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(54) Title: MULTIPLE TRANSGENE RECOMBINANT ADENOVIRUS

(57) Abstract: The invention provides a recombinant adenovirus comprising two (or more) therapeutic transgenes, e.g., CD80 and CD137L. The transgenes are preferably inserted into an Elb-19K insertion site and/or an E3 insertion site.

WO 2018/140973 A1

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 62/452,342, filed January 30, 2017 and U.S. Provisional Patent Application serial number 62/520,945, filed June 16, 2017.

FIELD OF THE INVENTION

[0002] The field of the invention is molecular biology and virology, specifically modified 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. WO2010/101921). 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 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 adenoviruses such as oncolytic viruses, unexpectedly can efficiently express, when inserted into particular insertion sites, multiple (two or more) therapeutic transgenes without the use of an exogenous promoter and that the viruses can replicate and efficiently express the two or more therapeutic transgenes despite the size of the transgenes incorporated into the viral genome.

[0007] Accordingly, in one aspect the invention provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K; and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

[0008] In certain embodiments, the recombinant adenovirus is a type 5 adenovirus (Ad5).

[0009] In certain embodiments, the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K. 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: 23). In certain embodiments, the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23). In certain

embodiments, the first therapeutic transgene is inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

[0010] In certain embodiments, the E3 insertion site 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 insertion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K. In certain embodiments, the E3 insertion site 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 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K, *e.g.,* the E3 insertion site comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 insertion site comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain embodiments, the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the second therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). In certain embodiments, the E3 insertion site is located between stop site of E3-gp19K and the stop site of E3-14.7K. 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: 23). In certain embodiments, the second therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the second therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

[0011] In another aspect, the invention provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site, wherein the E1b-19k insertion site is located between the start site of E1b-19k and the start site of E1b-55k, and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

[0012] In certain embodiments, the recombinant adenovirus is a type 5 adenovirus (Ad5).

[0013] In certain embodiments, the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K. 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: 23). In certain embodiments, the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1917 or

between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the first and second therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

[0014] 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: 23). 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 insertion site 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: 23).

[0015] In certain embodiments, the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene. The third therapeutic transgene may be inserted into the E1b-19k insertion site wherein, *e.g.*, the second nucleotide sequence and the third nucleotide sequence are separated by a second internal ribosome entry site (IRES). In certain embodiments, the first, second, and third therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, the second IRES, the third therapeutic transgene, and TCACCAGG (SEQ ID NO: 2). The third therapeutic transgene may also be inserted into the E3 deletion site, *i.e.,* in certain embodiments the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into an E3 insertion site. In certain embodiments, the third therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome. In certain embodiments, the third therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the third therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). In certain embodiments, the third therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the third therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the third therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

[0016] The IRES may, *e.g.,* be selected from the group consisting of the encephalomyocarditis virus (EMCV) IRES, the foot-and-mouth disease virus (FMDV) IRES, and the poliovirus IRES.

[0017] In certain embodiments, in any of the foregoing viruses, 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: 23).

[0018] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes, the first, second, and/or third therapeutic transgenes, or all of the therapeutic transgenes, are not operably linked to an exogenous promoter sequence.

[0019] In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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 about 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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.

[0020] In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 1650 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 3100 nucleotides.

[0021] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, p40, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

[0022] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of

CD80, CD137L, IL-23, IL-23A/p19, p40, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

[0023] In certain embodiments, the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

[0024] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of CD80 and CD137L, *e.g.,* the first therapeutic transgene encodes CD80 and the second therapeutic transgene encodes CD137L. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, and/or SEQ ID NO: 7, or comprises the nucleotide sequence of SEQ ID NO: 6, and/or SEQ ID NO: 8. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 27.

[0025] In certain embodiments, in any of the foregoing viruses, the first, second, and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1., *e.g.,* the first therapeutic transgene encodes CD80, the second therapeutic transgene encodes CD137L, and the third therapeutic transgene encodes ICAM-1. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, SEQ ID NO: 7, and/or SEQ ID NO: 32. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 31, SEQ ID NO: 9, or SEQ ID NO: 22.

[0026] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of IL-23A/p19 and p40, which make up the heterodimeric cytokine IL-23. For example, in certain embodiments, the first therapeutic transgene encodes IL-23A/p19 and the second therapeutic transgene encodes p40. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 12 and/or SEQ ID NO: 10, or comprises the nucleotide sequence of SEQ ID NO: 13.

[0027] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of IL-27A/p28 and IL-27B/EBI3, , which make up the heterodimeric cytokine IL-27. For example, in certain embodiments, the first therapeutic transgene encodes IL-27A/p28 and the second therapeutic transgene encodes IL-27B/EBI3.

[0028] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin *e.g.,* the first therapeutic transgene encodes endostatin and the second therapeutic transgene encodes angiostatin. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 37 or SEQ ID NO: 38. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 11.

[0029] In certain embodiments, any of the foregoing recombinant adenoviruses may comprise a deletion of at least one Pea3 binding site, or a functional portion thereof, *e.g.,* the virus may comprise a deletion of nucleotides corresponding to about -300 to about -250 upstream of the initiation site of E1a or a deletion of nucleotides corresponding to -305 to -255 or -304 to -255 upstream of the initiation site of E1a.

[0030] In certain embodiments, in any of the foregoing compositions, the recombinant oncolytic adenovirus may comprise a deletion of at least one E2F binding site, or a functional portion thereof. In certain embodiments, the recombinant oncolytic adenovirus may comprise a deletion of at least one E2F binding site, or a functional portion thereof, and not comprise a deletion of a Pea3 binding site.

[0031] In another aspect, the invention provides a recombinant adenovirus comprising SEQ ID NO: 14, or 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: 14.

[0032] In certain embodiments, each of the foregoing recombinant adenoviruses may selectively replicate in a hyperproliferative cell. In certain embodiments, any of the foregoing recombinant adenoviruses 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, each of the foregoing recombinant adenoviruses may be an oncolytic adenovirus.

[0033] 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.

[0034] 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 the group consisting of 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.

[0035] 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.

[0036] 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.

[0037] In each of the foregoing methods, the recombinant adenovirus can, *e.g.*, be administered in combination with one or more therapies selected from the group consisting of 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 each of the foregoing methods, the subject can, *e.g.,* be a human, *e.g.,* a pediatric human, or an animal.

[0038] In each of the foregoing methods, the effective amount of the recombinant virus may, *e.g.,* be identified by measuring an immune response to an antigen in the subject. 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 at the injection site.

[0039] In another aspect, the invention provides a method of expressing two or more therapeutic transgenes in a target cell. The method comprises exposing the cell to an effective amount of the recombinant adenovirus described herein to express the target transgenes.

[0040] These and other aspects and advantages of the invention are illustrated by the following figures, detailed description and claims.

DESCRIPTION OF THE DRAWINGS

[0041] The invention can be more completely understood with reference to the following drawings.

[0042] **FIGURE 1** depicts staining of ADS-12 cells for mouse CD80 or mouse CD137L two days following infection with the indicated virus at a multiplicity of infection (MOI) of 5.

[0043] **FIGURE 2** depicts staining of ADS-12 cells for mouse CD80 or mouse CD137L two days following infection with the indicated virus at a multiplicity of infection (MOI) of 5.

[0044] **FIGURE 3** depicts staining of 4T1 cells for mouse CD80 or mouse CD137L three days following infection with the indicated virus.

[0045] **FIGURE 4** depicts staining of 4T1 cells for mouse CD80 or mouse CD137L three days following infection with the indicated virus.

[0046] **FIGURE 5** depicts staining of non-cancerous (WI-38 and MRC5) or cancerous (A549) cells for human CD80 or human CD137L two days following infection with the indicated virus at a MOI of 2.

[0047] **FIGURE 6** depicts staining of A549 cells for human CD80 or human CD137L two days following infection with the indicated virus at a MOI of 5.

[0048] **FIGURE 7** depicts crystal violet staining of non-cancerous (WI-38 and MRC5) or cancerous (A549) cells at the indicated timepoints with or without infection with the TAV-hCD80-hCD137L virus at a MOI of 10.

[0049] **FIGURE 8** depicts crystal violet staining of ADS-12 cells at the indicated timepoints with or without infection with the indicated virus at a MOI of 10.

[0050] **FIGURE 9** depicts replication of the indicated viruses in ADS cells as determined by plaque assays.

[0051] **FIGURE 10** depicts mean tumor volume (± SEM) of subcutaneous ADS-12 tumors in mice following treatment with three intratumoral injections of $5 \cdot 10^7$ PFU of the indicated virus on days 0, 4, and 8 (n=10). Tumor volumes were estimated as length · width^2/2.

[0052] **FIGURE 11** depicts tumor volumes of subcutaneous ADS-12 tumors in mice following treatment with three intratumoral injections of $1 \cdot 10^7$ PFU of the indicated virus on days 0, 4, and 8 (n=3). Tumor volumes were estimated as length · width^2/2.

[0053] **FIGURE 12** depicts mean tumor volume (± SEM) of orthotopic 4T1 tumors in the mammary fat pad of mice following treatment with three intratumoral injections of $5 \cdot 10^7$ PFU of the indicated virus on days 0, 4, and 8 (n=10). Tumor volumes were estimated as length · width^2/2.

[0054] **FIGURE 13** depicts staining of ADS-12 cells for murine CD80, murine CD137L, and murine ICAM-1 four days following infection with the indicated virus at a MOI of 10.

[0055] **FIGURE 14** depicts staining of F244 cells for murine CD80, murine CD137L, and murine ICAM-1 three days following infection with the indicated virus at a MOI of 5.

[0056] **FIGURE 15** depicts staining of HT29 cells for murine CD80, murine CD137L, and murine ICAM-1 three days following infection with the indicated virus at a MOI of 5.

[0057] **FIGURE 16** depicts tumor volumes of 129S4 mice carrying subcutaneous ADS-12 tumors treated with intratumoral injections of either buffer (**FIGURE 16A**), TAV-mCD80-137L (**FIGURE 16B**), or TAV-mCD80-137L-ICAM (**FIGURE 16C**). Each treatment was dosed every

four days at 1 x 10^9 PFU per dose for a total of three doses. Each line represents the tumor volume of an individual mouse, with 10 mice per each treatment group.

DETAILED DESCRIPTION

[0058] The invention is based, in part, upon the discovery that adenoviruses such as oncolytic viruses, unexpectedly can efficiently express, when inserted into particular insertion sites, multiple (two or more) therapeutic transgenes without the use of an exogenous promoter and that the viruses can replicate and efficiently express the two or more therapeutic transgenes despite the size of the transgenes incorporated into the viral genome.

[0059] Accordingly, in one aspect the invention provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein 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: 23) 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: 23); and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site 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: 23) 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: 23). 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. The term "transgene" refers to an exogenous gene or polynucleotide sequence. The term "therapeutic transgene" refers to a

transgene, which when replicated and/or expressed in or by the virus imparts a therapeutic effect in a target cell, body fluid, tissue, organ, physiological system, or subject.

[0060] 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: 23) 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: 23). 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: 23). In certain embodiments, the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the first therapeutic transgene is inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, and TCACCAGG (SEQ ID NO: 2). CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2) define unique boundary sequences for the E1b-19K insertion site within the Ad5 genome (SEQ ID NO: 23). Throughout the description and claims, a deletion adjacent to a site, for example, a deletion adjacent to a start site of a gene or a deletion adjacent to 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.

[0061] In certain embodiments, the E3 insertion site 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 insertion site 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: 23) 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: 23). In certain embodiments, the E3 insertion site 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 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K, *e.g.,* the E3 insertion site comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain embodiments, the E3 insertion site comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain embodiments, the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the second therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4) define unique boundary sequences for an E3 insertion site within the Ad5 genome (SEQ ID NO: 23).

[0062] 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: 23) 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: 23). 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: 23). In certain embodiments, the second therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the second therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the second therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30). TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30) define unique boundary sequences for an E3 insertion site within the Ad5 genome (SEQ ID NO: 23).

[0063] In another aspect, the invention provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site, wherein the E1b-19k insertion site is located between the start 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: 23) 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: 23), and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

[0064] 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: 23) 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: 23). 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: 23). In certain embodiments, the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome. In certain embodiments, the first and second therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the IRES, the second therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

[0065] 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: 23) 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: 23). In certain embodiments, the E3 deletion site 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: 23) 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: 23). 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: 23).

[0066] 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: 23) 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: 23). 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: 23).

[0067] In certain embodiments, the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene. The third therapeutic transgene may be inserted into the E1b-19k insertion site wherein, *e.g.*, the second nucleotide sequence and the third nucleotide sequence are separated by a second internal ribosome entry site (IRES). In certain embodiments, the first, second, and third therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, the second IRES, the third therapeutic transgene, and TCACCAGG (SEQ ID NO: 2). The third therapeutic transgene may also be inserted into the E3 deletion site, *i.e.,* in certain embodiments the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into an E3 insertion site. In certain

embodiments, the third therapeutic transgene is inserted between nucleotides corresponding to 29772 and 30837 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the third therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the third therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). In certain embodiments, the third therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain embodiments, the third therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the third therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

[0068] The IRES may, *e.g.,* be selected from the group consisting of the encephalomyocarditis virus IRES, the foot-and-mouth disease virus IRES, and the poliovirus IRES.

[0069] In certain embodiments, in any of the foregoing viruses, the recombinant adenovirus further 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: 23) and the right inverted terminal repeat (ITR; *e.g.,* corresponding to nucleotides 35836-35938 of SEQ ID NO: 23). 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: 23). 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: 23).

[0070] In certain embodiments, a recombinant adenovirus of the invention is an oncolytic virus, *e.g.*, a virus that exhibits tumor-selective replication and/or viral mediated lysis. In certain embodiments, a recombinant adenovirus of the invention exhibits selective expression of a therapeutic transgene in a hyperproliferative cell, *e.g.*, a cancer cell, relative to a non-hyperproliferative cell. In certain embodiments, the expression of a 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 of the gene in the hyperproliferative cell. In certain embodiments, the virus exhibits no detectable expression of a 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.

[0071] 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.

[0072] Features of recombinant adenoviruses of the invention, *e.g.,* the lack of exogenous promoters, may allow for the expression of additional therapeutic transgenes or larger therapeutic transgenes relative to other recombinant adenoviruses. For example, in certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgenes, the first, second, and/or third therapeutic transgenes, or all of the therapeutic transgenes are not operably linked to an exogenous promoter sequence. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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 about 5000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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.

[0073] In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise 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, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 1650 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides. In certain embodiments, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 3100 nucleotides.

[0074] In certain embodiments, the recombinant adenovirus comprises SEQ ID NO: 14, 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: 14.

[0075] 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.

[0076] The invention also provides an adenovirus type 5 vector that expresses one or more therapeutic transgenes, in particular, immunomodulatory transgenes in E1, E3 and E4 sites, and right and left orientations. As used herein "immunomodulatory" refers to a therapeutic transgene that modulates the function of the immune system of a subject. Immunomodulatory transgenes may modulate the function of, *e.g.,* B-cells, T cells and/or the production of antibodies. Exemplary immunomodulatory transgenes include checkpoint inhibitors. Exemplary immunomodulatory transgenes may include, *e.g.,* PD-1, or PD-L1, or any transgene that modulates the activity thereof. Further exemplary immunomodulatory transgenes may include an anti PD-1 antibody, or anti-PD-L1 antibody. Certain immunomodulatory transgenes may comprise peptide linkers, *e.g.*, peptide linkers from 2 to 5000 or more amino acids in length that may be immunogenic, *i.e.*, that are vulnerable to neutralizing antibodies. It is contemplated that the immunogenicity of such linkers may be reduced by replacing the immunogenic sequences with non-immunogenic sequences.

[0077] The invention further provides methods of treatment comprising administering a disclosed recombinant adenovirus in combination with antibodies that, *e.g.,* block immune checkpoints or improve antigen presentation/engulfment of antigens and/or/enhance tumor-specific T-cell responsiveness.

I. Viruses

[0078] 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.

[0079] 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: 23.

[0080] 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.

[0081] 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.

[0082] In certain embodiments, the virus 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.

[0083] In certain embodiments, the modification of a regulatory sequence or promoter comprises a modification of 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 additional modified regulatory sequence enhances expression in neoplastic cells, but attenuates expression in normal cells.

[0084] 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 the modified regulatory sequence.

[0085] 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.

[0086] In certain embodiments, at least one of these seven binding sites, or a functional portion thereof, is deleted. A "functional portion" is a portion of the binding site that, when deleted, decreases or even eliminates the functionality, *e.g.* binding affinity, of the binding site to its respective transcription factor (Pea3 or E2F) by, for example, at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% relative to the complete sequence. In certain embodiments, one or more entire binding sites are deleted. In certain embodiments, a functional portion of one or more binding sites is deleted. A "deleted binding site" encompasses both the deletion of an entire binding site and the deletion of a functional portion. When two or more binding sites are deleted, any combination of entire binding site deletion and functional portion deletion may be used.

[0087] In certain embodiments, at least one Pea3 binding site, or a functional portion thereof, 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 portion thereof, is retained.

[0088] In certain embodiments, at least one E2F binding site, or a functional portion thereof, is deleted. In certain embodiments, at least one E2F binding site, or a functional portion thereof, 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, or functional portions thereof. 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: 23), 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: 28).

[0089] 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 adenovirus has a nucleotide sequence encoding a therapeutic transgene inserted into an E1b-19K insertion site. In certain embodiments, the adenovirus comprises a nucleotide sequence encoding a therapeutic transgene inserted into an E1b-19K insertion site, wherein 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: 23) 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: 23).

II. Methods of Viral Production

[0090] 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).

[0091] 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.

[0092] 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

[0093] A disclosed recombinant adenovirus may comprise a nucleotide sequence that encodes for a therapeutic transgene. In certain embodiments, a disclosed recombinant comprise virus may comprise a first nucleotide sequence and a second nucleotide sequence that encode for a first and a second therapeutic transgene, respectively. In certain embodiments, a disclosed recombinant comprise virus may comprise a first nucleotide sequence, a second nucleotide sequence, and a third nucleotide sequence that encode for a first, second, and third therapeutic transgene, respectively.

[0094] 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 oncoprotein, tumor suppressor peptide or polypeptide, enzyme, cytokine, immune modulating peptide or polypeptide, antibody, lytic peptide, vaccine antigen, a peptide or polypeptide which complements genetic defects in somatic cells, or a peptide or polypeptide which catalyzes processes leading to cell death.

