previously described in U.S. Patent No. 9,073,980). The resulting virus is hereafter referred to as TAV.

[00122] TAV was further modified to carry a SalI site at the start site of the E1b-19k region and an XhoI site 200 base pairs 3' of the SalI site to facilitate insertion of therapeutic transgenes. The resulting virus is hereafter referred to as TAV-Δ19k. The nucleotide sequence of the modified E1b-19k region is as follows, with the residual bases from the fused SalI and XhoI sites underlined:

ATCTTGGTTACATCTGACCTCGTCGAGTCACCAGGCGCTTTTCCAA (SEQ ID NO: 10).

[00123] TAV-Δ19k carried the dl309 disruption in the E3 region. To create viruses carrying therapeutic transgenes that would otherwise render the viral genome too large to be packaged into a viral capsid, TAV-Δ19k was further modified to delete the entire RIDα, RIDβ, and 14.7k genes of the E3 region. The resulting virus is hereafter referred to as TAV-Δ19k-ΔE3. The nucleotide sequence of the modified E3 region is as follows, with the hyphen indicating the point of deletion:

TCTTTTCTCTTACAGTATGA-TAATAAAAAAAAATAATAAAGCATCACTTA (SEQ ID NO: 13).

[00124] A nucleotide sequence encoding human IL-12B/p40 followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding human IL-12A/p35 was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-hIL-12-furin. The nucleotide sequence encoding human IL-12B/p40 followed by the nucleotide sequence encoding the furin cleavage site followed by the nucleotide sequence encoding human IL-12A/p35 inserted into the E1b-19k region is as follows, where the IL-12B/p40 and IL-12A/p35 coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCT
GGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTG
GTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTAT
CACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGT
CAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTC
GCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAA
AGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTG
CTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAA
CAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAG
TCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAG
CTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAA
GAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTA
CTTCTCCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGT
CTTCACGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGC
CCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTcgtgctaa
gcgaAGAAACCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCA
AAACCTGCTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCC
TTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGC
CTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCAT
AACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAG
TATTTATGAAGACTTGAAGATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGAT
GGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCA
GGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTA
TAAAACTAAAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGA
TAGAGTGATGAGCTATCTGAATGCTTCCTAACTCGAGTCACCAGGCG (SEQ ID NO: 8).

[00125] Additionally, a nucleotide sequence encoding human IL-12B/p40 was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3, and a nucleotide sequence encoding human IL-12A/p35 was cloned into the modified E3 region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-hIL-12-Separate. The nucleotide sequence encoding human IL-

12B/p40 inserted into the E1b-19k region is as follows, where the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCT
GGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTG
GTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTAT
CACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGT
CAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTC
GCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAA
AGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTG
CTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAA
CAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAG
TCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAG
CTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAA
GAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTA
CTTCTCCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGT
CTTCACGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGC
CCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTTAGTAACT
CGAGTCACCAGGCG (SEQ ID NO: 14).

[00126] The nucleotide sequence encoding human IL-12A/p35 inserted into the E3 region is as follows, where the flanking adenoviral sequence is underlined:

ATGTTCTTTTCTCTTACAGTATGATTAAATGAGACATGTGGCCCCCTGGGTCTGCCTCCCAAC
CACCGCCCTCACCTGCCGCGGCCACTGGTCTGCATCCTGCGGCTCGCCCTGTGTCCCTGCAAT
GCCGGCTCTCCATGTGTCCTGCGCGCTCCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACC
ACCTCTCTTTGGCCCGAAACCTCCCCGTGGCCACTCCTGACCCTGGAATGTTCCCATGCCTTC
ACCACTCCCAAAACCTGCTGCGGGCCGTCTCCAACATGCTCCAAAAAGCCCGACAAACTCTTG
AATTTTACCCTTGCACTTCTGAAGAAATTGATCATGAAGATATCACAAAAGATAAAACCTCCA
CTGTGGAAGCCTGTTTACCATTGGAATTAACCAAAAATGAATCTTGCCTAAATTCCCGAGAAA
CCTCTTTCATAACTAATGGGTCTTGCCTGGCCTCCCGAAAAACCTCTTTTATGATGGCCCTGT
GCCTTTCTTCTATTTATGAAGACTTGAAAATGTACCAAGTGGAATTCAAAACCATGAATGCAA
AACTTCTGATGGATCCTAAACGGCAAATCTTTCTTGATCAAAACATGCTGGCTGTTATTGATG
AACTGATGCAAGCCCTGAATTTCAACTCTGAAACTGTGCCACAAAAATCCTCCCTTGAAGAAC
CGGATTTTTATAAAACTAAAATCAAACTCTGCATACTTCTTCATGCTTTCCGAATTCGGGCTG
TGACTATTGATCGAGTGATGTCCTATCTGAATGCTTCCTAATGAGGTCTCAAAGATCTTATTC
CCTTTAACTAATAAA (SEQ ID NO: 15).

[00127] Details of the viruses tested are shown in **TABLE 2.**

TABLE 2

Virus	E1A Promoter	E1b-19k Modification	E3 (RIDα, RIDβ, and 14.7k) Modification
TAV-Δ19k	TAV-255	Deleted	Disrupted (containing the dl309 sequence)

TAV-hIL-12-Separate	TAV-255	Deleted and replaced with IL-12B/p40	Deleted and replaced with IL-12A/p35
TAV -hIL-12-furin	TAV-255	Deleted and replaced with IL-12B/p40-furin-IL-12A/p35	Deleted

[00128] Viral genomic DNA from lysate of those viruses, as well as a control virus (dl309), was PCR-amplified with primers flanking the E1A promoter, with primers flanking the E1b-19k region, and with primers flanking the E3 RIDα, RIDβ, and 14.7k region to confirm presence of the desired elements. As seen in **FIGURE 2**, the viruses had the desired features, namely, PCR products of the anticipated size. The IL-12 coding regions in both viruses were sequenced for confirmation.

Example 3: IL-23 Expressing Adenovirus

[00129] This Example describes a recombinant adenovirus type 5 (Ad5) that expresses the human IL-23 subunits IL-23A/p19 and IL-12B/p40.

[00130] A TAV-Δ19k-ΔE3 virus is generated as described in Example 2. A nucleotide sequence encoding human IL-12B/p40 followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding human IL-23A/p19 is cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-hIL-23-furin. The nucleotide sequence encoding human IL-12B/p40 followed by the nucleotide sequence encoding the furin cleavage site followed by the nucleotide sequence encoding human IL-23A/p19 inserted into the E1b-19k region is as follows, where the IL-12B/p40 and IL-23A/p19 coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

<u>ATCTGACCTCGTCGAC</u>ATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCT
GGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTG
GTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTAT
CACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGT
CAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTC
GCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAA
AGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTG
CTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAA
CAAGGAGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAG
TCTGCCCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAG
CTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAA

GAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTA
CTTCTCCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGT
CTTCACGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGC
CCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTcgtgctaa
gcgaATGCTGGGGAGCAGAGCTGTAATGCTGCTGTTGCTGCTGCCCTGGACAGCTCAGGGCAG
AGCTGTGCCTGGGGGCAGCAGCCCTGCCTGGACTCAGTGCCAGCAGCTTTCACAGAAGCTCTG
CACACTGGCCTGGAGTGCACATCCACTAGTGGGACACATGGATCTAAGAGAAGAGGGAGATGA
AGAGACTACAAATGATGTTCCCCATATCCAGTGTGGAGATGGCTGTGACCCCCAAGGACTCAG
GGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTTATGAGAAGCTGCT
AGGATCGGATATTTTCACAGGGGAGCCTTCTCTGCTCCCTGATAGCCCTGTGGGCCAGCTTCA
TGCCTCCCTACTGGGCCTCAGCCAACTCCTGCAGCCTGAGGGTCACCACTGGGAGACTCAGCA
GATTCCAAGCCTCAGTCCCAGCCAGCCATGGCAGCGTCTCCTTCTCCGCTTCAAAATCCTTCG
CAGCCTCCAGGCCTTTGTGGCTGTAGCCGCCCGGGTCTTTGCCCATGGAGCAGCAACCCTGAG
TCCCTAACTCGAGTCACCAGGCG (SEQ ID NO: 9).

Example 4: IL-12 Expressing Adenovirus

[00131] This Example describes a recombinant adenovirus type 5 (Ad5) that expresses the mouse IL-12 subunits IL-12A and IL-12B using an IRES.

[00132] TAV, TAV-Δ19k, and TAV-Δ19k-ΔE3 viruses were generated, as described in Example 2. A nucleotide sequence encoding mouse IL-12A followed by an EMCV IRES followed by a nucleotide sequence encoding mouse IL-12B was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-mIL-12-IRES. The nucleotide sequence encoding mouse IL-12A followed by the EMCV IRES followed by the nucleotide sequence encoding mouse IL-12B inserted into the E1b-19k region is as follows, where the mouse IL-12B and IL-12A coding regions are capitalized, the IRES is lowercase, and the flanking restriction sites and adenoviral sequences are underlined:

CTGACCTCGTCGACATGTGTCAATCACGCTACCTCCTCTTTTTGGCCACCCTTGCCCTCCTAA
ACCACCTCAGTTTGGCCAGAGTGATCCCTGTGTCCGGCCCTGCCAGATGCCTGAGCCAGAGCA
GAAACCTGCTGAAAACCACCGACGACATGGTGAAAACCGCCAGAGAGAAGCTGAAGCACTACA
GCTGCACAGCCGAGGACATCGACCACGAGGACATCACCCGGGACCAGACCTCCACCCTGAAAA
CCTGCCTGCCCCTGGAACTGCATAAGAACGAGAGCTGCCTGGCCACCCGCGAGACAAGCAGCA
CCACCAGAGGCAGCTGTCTGCCCCCCCAGAAAACCAGCCTGATGATGACCCTGTGCCTGGGCA
GCATCTACGAGGACCTGAAGATGTACCAGACCGAGTTCCAGGCCATCAACGCCGCCCTGCAGA
ACCACAACCACCAGCAGATCATCCTGGACAAGGGCATGCTGGTGGCCATCGACGAGCTGATGC
AGAGCCTGAACCACAACGGCGAAACCCTGAGACAGAAACCCCCCGTGGGCGAGGCCGACCCCT
ACAGAGTGAAGATGAAGCTGTGCATCCTGCTGCACGCCTTCAGCACCAGAGTGGTGACAATCA
ACAGAGTGATGGGCTACCTGAGCAGCGCCTGAtaacgttactggccgaagccgcttggaataa
ggccggtgtgcgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagg
gcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaa
ggaatgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaa
acaacgtctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgc

ggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtg
agttggatagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaag
gatgcccagaaggtaccccattgtatgggatctgatctggggcctcggtgcacatgctttaca
tgtgtttagtcgaggttaaaaaacgtctaggcccccgaaccacggggacgtggttttccttt
gaaaaacacgatgataatATGTGCCCCCAGAAGCTGACCATCAGTTGGTTCGCCATCGTGCTG
CTGGTGTCCCCCCTGATGGCCATGTGGGAGCTGGAAAAGGACGTGTACGTGGTGGAAGTGGAC
TGGACCCCCGACGCCCCTGGCGAGACAGTGAACCTGACCTGCGACACCCCCGAAGAGGACGAC
ATCACCTGGACCAGCGACCAGAGACACGGCGTGATCGGCAGCGGCAAGACCCTGACAATCACC
GTGAAAGAGTTTCTGGACGCCGGCCAGTACACCTGTCACAAGGGCGGCGAGACACTGAGCCAC
TCCCATCTGCTGCTGCACAAGAAAGAGAACGGCATCTGGTCCACCGAGATCCTGAAGAACTTC
AAGAACAAGACCTTCCTGAAGTGCGAGGCCCCCAACTACAGCGGCAGATTCACCTGTAGCTGG
CTGGTGCAGAGAAACATGGACCTGAAGTTCAACATCAAGAGCAGCAGCAGCTCCCCCGACAGC
AGAGCCGTGACCTGTGGCATGGCCAGCCTGAGCGCCGAGAAAGTGACCCTGGACCAGAGAGAC
TACGAGAAGTACAGCGTGTCCTGCCAGGAAGATGTCACCTGCCCCACCGCCGAGGAAACCCTG
CCTATCGAGCTGGCCCTGGAAGCCAGACAGCAGAACAAATACGAGAACTACTCTACCAGCTTC
TTCATCCGGGACATCATCAAGCCCGACCCCCCCCAAGAACCTGCAGATGAAGCCCCTGAAGAAC
AGCCAGGTGGAAGTGTCCTGGGAGTACCCCGACAGCTGGTCCACCCCCCACAGCTACTTCAGC
CTGAAGTTCTTCGTGCGGATCCAGCGCAAGAAAGAAAAGATGAAGGAAACCGAGGAAGGCTGC
AACCAGAAAGGCGCTTTCCTGGTGGAAAAGACCAGCACCGAGGTGCAGTGCAAGGGCGGCAAC
GTGTGCGTGCAGGCCCAGGACCGGTACTACAACAGCAGCTGCAGCAAGTGGGCCTGCGTGCCC
TGTAGAGTGCGCTCTTGACTCGAGTCACCAGGCGCTT (SEQ ID NO: 23).

[00133] Cytotoxic activity of TAV-mIL-12-IRES was tested. ADS-12 (mouse lung cancer) cells were infected at a multiplicity of infection (MOI) of 10 with TAV-mIL12-IRES, TAV-Δ19k virus, or no virus, and stained with crystal violet, which stains viable cells purple. As depicted in **FIGURE 3**, TAV-mIL12-IRES retained cytotoxic activity, although at a lower level than the control TAV-Δ19k virus.

[00134] IL-12 expression from TAV-mIL-12-IRES was tested. ADS-12 cells were infected at an MOI of 5 with TAV-Δ19k or TAV-mIL-12-IRES. Conditioned media was collected five days after infection and IL-12 concentration was measured with an ELISA specific for the IL-12 heterodimer. As depicted in **FIGURE 4**, there was no detectable expression from cells infected with TAV-Δ19k while cells infected with TAV-mIL-12-IRES expressed 67 ng/ml of the IL-12 heterodimer.

[00135] Viral replication of TAV-mIL-12-IRES was tested. ADS-12 cells were infected at an MOI of 5 with TAV-mIL12-IRES or TAV-Δ19k. Media and cell lysate were harvested five days after infection and titered. Viral replication was assayed by measuring the number of plaque-forming units (PFU) of virus per infected ADS-12 cell. As depicted in **FIGURE 5**, TAV-mIL-12-replicated at lower levels in ADS-12 cells compared to TAV-Δ19k.

Example 5: IL-12 Expressing Adenoviruses

[00136] This Example describes recombinant adenoviruses that expresses the human IL-12 subunits IL-12A and IL-12B using an IRES and recombinant adenoviruses that expresses the human IL-12 subunits IL-12A and IL-12B using a furin cleavage site.

[00137] TAV, TAV-Δ19k, TAV-Δ19k-ΔE3, and TAV-hIL-12-furin viruses were generated, as described in Example 2. Additionally, a nucleotide sequence encoding human IL-12A/p35 followed by an EMCV IRES followed by a nucleotide sequence encoding human IL-12B/p40 was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting adenovirus is hereafter referred to as TAV-hIL-12-IRES. The nucleotide sequence encoding human IL-12A/p35 followed by the EMCV IRES followed by the nucleotide sequence encoding human IL-12B/p40 inserted into the E1b-19k region is as follows, where the IL-12A/p35 and IL-12B/p40 coding regions are capitalized, the IRES is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTGGCCCCCTGGGTCAGCCTCCCAGCCACCGCCCTCACCTGCCGC
GGCCACAGGTCTGCATCCAGCGGCTCGCCCTGTGTCCCTGCAGTGCCGGCTCAGCATGTGTCC
AGCGCGCAGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAA
CCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCT
GAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCTTGCACTTC
TGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCACAGTGGAGGCCTGTTTACC
ATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCATAACTAATGG
GAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGA
AGACTTGAAGATGTACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAA
GAGGCAGATCTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAA
TTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAA
AATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGAT
GAGCTATCTGAATGCTTCCTAATAAtaacgttactggccgaagccgcttggaataaggccggt
gtgcgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagggcccgga
aacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgc
aaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgt
ctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaa
agccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttgga
tagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgccc
agaaggtaccccattgtatgggatctgatctggggcctcggtgcacatgctttacatgtgttt
agtcgaggttaaaaaacgtctaggccccccgaaccacggggacgtggttttcctttgaaaaac
acgatgataatATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCTGGCAT
CTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTGGTATC
CGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTATCACCT
GGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGTCAAAG
AGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTCGCTCC
TGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAAC
CCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTGCTGGT
GGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTCTGACC

CCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGG
AGTATGAGTACTCAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAGTCTGC
CCATTGAGGTCATGGTGGATGCCGTTCACAAGCTCAAGTATGAAAACTACACCAGCAGCTTCT
TCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATT
CTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCT
CCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAAGAAAGATAGAGTCTTCA
CGGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGCCCAGG
ACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTTAGTAACTCGAGT
CACCAGGCG (SEQ ID NO: 11).

[00138] IL-12 expression from TAV-hIL-12-furin and TAV-hIL-12-IRES were tested. A549 cells were infected at an MOI of 5 with TAV-Δ19k, TAV-hIL-12-IRES, TAV-hIL-12-furin, or no virus. Conditioned media was collected four days after infection and IL-12 concentration was measured by ELISA (Biolegend 431704). As depicted in **FIGURE 6**, expression from TAV-hIL-12-furin (170 ng/ml) was higher than from TAV-hIL-12-IRES (23 ng/ml).

[00139] Cytotoxic activity of TAV-hIL-12-furin was tested. A549 cells were infected at an MOI of 5 with TAV-Δ19k, TAV-hIL12-furin, or no virus, and stained with crystal violet, which stains viable cells purple. As depicted in **FIGURE 7**, TAV-hIL12-furin retained cytotoxic activity at a similar level to the control TAV-Δ19k virus.

[00140] Viral replication of TAV-hIL-12-furin and TAV-hIL-12-IRES were tested. A549 cells were infected at an MOI of 5 with TAV-hIL-12-furin, TAV-hIL-12-IRES, or TAV-Δ19k. Media and cell lysate were harvested six days after infection and titered. Viral replication was assayed by measuring the number of plaque-forming units (PFU) of virus per infected A549 cell. As depicted in **FIGURE 8**, TAV-hIL-12-furin replicated more efficiently than TAV-hIL-12-IRES.

Example 6: IL-12, IL-23, and IL-27 Expressing Adenoviruses

[00141] This Example describes recombinant adenoviruses that expresses the two subunits of mouse IL-12, IL-23, and IL-27 using a furin cleavage site.