[0095] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, p40, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

[0096] In certain embodiments, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23, IL-23A/p19, p40, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin,

angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

[0097] In certain embodiments, the first therapeutic transgene encodes CD80, and/or the second therapeutic transgene encodes CD137L. In further embodiments, the first therapeutic transgene encodes CD137L, and/or the second therapeutic transgene encodes CD80. CD80 is a costimulatory molecule that can play a role in activating naive CD8+ T cells. CD8+ T cells are activated when the T cell receptor (TCR) binds to a class I major histocompatibility complex (MHC) on an antigen presenting cell (APC) presenting a peptide that the TCR recognizes. In addition to the TCR – MHC interaction, the T cell must also receive a costimulatory signal through a CD28 molecule on the T cell binding to either CD80 or CD86 on the APC. The T cell can then become activated, dividing and gaining the ability to mount a response against other cells that display the same peptide. Activation also leads to expression of other molecules including CTLA-4 and CD137 on the T cell. CTLA-4 binds to CD80 with higher affinity than CD28, and CTLA-4 binding to CD80 leads to inactivation of the T cell. CD137 binds to CD137L, and upon binding it further activates the T cell and promotes cell division and persistence of an immune response.

[0098] In certain embodiments the first and/or second therapeutic transgenes are selected from the group consisting of CD80 and CD137L, *e.g.,* the first therapeutic transgene encodes CD80 and the second therapeutic transgene encodes CD137L. An exemplary nucleotide sequence encoding human CD80 is depicted in SEQ ID NO: 5, and an exemplary nucleotide sequence encoding human CD137L is depicted in SEQ ID NO: 7. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, and/or SEQ ID NO: 7, or 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: 5, and/or SEQ ID NO: 7. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 6, and/or SEQ ID NO: 8, or 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: 6, and/or SEQ ID NO: 8. In certain embodiments, the recombinant

adenovirus comprises the nucleotide sequence of SEQ ID NO: 27, or 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: 27.

[0099] In certain embodiments, in any of the foregoing viruses, the first, second, and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1., *e.g.,* the first therapeutic transgene encodes CD80, the second therapeutic transgene encodes CD137L, and the third therapeutic transgene encodes ICAM-1. An exemplary nucleotide sequence encoding human ICAM1 is depicted in SEQ ID NO: 32. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, SEQ ID NO: 7, and/or SEQ ID NO: 32, or 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: 5, SEQ ID NO: 7, and/or SEQ ID NO: 32. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 31, SEQ ID NO: 9, or SEQ ID NO: 22, or 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: 31, SEQ ID NO: 9, or SEQ ID NO: 22.

[00100] In certain embodiments, the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine. For example, in certain embodiments the first and/or second therapeutic transgenes are selected from the group consisting of IL-23A/p19 and p40, which make up the heterodimeric cytokine IL-23. For example, the first therapeutic transgene may encode IL-23A/p19 and the second therapeutic transgene may encode p40. An exemplary nucleotide sequence encoding human IL-23A/p19 is depicted in SEQ ID NO: 12, and an exemplary nucleotide sequence encoding human p40 is depicted in SEQ ID NO: 10. In certain embodiments, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 12 and/or SEQ ID NO: 10, or 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: 12 and/or SEQ ID NO: 10. In certain embodiments, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 13, or 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: 13.

[00101] Additionally, in certain embodiments, the first and/or second therapeutic transgenes are selected from the group consisting of IL-27A/p28 and IL-27B/EBI3, which make up the heterodimeric cytokine IL-27. For example, the first therapeutic transgene may encode IL- IL-27A/p28 and the second therapeutic transgene may encode IL-27B/EBI3.

[00102] When tumors grow beyond approximately 2 mm^3 in diameter, they require the proliferation of an independent network of blood vessels to supply nutrients and oxygen and remove waste products. This new vessel formation, *i.e.,* neovascularization, is known as tumor angiogenesis. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), interleukin 8 (IL-8), and the angiopoietins. Endostatin and angiostatin are naturally occurring anti-angiogenic proteins that are reported to inhibit neovascularization.

[00103] In certain embodiments, the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin. In certain embodiments, the first therapeutic transgene is endostatin and the second therapeutic transgene is angiostatin. In certain embodiments, the first therapeutic transgene is angiostatin and the second therapeutic transgene is endostatin.

[00104] Endostatin is a proteolytic fragment of collagen XVIII. An exemplary human collagen XVIII amino acid sequence, corresponding to NCBI Reference Sequence NP_085059.2, is depicted in SEQ ID NO: 33. Endostatin can result from proteolytic cleavage of collagen XVIII at different sites. The non-collagenous 1 (NC1) domain at the C-terminus of collagen XVIII is generally considered responsible for the anti-angiogenic effects of endostatin. An exemplary human collagen XVIII NC1 domain amino acid sequence is depicted in SEQ ID NO: 37. Accordingly, as used herein, the term "endostatin" is understood to mean a protein comprising the amino acid sequence of SEQ ID NO: 37, or comprising an amino acid sequence having greater than 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 37, or a fragment of any of the forgoing that is capable of noncovalently oligomerizing into trimers, for example, through an association domain present in SEQ ID NO: 37. Oligomerization can be assayed by any method known in the art, including, for example, size exclusion chromatography, analytical ultracentrifugation, scattering techniques,

NMR spectroscopy, isothermal titration calorimetry, fluorescence anisotropy and mass spectrometry.

[00105] In certain embodiments, a disclosed recombinant virus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 37 or SEQ ID NO: 38, or 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: 37 or SEQ ID NO: 38.

[00106] Angiostatin is a proteolytic fragment of plasminogen. An exemplary human plasminogen amino acid sequence, corresponding to NCBI Reference Sequence NP_000292.1, is depicted in SEQ ID NO: 34.

[00107] Angiostatin can result from proteolytic cleavage of plasminogen at different sites. Plasminogen has five kringle domains, which are generally considered responsible for the anti-angiogenic effects of angiostatin. An exemplary amino acid sequence of the first kringle domain of human plasminogen is depicted in SEQ ID NO: 39, an exemplary amino acid sequence of the second kringle domain of human plasminogen is depicted in SEQ ID NO: 40, an exemplary amino acid sequence of the third kringle domain of human plasminogen is depicted in SEQ ID NO: 41, an exemplary amino acid sequence of the fourth kringle domain of human plasminogen is depicted in SEQ ID NO: 42, and an exemplary amino acid sequence of the fifth kringle domain of human plasminogen is depicted in SEQ ID NO: 43. Accordingly, as used herein, the term "angiostatin" is understood to mean a protein comprising the amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, or SEQ ID NO: 43, or comprising an amino acid sequence having greater than 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, or SEQ ID NO: 43, or a fragment of any of the foregoing that is capable of antagonizing endothelial cell migration and/or endothelial cell proliferation. Endothelial cell migration and/or proliferation can be assayed by any method known in the art, including, for example, those described in Guo *et al.* (2014) METHODS MOL. BIOL. 1135: 393-402.

[00108] In certain embodiments, a disclosed recombinant virus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44 or 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: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

[00109] In certain embodiments, a disclosed recombinant virus comprises the nucleotide sequence of SEQ ID NO: 11, or 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: 11.

IV. Methods of Treatment

[00110] For therapeutic use, a recombinant adenovirus is preferably is 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.

[00111] Pharmaceutical compositions containing recombinant adenoviruses disclosed herein 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. A preferred route of administration for fusion proteins is IV infusion. 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.

[00112] 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.

[00113] 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.

[00114] The term "effective amount" as used herein refers to the amount of an active component (*e.g.*, the amount of a recombinant adenovirus of the present invention) 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.

[00115] 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 antibody, 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 antibody, and the disease being treated. Exemplary dosing frequencies are once per day, once per week and once every two weeks. A preferred route of administration is parenteral, *e.g.*, intravenous infusion. Formulation of monoclonal antibody-based drugs is within ordinary skill in the art. In certain embodiments, a recombinant adenovirus is lyophilized, and then reconstituted in buffered saline, at the time of administration.

[00116] The recombinant adenoviruses disclosed herein can be used to treat various medical indications. For example, the recombinant adenoviruses can be used to treat cancers. The cancer cells are exposed to a therapeutically effective amount of the recombinant adenovirus so as to inhibit or reduce proliferation of the cancer cells. The invention provides a method of treating a cancer in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus of the invention either alone or in a combination with another therapeutic agent to treat the cancer in the subject. In certain embodiments, administering an effective amount of a recombinant adenovirus to a subject reduces tumor load in that subject by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%.

[00117] 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.

[00118] 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).

[00119] In certain embodiments, the cancer is selected from the group consisting of 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, and pancreatic cancer.

[00120] 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.

[00121] In certain embodiments, a recombinant adenovirus of the invention is administered in combination with a tyrosine kinase inhibitor, *e.g.*, erlotinib.

[00122] In certain embodiments, a recombinant adenovirus of the invention 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.).

[00123] 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.

[00124] 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. Hyperproliferative diseases, *e.g.*, 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.

[00125] 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.

[00126] 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.

[00127] 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.

[00128] 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.

[00129] 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.

[00130] 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.

[00131] 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.

[00132] 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.

[00133] 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

[00134] The following Examples are merely illustrative and are not intended to limit the scope or content of the invention in any way.

Example 1: Construction Of A CD80 And CD137L Expressing Adenovirus

[00135] This Example describes the production of a recombinant adenovirus type 5 (Ad5) that expresses the murine forms of CD80 and CD137L.

[00136] 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.

[00137] 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 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: 15).

[00138] TAV was further modified to carry the dl309 disruption of the E3 region's RIDα, RIDβ, and 14.7k genes The nucleotide sequence of the modified E3 region is as follows, with the hyphen indicating the point of deletion:

TCTTTTCTCTTACAGTATGA-TAATAAAAAAAAATAATAAAGCATCACTTAC (SEQ ID NO: 16).

[00139] The resulting virus, including both the modified E1b-19k region and the modified E3 region is hereafter referred to as TAV- Δ19k.

[00140] Where indicated, murine CD80 (mCD80) or human CD80 (hCD80) was cloned into the modified E1b-19k region.

[00141] The sequence of mCD80 in the modified E1b-19k region is as follows, with the coding region in lower case, and the flanking adenoviral sequences including the SalI and XhoI sites capitalized:

ATCTGACCTCGTCGACatggcttgcaattgtcagttgatgcaggatacaccactcctcaagtttcc
atgtccaaggctcattcttctctttgtgctgctgattcgtctttcacaagtgtcttcagatgttga
tgaacaactgtccaagtcagtgaaagataaggtattgctgccttgccgttacaactctcctcatga
agatgagtctgaagaccgaatctactggcaaaaacatgacaaagtggtgctgtctgtcattgctgg
gaaactaaaagtgtggcccgagtataagaaccggactttatatgacaacactacctactctcttat
catcctgggcctggtcctttcagaccggggcacatacagctgtgtcgttcaaaagaaggaaagagg
aacgtatgaagttaaacacttggctttagtaaagttgtccatcaaagctgacttctctaccccaa
cataactgagtctggaaacccatctgcagacactaaaaggattacctgctttgcttccgggggttt
cccaaagcctcgcttctcttggttggaaaatggaagagaattacctggcatcaatacgacaatttc
ccaggatcctgaatctgaattgtacaccattagtagccaactagatttcaatacgactcgcaacca
caccattaagtgtctcattaaatatggagatgctcacgtgtcagaggacttcacctgggaaaaacc
cccagaagaccctcctgatagcaagaacacacttgtgctctttggggcaggattcggcgcagtaat
aacagtcgtcgtcatcgttgtcatcatcaaatgcttctgtaagcacagaagctgtttcagaagaaa

tgaggcaagcagagaaacaaacaacagccttaccttcgggcctgaagaagcattagctgaacagac
cgtcttcctttagCTCGAGTCACCAGGCG (SEQ ID NO: 17).

[00142] The sequence of hCD80 in the modified E1b-19k region is as follows, with the coding region in lower case, and the flanking adenoviral sequences including the SalI and XhoI sites capitalized:

GCGCCGTGGGCTAATCTTGGTTACATCTGACCTCGTCGACatgggccacacacggaggcagggaac
atcaccatccaagtgtccatacctcaatttctttcagctcttggtgctggctggtctttctcactt
ctgttcaggtgttatccacgtgaccaaggaagtgaaagaagtggcaacgctgtcctgtggtcacaa
tgtttctgttgaagagctggcacaaactcgcatctactggcaaaaggagaagaaaatggtgctgac
tatgatgtctggggacatgaatatatggcccgagtacaagaaccggaccatctttgatatcactaa
taacctctccattgtgatcctggctctgcgcccatctgacgagggcacatacgagtgtgttgttct
gaagtatgaaaaagacgctttcaagcgggaacacctggctgaagtgacgttatcagtcaaagctga
cttccctacacctagtatatctgactttgaaattccaacttctaatattagaaggataatttgctc
aacctctggaggttttccagagcctcacctctcctggttggaaaatggagaagaattaaatgccat
caacacaacagtttcccaagatcctgaaactgagctctatgctgttagcagcaaactggatttcaa
tatgacaaccaaccacagcttcatgtgtctcatcaagtatggacatttaagagtgaatcagacctt
caactggaatacaaccaagcaagagcattttcctgataacctgctcccatcctgggccattacctt
aatctcagtaaatggaatttttgtgatatgctgcctgacctactgctttgccccaagatgcagaga
gagaaggaggaatgagagattgagaagggaaagtgtacgccctgtataaCTCGAGTCACCAGGCGC
TTTTCCAAGAGAAGGTCATCAAG (SEQ ID NO: 18).

[00143] Where indicated murine CD137L (mCD137L) or human CD137L (hCD137L) were cloned into the modified E3 region.

[00144] The sequence of mCD137L in the modified E3 region is as follows, with the coding region in lower case, and the flanking adenoviral sequences capitalized:

ATGTTCTTTTCTCTTACAGTATGATTAAATGAGACatggaccagcacacacttgatgtggaggata
ccgcggatgccagacatccagcaggtacttcgtgcccctcggatgcggcgctcctcagagataccg
ggctcctcgcggacgctgcgctcctctcagatactgtgcgccccacaaatgccgcgctccccacgg
atgctgcctaccctgcggttaatgttcgggatcgcgaggccgcgtggccgcctgcactgaacttct
gttcccgccacccaaagctctatggcctagtcgctttggttttgctgcttctgatcgccgcctgtg
ttcctatcttcacccgcaccgagcctcggccagcgctcacaatcaccacctcgcccaacctgggta
cccgagagaataatgcagaccaggtcacccctgtttcccacattggctgccccaacactacacaac
agggctctcctgtgttcgccaagctactggctaaaaaccaagcatcgttgtgcaatacaactctga
actggcacagccaagatggagctgggagctcatacctatctcaaggtctgaggtacgaagaagaca
aaaaggagttggtggtagacagtcccgggctctactacgtatttttggaactgaagctcagtccaa
cattcacaaacacaggccacaaggtgcagggctgggtctctcttgttttgcaagcaaagcctcagg
tagatgactttgacaacttggccctgacagtggaactgttcccttgctccatggagaacaagttag
tggaccgttcctggagtcaactgttgctcctgaaggctggccaccgcctcagtgtgggtctgaggg
cttatctgcatggagcccaggatgcatacagagactgggagctgtcttatcccaacaccaccagct

ttggactctttcttgtgaaacccgacaacccatgggaatgaGGTCTCAAAGATCTTATTCCCTTTA
ACTAATAAA (SEQ ID NO: 19).

[00145] The sequence of hCD137L in the modified E3 region is as follows, with the coding region in lower case, and the flanking adenoviral sequences capitalized:

ATGTTCTTTTCTCTTACAGTATGATTAAATGAGACatggaatacgcctctgacgcttcactggacc
ccgaagccccgtggcctcctgcacctcgcgctcgcgcctgccgcgtactgccttgggccctggtcg
cggggctgctgctcctgctcctgctcgctgctgcatgcgctgtatttcttgcatgcccatgggctg
tgtctggggctcgcgcatcacctggctccgcggccagcccgagactccgcgagggtcccgagcttt
cgcccgacgatcccgccggcctcttggacctgcggcagggcatgtttgcgcagctggtggcccaaa
atgttctgctgatcgatgggcccctgagctggtacagtgacccaggcctggcaggcgtgtccctga
cgggggcctgagctacaaagaggacacgaaggagctggtggtggccaaggctggagtctactatg
tcttctttcaactagagctgcggcgcgtggtggccggcgagggctcaggctccgtttcacttgcgc
tgcacctgcagccactgcgctctgctgctggggccgccgccctggctttgaccgtggacctgccac
ccgcctcctccgaggctcggaactcggccttcggtttccagggccgcttgctgcacctgagtgccg
gccagcgcctgggcgtccatcttcacactgaggccagggcacgccatgcctggcagcttacccagg
gcgccacagtcttgggactcttccgggtgacccccgaaatcccagccggactcccttcaccgaggt
cggaataaGGTCTCAAAGATCTTATTCCCTTTAACTAATAAA (SEQ ID NO: 20).

[00146] Additionally, where indicated, both human CD80 and CD137L were cloned into the modified E1b-19k region, separated by an internal ribosome entry site (IRES). In these instances, the E1b-19k region contained the human CD80 gene including a stop codon, followed by the IRES from encephalomyocarditis virus, followed by the human CD137L gene. Because the insertion of both the CD80 and CD137L genes in the E1b-19k region would make the viral genome size exceed the packaging limits for an adenovirus, this virus still has the RIDα, RIDβ, and 14.7k gene deletion in the E3 region.

[00147] The sequence of hCD80 and hCD137L in the modified E1b-19k region, separated by IRES, is as follows, with the coding region in lower case, the flanking adenoviral sequences capitalized, and the central IRES capitalized:

[00148] GCGCCGTGGGCTAATCTTGGTTACATCTGACCTCGTCGACatgggccacacacggagg
cagggaacatcaccatccaagtgtccatacctcaatttctttcagctcttggtgctggctggtctt
tctcacttctgttcaggtgttatccacgtgaccaaggaagtgaaagaagtggcaacgctgtcctgt
ggtcacaatgtttctgttgaagagctggcacaaactcgcatctactggcaaaaggagaagaaaatg
gtgctgactatgatgtctggggacatgaatatatggcccgagtacaagaaccggaccatctttgat
atcactaataacctctccattgtgatcctggctctgcgcccatctgacgagggcacatacgagtgt
gttgttctgaagtatgaaaaagacgctttcaagcgggaacacctggctgaagtgacgttatcagtc
aaagctgacttccctacacctagtatatctgactttgaaattccaacttctaatattagaaggata

atttgctcaacctctggaggttttccagagcctcacctctcctggttggaaaatggagaagaatta
aatgccatcaacacaacagtttcccaagatcctgaaactgagctctatgctgttagcagcaaactg
gatttcaatatgacaaccaaccacagcttcatgtgtctcatcaagtatggacatttaagagtgaat
cagaccttcaactggaatacaaccaagcaagagcattttcctgataacctgctcccatcctgggcc
attaccttaatctcagtaaatggaatttttgtgatatgctgcctgacctactgctttgccccaaga
tgcagagagagaaggaggaatgagagattgagaagggaaagtgtacgccctgtataaTAACGTTAC
TGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCC
GTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCT
TTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGC
TTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAG
GTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCA
CGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCT
GAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTAC
ATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGA
AAAACACGATGATAATatggaatacgcctctgacgcttcactggaccccgaagccccgtggcctcc
tgcacctcgcgctcgcgcctgccgcgtactgccttgggccctggtcgcggggctgctgctcctgct
cctgctcgctgctgcatgcgctgtatttcttgcatgcccatgggctgtgtctggggctcgcgcatc
acctggctccgcggccagcccgagactccgcgagggtcccgagctttcgcccgacgatcccgccgg
cctcttggacctgcggcagggcatgtttgcgcagctggtggcccaaaatgttctgctgatcgatgg
gcccctgagctggtacagtgacccaggcctggcaggcgtgtccctgacgggggcctgagctacaa
agaggacacgaaggagctggtggtggccaaggctggagtctactatgtcttctttcaactagagct
gcggcgcgtggtggccggcgagggctcaggctccgtttcacttgcgctgcacctgcagccactgcg
ctctgctgctggggccgccgccctggctttgaccgtggacctgccacccgcctcctccgaggctcg
gaactcggccttcggtttccagggccgcttgctgcacctgagtgccggccagcgcctgggcgtcca
tcttcacactgaggccagggcacgccatgcctggcagcttacccagggcgccacagtcttgggact
cttccgggtgacccccgaaatcccagccggactcccttcaccgaggtcggaataaCTCGAGTCACC
AGGCGCTTTTCCAAGAGAAGGTCATCAAG (SEQ ID NO: 21).

[00149] Details of the viruses tested are shown in **TABLE 1.**

TABLE 1

Virus	E1A Promoter	E1b-19k Modification	E3 (RIDα, RIDβ, and 14.7k) Modification
TAV-Δ19k	TAV-255	Deleted	Disrupted (containing the dl309 sequence)
TAV-mCD80	TAV-255	Deleted and Replaced with murine CD80	Disrupted (containing the dl309 sequence)
TAV-mCD137L	TAV-255	Deleted	Deleted and Replaced with murine CD137L
TAV-mCD80-137L	TAV-255	Deleted and Replaced with murine CD80	Deleted and Replaced with murine CD137L
TAV-hCD80-137L	TAV-255	Deleted and Replaced with human CD80	Deleted and Replaced with human CD137L

TAV-hCD80-IRES-137L	TAV-255	Deleted and Replaced with human CD80, IRES, and human CD137L	Deleted

Example 2: CD80 and CD137L Gene Expression

[00150] This example describes the expression of CD80 and/or CD137L from the recombinant adenoviruses produced as described in Example 1.

[00151] ADS-12 cells (mouse lung adenocarcinoma cells) were infected with the TAV-Δ19k, TAV-mCD80. TAV-mCD137L, and TAV-mCD80-137L viruses, and infected cells were stained for CD80 and CD137L with immunocytochemistry. As depicted in **FIGURE 1** and **FIGURE 2**, mCD80 was expressed after infection with either TAV-mCD80 or TAV-mCD80-137L, and CD137L was expressed after infection with either TAV-mCD137L or TAV-mCD80-137L. Importantly, both genes were expressed with the TAV-mCD80-137L virus, demonstrating that the single virus drove expression of two therapeutic genes.

[00152] 4T1 cells (mouse mammary carcinoma cells) were infected with the TAV-Δ19k and TAV-mCD80-137L viruses, and infected cells were stained for CD80 and CD137L with immunocytochemistry. As with the ADS-12 cells, both CD80 and CD137L were expressed after infection with TAV-mCD80-137L (**FIGURE 3** and **FIGURE 4**).

[00153] A549 cells (human lung carcinoma cells), WI-38 cells (non-cancerous human lung fibroblasts), and MRC5 cells (non-cancerous human lung fibroblasts) were infected with the TAV-Δ19k and TAV-hCD80-137L viruses, and infected cells were stained for CD80 and CD137L with immunocytochemistry. As depicted in **FIGURE 5**, the TAV-hCD80-137L virus induced expression of human CD80 and human CD137L in cancerous A549 cells with little to no expression in non-cancerous WI-38 and MRC5 cells. These results demonstrate that dual transgene expression can be achieved in human as well as murine cells, and that transgene expression can be selective for cancerous cells.

[00154] A549 cells (human lung carcinoma cells) were infected with the TAV-Δ19k and TAV-hCD80-IRES-137L viruses, and infected cells were stained for CD80 and CD137L with

immunocytochemistry. As depicted in **FIGURE 6**, the TAV-hCD80-IRES-137L virus induced expression of both human CD80 and human CD137L in cancerous A549 cells. These results demonstrate dual transgene expression can be achieved by inserting both transgenes into a single genome region, *e.g.*, the E1b-19k region, separated by an internal ribosome entry site (IRES).

Example 3: Cytotoxicity Of CD80 And CD137L Expressing Adenoviruses

[00155] This Example describes the cytotoxicity of CD80 and CD137L expressing recombinant adenoviruses produced as described in Example 1

[00156] A549 cells (human lung carcinoma cells), WI-38 cells (non-cancerous human lung fibroblasts), and MRC5 cells (non-cancerous human lung fibroblasts) were infected with the TAV-Δ19k and TAV-hCD80-137L viruses, and infected cells were stained with crystal violet, which stains viable cells blue, at the indicated time points after infection.

[00157] As depicted in **FIGURE 7**, TAV-hCD80-137L was lytic in A549 but not WI-38 or MRC5 cells. These results demonstrate that the TAV-hCD80-137L virus can selectively lyse cancerous cells compared to non-cancerous cells.

[00158] ADS-12 cells were infected with the TAV-Δ19k, TAV-mCD80, TAV-mCD137L, and TAV-mCD80-137L viruses, and infected cells were stained with crystal violet, which stains viable cells blue, at the indicated time points after infection. Results, depicted in **FIGURE 8**, demonstrate that the TAV-mCD80, TAV-mCD137L, and TAV-mCD80-137L viruses can selectively lyse cancerous cells compared to non-cancerous cells.

Example 4: Replication Of CD80 And CD137L Expressing Adenoviruses

[00159] This Example describes the replication in cells of CD80 and CD137L expressing recombinant adenoviruses produced as described in Example 1 in cancerous cells.

[00160] ADS cells were infected in triplicate with TAV-Δ19k, TAV-CD80, TAV-CD137L and TAV-CD80-137L viruses at a MOI of 1. Cells and media were harvested five days after infection and virus titer was determined by plaque assay.

[00161] As depicted in **FIGURE 9**, the viruses can effectively replicate in cancerous cells.

Example 5: Anti-Cancer Activity Of CD80 And CD137L Expressing Adenoviruses

[00162] This example describes the anti-cancer activity of CD80 and/or CD137L expressing recombinant adenoviruses produced as described in Example 1.

[00163] 129S4 mice carrying ADS-12 tumors were treated with three intratumoral injections of TAV-Δ19k, TAV-mCD80, TAV-mCD137L, or TAV-mCD80-137L. Results are depicted in **FIGURE 10**. Mice treated with TAV-mCD80 had comparable tumor growth to mice treated with TAV-Δ19k. Mice treated with TAV-mCD137L showed a trend toward smaller tumor size that did not reach statistical significance, and tumors of mice treated with TAV-mCD80-137L were significantly smaller. These results demonstrate that the dual-gene adenovirus expressing CD80 and 137L was most effective in reducing tumor size.

[00164] In a separate experiment, 129S4 mice carrying ADS-12 tumors were treated with three intratumoral injections of TAV-Δ19k, TAV-mCD80, TAV-mCD137L, or TAV-mCD80-137L. Results are depicted in **FIGURE 11**. Mice treated with TAV-mCD80-137L had smaller tumor size. These results demonstrate that the dual-gene adenovirus expressing CD80 and 137L was most effective in reducing tumor size.

[00165] BALB/c mice carrying 4T1 tumors orthotopically implanted in the mammary fat pad were treated with three intratumoral doses of TAV-Δ19k or TAV-mCD80-137L. Again, mice treated with TAV-mCD80-137L had significantly smaller tumors than mice treated with the control virus TAV-Δ19k (**FIGURE 12**).

Example 6: Construction Of A CD80, CD137L, And ICAM-1 Expressing Adenovirus

[00166] This Example describes the production of a recombinant adenovirus type 5 (Ad5) that expresses the murine forms of CD80, CD137L, and ICAM-1. ICAM-1 is an intracellular adhesion molecule that is expressed by antigen presenting cells (APCs) and stabilizes interactions between APCs and T-cells by binding to LFA1 on the T cell surface

[00167] 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.

[00168] 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 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: 15)

[00169] TAV was further modified to delete the adenoviral death protein (ADP), RIDα, RIDβ, and 14.7k genes from the E3 region. The nucleotide sequence of the modified E3 region is as follows, with the hyphen indicating the point of deletion:

TTATTGAGGAAAAGAAAATGCCTTAA-TAAAAAAAAATAATAAAGCATCACTTAC (SEQ ID NO: 24).

[00170] TAV was further modified to delete the E4 region except for E4-ORF6/7. The nucleotide sequence of the modified E4 region is as follows, with the hyphen indicating the point of deletion:

GAACGCCGGACGTAGTCAT-AACAGTCAGCCTTACCAGTAAA (SEQ ID NO: 25).

[00171] The protein coding region of murine CD80 (mCD80), followed by the EMCV IRES, followed by the protein coding region of murine CD137L (mCD137L), followed by the FMDV IRES, followed by the protein coding region of murine ICAM-1 (mICAM-1) was cloned in to the E1b-19k site. The resulting virus is hereafter referred to as TAV-mCD80-137L-ICAM.

[00172] The nucleotide sequence of the mCD80–EMCV IRES–137L–FMDV IRES–ICAM insert in the E1b-19k region is as follows, where the coding regions are capitalized, the IRESs are lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGGCTTGCAATTGTCAGTTGATGCAGGATACACCACTCCTCAAGTTTCC
ATGTCCAAGGCTCATTCTTCTCTTTGTGCTGCTGATTCGTCTTTCACAAGTGTCTTCAGATGTTGA
TGAACAACTGTCCAAGTCAGTGAAAGATAAGGTATTGCTGCCTTGCCGTTACAACTCTCCTCATGA
AGATGAGTCTGAAGACCGAATCTACTGGCAAAAACATGACAAAGTGGTGCTGTCTGTCATTGCTGG
GAAACTAAAAGTGTGGCCCGAGTATAAGAACCGGACTTTATATGACAACACTACCTACTCTCTTAT
CATCCTGGGCCTGGTCCTTTCAGACCGGGGCACATACAGCTGTGTCGTTCAAAAGAAGGAAAGAGG
AACGTATGAAGTTAAACACTTGGCTTTAGTAAAGTTGTCCATCAAAGCTGACTTCTCTACCCCCAA
CATAACTGAGTCTGGAAACCCATCTGCAGACACTAAAAGGATTACCTGCTTTGCTTCCGGGGGTTT
CCCAAAGCCTCGCTTCTCTTGGTTGGAAAATGGAAGAGAATTACCTGGCATCAATACGACAATTTC
CCAGGATCCTGAATCTGAATTGTACACCATTAGTAGCCAACTAGATTTCAATACGACTCGCAACCA
CACCATTAAGTGTCTCATTAAATATGGAGATGCTCACGTGTCAGAGGACTTCACCTGGGAAAAACC
CCCAGAAGACCCTCCTGATAGCAAGAACACACTTGTGCTCTTTGGGGCAGGATTCGGCGCAGTAAT
AACAGTCGTCGTCATCGTTGTCATCATCAAATGCTTCTGTAAGCACAGAAGCTGTTTCAGAAGAAA
TGAGGCAAGCAGAGAAACAAACAACAGCCTTACCTTCGGGCCTGAAGAAGCATTAGCTGAACAGAC
CGTCTTCCTTTAGtaacgttactggccgaagccgcttggaataaggccggtgtgcgtttgtctata
tgttattttccaccatattgccgtcttttggcaatgtgagggcccggaaacctggccctgtcttct
tgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgttgaatgtcgtga
aggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgacccctttgcaggcagc
ggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgcaa
aggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatggctctcct
caagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgtatgggatctgatctgg
ggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaacgtctaggccccccgaacca
cggggacgtggttttcctttgaaaaacacgatgataatATGGACCAGCACACACTTGATGTGGAGG
ATACCGCGGATGCCAGACATCCAGCAGGTACTTCGTGCCCCTCGGATGCGGCGCTCCTCAGAGATA
CCGGGCTCCTCGCGGACGCTGCGCTCCTCTCAGATACTGTGCGCCCCACAAATGCCGCGCTCCCCA
CGGATGCTGCCTACCCTGCGGTTAATGTTCGGGATCGCGAGGCCGCGTGGCCGCCTGCACTGAACT
TCTGTTCCCGCCACCCAAAGCTCTATGGCCTAGTCGCTTTGGTTTTGCTGCTTCTGATCGCCGCCT
GTGTTCCTATCTTCACCCGCACCGAGCCTCGGCCAGCGCTCACAATCACCACCTCGCCCAACCTGG
GTACCCGAGAGAATAATGCAGACCAGGTCACCCCTGTTTCCCACATTGGCTGCCCCAACACTACAC
AACAGGGCTCTCCTGTGTTCGCCAAGCTACTGGCTAAAAACCAAGCATCGTTGTGCAATACAACTC
TGAACTGGCACAGCCAAGATGGAGCTGGGAGCTCATACCTATCTCAAGGTCTGAGGTACGAAGAAG
ACAAAAAGGAGTTGGTGGTAGACAGTCCCGGGCTCTACTACGTATTTTTGGAACTGAAGCTCAGTC
CAACATTCACAAACACAGGCCACAAGGTGCAGGGCTGGGTCTCTCTTGTTTTGCAAGCAAAGCCTC
AGGTAGATGACTTTGACAACTTGGCCCTGACAGTGGAACTGTTCCCTTGCTCCATGGAGAACAAGT
TAGTGGACCGTTCCTGGAGTCAACTGTTGCTCCTGAAGGCTGGCCACCGCCTCAGTGTGGGTCTGA
GGGCTTATCTGCATGGAGCCCAGGATGCATACAGAGACTGGGAGCTGTCTTATCCCAACACCACCA
GCTTTGGACTCTTTCTTGTGAAACCCGACAACCCATGGGAATGAggtttccacaactgataaaact
cgtgcaacttgaaactccgcctggtctttccaggtctagaggggttacactttgtactgtgctcga
ctccacgcccggtccactggcgggtgttagtagcagcactgttgtttcgtagcggagcatggtggc
cgtgggaactcctccttggtgacaagggcccacggggccgaaagccacgtccagacggacccacca
tgtgtgcaaccccagcacggcaacttttactgcgaacaccaccttaaggtgacactggtactggta
ctcggtcactggtgacaggctaaggatgcccttcaggtaccccgaggtaacacgggacactcggga
tctgagaaggggattgggacttctttaaaagtgcccagtttaaaaagcttctacgcctgaataggc
gaccggaggccggcgcctttccattacccactactaaatccATGGCTTCAACCCGTGCCAAGCCCA
CGCTACCTCTGCTCCTGGCCCTGGTCACCGTTGTGATCCCTGGGCCTGGTGATGCTCAGGTATCCA

TCCATCCCAGAGAAGCCTTCCTGCCCCAGGGTGGGTCCGTGCAGGTGAACTGTTCTTCCTCATGCA
AGGAGGACCTCAGCCTGGGCTTGGAGACTCAGTGGCTGAAAGATGAGCTCGAGAGTGGACCCAACT
GGAAGCTGTTTGAGCTGAGCGAGATCGGGGAGGACAGCAGTCCGCTGTGCTTTGAGAACTGTGGCA
CCGTGCAGTCGTCCGCTTCCGCTACCATCACCGTGTATTCGTTTCCGGAGAGTGTGGAGCTGAGAC
CTCTGCCAGCCTGGCAGCAAGTAGGCAAGGACCTCACCCTGCGCTGCCACGTGGATGGTGGAGCAC
CGCGGACCCAGCTCTCAGCAGTGCTGCTCCGTGGGGAGGAGATACTGAGCCGCCAGCCAGTGGGTG
GGCACCCCAAGGACCCCAAGGAGATCACATTCACGGTGCTGGCTAGCAGAGGGGACCACGGAGCCA
ATTTCTCATGCCGCACAGAACTGGATCTCAGGCCGCAAGGGCTGGCATTGTTCTCTAATGTCTCCG
AGGCCAGGAGCCTCCGGACTTTCGATCTTCCAGCTACCATCCCAAAGCTCGACACCCCTGACCTCC
TGGAGGTGGGCACCCAGCAGAAGTTGTTTTGCTCCCTGGAAGGCCTGTTTCCTGCCTCTGAAGCTC
GGATATACCTGGAGCTGGGAGGCCAGATGCCGACCCAGGAGAGCACAAACAGCAGTGACTCTGTGT
CAGCCACTGCCTTGGTAGAGGTGACTGAGGAGTTCGACAGAACCCTGCCGCTGCGCTGCGTTTTGG
AGCTAGCGGACCAGATCCTGGAGACGCAGAGGACCTTAACAGTCTACAACTTTTCAGCTCCGGTCC
TGACCCTGAGCCAGCTGGAGGTCTCGGAAGGGAGCCAAGTAACTGTGAAGTGTGAAGCCCACAGTG
GGTCGAAGGTGGTTCTTCTGAGCGGCGTCGAGCCTAGGCCACCCACCCCGCAGGTCCAATTCACAC
TGAATGCCAGCTCGGAGGATCACAAACGAAGCTTCTTTTGCTCTGCCGCTCTGGAGGTGGCGGGAA
AGTTCCTGTTTAAAAACCAGACCCTGGAACTGCACGTGCTGTATGGTCCTCGGCTGGACGAGACGG
ACTGCTTGGGGAACTGGACCTGGCAAGAGGGGTCTCAGCAGACTCTGAAATGCCAGGCCTGGGGGA
ACCCATCTCCTAAGATGACCTGCAGACGGAAGGCAGATGGTGCCCTGCTGCCCATCGGGGTGGTGA
AGTCTGTCAAACAGGAGATGAATGGTACATACGTGTGCCATGCCTTTAGCTCCCATGGGAATGTCA
CCAGGAATGTGTACCTGACAGTACTGTACCACTCTCAAAATAACTGGACTATAATCATTCTGGTGC
CAGTACTGCTGGTCATTGTGGGCCTCGTGATGGCAGCCTCTTATGTTTATAACCGCCAGAGAAAGA
TCAGGATATACAAGTTACAGAAGGCTCAGGAGGAGGCCATAAAACTCAAGGGACAAGCCCCACCTC
CCTGACTCGAGTCACCAGGCG (SEQ ID NO: 26).