[00142] TAV, TAV-Δ19k, and TAV-Δ19k-ΔE3 viruses were generated, as described in Example 2. A nucleotide sequence encoding mouse IL-12B followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding mouse IL-12A was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-mIL-12-furin. The nucleotide sequence encoding mouse IL-12B followed by the nucleotide sequence encoding the furin cleavage site followed by the

nucleotide sequence encoding mouse IL-12A inserted into the E1b-19k region is as follows, where the IL-12B and IL-12A coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTGTCCTCAGAAGCTAACCATCTCCTGGTTTGCCATCGTTTTGCT
GGTGTCTCCACTCATGGCCATGTGGGAGCTGGAGAAAGACGTTTATGTTGTAGAGGTGGACTG
GACTCCCGATGCCCCTGGAGAAACAGTGAACCTCACCTGTGACACGCCTGAAGAAGATGACAT
CACCTGGACCTCAGACCAGAGACATGGAGTCATAGGCTCTGGAAAGACCCTGACCATCACTGT
CAAAGAGTTTCTAGATGCTGGCCAGTACACCTGCCACAAAGGAGGCGAGACTCTGAGCCACTC
ACATCTGCTGCTCCACAAGAAGGAAAATGGAATTTGGTCCACTGAAATTTTAAAAAATTTCAA
AAACAAGACTTTCCTGAAGTGTGAAGCACCAAATTACTCCGGACGGTTCACGTGCTCATGGCT
GGTGCAAAGAAACATGGACTTGAAGTTCAACATCAAGAGCAGTAGCAGTTCCCCTGACTCTCG
GGCAGTGACATGTGGAATGGCGTCTCTGTCTGCAGAGAAGGTCACACTGGACCAAAGGGACTA
TGAGAAGTATTCAGTGTCCTGCCAGGAGGATGTCACCTGCCCAACTGCCGAGGAGACCCTGCC
CATTGAACTGGCGTTGGAAGCACGGCAGCAGAATAAATATGAGAACTACAGCACCAGCTTCTT
CATCAGGGACATCATCAAACCAGACCCGCCCAAGAACTTGCAGATGAAGCCTTTGAAGAACTC
ACAGGTGGAGGTCAGCTGGGAGTACCCTGACTCCTGGAGCACTCCCCATTCCTACTTCTCCCT
CAAGTTCTTTGTTCGAATCCAGCGCAAGAAAGAAAAGATGAAGGAGACAGAGGAGGGGTGTAA
CCAGAAAGGTGCGTTCCTCGTAGAGAAGACATCTACCGAAGTCCAATGCAAAGGCGGGAATGT
CTGCGTGCAAGCTCAGGATCGCTATTACAATTCCTCATGCAGCAAGTGGGCATGTGTTCCCTG
CAGGGTCCGATCCcgtgctaagcgaAGGGTCATTCCAGTCTCTGGACCTGCCAGGTGTCTTAG
CCAGTCCCGAAACCTGCTGAAGACCACAGATGACATGGTGAAGACGGCCAGAGAAAAACTGAA
ACATTATTCCTGCACTGCTGAAGACATCGATCATGAAGACATCACACGGGACCAAACCAGCAC
ATTGAAGACCTGTTTACCACTGGAACTACACAAGAACGAGAGTTGCCTGGCTACTAGAGAGAC
TTCTTCCACAACAAGAGGGAGCTGCCTGCCCCCACAGAAGACGTCTTTGATGATGACCCTGTG
CCTTGGTAGCATCTATGAGGACTTGAAGATGTACCAGACAGAGTTCCAGGCCATCAACGCAGC
ACTTCAGAATCACAACCATCAGCAGATCATTCTAGACAAGGGCATGCTGGTGGCCATCGATGA
GCTGATGCAGTCTCTGAATCATAATGGCGAGACTCTGCGCCAGAAACCTCCTGTGGGAGAAGC
AGACCCTTACAGAGTGAAAATGAAGCTCTGCATCCTGCTTCACGCCTTCAGCACCCGCGTCGT
GACCATCAACAGGGTGATGGGCTATCTGAGCTCCGCCTGACTCGAGTCACCAGGCG (SEQ ID NO: 24).

[00143] Additionally, a nucleotide sequence encoding mouse IL-12B followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding mouse IL-23A was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-mIL-23-furin. The nucleotide sequence encoding mouse IL-12B followed by the nucleotide sequence encoding the furin cleavage site followed by the nucleotide sequence encoding mouse IL-23A inserted into the E1b-19k region is as follows, where the IL-12B and IL-23A coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTGTCCTCAGAAGCTAACCATCTCCTGGTTTGCCATCGTTTTGCT
GGTGTCTCCACTCATGGCCATGTGGGAGCTGGAGAAAGACGTTTATGTTGTAGAGGTGGACTG
GACTCCCGATGCCCCTGGAGAAACAGTGAACCTCACCTGTGACACGCCTGAAGAAGATGACAT
CACCTGGACCTCAGACCAGAGACATGGAGTCATAGGCTCTGGAAAGACCCTGACCATCACTGT
CAAAGAGTTTCTAGATGCTGGCCAGTACACCTGCCACAAAGGAGGCGAGACTCTGAGCCACTC
ACATCTGCTGCTCCACAAGAAGGAAAATGGAATTTGGTCCACTGAAATTTTAAAAAATTTCAA
AAACAAGACTTTCCTGAAGTGTGAAGCACCAAATTACTCCGGACGGTTCACGTGCTCATGGCT
GGTGCAAAGAAACATGGACTTGAAGTTCAACATCAAGAGCAGTAGCAGTTCCCCTGACTCTCG
GGCAGTGACATGTGGAATGGCGTCTCTGTCTGCAGAGAAGGTCACACTGGACCAAAGGGACTA
TGAGAAGTATTCAGTGTCCTGCCAGGAGGATGTCACCTGCCCAACTGCCGAGGAGACCCTGCC
CATTGAACTGGCGTTGGAAGCACGGCAGCAGAATAAATATGAGAACTACAGCACCAGCTTCTT
CATCAGGGACATCATCAAACCAGACCCGCCCAAGAACTTGCAGATGAAGCCTTTGAAGAACTC
ACAGGTGGAGGTCAGCTGGGAGTACCCTGACTCCTGGAGCACTCCCCATTCCTACTTCTCCCT
CAAGTTCTTTGTTCGAATCCAGCGCAAGAAAGAAAAGATGAAGGAGACAGAGGAGGGGTGTAA
CCAGAAAGGTGCGTTCCTCGTAGAGAAGACATCTACCGAAGTCCAATGCAAAGGCGGGAATGT
CTGCGTGCAAGCTCAGGATCGCTATTACAATTCCTCATGCAGCAAGTGGGCATGTGTTCCCTG
CAGGGTCCGATCCcgtgctaagcgaGTGCCTAGGAGTAGCAGTCCTGACTGGGCTCAGTGCCA
GCAGCTCTCTCGGAATCTCTGCATGCTAGCCTGGAACGCACATGCACCAGCGGGACATATGAA
TCTACTAAGAGAAGAAGAGGATGAAGAGACTAAAAATAATGTGCCCCGTATCCAGTGTGAAGA
TGGTTGTGACCCACAAGGACTCAAGGACAACAGCCAGTTCTGCTTGCAAAGGATCCGCCAAGG
TCTGGCTTTTTATAAGCACCTGCTTGACTCTGACATCTTCAAAGGGGAGCCTGCTCTACTCCC
TGATAGCCCCATGGAGCAACTTCACACCTCCCTACTAGGACTCAGCCAACTCCTCCAGCCAGA
GGATCACCCCCGGGAGACCCAACAGATGCCCAGCCTGAGTTCTAGTCAGCAGTGGCAGCGCCC
CCTTCTCCGTTCCAAGATCCTTCGAAGCCTCCAGGCCTTTTTGGCCATAGCTGCCCGGGTCTT
TGCCCACGGAGCAGCAACTCTGACTGAGCCCTTAGTGCCAACAGCTTAACTCGAGTCACCAGG
CG (SEQ ID NO: 25).

[00144] Additionally, a nucleotide sequence encoding mouse IL-27B followed by a nucleotide sequence encoding a furin cleavage site (RAKR) followed by a nucleotide sequence encoding mouse IL-27A was cloned into the modified E1b-19k region of TAV-Δ19k-ΔE3. The resulting virus is hereafter referred to as TAV-mIL-27-furin. The nucleotide sequence encoding mouse IL-27B followed by the nucleotide sequence encoding the furin cleavage site followed by the nucleotide sequence encoding mouse IL-27A inserted into the E1b-19k region is as follows, where the IL-27B and IL-27A coding regions are capitalized, the furin cleavage site coding region is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGTCCAAGCTGCTCTTCCTGTCACTTGCCCTCTGGGCCAGCCGCTC
CCCTGGTTACACTGAAACAGCTCTCGTGGCTCTAAGCCAGCCCAGAGTGCAATGCCATGCTTC
TCGGTATCCCGTGGCCGTGGACTGCTCCTGGACTCCTCTCCAGGCTCCCAACTCCACCAGATC
CACGTCCTTCATTGCCACTTACAGGCTCGGTGTGGCCACCCAGCAGCAGAGCCAGCCCTGCCT
ACAACGGAGCCCCCAGGCCTCCCGATGCACCATCCCCGACGTGCACCTGTTCTCCACGGTGCC
CTACATGCTAAATGTCACTGCAGTGCACCCAGGCGGCGCCAGCAGCAGCCTCCTAGCCTTTGT
GGCTGAGCGAATCATCAAGCCGGACCCTCCGGAAGGCGTGCGCCTGCGCACAGCGGGACAGCG

CCTGCAGGTGCTCTGGCATCCCCCTGCTTCCTGGCCCTTCCCGGACATCTTCTCTCTCAAGTA
CCGACTCCGCTACCGGCGCCGAGGAGCCTCTCACTTCCGCCAGGTGGGACCCATTGAAGCCAC
GACTTTCACCCTCAGGAACTCGAAACCCCATGCCAAGTATTGCATCCAGGTGTCAGCTCAGGA
CCTCACAGATTATGGGAAACCAAGTGACTGGAGCCTCCCTGGGCAAGTAGAAAGTGCACCCCA
TAAGCCCcgtgctaagcgaTTCCCAACAGACCCCCTGAGCCTTCAAGAGCTGCGCAGGGAATT
CACAGTCAGCCTGTACCTTGCCAGGAAGCTGCTCTCTGAGGTTCAGGGCTATGTCCACAGCTT
TGCTGAATCTCGATTGCCAGGAGTGAACCTGGACCTCCTGCCCCTGGGATACCATCTTCCCAA
TGTTTCCCTGACTTTCCAGGCATGGCATCACCTCTCTGACTCTGAGAGACTCTGCTTCCTCGC
TACCACACTTCGGCCCTTCCCTGCCATGCTGGGAGGGCTGGGGACCCAGGGGACCTGGACCAG
CTCAGAGAGGGAGCAGCTGTGGGCCATGAGGCTGGATCTCCGGGACCTGCACAGGCACCTCCG
CTTTCAGGTGCTGGCTGCAGGATTCAAATGTTCAAAGGAAGAGGAAGACAAGGAGGAAGAGGA
AGAGGAGGAAGAAGAAGAAAAGAAGCTGCCCCTAGGGGCTCTGGGTGGCCCCAATCAGGTGTC
ATCCCAAGTGTCCTGGCCCCAGCTGCTCTATACCTACCAGCTCCTTCACTCCCTGGAGCTTGT
CCTGTCTCGGGCTGTTCGGGACCTGCTGCTGCTGTCCCTGCCCAGGCGCCCAGGCTCAGCCTG
GGATTCCTAACTCGAGTCACCAGGCG (SEQ ID NO: 26).