[00173] Additionally, the protein coding region of human CD80 (hCD80), followed by the EMCV IRES, followed by the protein coding region of human CD137L (hCD137L), followed by the FMDV IRES, followed by the protein coding region of human ICAM-1 (hICAM-1) is cloned in to the E1b-19k site. The resulting virus is hereafter referred to as TAV-hCD80-137L-ICAM.

[00174] The nucleotide sequence of the hCD80–EMCV IRES–137L–FMDV IRES–ICAM insert in the E1b-19k region is as follows, where the coding regions are capitalized, the IRESs are lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGGGCCACACACGGAGGCAGGGAACATCACCATCCAAGTGTCCATACCT
CAATTTCTTTCAGCTCTTGGTGCTGGCTGGTCTTTCTCACTTCTGTTCAGGTGTTATCCACGTGAC
CAAGGAAGTGAAAGAAGTGGCAACGCTGTCCTGTGGTCACAATGTTTCTGTTGAAGAGCTGGCACA
AACTCGCATCTACTGGCAAAAGGAGAAGAAAATGGTGCTGACTATGATGTCTGGGGACATGAATAT
ATGGCCCGAGTACAAGAACCGGACCATCTTTGATATCACTAATAACCTCTCCATTGTGATCCTGGC
TCTGCGCCCATCTGACGAGGGCACATACGAGTGTGTTGTTCTGAAGTATGAAAAAGACGCTTTCAA
GCGGGAACACCTGGCTGAAGTGACGTTATCAGTCAAAGCTGACTTCCCTACACCTAGTATATCTGA

CTTTGAAATTCCAACTTCTAATATTAGAAGGATAATTTGCTCAACCTCTGGAGGTTTTCCAGAGCC
TCACCTCTCCTGGTTGGAAAATGGAGAAGAATTAAATGCCATCAACACAACAGTTTCCCAAGATCC
TGAAACTGAGCTCTATGCTGTTAGCAGCAAACTGGATTTCAATATGACAACCAACCACAGCTTCAT
GTGTCTCATCAAGTATGGACATTTAAGAGTGAATCAGACCTTCAACTGGAATACAACCAAGCAAGA
GCATTTTCCTGATAACCTGCTCCCATCCTGGGCCATTACCTTAATCTCAGTAAATGGAATTTTTGT
GATATGCTGCCTGACCTACTGCTTTGCCCCAAGATGCAGAGAGAGAAGGAGGAATGAGAGATTGAG
AAGGGAAAGTGTACGCCCTGTATAAtaacgttactggccgaagccgcttggaataaggccggtgtg
cgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagggcccggaaacctg
gccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgt
tgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgaccc
tttgcaggcagcggaacccccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataag
atacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtca
aatggctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgtatgg
gatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaacgtctagg
ccccccgaaccacggggacgtggttttcctttgaaaaacacgatgataatATGGAATACGCCTCTG
ACGCTTCACTGGACCCCGAAGCCCCGTGGCCTCCTGCACCTCGCGCTCGCGCCTGCCGCGTACTGC
CTTGGGCCCTGGTCGCGGGGCTGCTGCTCCTGCTCCTGCTCGCTGCTGCATGCGCTGTATTTCTTG
CATGCCCATGGGCTGTGTCTGGGGCTCGCGCATCACCTGGCTCCGCGGCCAGCCCGAGACTCCGCG
AGGGTCCCGAGCTTTCGCCCGACGATCCCGCCGGCCTCTTGGACCTGCGGCAGGGCATGTTTGCGC
AGCTGGTGGCCCAAAATGTTCTGCTGATCGATGGGCCCCTGAGCTGGTACAGTGACCCAGGCCTGG
CAGGCGTGTCCCTGACGGGGGGCCTGAGCTACAAAGAGGACACGAAGGAGCTGGTGGTGGCCAAGG
CTGGAGTCTACTATGTCTTCTTTCAACTAGAGCTGCGGCGCGTGGTGGCCGGCGAGGGCTCAGGCT
CCGTTTCACTTGCGCTGCACCTGCAGCCACTGCGCTCTGCTGCTGGGGCCGCCGCCCTGGCTTTGA
CCGTGGACCTGCCACCCGCCTCCTCCGAGGCTCGGAACTCGGCCTTCGGTTTCCAGGGCCGCTTGC
TGCACCTGAGTGCCGGCCAGCGCCTGGGCGTCCATCTTCACACTGAGGCCAGGGCACGCCATGCCT
GGCAGCTTACCCAGGGCGCCACAGTCTTGGGACTCTTCCGGGTGACCCCCGAAATCCCAGCCGGAC
TCCCTTCACCGAGGTCGGAATAAggtttccacaactgataaaactcgtgcaacttgaaactccgcc
tggtctttccaggtctagaggggttacactttgtactgtgctcgactccacgcccggtccactggc
gggtgttagtagcagcactgttgtttcgtagcggagcatggtggccgtgggaactcctccttggtg
acaagggcccacggggccgaaagccacgtccagacggacccaccatgtgtgcaaccccagcacggc
aacttttactgcgaacaccaccttaaggtgacactggtactggtactcggtcactggtgacaggct
aaggatgcccttcaggtaccccgaggtaacacgggacactcgggatctgagaaggggattgggact
tctttaaaagtgcccagtttaaaaagcttctacgcctgaataggcgaccggaggccggcgcctttc
cattacccactactaaatccATGGCTCCCAGCAGCCCCCGGCCCGCGCTGCCCGCACTCCTGGTCC
TGCTCGGGGCTCTGTTCCCAGGACCTGGCAATGCCCAGACATCTGTGTCCCCCTCAAAAGTCATCC
TGCCCCGGGGAGGCTCCGTGCTGGTGACATGCAGCACCTCCTGTGACCAGCCCAAGTTGTTGGGCA
TAGAGACCCCGTTGCCTAAAAAGGAGTTGCTCCTGCCTGGGAACAACCGGAAGGTGTATGAACTGA
GCAATGTGCAAGAAGATAGCCAACCAATGTGCTATTCAAACTGCCCTGATGGGCAGTCAACAGCTA
AAACCTTCCTCACCGTGTACTGGACTCCAGAACGGGTGGAACTGGCACCCCTCCCCTCTTGGCAGC
CAGTGGGCAAGAACCTTACCCTACGCTGCCAGGTGGAGGGTGGGGCACCCCGGGCCAACCTCACCG
TGGTGCTGCTCCGTGGGGAGAAGGAGCTGAAACGGGAGCCAGCTGTGGGGGAGCCCGCTGAGGTCA
CGACCACGGTGCTGGTGAGGAGAGATCACCATGGAGCCAATTTCTCGTGCCGCACTGAACTGGACC
TGCGGCCCCAAGGGCTGGAGCTGTTTGAGAACACCTCGGCCCCCTACCAGCTCCAGACCTTTGTCC
TGCCAGCGACTCCCCCACAACTTGTCAGCCCCCGGGTCCTAGAGGTGGACACGCAGGGGACCGTGG
TCTGTTCCCTGGACGGGCTGTTCCCAGTCTCGGAGGCCCAGGTCCACCTGGCACTGGGGGACCAGA

GGTTGAACCCCACAGTCACCTATGGCAACGACTCCTTCTCGGCCAAGGCCTCAGTCAGTGTGACCG
CAGAGGACGAGGGCACCCAGCGGCTGACGTGTGCAGTAATACTGGGGAACCAGAGCCAGGAGACAC
TGCAGACAGTGACCATCTACAGCTTTCCGGCGCCCAACGTGATTCTGACGAAGCCAGAGGTCTCAG
AAGGGACCGAGGTGACAGTGAAGTGTGAGGCCCACCCTAGAGCCAAGGTGACGCTGAATGGGGTTC
CAGCCCAGCCACTGGGCCCGAGGGCCCAGCTCCTGCTGAAGGCCACCCCAGAGGACAACGGGCGCA
GCTTCTCCTGCTCTGCAACCCTGGAGGTGGCCGGCCAGCTTATACACAAGAACCAGACCCGGGAGC
TTCGTGTCCTGTATGGCCCCCGACTGGACGAGAGGGATTGTCCGGGAAACTGGACGTGGCCAGAAA
ATTCCCAGCAGACTCCAATGTGCCAGGCTTGGGGGAACCCATTGCCCGAGCTCAAGTGTCTAAAGG
ATGGCACTTTCCCACTGCCCATCGGGGAATCAGTGACTGTCACTCGAGATCTTGAGGGCACCTACC
TCTGTCGGGCCAGGAGCACTCAAGGGGAGGTCACCCGCAAGGTGACCGTGAATGTGCTCTCCCCCC
GGTATGAGATTGTCATCATCACTGTGGTAGCAGCCGCAGTCATAATGGGCACTGCAGGCCTCAGCA
CGTACCTCTATAACCGCCAGCGGAAGATCAAGAAATACAGACTACAACAGGCCCAAAAAGGGACCC
CCATGAAACCGAACACACAAGCCACGCCTCCCTGACTCGAGTCACCAGGCG (SEQ ID NO: 31).

Example 7: CD80, CD137L, And ICAM-1 Gene Expression

[00175] This example describes the expression of CD80, CD137L, and ICAM-1 from the recombinant adenovirus produced as described in Example 6.

[00176] ADS-12 cells (mouse lung adenocarcinoma cells) were infected with the TAV-mCD80-137L-ICAM virus at a MOI of 10 or kept as non-infected controls and stained four days after infection for CD80, CD137L, and ICAM-1 by immunocytochemistry. As depicted in **FIGURE 13**, each gene was expressed with the TAV-mCD80-137L-ICAM virus, demonstrating that the single virus drove expression of three therapeutic genes.

[00177] F244 cells (mouse sarcoma cells) were infected with the TAV-mCD80-137L-ICAM virus at a MOI of 5 or kept as non-infected controls and stained three days after infection for CD80, CD137L, and ICAM-1 by immunocytochemistry. As depicted in **FIGURE 14**, each gene was expressed with the TAV-mCD80-137L-ICAM virus, demonstrating that the single virus drove expression of three therapeutic genes.

[00178] HT29 (human colorectal adenocarcinoma cells) were infected with the TAV-mCD80-mCD137L-mICAM-1 virus at a MOI of 5 or kept as non-infected controls and stained three days after infection for CD80, CD137L, and ICAM-1 by immunocytochemistry. As depicted in **FIGURE 15**, each gene was expressed with the TAV-mCD80-137L-ICAM virus, demonstrating that the single virus drove expression of three therapeutic genes.

Example 8: Anti-Cancer Activity Of CD80, CD137L, And ICAM-1 Expressing Adenoviruses

[00179] This example describes the anti-cancer activity of CD80 and CD137L expressing recombinant adenoviruses and CD80, CD137L, and ICAM-1 expressing adenoviruses.

[00180] 129S4 mice carrying ADS-12 tumors were treated with three intratumoral injections of buffer, TAV-mCD80-137L (produced as described in Example 1), or TAV-mCD80-137L-ICAM (produced as described in Example 6). Results are depicted in **FIGURE 16**. Tumors in mice treated with TAV-mCD80-137L were smaller than those treated with buffer. Tumors of mice treated with TAV-mCD80-137L-ICAM were smaller than those treated with TAV-mCD80-m137L or buffer, with many mice showing complete loss of tumor volume. These results demonstrate that CD80 and 137L expressing viruses and CD80, CD137L, and mICAM-1 expressing viruses are effective in reducing tumor size.

Example 9: Construction Of Endostatin And Angiostatin Expressing Adenoviruses

[00181] This Example describes the construction of a recombinant adenovirus type 5 (Ad5) that expresses endostatin and angiostatin.

[00182] A plasmid carrying the 5' portion of the adenovirus type 5 genomic sequence is modified to carry the deletion of a nucleotide region located from -304 to -255 upstream of the E1a initiation site, which renders E1a expression cancer-selective (as previously described in U.S. Patent No. 9,073,980). The modified plasmid is hereafter referred to as the TAV plasmid, and any resulting viral particles produced therefrom are hereafter referred to as the TAV virus.

[00183] The TAV plasmid is further modified to carry a SalI site at the start of the E1b-19k region and an XhoI site 200 base pairs 3' of the SalI site to facilitate insertion of therapeutic transgenes. To delete the 200 base pair E1b-19k region the plasmid is cut with SalI and XhoI and self-ligated. 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: 15).

[00184] Additionally, a nucleotide sequence encoding amino acid residues 1-23 of human collagen XVIII (corresponding to the signal peptide) followed by residues 1318-1516 of human collagen XVIII (corresponding to a C-terminal fragment) followed by an encephalomyocarditis virus (EMCV) IRES followed by a nucleotide sequence encoding amino acid residues 1-19 of human plasminogen (corresponding to the signal peptide) followed by residues 97-549 of human plasminogen (corresponding to kringle domains 1-5) is cloned in to the modified E1b-19k region. All human collagen XVIII amino acid residue numbers are relative to NCBI Reference Sequence: NP_085059.2, depicted herein as SEQ ID NO: 33. All human plasminogen amino acid residue numbers are relative to NCBI Reference Sequence: NP_000292.1, depicted herein as SEQ ID NO: 34.The modified plasmid is hereafter referred to as the TAV-hEndo-IRES-hAng plasmid, and any resulting viral particles produced therefrom are hereafter referred to as the TAV-hEndo-IRES-hAng virus. The nucleotide sequence of the TAV-hEndo-IRES-hAng plasmid in the E1b-19k region is as follows, where the coding regions are capitalized, the IRES is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGGCTCCCTACCCCTGTGGCTGCCACATCCTGCTGCTGCTCTTCTGCTG
CCTGGCGGCTGCCCGGGCCAGCTCCTACGTGCACCTGCGGCCGGCGCGACCCACAAGCCCACCCGC
CCACAGCCACCGCGACTTCCAGCCGGTGCTCCACCTGGTTGCGCTCAACAGCCCCCTGTCAGGCGG
CATGCGGGGCATCCGCGGGGCCGACTTCCAGTGCTTCCAGCAGGCGCGGGCCGTGGGGCTGGCGGG
CACCTTCCGCGCCTTCCTGTCCTCGCGCCTGCAGGACCTGTACAGCATCGTGCGCCGTGCCGACCG
CGCAGCCGTGCCCATCGTCAACCTCAAGGACGAGCTGCTGTTTCCCAGCTGGGAGGCTCTGTTCTC
AGGCTCTGAGGGTCCGCTGAAGCCCGGGGCACGCATCTTCTCCTTTGACGGCAAGGACGTCCTGAG
GCACCCCACCTGGCCCCAGAAGAGCGTGTGGCATGGCTCGGACCCCAACGGGCGCAGGCTGACCGA
GAGCTACTGTGAGACGTGGCGGACGGAGGCTCCCTCGGCCACGGGCCAGGCCTCCTCGCTGCTGGG
GGGCAGGCTCCTGGGGCAGAGTGCCGCGAGCTGCCATCACGCCTACATCGTGCTCTGCATTGAGAA
CAGCTTCATGACTGCCTCCAAGTAGtaacgttactggccgaagccgcttggaataaggccggtgtg
cgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagggcccggaaacctg
gccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgt
tgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgaccc
tttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataag
atacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtca
aatggctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgtatgg
gatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaacgtctagg
ccccccgaaccacggggacgtggttttcctttgaaaaacacgatgataatATGGAACATAAGGAAG
TGGTTCTTCTACTTCTTTTATTTCTGAAATCAGGTCAAGGAAAAGTGTATCTCTCAGAGTGCAAGA
CTGGGAATGGAAAGAACTACAGAGGGACGATGTCCAAAACAAAAAATGGCATCACCTGTCAAAAAT
GGAGTTCCACTTCTCCCCACAGACCTAGATTCTCACCTGCTACACACCCCTCAGAGGGACTGGAGG

AGAACTACTGCAGGAATCCAGACAACGATCCGCAGGGGCCCTGGTGCTATACTACTGATCCAGAAA
AGAGATATGACTACTGCGACATTCTTGAGTGTGAAGAGGAATGTATGCATTGCAGTGGAGAAAACT
ATGACGGCAAAATTTCCAAGACCATGTCTGGACTGGAATGCCAGGCCTGGGACTCTCAGAGCCCAC
ACGCTCATGGATACATTCCTTCCAAATTTCCAAACAAGAACCTGAAGAAGAATTACTGTCGTAACC
CCGATAGGGAGCTGCGGCCTTGGTGTTTCACCACCGACCCCAACAAGCGCTGGGAACTTTGTGACA
TCCCCCGCTGCACAACACCTCCACCATCTTCTGGTCCCACCTACCAGTGTCTGAAGGGAACAGGTG
AAAACTATCGCGGGAATGTGGCTGTTACCGTGTCCGGGCACACCTGTCAGCACTGGAGTGCACAGA
CCCCTCACACACATAACAGGACACCAGAAAACTTCCCCTGCAAAAATTTGGATGAAAACTACTGCC
GCAATCCTGACGGAAAAAGGGCCCCATGGTGCCATACAACCAACAGCCAAGTGCGGTGGGAGTACT
GTAAGATACCGTCCTGTGACTCCTCCCCAGTATCCACGGAACAATTGGCTCCCACAGCACCACCTG
AGCTAACCCCTGTGGTCCAGGACTGCTACCATGGTGATGGACAGAGCTACCGAGGCACATCCTCCA
CCACCACCACAGGAAAGAAGTGTCAGTCTTGGTCATCTATGACACCACACCGGCACCAGAAGACCC
CAGAAAACTACCCAAATGCTGGCCTGACAATGAACTACTGCAGGAATCCAGATGCCGATAAAGGCC
CCTGGTGTTTTACCACAGACCCCAGCGTCAGGTGGGAGTACTGCAACCTGAAAAAATGCTCAGGAA
CAGAAGCGAGTGTTGTAGCACCTCCGCCTGTTGTCCTGCTTCCAGATGTAGAGACTCCTTCCGAAG
AAGACTGTATGTTTGGGAATGGGAAAGGATACCGAGGCAAGAGGGCGACCACTGTTACTGGGACGC
CATGCCAGGACTGGGCTGCCCAGGAGCCCCATAGACACAGCATTTTCACTCCAGAGACAAATCCAC
GGGCGGGTCTGGAAAAAAATTACTGCCGTAACCCTGATGGTGATGTAGGTGGTCCCTGGTGCTACA
CGACAAATCCAAGATAGCTCGAGTCACCAGGCG (SEQ ID NO: 35).