[00145] IL-12 expression from TAV-mIL-12-furin was tested. A549 cells were infected at an MOI of 1, 5, or 10 with TAV-mIL-12-furin or no virus. Conditioned media was collected at various times after infection and IL-12 concentration was measured by ELISA. As depicted in **FIGURE 9**, IL-12 was expressed in a dose-dependent and time-dependent manner.

[00146] IL-23 and IL-27 expression from TAV-mIL-23-furin and TAV-mIL-27-furin were tested. A549 cells were infected at an MOI 5 with TAV-mIL-23-furin, TAV-mIL-27-furin, or no virus. Conditioned media was collected four days after infection and IL-23 and IL-27 concentrations were measured by ELISA. As depicted in **FIGURE 10**, both viruses expressed the corresponding heterodimeric cytokine.

INCORPORATION BY REFERENCE

[00147] The entire disclosure of each of the patent documents and scientific articles referred to herein is incorporated by reference for all purposes.

EQUIVALENTS

[00148] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and the range of equivalency of the claims are intended to be embraced therein.

What is claimed is:

1. A recombinant adenovirus comprising a first nucleotide sequence encoding a first therapeutic transgene, a second nucleotide sequence encoding a second therapeutic transgene, and a third nucleotide sequence encoding a cleavage site disposed between the first nucleotide sequence and the second nucleotide sequence.

2. The recombinant adenovirus of claim 1, wherein the recombinant adenovirus comprises a polynucleotide sequence comprising, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence.

3. The recombinant adenovirus of claims 1 or 2, wherein the recombinant adenovirus comprises a polynucleotide sequence comprising, consecutively, in a 5' to 3' orientation, the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence.

4. The recombinant adenovirus of any one of claims 1-3, wherein the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are operably linked to a single promoter, optionally positioned 5' to the first nucleotide of the first nucleotide sequence.

5. The recombinant adenovirus of any one of claims 1-4, wherein the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are expressed as a single polypeptide chain.

6. The recombinant adenovirus of any one of claims 1-5, wherein the recombinant adenovirus is a type 5 adenovirus (Ad5) or a type 2 adenovirus (Ad2).

7. The recombinant adenovirus of claim 6, wherein the recombinant adenovirus is a type 5 adenovirus (Ad5).

8. The recombinant adenovirus of any one of claims 1-7, wherein the cleavage site is a proteolytic cleavage site.

9. The recombinant adenovirus of claim 8, wherein the proteolytic cleavage site is cleaved by a protease that is present in an endoplasmic reticulum or golgi of a eukaryotic cell.

10. The recombinant adenovirus of claims 8 or 9, wherein the proteolytic cleavage site is a furin cleavage site.

11. The recombinant adenovirus of claim 10, wherein the furin cleavage site comprises RX_1X_2R (SEQ ID NO: 6), wherein X_1 is any amino acid, and X_2 is Lys or Arg.

12. The recombinant adenovirus of claim 11, wherein the furin cleavage site comprises RAKR (SEQ ID NO: 7).

13. The recombinant adenovirus of any one of claims 1-12, wherein the first, second, and third nucleotide sequences are inserted into an E1b-19k insertion site located between the start site of E1b-19K and the stop site of E1b-19K.

14. The recombinant adenovirus of claim 13, wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K.

15. The recombinant adenovirus of claim 13 or 14, wherein the E1b-19K insertion site comprises a deletion of about 200 nucleotides adjacent the start site of E1b-19K.

16. The recombinant adenovirus of claim 15, wherein the E1b-19K insertion site comprises a deletion of 202 nucleotides adjacent the start site of E1b-19K.

17. The recombinant adenovirus of claim 15, wherein the E1b-19K insertion site comprises a deletion of 203 nucleotides adjacent the start site of E1b-19K.

18. The recombinant adenovirus of any one of claims 13-17, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1916 of the Ad5 genome (SEQ ID NO: 1).

19. The recombinant adenovirus of any one of claims 13-17, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 of the Ad5 genome (SEQ ID NO: 1).

20. The recombinant adenovirus of any one of claims 13-19, wherein the first nucleotide sequence, the third nucleotide sequence, and the second nucleotide sequence are inserted between CTGACCTC (SEQ ID NO: 2) and TCACCAGG (SEQ ID NO: 3).

21. The recombinant adenovirus of any one of claims 13-20, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 2), the first nucleotide sequence, the third nucleotide sequence, the second nucleotide sequence, and TCACCAGG (SEQ ID NO: 3).

22. The recombinant adenovirus of any one of claims 1-21, wherein the recombinant adenovirus comprises an E1a promoter having a deletion of a functional Pea3 binding site.

23. The recombinant adenovirus of claim 22, wherein the deletion comprises a deletion of nucleotides corresponding to about -300 to about -250 upstream of the initiation site of E1a.

24 The recombinant adenovirus of claims 22 or 23, wherein the deletion comprises a deletion of nucleotides corresponding to -304 to -255 upstream of the initiation site of E1a.

25. The recombinant adenovirus of claims 22 or 23, wherein the deletion comprises a deletion of nucleotides corresponding to -305 to -255 upstream of the initiation site of E1a.

26. The recombinant adenovirus of any one of claims 22-25, wherein the deletion comprises a deletion of nucleotides corresponding to 195-244 of the Ad5 genome (SEQ ID NO: 1).

27. The recombinant adenovirus of any one of claims 22-26, wherein the E1a promoter comprises the sequence GGTGTTTTGG (SEQ ID NO: 4).

28. The recombinant adenovirus of any one of claims 1-27, wherein the recombinant adenovirus comprises an E1a promoter having a deletion of a functional TATA box.

29. The recombinant adenovirus of claim 28, wherein the deletion comprises a deletion of the entire TATA box.

30. The recombinant adenovirus of claims 28 or 29, wherein the deletion comprises a deletion of nucleotides corresponding to -27 to -24 of the E1a promoter.

31. The recombinant adenovirus of any one of claims 28-30, wherein the deletion comprises a deletion of nucleotides corresponding to -31 to -24 of the E1a promoter.

32. The recombinant adenovirus of any one of claims 28-31, wherein the deletion comprises a deletion of nucleotides corresponding to -44 to +54 of the E1a promoter.

33. The recombinant adenovirus of any one of claims 28-32, wherein the deletion comprises a deletion of nucleotides corresponding to -146 to +54 of the E1a promoter.

34. The recombinant adenovirus of any one of claims 28-33, wherein the deletion comprises a deletion of nucleotides corresponding to 472 to 475 of the Ad5 genome (SEQ ID NO: 1).

35. The recombinant adenovirus of any one of claims 28-34, wherein the deletion comprises a deletion of nucleotides corresponding to 468 to 475 of the Ad5 genome (SEQ ID NO: 1).

36. The recombinant adenovirus of any one of claims 28-35, wherein the deletion comprises a deletion of nucleotides corresponding to 455 to 552 of the Ad5 genome (SEQ ID NO: 1).

37. The recombinant adenovirus of any one of claims 28-36, wherein the deletion comprises a deletion of nucleotides corresponding to 353 to 552 of the Ad5 genome (SEQ ID NO: 1).

38. The recombinant adenovirus of any one of claims 28-37, wherein the recombinant adenovirus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5), AGTGCCCG (SEQ ID NO: 16) and/or TATTCCCG (SEQ ID NO: 17).

39. The recombinant adenovirus of claim 38, wherein the recombinant adenovirus comprises a polynucleotide deletion that results in a virus comprising the sequence CTAGGACTG (SEQ ID NO: 5).

40. The recombinant adenovirus of any one of claims 1-39, wherein the recombinant adenovirus comprises an E1a promoter having a deletion of a functional CAAT box.

41. The recombinant adenovirus of claim 40, wherein the deletion comprises a deletion of the entire CAAT box.

42. The recombinant adenovirus of claim 40 or 41, wherein the deletion comprises a deletion of nucleotides corresponding to -76 to -68 of the E1a promoter.

43. The recombinant adenovirus of any one of claims 40-42, wherein the deletion comprises a deletion of nucleotides corresponding to 423 to 431 of the Ad5 genome (SEQ ID NO: 1).

44. The recombinant adenovirus of any one of claims 40-43, wherein the E1a promoter comprises the sequence TTCCGTGGCG (SEQ ID NO: 18).

45. The recombinant adenovirus of any of claims 1-44, wherein the recombinant adenovirus further comprises an E3 deletion, wherein the E3 deletion is located between the stop site of pVIII and the start site of Fiber.

46. The recombinant adenovirus claim 45, wherein the E3 deletion is located between the stop site of E3-10.5K and the stop site of E3-14.7K.

47. The recombinant adenovirus of claim 45 or 46, wherein the E3 deletion comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides.

48. The recombinant adenovirus of any one of claims 45-47, wherein the E3 deletion comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K.

49. The recombinant adenovirus of any one of claims 45-48, wherein the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

50. The recombinant adenovirus of any one of claims 45-49, wherein the E3 deletion comprises a deletion of 1063 nucleotides adjacent the stop site of E3-10.5K.

51. The recombinant adenovirus of any one of claims 45-49, wherein the E3 deletion comprises a deletion of 1064 nucleotides adjacent the stop site of E3-10.5K.

52. The recombinant adenovirus of any one of claims 45-51, wherein the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion.

53. The recombinant adenovirus of any one of claims 45-52, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 1).

54. The recombinant adenovirus of claim 45, wherein the E3 deletion is located between the stop site of E3-gp19K and the stop site of E3-14.7K.

55. The recombinant adenovirus of claim 54, wherein the E3 deletion comprises a deletion of from about 500 to about 1824, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K.

56. The recombinant adenovirus of claim 54 or 55, wherein the E3 deletion comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

57. The recombinant adenovirus of any one of claims 54-56, wherein the E3 deletion comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K.

58. The recombinant adenovirus of any one of claims 54-58, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 1).

59. The recombinant adenovirus of any one of claims 1-58, wherein the recombinant adenovirus further comprises an E4 deletion, wherein the E4 deletion is located between the start site of E4-ORF6/7 and right inverted terminal repeat (ITR).

60. The recombinant adenovirus of claim 59, wherein the E4 deletion is located between the start site of E4-ORF6/7 and the start site of E4-ORF1.

61. The recombinant adenovirus of claims 59 or 60, wherein the E4 deletion comprises a deletion of from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 2500, from about 1500 to about 2000, or from about 2000 to about 2500 nucleotides.

62. The recombinant adenovirus of any one of claims 59-61, wherein the E4 deletion comprises a deletion of from about 250 to about 1500, from about 250 to about 1250, from about 250 to about 1000, from about 250 to about 750, from about 250 to about 500, from 500 to about 1500, from about 500 to about 1250, from about 500 to about 1000, from about 500 to about 750, from 750 to about 1500, from about 750 to about 1250, from about 750 to about 1000, from about 1000 to about 1500, from about 1000 to about 1250, or from about 1250 to about 1500 nucleotides adjacent the start site of E4-ORF6/7.

63. The recombinant adenovirus of any one of claims 59-62, wherein the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7.

64. The recombinant adenovirus of any one of claims 59-63, wherein the E4 deletion comprises a deletion of 1449 nucleotides adjacent the start site of E4-ORF6/7.

65. The recombinant adenovirus of any one of claims 59-64, wherein the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 1).

66. The recombinant adenovirus of any one of claims 1-65, wherein the first and/or the second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, ICAM, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-17, IL-23, IL-23A/p19, p40, IL-24, IL-27, IL-27A/p28, IL-27B/EBI3, IL-35, interferon-gamma, MAGE, NY-ESO-1, p53, TGF-β, a TGF-β trap, and thymidine kinase.

67. The recombinant adenovirus of any one of claims 1-65, wherein the first and/or the second therapeutic transgene encodes a polypeptide selected from acetylcholine, an anti-PD-1 antibody heavy chain or light chain, an anti-PD-L1 antibody heavy chain or light, BORIS/CTCFL, CD19, CD20, CD80, CD86, CD137, CD137L, CD154, DKK1/Wnt, FGF, ICAM, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-23A/p19, p40, interferon-gamma, MAGE, NY-ESO-1, p53, TGF-β, a TGF-β trap, and thymidine kinase.

68. The recombinant adenovirus of any one of claims 1-67, wherein the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

69. The recombinant adenovirus of claim 68, wherein the first and/or second therapeutic transgenes are selected from IL-23A/p19 and p40.

70. The recombinant adenovirus of claim 69, wherein the first therapeutic transgene encodes p40.

71. The recombinant adenovirus of claims 69 or 70, wherein the second therapeutic transgene encodes IL-23A/p19.

72. The recombinant adenovirus of any one of claims 69-71, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1000 of SEQ ID NO: 9.

73. The recombinant adenovirus of any one of claims 69-72, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 1013-1582 of SEQ ID NO: 9.

74. The recombinant adenovirus of any one of claims 69-73, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by nucleotides 17-1582 of SEQ ID NO: 9.

75. The recombinant adenovirus of any one of claims 69-74, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 9, or comprises a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 9.

76. The recombinant adenovirus of any one of claims 1-75, wherein the first and/or second therapeutic transgenes are not operably linked to an exogenous promoter sequence.

77. The recombinant adenovirus of any one of claims 1-76, wherein the combined size of the first and second therapeutic transgenes comprises from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides.

78. The recombinant adenovirus of any one of claims 1-76, wherein the combined size of the first and second therapeutic transgenes comprises from about 500 to about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to

about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

79. The recombinant adenovirus of any one of claims 1-78, wherein the combined size of the first and second therapeutic transgenes comprises at least from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to 5000 nucleotides.

80. The recombinant adenovirus of any one of claims 1-78, wherein the combined size of the first and second therapeutic transgenes comprises at least from about 500 to about 7000, from about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

81. The recombinant adenovirus of any one of claims 1-80, wherein the combined size of the first and second therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides.

82. The recombinant adenovirus of any one of claims 1-80, wherein the combined size of the first and second therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000 nucleotides, about 6000, or about 7000 nucleotides.

83. The recombinant adenovirus of any one of claims 1-82, wherein the combined size of the first and second therapeutic transgenes comprises about 1600 nucleotides.

84. The recombinant adenovirus of any one of claims 1-83, wherein the combined size of the first and second therapeutic transgenes comprises about 1650 nucleotides.

85. The recombinant adenovirus of any one of claims 1-82, wherein the combined size of the first and second therapeutic transgenes comprises about 3100 nucleotides.

86. The recombinant adenovirus of any one of claims 1-85, wherein the recombinant adenovirus selectively replicates in a hyperproliferative cell.

87. The recombinant adenovirus of any one of claims 1-86, wherein the recombinant adenovirus selectively expresses the first and/or the second therapeutic transgene in a hyperproliferative cell.

88. The recombinant adenovirus of claims 86 or 87, wherein the hyperproliferative cell is a cancer cell.

89. The recombinant adenovirus of any one of claims 1-88, wherein the recombinant adenovirus is an oncolytic virus.

90. A pharmaceutical composition comprising the recombinant adenovirus of any one of claims 1-89 and at least one pharmaceutically acceptable carrier or diluent.

91. A method of expressing two therapeutic transgenes in a target cell comprising exposing the cell to an effective amount of the recombinant adenovirus of any one of claims 1-89 to express the two therapeutic transgenes.

92. The method of claim 91, wherein the two therapeutic transgenes, when expressed, produce a single polypeptide chain.

93. The method of claim 92, wherein the single polypeptide chain is cleaved posttranslationally into two polypeptide chains.

94. A method of inhibiting proliferation of a tumor cell comprising exposing the cell to an effective amount of the recombinant adenovirus of any one of claims 1-89 to inhibit proliferation of the tumor cell.

95. A method of inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject to an effective amount of the recombinant adenovirus of any one of claims 1-89 to inhibit growth of the tumor.

96. A method of treating cancer in a subject in need thereof, the method comprising administering to the subject an effective amount of the recombinant adenovirus of any one of claims 1-89 to treat the cancer in the subject.

97. The method of claim 96, wherein the cancer is selected from anal cancer, basal cell carcinoma, bladder cancer, bone cancer, brain cancer, breast cancer, carcinoma, cholangiocarcinoma, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, gastroesophageal cancer, gastrointestinal (GI) cancer, gastrointestinal stromal tumor, hepatocellular carcinoma, gynecologic cancer, head and neck cancer, hematologic cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, merkel cell carcinoma, mesothelioma, neuroendocrine cancer, non-small cell lung cancer, ovarian cancer, pancreatic cancer, pediatric cancer, prostate cancer, renal cell carcinoma, sarcoma, skin cancer, small cell lung cancer, squamous cell carcinoma of the skin, stomach cancer, testicular cancer and thyroid cancer.

98. The method of claim 96, wherein the cancer is selected from melanoma, squamous cell carcinoma of the skin, basal cell carcinoma, head and neck cancer, breast cancer, anal cancer, cervical cancer, non-small cell lung cancer, mesothelioma, small cell lung cancer, renal cell carcinoma, prostate cancer, gastroesophageal cancer, colorectal cancer, testicular cancer, bladder cancer, ovarian cancer, hepatocellular carcinoma, cholangiocarcinoma, brain cancer, endometrial cancer, neuroendocrine cancer, merkel cell carcinoma, gastrointestinal stromal tumors, a sarcoma, and pancreatic cancer.

99. The method of claims 95-98, wherein the recombinant adenovirus is administered in combination with one or more therapies selected from surgery, radiation, chemotherapy, immunotherapy, hormone therapy, and virotherapy.

100. The method of any one of claims 94-99, wherein the effective amount of the recombinant adenovirus is 10^2-10^{15} plaque forming units (pfus).

101. The method of any one of claims 95-100, wherein the subject is a human.

102. The method of claim 101, wherein the subject is a pediatric human.

FIGURE 1

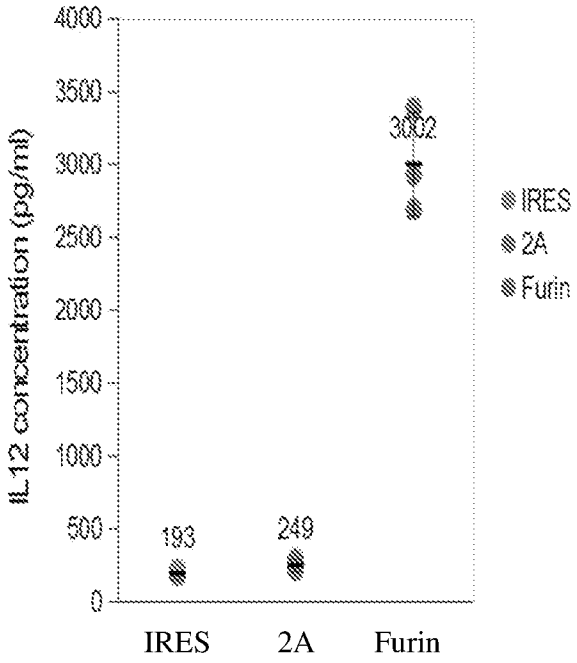

IL12 concentration (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
193
249
3002
IRES
2A
Furin
IRES
2A
Furin