[00185] Additionally, a nucleotide sequence encoding amino acid residues 1-26 of mouse collagen XVIII (corresponding to the signal peptide) followed by residues 1577-1774 of mouse collagen XVIII (corresponding to a C-terminal fragment) followed by an encephalomyocarditis virus (EMCV) IRES followed by a nucleotide sequence encoding amino acid residues 1-19 of mouse plasminogen (corresponding to the signal peptide) followed by residues 96-549 of mouse plasminogen (corresponding to kringle domains 1-5) is cloned in to the modified E1b-19k region. The modified plasmid is hereafter referred to as the TAV-Endo-IRES-Ang plasmid, and any resulting viral particles produced therefrom are hereafter referred to as the TAV-Endo-IRES-Ang virus. The nucleotide sequence of the TAV-Endo-IRES-Ang plasmid in the E1b-19k region is as follows, where the coding regions are capitalized, the IRES is lowercase, and the flanking E1b-19k sequence including the SalI and XhoI restriction sites is underlined:

ATCTGACCTCGTCGACATGGCTCCCGACCCCAGCAGACGCCTCTGCCTGCTGCTGCTGTTGCTGCT
CTCCTGCCGCCTTGTGCCTGCCAGCGCTTATGTGCACCTGCCGCCAGCCCGCCCCACCCTCTCACT
TGCTCATACTCATCAGGACTTTCAGCCAGTGCTCCACCTGGTGGCACTGAACACCCCCCTGTCTGG
AGGCATGCGTGGTATCCGTGGAGCAGATTTCCAGTGCTTCCAGCAAGCCCGAGCCGTGGGGCTGTC
GGGCACCTTCCGGGCTTTCCTGTCCTCTAGGCTGCAGGATCTCTATAGCATCGTGCGCCGTGCTGA
CCGGGGGTCTGTGCCCATCGTCAACCTGAAGGACGAGGTGCTATCTCCCAGCTGGGACTCCCTGTT
TTCTGGCTCCCAGGGTCAACTGCAACCCGGGGCCCGCATCTTTTCTTTTGACGGCAGAGATGTCCT
GAGACACCCAGCCTGGCCGCAGAAGAGCGTATGGCACGGCTCGGACCCCAGTGGGCGGAGGCTGAT

GGAGAGTTACTGTGAGACATGGCGAACTGAAACTACTGGGGCTACAGGTCAGGCCTCCTCCCTGCT
GTCAGGCAGGCTCCTGGAACAGAAAGCTGCGAGCTGCCACAACAGCTACATCGTCCTGTGCATTGA
GAATAGCTTCATGACCTCTTTCTCCAAATAGtaacgttactggccgaagccgcttggaataaggcc
ggtgtgcgtttgtctatatgttattttccaccatattgccgtcttttggcaatgtgagggcccgga
aacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaag
gtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtag
cgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtg
tataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaa
gagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtaccccatt
gtatgggatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaacg
tctaggccccccgaaccacggggacgtggttttcctttgaaaaacacgatgataatATGGACCACA
AGGAAGTAATCCTTCTGTTTCTCTTGCTTCTGAAACCAGGACAAGGGAAGAGAGTGTATCTGTCAG
AATGTAAGACCGGCATCGGCAACGGCTACAGAGGAACAATGTCCAGGACAAAGAGTGGTGTTGCCT
GTCAAAAGTGGGGTGCCACGTTCCCCCACGTACCCAACTACTCTCCCAGTACACATCCCAATGAGG
GACTAGAAGAAAATTACTGTAGGAACCCAGACAATGATGAACAAGGGCCTTGGTGCTACACTACAG
ATCCGGACAAGAGATATGACTACTGCAACATTCCTGAATGTGAAGAAGAATGCATGTACTGCAGTG
GCGAAAAGTATGAGGGGAAAATCTCCAAGACCATGTCTGGACTTGACTGCCAGGCCTGGGATTCTC
AGAGCCCACATGCTCATGGATACATCCCTGCCAAATTCCCAAGCAAGAACCTGAAGATGAATTATT
GCCGCAACCCTGACGGGGAGCCAAGGCCCTGGTGCTTCACAACAGACCCCACCAAACGCTGGGAAT
ACTGTGACATCCCCCGCTGCACAACACCCCCGCCCCCACCCAGCCCAACCTACCAATGTCTGAAAG
GAAGAGGTGAAAATTACCGAGGGACCGTGTCTGTCACCGTGTCTGGGAAAACCTGTCAGCGCTGGA
GTGAGCAAACCCCTCATAGGCACAACAGGACACCAGAAAATTTCCCCTGCAAAAATCTGGAGGAGA
ATTACTGCCGGAACCCGGATGGAGAAACTGCTCCCTGGTGCTATACCACTGACAGCCAGCTGAGGT
GGGAGTACTGTGAGATTCCATCCTGCGAGTCCTCAGCATCACCAGACCAGTCAGATTCCTCAGTTC
CACCAGAGGAGCAAACACCTGTGGTCCAGGAATGCTACCAGAGCGATGGGCAGAGCTATCGGGGTA
CATCGTCCACTACCATCACAGGGAAGAAGTGCCAGTCCTGGGCAGCTATGTTTCCACATAGGCATT
CGAAGACGCCAGAGAACTTCCCAGATGCTGGCTTGGAGATGAACTATTGCAGGAACCCGGATGGTG
ACAAGGGCCCTTGGTGCTACACCACTGACCCGAGCGTCAGGTGGGAATACTGCAACCTGAAGCGGT
GCTCAGAGACAGGAGGGAGTGTTGTGGAATTGCCCACAGTTTCCCAGGAACCAAGTGGGCCGAGCG
ACTCTGAGACAGACTGCATGTATGGGAATGGCAAAGACTACCGGGGCAAAACGGCCGTCACTGCAG
CTGGCACCCCTTGCCAAGGATGGGCTGCCCAGGAGCCCCACAGGCACAGCATCTTCACCCCACAGA
CAAACCCACGGGCAGGTCTGGAAAAGAATTATTGCCGAAACCCCGATGGGGATGTGAATGGTCCTT
GGTGCTATACAACAAACCCTAGATGATAGCTCGAGTCACCAGGCG (SEQ ID NO: 36).

[00186] The various plasmids described are used along with other plasmids carrying the remainder of the adenovirus type 5 genomic sequence (based on strain dl309) to generate recombinant adenoviruses.

INCORPORATION BY REFERENCE

[00187] The entire disclosure of each of the patent documents and scientific articles referred to herein is incorporated by reference for all purposes.

EQUIVALENTS

[00188] 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) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

2. The recombinant adenovirus of claim 1, wherein the recombinant adenovirus is a type 5 adenovirus (Ad5).

3. The recombinant adenovirus of claims 1 or 2, wherein the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K.

4. The recombinant adenovirus of any one of claims 1-3, wherein 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.

5. The recombinant adenovirus of any one of claims 1-4, wherein the E1b-19K insertion site comprises a deletion of about 200 nucleotides adjacent the start site of E1b-19K.

6. The recombinant adenovirus of any one of claims 1-5, wherein the E1b-19K insertion site comprises a deletion of 202 nucleotides adjacent the start site of E1b-19K.

7 The recombinant adenovirus of any one of claims 1-5, wherein the E1b-19K insertion site comprises a deletion of 203 nucleotides adjacent the start site of E1b-19K.

8. The recombinant adenovirus of any one of claims 1-7, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 of the Ad5 genome (SEQ ID NO: 23).

9. The recombinant adenovirus of any one of claims 1-7, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1916 of the Ad5 genome (SEQ ID NO: 23).

10. The recombinant adenovirus of any one of claims 1-9, wherein the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1917 of the Ad5 genome (SEQ ID NO: 23).

11. The recombinant adenovirus of any one of claims 1-9, wherein the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23).

12. The recombinant adenovirus of any one of claims 1-11, wherein the first therapeutic transgene is inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2).

13. The recombinant adenovirus of any one of claims 1-12, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

14. The recombinant adenovirus of any one of claims 1-13, wherein the E3 insertion site 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.

15. The recombinant adenovirus of any one of claims 1-14, wherein the E3 insertion site is located between the stop site of E3-gp19K and the stop site of E3-14.7K.

16. The recombinant adenovirus of any one of claims 1-15, wherein the E3 insertion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K.

17. The recombinant adenovirus of any one of claims 1-16, wherein the E3 insertion site 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.

18. The recombinant adenovirus of any one of claims 1-17, wherein the E3 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

19. The recombinant adenovirus of any one of claims 1-18, wherein the E3 insertion site comprises a deletion of 1063 nucleotides adjacent the stop site of E3-10.5K.

20. The recombinant adenovirus of any one of claims 1-18, wherein the E3 insertion site comprises a deletion of 1064 nucleotides adjacent the stop site of E3-10.5K

21. The recombinant adenovirus of any one of claims 1-18, wherein the E3 insertion site comprises a deletion corresponding to the Ad5 dl309 E3 deletion.

22. The recombinant adenovirus of any one of claims 1-21, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

23. The recombinant adenovirus of any one of claims 1-22, wherein the second therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23).

24. The recombinant adenovirus of any one of claims 1-23, wherein the second therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4).

25. The recombinant adenovirus of any one of claims 1-24, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the second therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4).

26. The recombinant adenovirus of claim 15, wherein 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.

27. The recombinant adenovirus of claim 26, wherein the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

28. The recombinant adenovirus of claims 26 or 27, wherein the E3 insertion site comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K.

29. The recombinant adenovirus of any one of claims 26-28, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

30. The recombinant adenovirus of any one of claims 26-29, wherein the second therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23).

31. The recombinant adenovirus of any one of claims 26-30, wherein the second therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30).

32. The recombinant adenovirus of any one of claims 26-31, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the second therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

33. A recombinant adenovirus comprising:

(a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site,

wherein the E1b-19k insertion site is located between the start site of E1b-19k and the start site of E1b-55k, and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

34. The recombinant adenovirus of claim 33, wherein the adenovirus is a type 5 adenovirus (Ad5).

35. The recombinant adenovirus of claims 33 or 34, wherein the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K.

36. The recombinant adenovirus of any one of claims 33-35, wherein 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.

37. The recombinant adenovirus of any one of claims 33-36, wherein the E1b-19K insertion site comprises a deletion of about 200 nucleotides adjacent the start site of E1b-19K.

38. The recombinant adenovirus of any one of claims 33-37, wherein the E1b-19K insertion site comprises a deletion of 202 nucleotides adjacent the start site of E1b-19K.

39. The recombinant adenovirus of any one of claims 33-37, wherein the E1b-19K insertion site comprises a deletion of 203 nucleotides adjacent the start site of E1b-19K.

40. The recombinant adenovirus of any one of claims 33-39, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 of the Ad5 genome (SEQ ID NO: 23).

41. The recombinant adenovirus of any one of claims 33-39, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1916 of the Ad5 genome (SEQ ID NO: 23).

42. The recombinant adenovirus of any one of claims 33-41, wherein the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1917 of the Ad5 genome (SEQ ID NO: 23).

43. The recombinant adenovirus of any one of claims 33-41, wherein the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23).

44. The recombinant adenovirus of any one of claims 33-43, wherein the first and second therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2).

45. The recombinant adenovirus of any one of claims 33-44, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the IRES, the second therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

46. The recombinant adenovirus of any one of claims 33-45, wherein the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into the E1b-19k insertion site wherein the second nucleotide sequence and the third nucleotide sequence are separated by a second internal ribosome entry site (IRES).

47. The recombinant adenovirus of claim 46, wherein the first, second, and third therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2).

48. The recombinant adenovirus of claims 46 or 47, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, the second IRES, the third therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

49. The recombinant adenovirus of any of claims 33-48, 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.

50. The recombinant adenovirus of claim 49, 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.

51. The recombinant adenovirus of claims 49 or 50, wherein the E3 insertion site is located between the stop site of E3-gp19K and the stop site of E3-14.7K.

52. The recombinant adenovirus of any one of claims 49-51, wherein the E3 deletion is located between the stop site of E3-10.5K and the stop site of E3-14.7K and the start site of Fiber.

53. The recombinant adenovirus of any one of claims 49-52, 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.

54. The recombinant adenovirus of any one of claims 49-53, wherein the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

55. The recombinant adenovirus of any one of claims 49-54, wherein the E3 deletion comprises a deletion of 1063 nucleotides adjacent the stop site of E3-10.5K.

56. The recombinant adenovirus of any one of claims 49-54, wherein the E3 deletion comprises a deletion of 1064 nucleotides adjacent the stop site of E3-10.5K.

57. The recombinant adenovirus of any one of claims 49-54, wherein the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion.

58. The recombinant adenovirus of any one of claims 49-57, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

59. The recombinant adenovirus of any one of claims 49-51, 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.

60. The recombinant adenovirus of claim 59, wherein the E3 deletion comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

61. The recombinant adenovirus of claims 59 or 60, wherein the E3 deletion comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K.

62. The recombinant adenovirus of any one of claims 59-61, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

63. The recombinant adenovirus of any of claims 33-45, wherein the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

64. The recombinant adenovirus of claim 63, wherein the E3 insertion site 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.

65. The recombinant adenovirus claims 63 or 64, wherein the E3 insertion site is located between the stop site of E3-gp19K and the stop site of E3-14.7K.

66. The recombinant adenovirus of any one of claims 63-65, wherein the E3 insertion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K.

67. The recombinant adenovirus of any one of claims 63-66, wherein the E3 insertion site 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.

68. The recombinant adenovirus of any one of claims 63-67, wherein the E3 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

69. The recombinant adenovirus of any one of claims 63-68, wherein the E3 insertion site comprises a deletion of 1063 nucleotides adjacent the stop site of E3-10.5K.

70. The recombinant adenovirus of any one of claims 63-68, wherein the E3 insertion site comprises a deletion of 1064 nucleotides adjacent the stop site of E3-10.5K.

71. The recombinant adenovirus of any one of claims 63-68, wherein the E3 insertion site comprises a deletion corresponding to the Ad5 dl309 E3 deletion.

72. The recombinant adenovirus of any one of claims 63-71, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

73. The recombinant adenovirus of any one of claims 63-72, wherein the third therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23).

74. The recombinant adenovirus of any one of claims 63-73, wherein the third therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4).

75. The recombinant adenovirus of any one of claims 63-74, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the third therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4).

76. The recombinant adenovirus of any one of claims 63-65, wherein 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.

77. The recombinant adenovirus of claim 76, wherein the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

78. The recombinant adenovirus of claims 76 or 77, wherein the E3 insertion site comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K.

79. The recombinant adenovirus of any one of claims 76-78, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

80. The recombinant adenovirus of any one of claims 76-79, wherein the third therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23).

81. The recombinant adenovirus of any one of claims 76-80, wherein the third therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30).

82. The recombinant adenovirus of any one of claims 76-81, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the third therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

83. The recombinant adenovirus of any one of claims 33-82, wherein the IRES is selected from the group consisting the encephalomyocarditis virus IRES, the foot-and-mouth disease virus IRES, and the poliovirus IRES.

84. The recombinant adenovirus of any of claims 1-83, 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).

85. The recombinant adenovirus of claim 84, wherein the E4 deletion is located between the start site of E4-ORF6/7 and the start site of E4-ORF1.

86. The recombinant adenovirus of claims 84 or 85, 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.

87. The recombinant adenovirus of any one of claims 84-86, 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.

88. The recombinant adenovirus of any one of claims 84-87, wherein the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7.

89. The recombinant adenovirus of any one of claims 84-88, wherein the E4 deletion comprises a deletion of 1449 nucleotides adjacent the start site of E4-ORF6/7.

90. The recombinant adenovirus of any one of claims 84-89, wherein the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 23).

91. The recombinant adenovirus of any one of claims 1-90, wherein the first and/or second therapeutic transgenes are not operably linked to an exogenous promoter sequence.

92. The recombinant adenovirus of any one of claims 46-90, wherein the first, second, and/or third therapeutic transgenes are not operably linked to an exogenous promoter sequence.

93. The recombinant adenovirus of any one of claims 1-90, wherein none of the therapeutic transgenes are operably linked to an exogenous promoter sequence.

94. The recombinant adenovirus of any one of claims 1-93, 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 about 5000 nucleotides.

95. The recombinant adenovirus of any one of claims 1-93, 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.

96. The recombinant adenovirus of any one of claims 46-93, wherein the combined size of the first, second, and third 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 about 5000 nucleotides.

97. The recombinant adenovirus of any one of claims 46-93, wherein the combined size of the first, second, and third 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.

98. The recombinant adenovirus of any one of claims 1-97, wherein the combined size of each of the 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 about 5000 nucleotides.

99. The recombinant adenovirus of any one of claims 1-97, wherein the combined size of each of the 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.

100. The recombinant adenovirus of any one of claims 1-99, 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 about 5000 nucleotides.

101. The recombinant adenovirus of any one of claims 1-99, 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.

102. The recombinant adenovirus of any one of claims 46-99, wherein the combined size of the first, second, and third 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 about 5000 nucleotides.

103. The recombinant adenovirus of any one of claims 46-99, wherein the combined size of the first, second, and third 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.

104. The recombinant adenovirus of any one of claims 1-99, wherein the combined size of each of the 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 about 5000 nucleotides.

105. The recombinant adenovirus of any one of claims 1-99, wherein the combined size of each of the 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.

106. The recombinant adenovirus of any one of claims 1-105, 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.

107. The recombinant adenovirus of any one of claims 1-105, 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, about 6000, or about 7000 nucleotides.