FIGURE 2

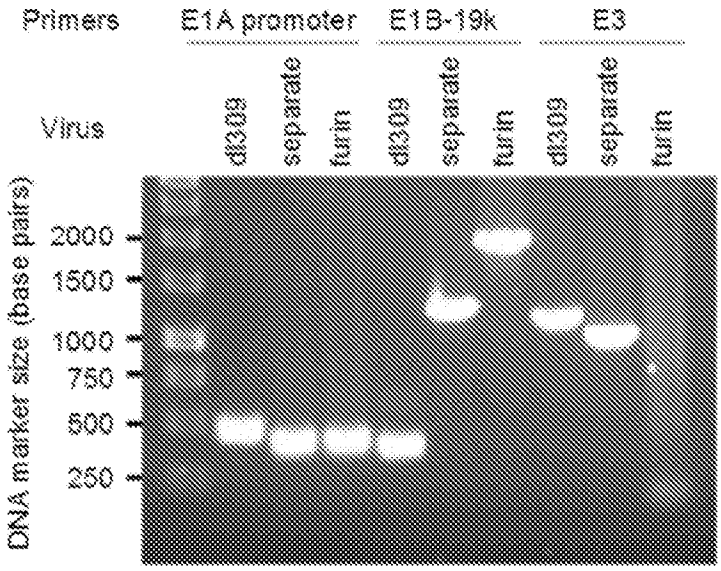

Primers
E1A promoter
E1B-19k
E3
Virus
d309
separate
turin
d309
separate
turin
d309
separate
turin
DNA marker size (base pairs)
2000
1500
1000
750
500
250

FIGURE 3

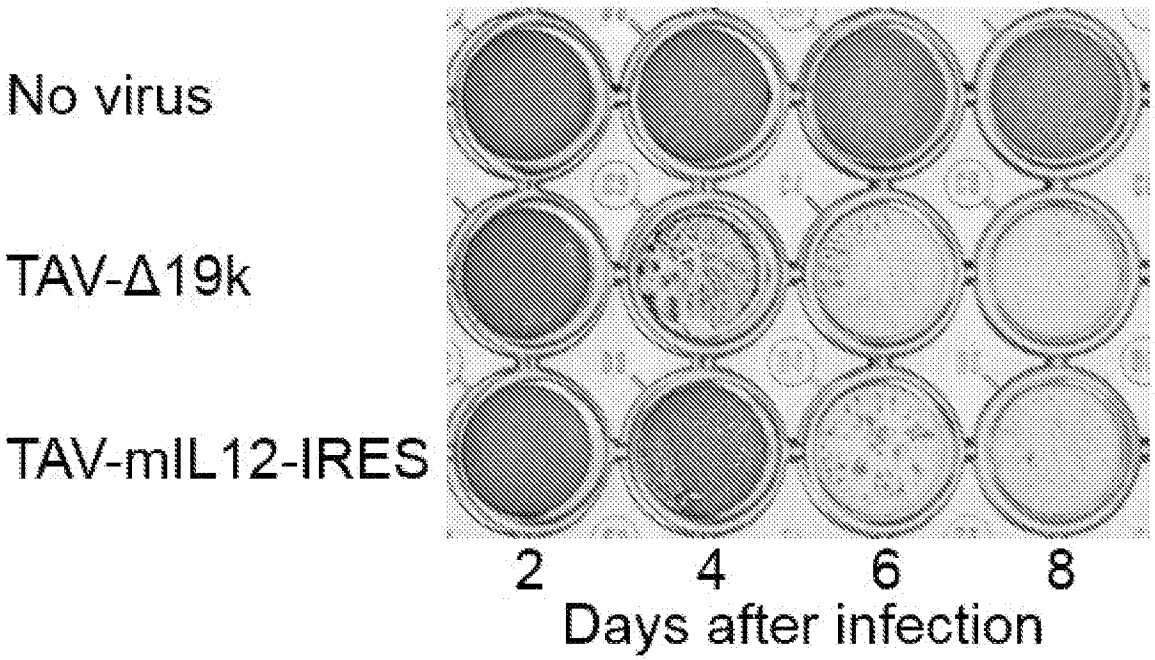


FIGURE 4

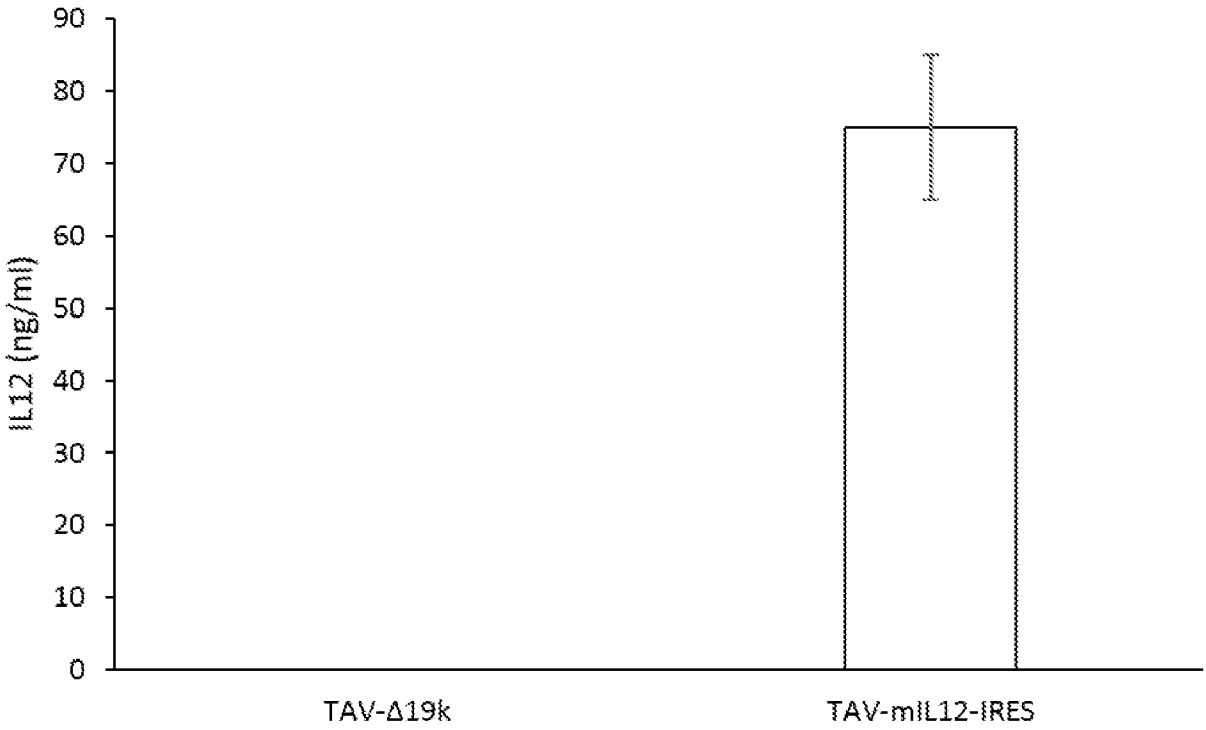


FIGURE 5

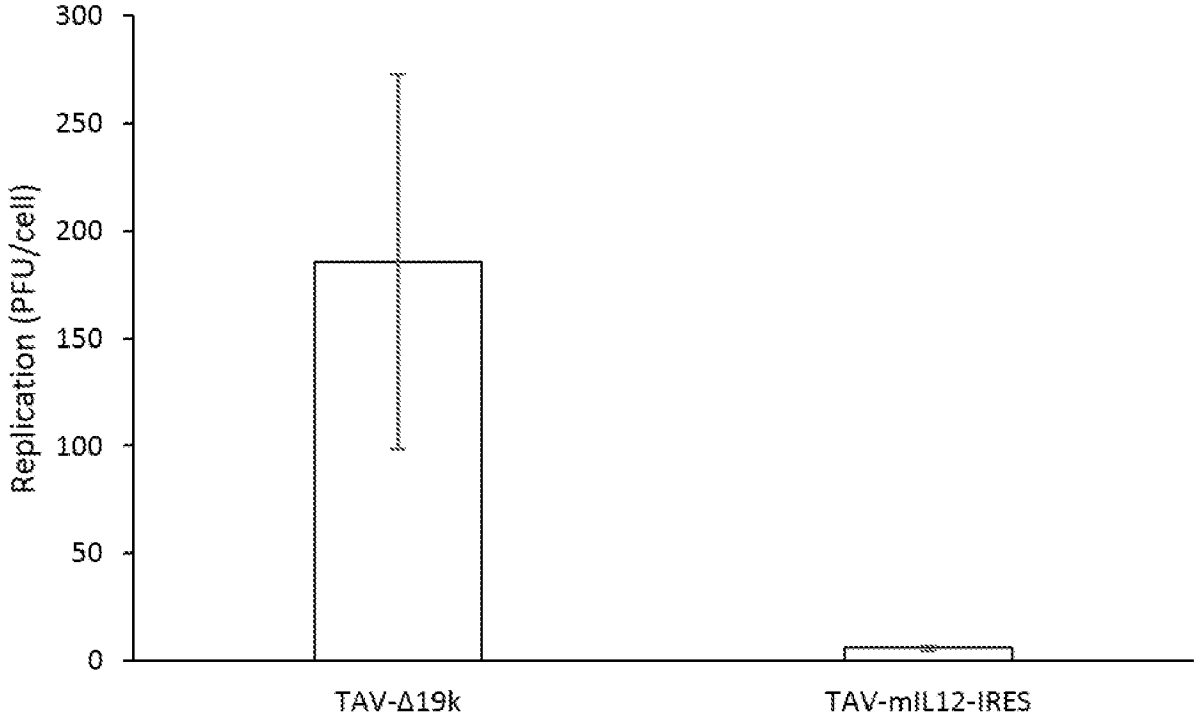

300
250
200
150
100
50
0
Replication (PFU/cell)
TAV-Δ19k
TAV-mIL12-IRES

FIGURE 6

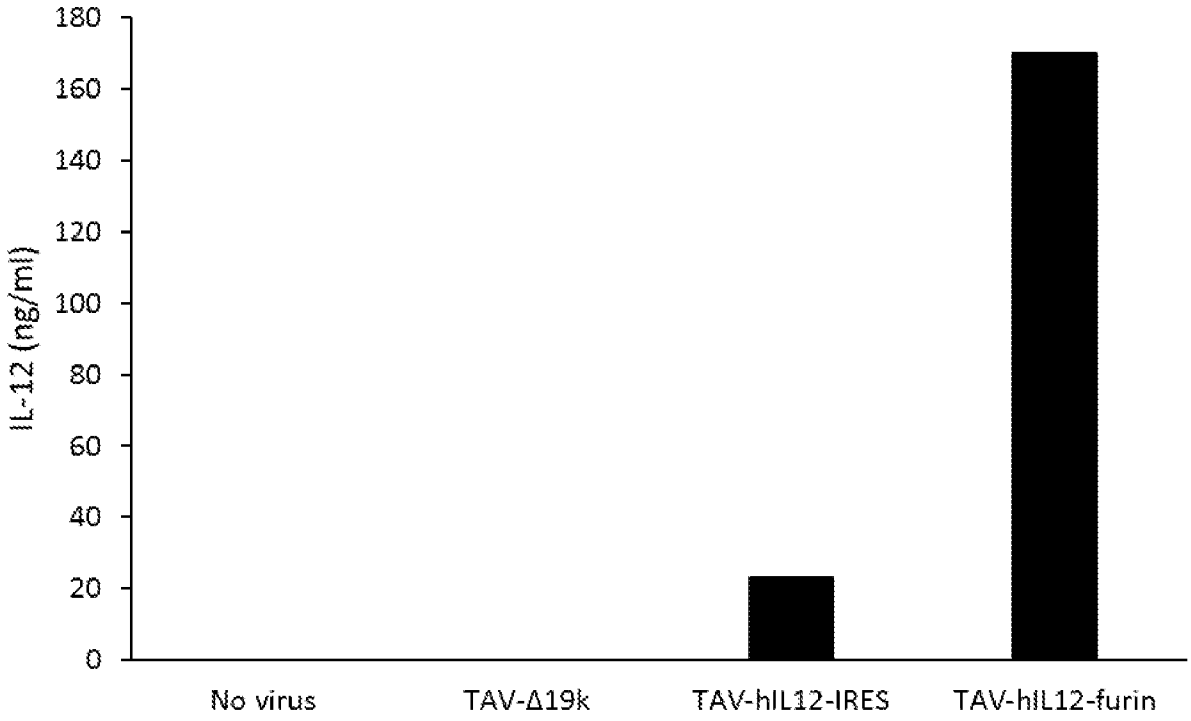

180
160
140
120
100
80
60
40
20
0
IL-12 (ng/ml)
No virus
TAV-Δ19k
TAV-hIL12-IRES
TAV-hIL12-furin

FIGURE 7

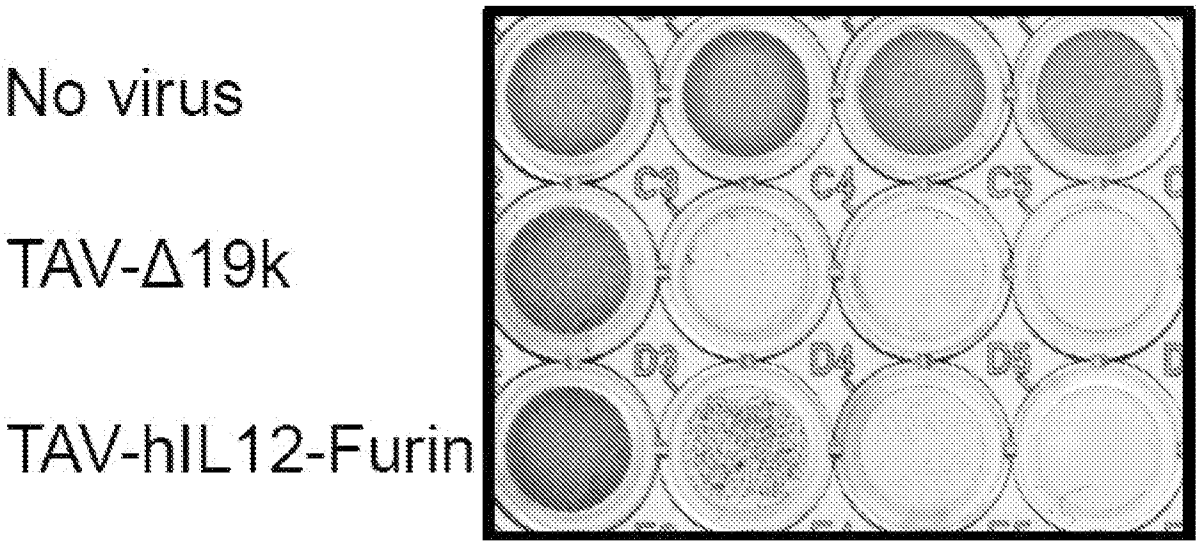


FIGURE 8

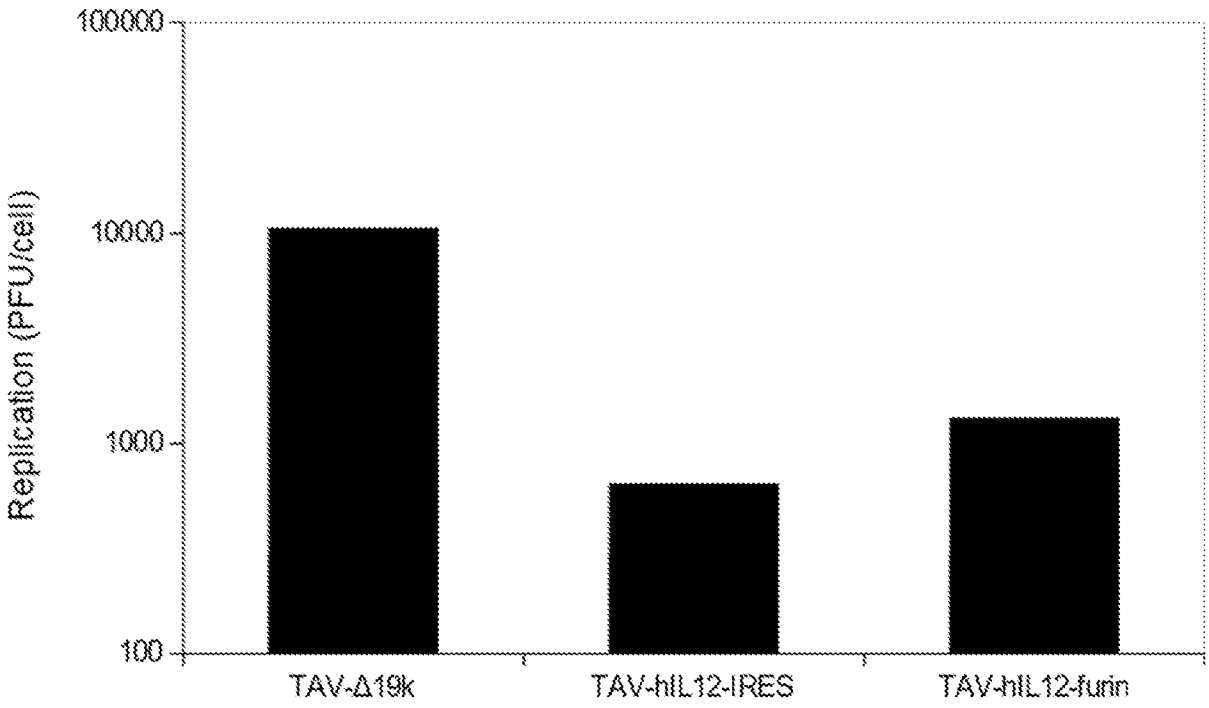

100000
10000
1000
100
Replication (PFU/cell)
TAV-Δ19k
TAV-hIL12-IRES
TAV-hIL12-furin

FIGURE 9

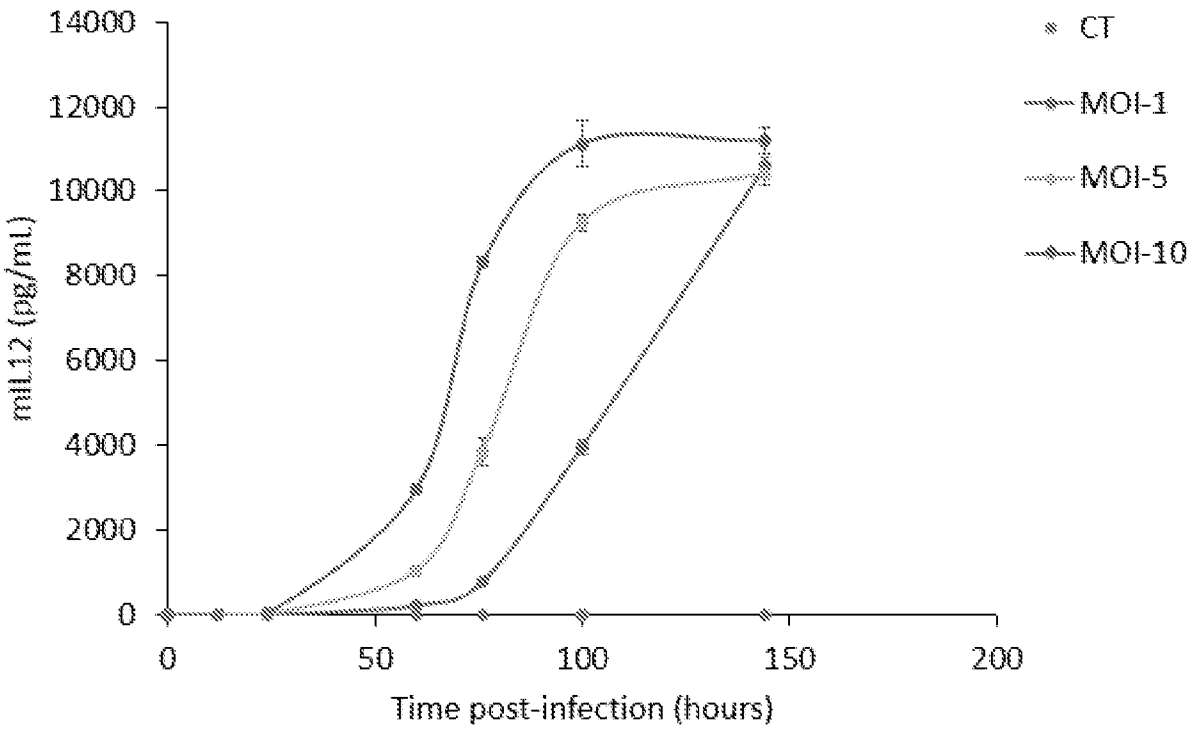
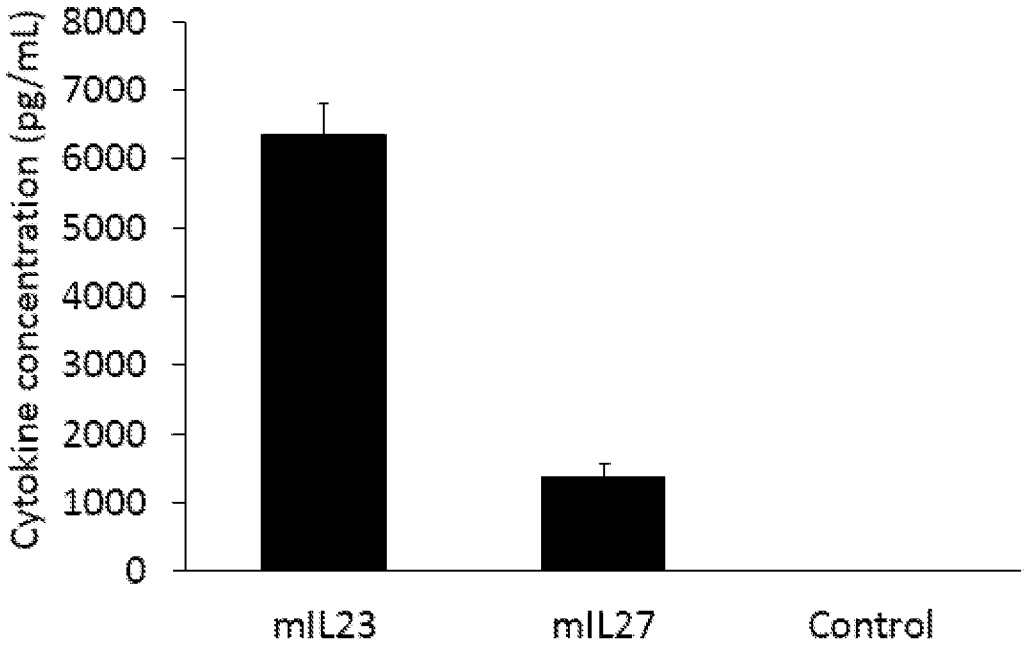