108. The recombinant adenovirus of any one of claims 46-105, wherein the combined size of the first, second, and third therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides.

109. The recombinant adenovirus of any one of claims 46-105, wherein the combined size of the first, second, and third therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides.

110. The recombinant adenovirus of any one of claims 1-109, wherein the combined size of each of the therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides.

111. The recombinant adenovirus of any one of claims 1-109, wherein the combined size of each of the therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides.

112. The recombinant adenovirus of any one of claims 1-111, wherein the combined size of the first and second therapeutic transgenes comprises about 1650 nucleotides.

113. The recombinant adenovirus of any one of claims 1-111, wherein the combined size of the first and second therapeutic transgenes comprises about 3100 nucleotides.

114. The recombinant adenovirus of any one of claims 46-111, wherein the combined size of the first, second, and third therapeutic transgenes comprises about 1650 nucleotides.

115. The recombinant adenovirus of any one of claims 46-111, wherein the combined size of the first, second, and third therapeutic transgenes comprises about 3100 nucleotides.

116. The recombinant adenovirus of any one of claims 1-115, wherein the combined size of each of the therapeutic transgenes comprises about 1650 nucleotides.

117. The recombinant adenovirus of any one of claims 1-115, wherein the combined size of each of the therapeutic transgenes comprises about 3100 nucleotides.

118. The recombinant adenovirus of any one of claims 1-117, wherein the first and/or second therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

119. The recombinant adenovirus of any one of claims 46-117, wherein the first, second and/or third therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

120. The recombinant adenovirus of any one of claims 1-117, wherein any one of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

121. The recombinant adenovirus of any one of claims 1-117, wherein the first and/or second therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19,

CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

122. The recombinant adenovirus of any one of claims 46-117, wherein the first, second and/or third therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

123. The recombinant adenovirus of any one of claims 1-117, wherein any one of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

124. The recombinant adenovirus of any one of claims 1-117, wherein the first and/or second therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23, IL-23A/p19, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

125. The recombinant adenovirus of any one of claims 46-117, wherein the first, second and/or third therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23, IL-23A/p19, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

126. The recombinant adenovirus of any one of claims 1-117, wherein any one of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23, IL-23A/p19, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

127. The recombinant adenovirus of any one of claims 1-126, wherein the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

128. The recombinant adenovirus of any one of claim 1-126, wherein the first and/or second therapeutic transgenes are selected from the group consisting of CD80 and CD137L.

129. The recombinant adenovirus of any one of claim 46-126, wherein the first, second and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1.

130. The recombinant adenovirus of claims 127 or 128, wherein the first therapeutic transgene encodes CD80.

131. The recombinant adenovirus of any one of claims 127-130, wherein the second therapeutic transgene encodes CD137L.

132. The recombinant adenovirus of any one of claims 128-131, wherein the third therapeutic transgene encodes ICAM-1.

133. The recombinant adenovirus of any one of claims 128-132, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5.

134. The recombinant adenovirus of any one of claims 128-133, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 6.

135. The recombinant adenovirus of any one of claims 128-134, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 7.

136. The recombinant adenovirus of any one of claims 128-135, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 8.

137. The recombinant adenovirus of any one of claims 128-136, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 27.

138. The recombinant adenovirus of any one of claims 128-137, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 32.

139. The recombinant adenovirus of any one of claims 128-138, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 31 or SEQ ID NO: 9.

140. The recombinant adenovirus of any one of claims 128-137, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 31.

141. The recombinant adenovirus of any one of claims 128-137, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 22.

142. The recombinant adenovirus of any one of claims 1-127, wherein the first and/or second therapeutic transgenes are selected from the group consisting of IL-27A/p28 and IL-27B/EBI3.

143. The recombinant adenovirus of claim 142, wherein the first therapeutic transgene encodes IL-27A/p28.

144. The recombinant adenovirus of claims 142 or 143, wherein the second therapeutic transgene encodes IL-27B/EBI3.

145. The recombinant adenovirus of any one of claims 1-126, wherein the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin.

146. The recombinant adenovirus of claim 145, wherein the first therapeutic transgene encodes endostatin.

147. The recombinant adenovirus of claims 145 or 146, wherein the second therapeutic transgene encodes angiostatin

148. The recombinant adenovirus of any one of claims 145-147, wherein the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 37 or SEQ ID NO: 38.

149. The recombinant adenovirus of any one of claims 145-148, wherein the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44.

150. The recombinant adenovirus of any one of claims 145-149, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 11.

151. The recombinant adenovirus of any one of claims 1-150, wherein the recombinant adenovirus further comprises a deletion of a Pea3 binding site, or a functional fragment thereof.

152. The recombinant adenovirus of claim 151, wherein the recombinant adenovirus comprises a deletion of nucleotides corresponding to about -300 to about -250 upstream of the initiation site of E1a.

153. The recombinant adenovirus of claims 151 or 152, wherein the recombinant adenovirus comprises a deletion of nucleotides corresponding to -305 to -255 upstream of the initiation site of E1a.

154. The recombinant adenovirus of claims 151 or 152, wherein the recombinant adenovirus comprises a deletion of nucleotides corresponding to -304 to -255 upstream of the initiation site of E1a.

155. A recombinant adenovirus comprising SEQ ID NO: 14, or 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: 14.

156. The recombinant adenovirus of any one of claims 1-155, wherein the recombinant adenovirus selectively replicates in a hyperproliferative cell.

157. The recombinant adenovirus of any one of claims 1-156, wherein the recombinant adenovirus selectively expresses the first and/or the second therapeutic transgene in a hyperproliferative cell.

158. The recombinant adenovirus of any one of claims 46-157, wherein the recombinant adenovirus selectively expresses the first, second, and/or third therapeutic transgene in a hyperproliferative cell.

159. The recombinant adenovirus of any one of claims 156-158, wherein the hyperproliferative cell is a cancer cell.

160. The recombinant adenovirus of any one of claims 1-159, wherein the recombinant adenovirus is an oncolytic virus.

161. A pharmaceutical composition comprising the recombinant adenovirus of any one of claims 1-160 and at least one pharmaceutically acceptable carrier or diluent.

162. 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 1-160 to express the two therapeutic transgenes.

163. A method of expressing three therapeutic transgenes in a target cell comprising exposing the cell to an effective amount of the recombinant adenovirus of any one of claims 46-160 to express the two therapeutic transgenes.

164. 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 1-160 to inhibit proliferation of the tumor cell.

165. 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 1-160 to inhibit growth of the tumor.

166. 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 1-160 to treat the cancer in the subject.

167. The method of claim 166, wherein the cancer is selected from the group consisting of 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.

168. The method of claims 165-167, wherein the recombinant adenovirus is administered in combination with one or more therapies selected from the group consisting of surgery, radiation, chemotherapy, immunotherapy, hormone therapy, and virotherapy.

169. The method of any one of claims 162-168, wherein the effective amount of the recombinant adenovirus is 10^2-10^{15} plaque forming units (pfus).

170. The method of any one of claims 165-169, wherein the subject is a human.

171. The method of claim 170, wherein the subject is a pediatric human.

172. The method of any one of claims 165-171, wherein the method further comprises measuring an immune response to an antigen in the subject.

173. The method of any one of claims 165-172, wherein the effective amount of the recombinant virus is identified by measuring an immune response to an antigen in the subject.

174. The method of claim 172 or 173, wherein 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 at the injection site.

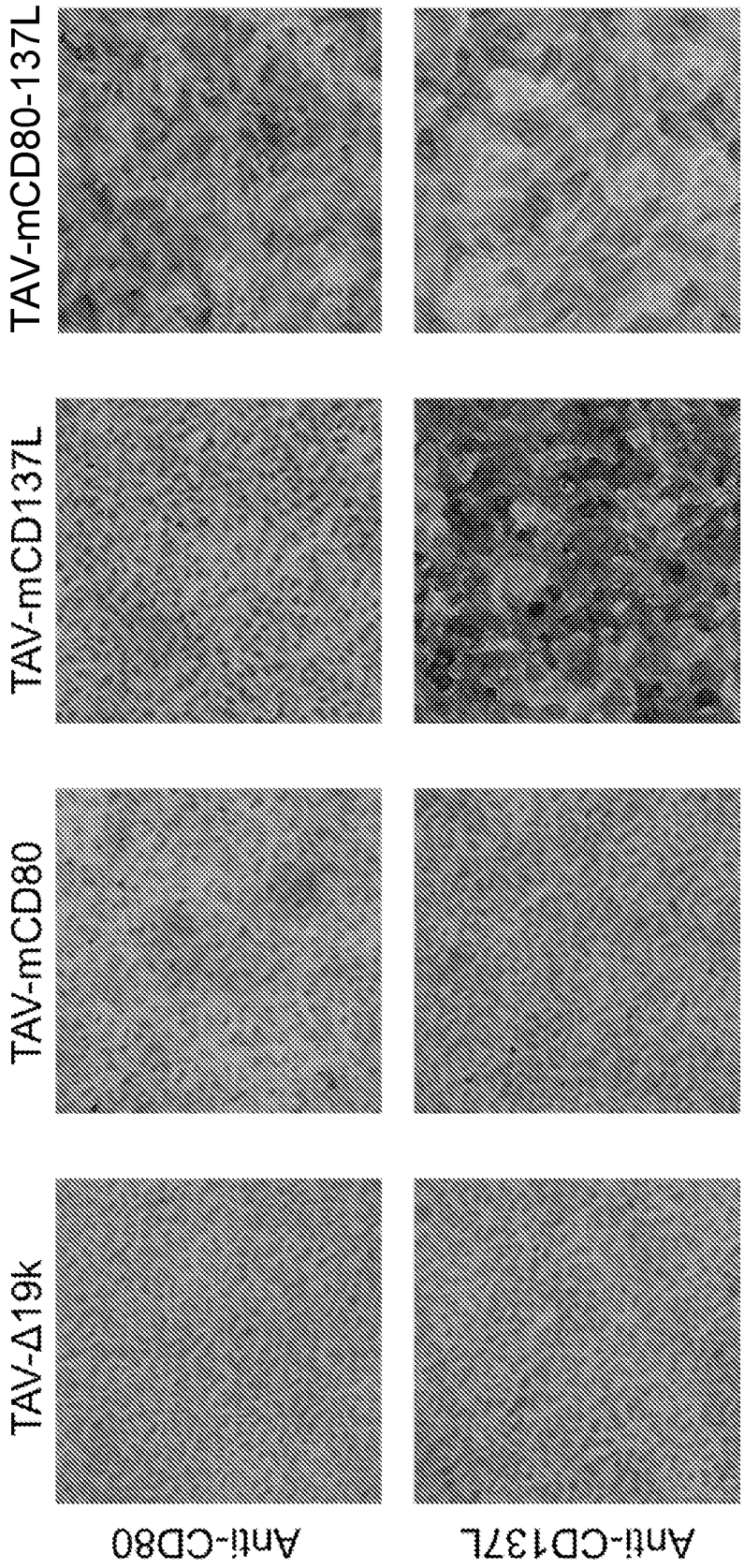

TAV-mCD80-137L
TAV-mCD137L
TAV-mCD80
TAV-Δ19k
Anti-CD80
Anti-CD137L

FIGURE 1

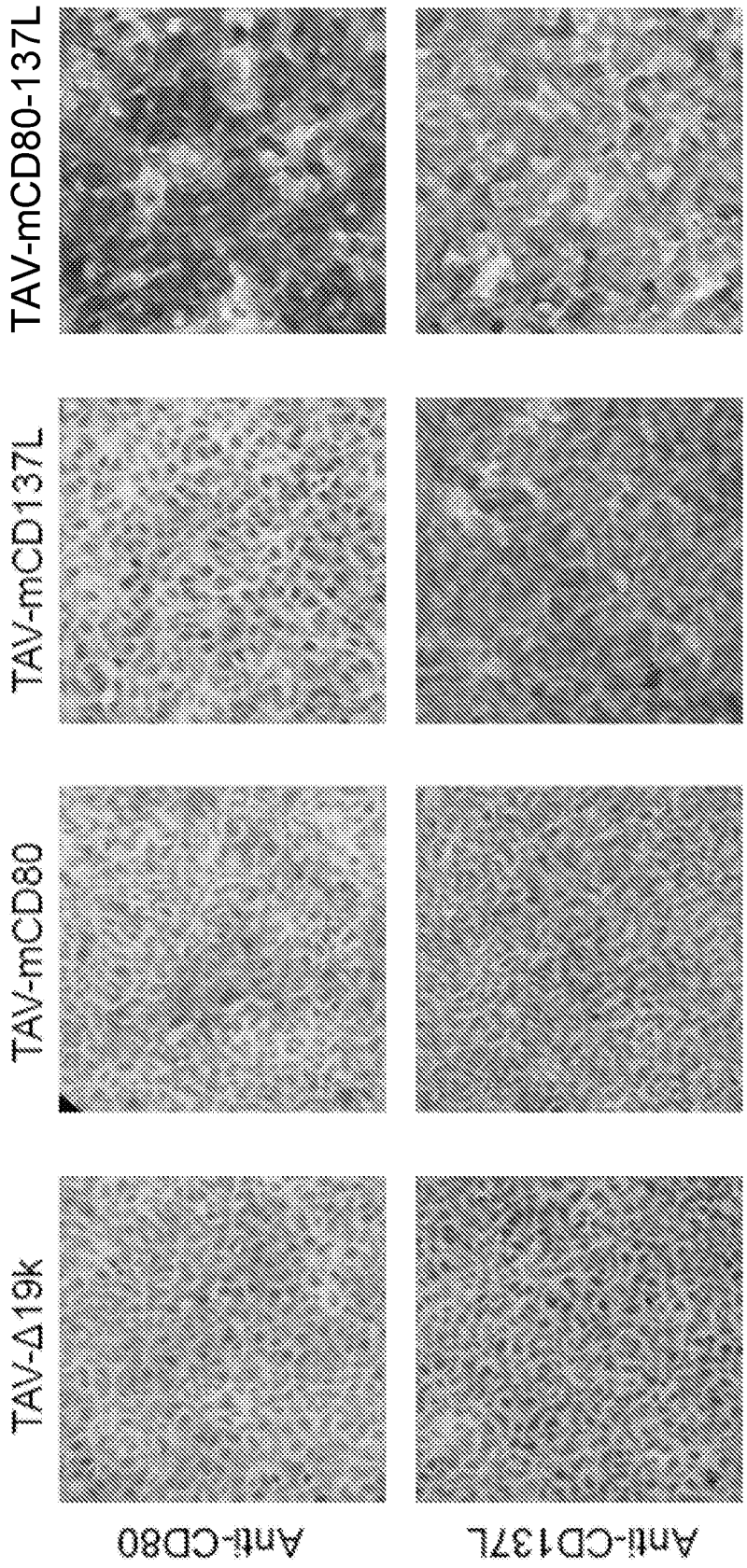

TAV-mCD80-137L
TAV-mCD137L
TAV-mCD80
TAV-Δ19k
Anti-CD80
Anti-CD137L

FIGURE 2

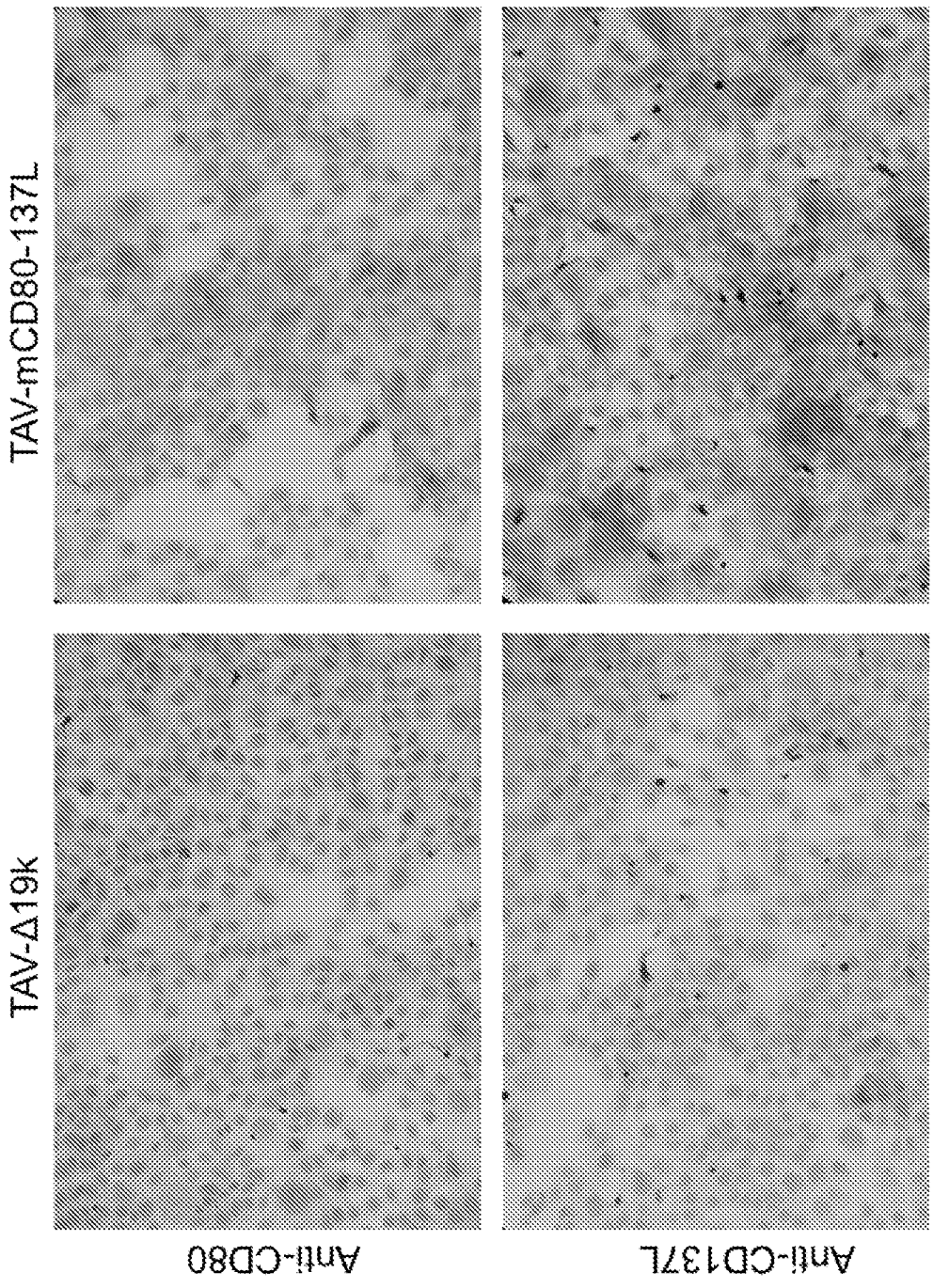


FIGURE 3

FIGURE 4

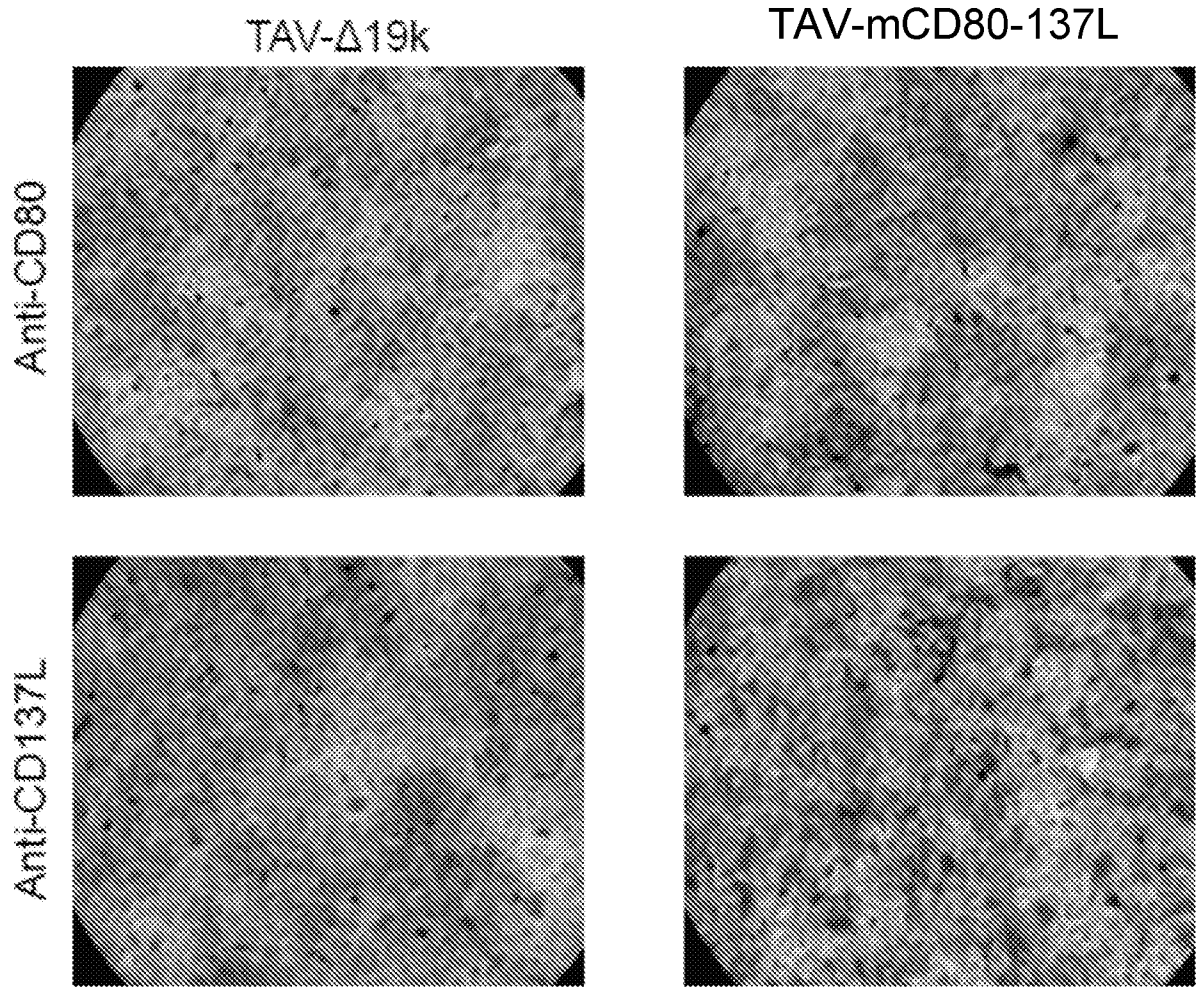


FIGURE 5

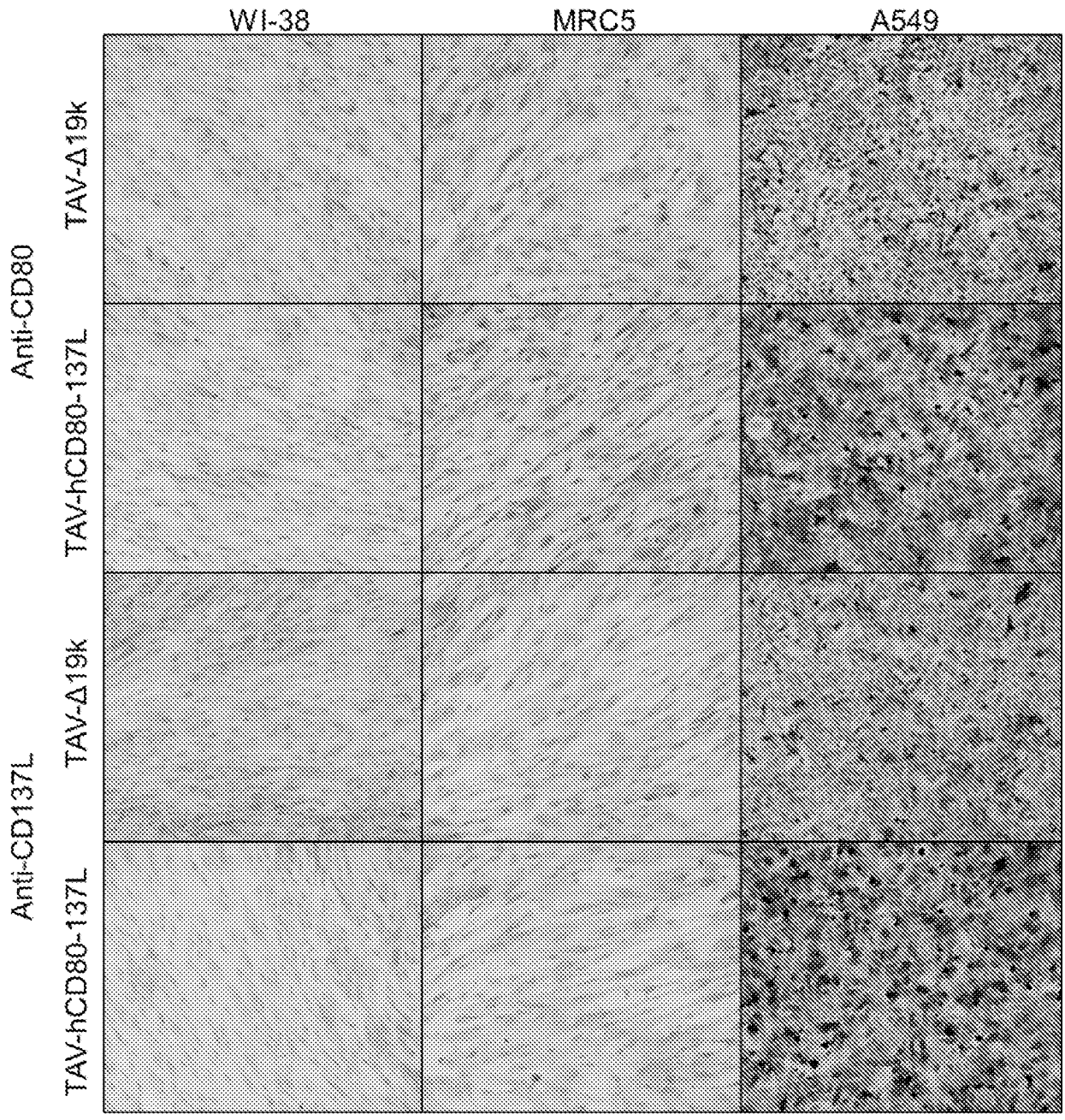

WI-38
MRC5
A549
Anti-CD80
TAV-Δ19k
TAV-hCD80-137L
Anti-CD137L
TAV-Δ19k
TAV-hCD80-137L

FIGURE 6

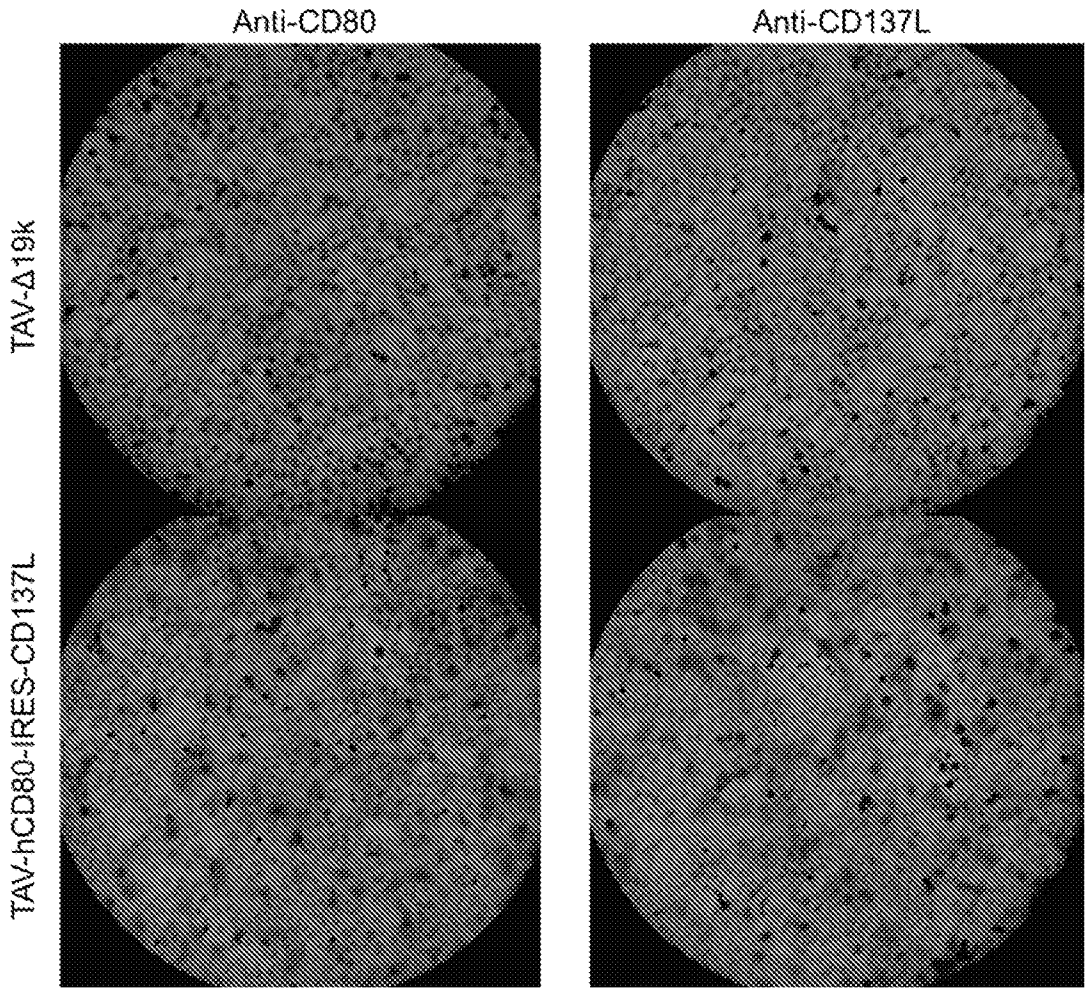


FIGURE 7

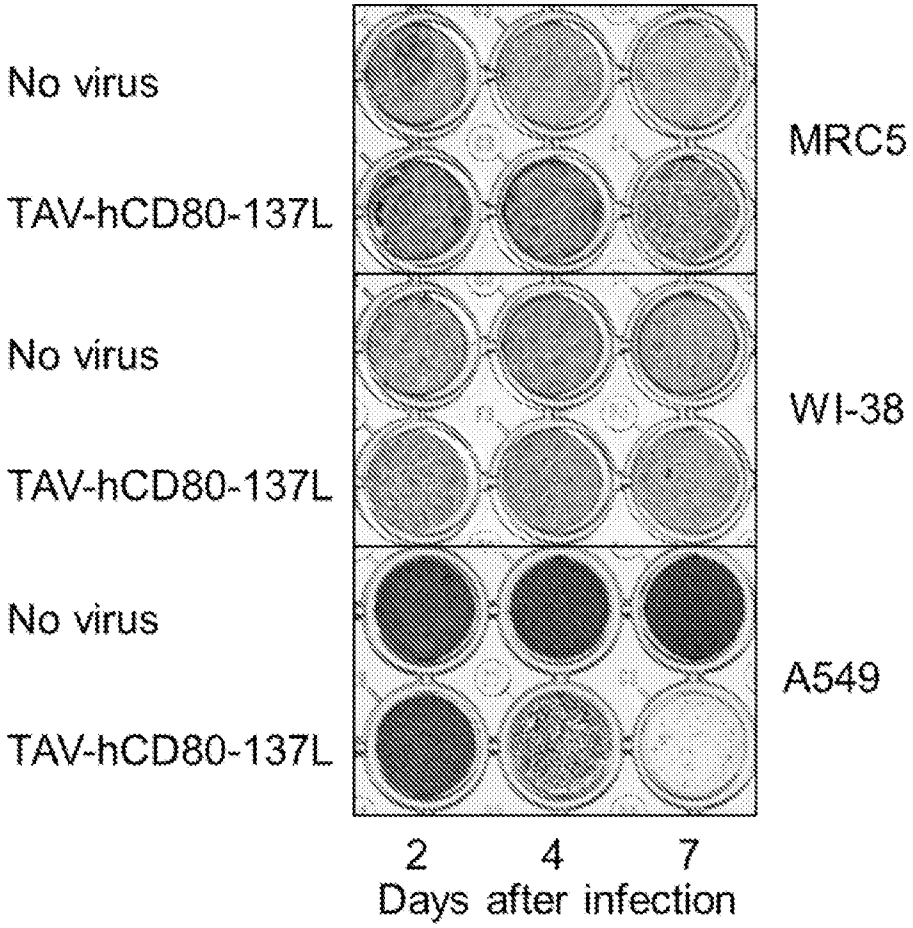

No virus
TAV-hCD80-137L
MRC5
No virus
TAV-hCD80-137L
WI-38
No virus
TAV-hCD80-137L
A549
2
4
7
Days after infection

FIGURE 8

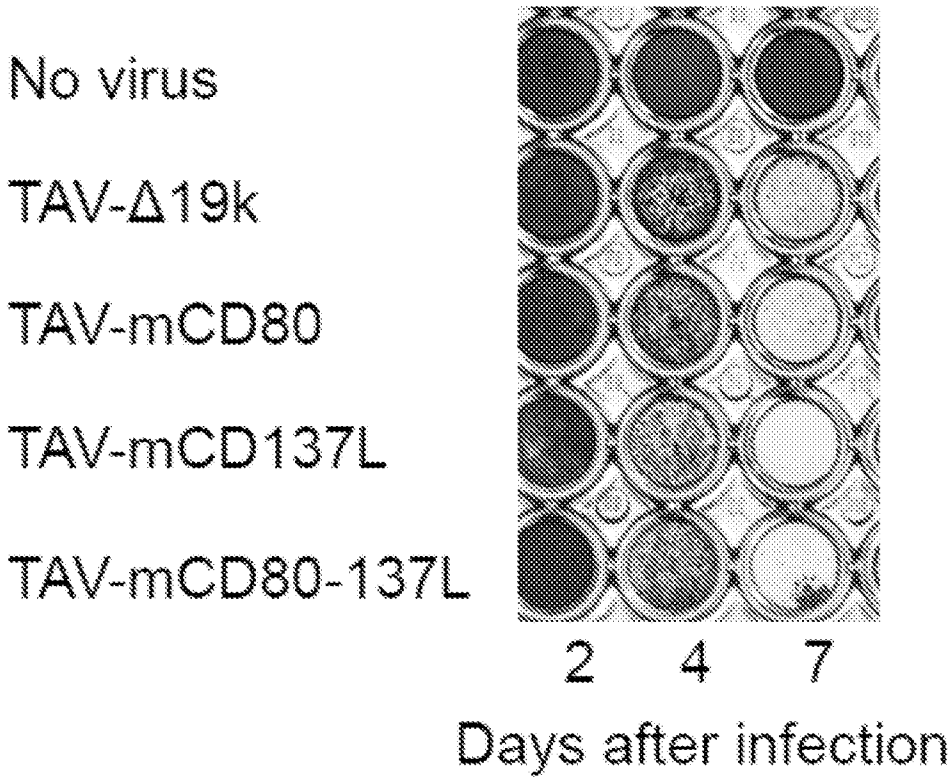
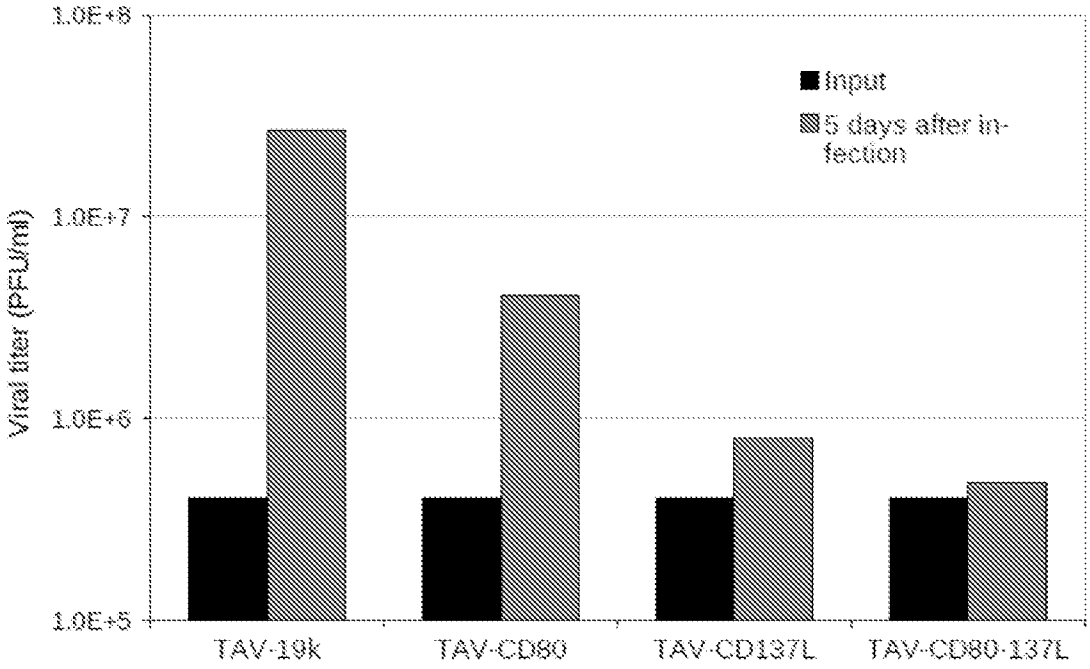