14000
12000
10000
8000
6000
4000
2000
0
mIL12 (pg/mL)
0
50
100
150
200
Time post-infection (hours)
CT
MOI-1
MOI-5
MOI-10

FIGURE 10


Cytokine concentration (pg/mL)
8000
7000
6000
5000
4000
3000
2000
1000
0
mIL23
mIL27
Control

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/027375

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/861 (2006.01) A61K 35/761 (2015.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

MEDLINE, CAPLUS, BIOSIS, EMBASE (adenovirus, AAV, Ad5, Ad2, recombinant, transgenic, vector, cleavage, furin, proteolytic, post translational, second, two, multiple, transgenes, oncolytic, p40, IL-23A, p19, E1b-19K, E1a, promoter, Pea3, TATA box, CAAT box, E3, E4, cancer, tumour, therapy, treatment, and other synonyms and like terms) EPODOC, WPIAP and all English language databases (A61K35/761, C12N15/861, and keywords listed above) GOOGLE (recombinant, adenovirus, Ad5, multiple transgenes, cleavage, therapeutic, and other synonyms and like terms) ESPACENET, PUBMED, AUSPAT (Applicant and Inventor Search)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2018	18 July 2018
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Damian Triffett AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832845

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 2004/108893 A2 (CELL GENESYS, INC.) 16 December 2004	
X	See whole document especially Abstract; Page 4, lines 7-12 and 18-22 Page 5, lines 15-21; Page 15, lines 20-30; Page 16, lines 8 and 9; Page 20, lines 8-10; Page 22, lines 18-23; Page 36, lines 18-22; Page 40, lines 11 and 12; Page 43, lines 13 and 14; Page 45, lines 14-16; Page 46, lines 11-20; Page 48, lines 23-31; Page 55, lines 6-9 and 20-22; Claim 58; SEQ ID NO's 10-12	1-14, 22-26, 28-58, 66, 67, 76-102
Y	As above	15-21, 27, 59-65, 68-75
	WO 2014/170389 A1 (TILT BIOTHERAPEUTICS OY) 23 October 2014	
X	See whole document especially Abstract; Page 5, lines 3 and 4; Page 12, lines 15-17 and 21 - 29; Page 18, lines 14-16; Page 22, line 36 - Page 23, line 11; Page 23, line 21; Page 25, line 20 - Page 26, line 8; Page 26, lines 14-23; Figures 33 and 34; Claims 6, 7 and 32	1-9, 22-26, 28-58, 66, 67, 76-102
Y	As above	27, 59-65, 68-75
	WO 2010/101921 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 September 2010	
Y	Claims 17-19 and 23	15-21
	HEDJRAN, F. et al., "Deletion analysis of Ad5 E1a transcriptional control region: impact on tumor-selective expression of E1a and E1b", Cancer Gene Therapy, 2011, Vol. 18, pages 717-723	
Y	See whole document especially Abstract; Page 718, left hand column, second paragraph	27
	US 5670488 A (Gregory et al.) 23 September 1997	
Y	See whole document especially Column 47, lines 37-42, 45-48, 50 and 51; Claim 1	59-65
	CHOI, I. et al., "Oncolytic Adenovirus Expressing IL-23 and p35 Elicits IFN-γ- and TNF-α-Co-Producing T Cell-Mediated Antitumor Immunity", PLOS ONE, 3 July 2013, Vol. 87, No. 7, e67512	
Y	See whole document especially Abstract	68-75
	WO 2018/083259 A1 (PSIOXUS THERAPEUTICS LIMITED) 11 May 2018	
E	See whole document especially Abstract; Page 4, claim 22; Page 13, lines 30-37; Page 18, lines 24 and 32; Page 21, lines 13-27; Page 25, line 37 - Page 26, line 4; Page 30, lines 20-22; Page 33, lines 4-10; Page 36, lines 5-20; Page 46, lines 6-10; Claim 2	1-12, 45-67, 76-102
E	As above	68-75

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/027375

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   There was a sequence listing originally filed but it was not used for the purposes of this search and opinion.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2018/027375**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2004/108893 A2	16 December 2004	WO 2004108893 A2	16 Dec 2004
		CA 2525401 A1	16 Dec 2004
		EP 1628535 A2	01 Mar 2006
		JP 2007525949 A	13 Sep 2007
		US 2005136035 A1	23 Jun 2005
		US 2009270485 A1	29 Oct 2009
WO 2014/170389 A1	23 October 2014	WO 2014170389 A1	23 Oct 2014
		AU 2014255733 A1	26 Nov 2015
		CA 2909432 A1	23 Oct 2014
		CN 105307671 A	03 Feb 2016
		EP 2986311 A1	24 Feb 2016
		JP 2016522805 A	04 Aug 2016
		KR 20160002971 A	08 Jan 2016
		RU 2015148920 A	24 May 2017
		SG 11201508585P A	27 Nov 2015
		US 2015232880 A1	20 Aug 2015
		US 2017137786 A1	18 May 2017
		ZA 201507790 B	21 Dec 2016
WO 2010/101921 A2	10 September 2010	WO 2010101921 A2	10 Sep 2010
		EP 2403951 A2	11 Jan 2012
		EP 2403951 B1	30 Sep 2015
		EP 3029144 A1	08 Jun 2016
		JP 2012519014 A	23 Aug 2012
		JP 6072414 B2	01 Feb 2017
		JP 2016028035 A	25 Feb 2016
		KR 20110122866 A	11 Nov 2011
		KR 101752910 B1	30 Jun 2017
		KR 20170077278 A	05 Jul 2017
		KR 20180049188 A	10 May 2018
		SM T201500329 B	25 Feb 2016
		US 2011318311 A1	29 Dec 2011
		US 9073980 B2	07 Jul 2015
		US 2016017294 A1	21 Jan 2016

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2018/027375**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 5670488 A	23 September 1997	US 5670488 A	23 Sep 1997
		AU 5734994 A	22 Jun 1994
		AU 680459 B2	31 Jul 1997
		AU 5092793 A	15 Mar 1994
		AU 684049 B2	04 Dec 1997
		AU 7630694 A	21 Mar 1995
		AU 7644494 A	27 Mar 1995
		CA 2037478 A1	06 Sep 1991
		CA 2143306 A1	03 Mar 1994
		CA 2145641 A1	09 Jun 1994
		CA 2592997 A1	09 Jun 1994
		EP 0446017 A1	11 Sep 1991
		EP 0659211 A1	28 Jun 1995
		EP 0673431 A1	27 Sep 1995
		EP 0905253 A2	31 Mar 1999
		EP 0911413 A2	28 Apr 1999
		EP 1024198 A2	02 Aug 2000
		JP H06303978 A	01 Nov 1994
		JP H08500596 A	23 Jan 1996
		JP H08503855 A	30 Apr 1996
		US 5674898 A	07 Oct 1997
		US 5750571 A	12 May 1998
		US 5876974 A	02 Mar 1999
		US 5882877 A	16 Mar 1999
		US 5939536 A	17 Aug 1999
		US 5981714 A	09 Nov 1999
		US 6093567 A	25 Jul 2000
		US 7118911 B1	10 Oct 2006
		US 2003147854 A1	07 Aug 2003
		US 7318919 B2	15 Jan 2008
		US 2002164782 A1	07 Nov 2002
		US 2007053879 A1	08 Mar 2007
		WO 9404671 A1	03 Mar 1994
		WO 9412649 A2	09 Jun 1994
		WO 9506066 A1	02 Mar 1995
		WO 9507453 A1	16 Mar 1995

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2018/027375**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2018/083259 A1	11 May 2018	WO 2018083259 A1	11 May 2018

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.