No virus
TAV-Δ19k
TAV-mCD80
TAV-mCD137L
TAV-mCD80-137L
2 4 7
Days after infection

FIGURE 9


1.0E+8
1.0E+7
1.0E+6
1.0E+5
Viral titer (PFU/ml)
Input
5 days after infection
TAV-19k
TAV-CD80
TAV-CD137L
TAV-CD80-137L

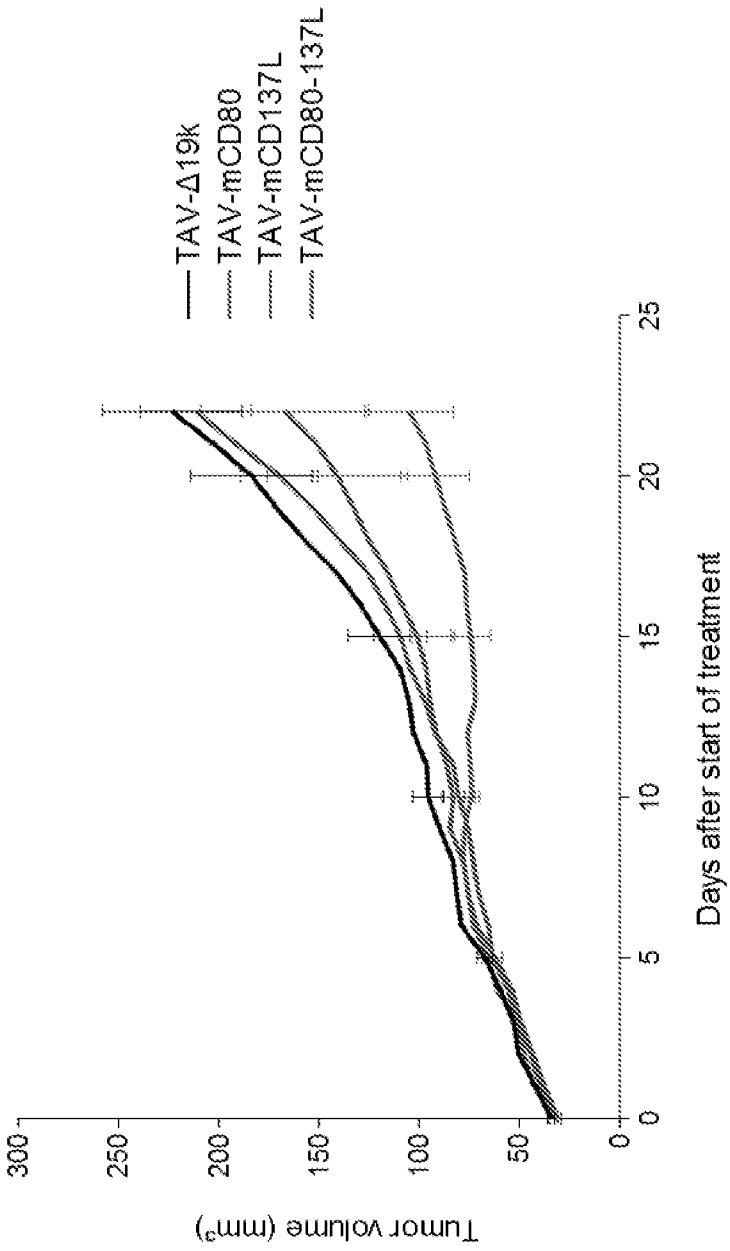

TAV-Δ19k
TAV-mCD80
TAV-mCD137L
TAV-mCD80-137L
Tumor volume (mm³)
300
250
200
150
100
50
0
0
5
10
15
20
25
Days after start of treatment

FIGURE 10

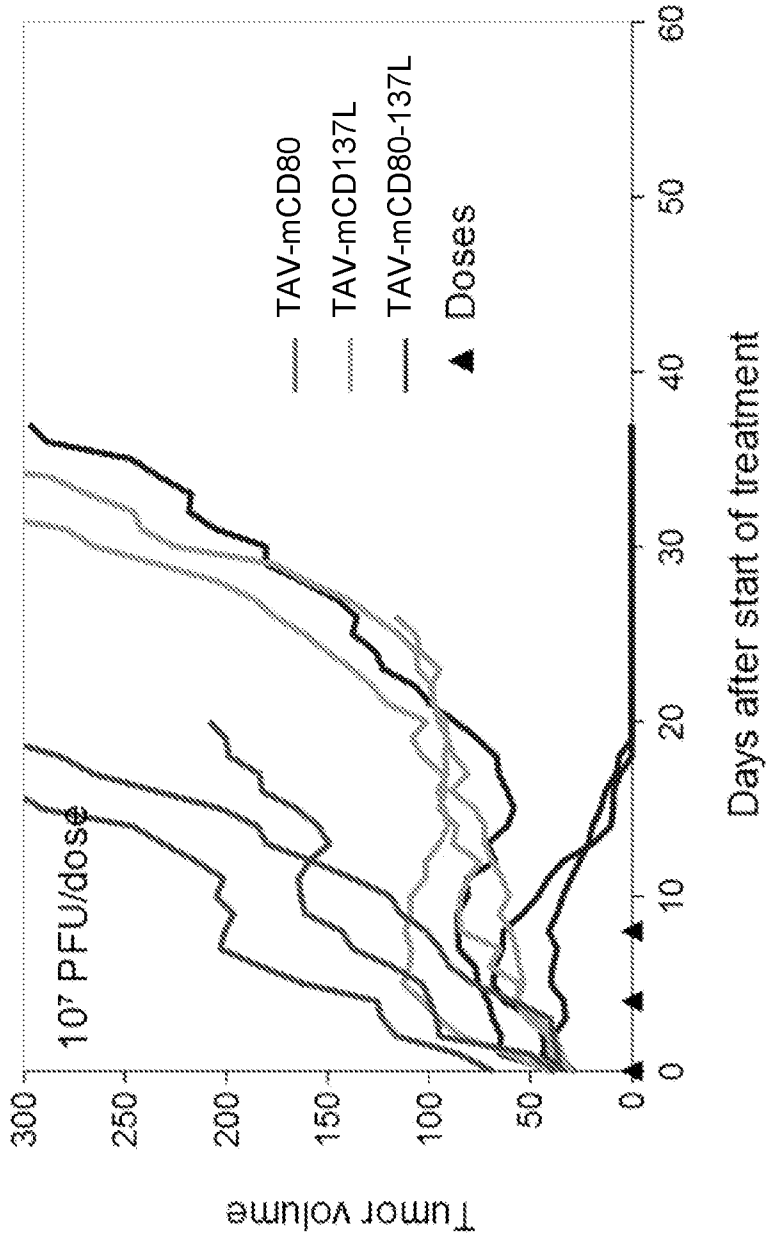

Tumor volume
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Days after start of treatment
10⁷ PFU/dose
TAV-mCD80
TAV-mCD137L
TAV-mCD80-137L
Doses

FIGURE 11

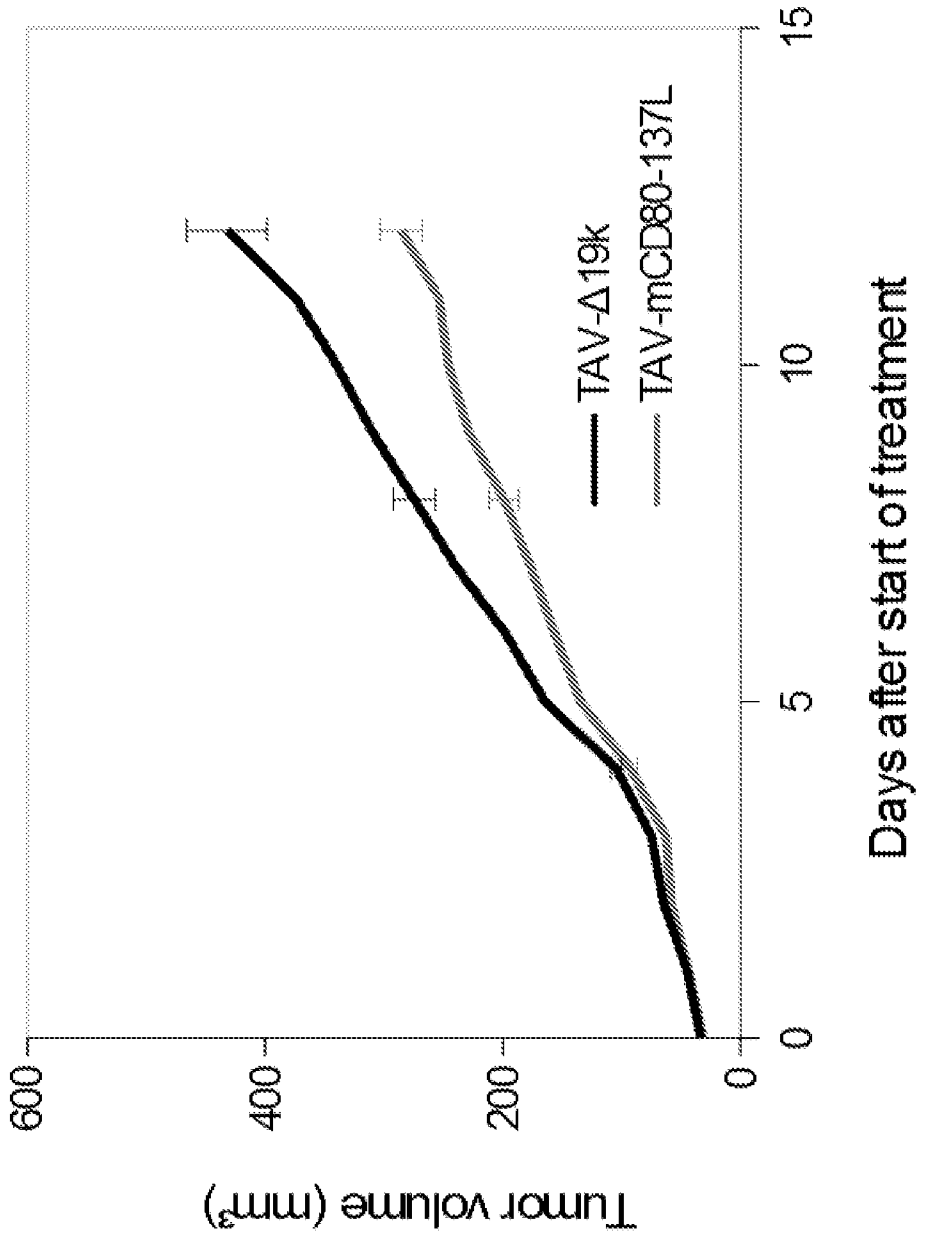

Tumor volume (mm³)
600
400
200
0
0
5
10
15
Days after start of treatment
TAV-Δ19k
TAV-mCD80-137L

FIGURE 12

FIGURE 13

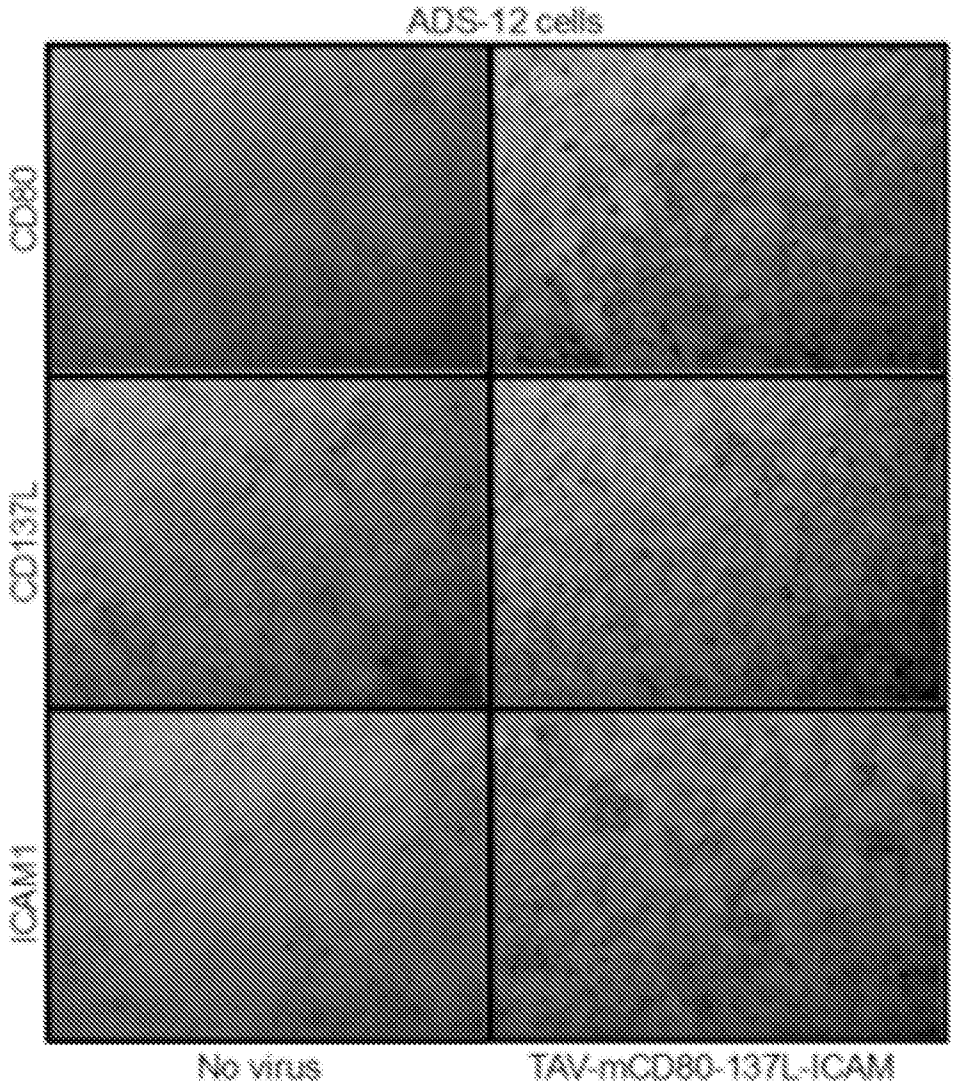


FIGURE 14

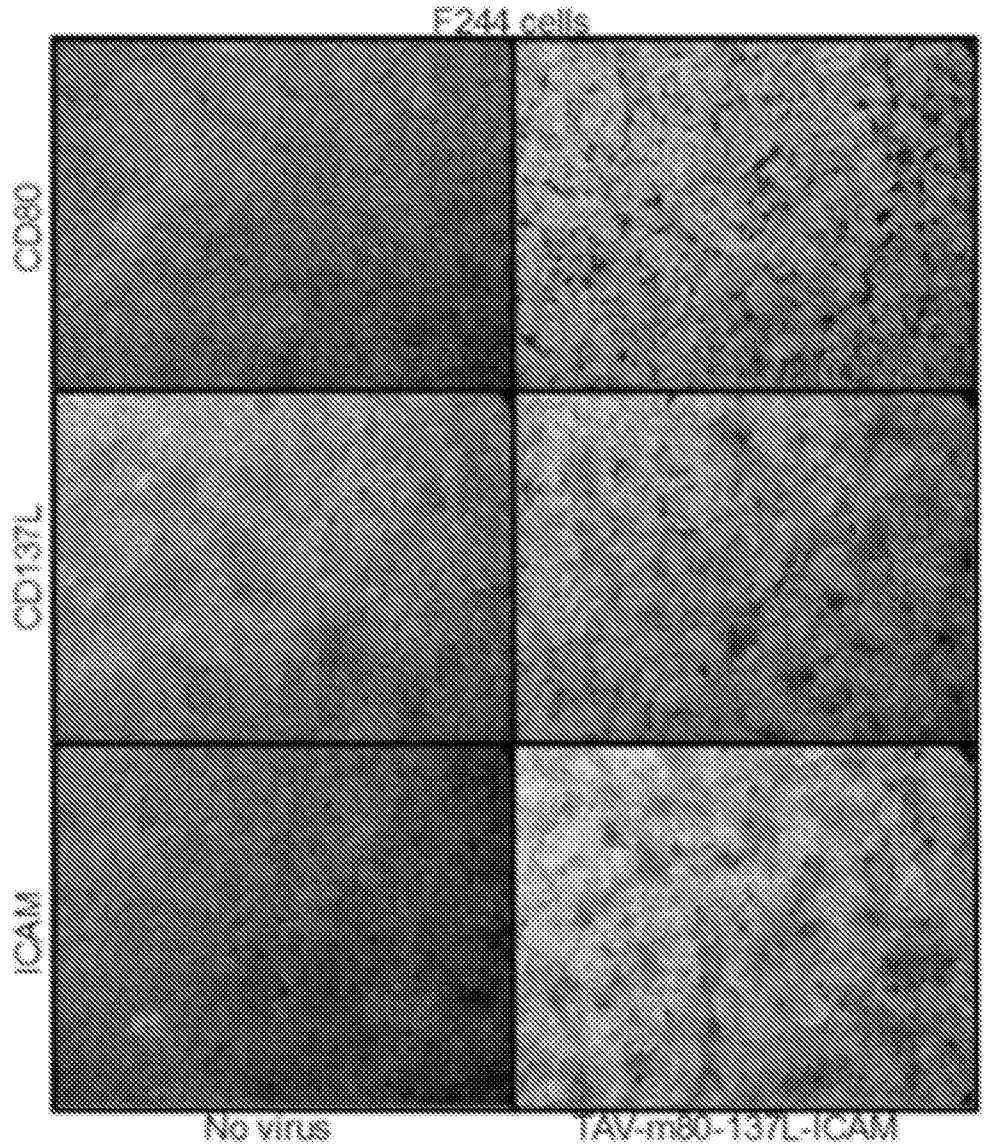


FIGURE 15

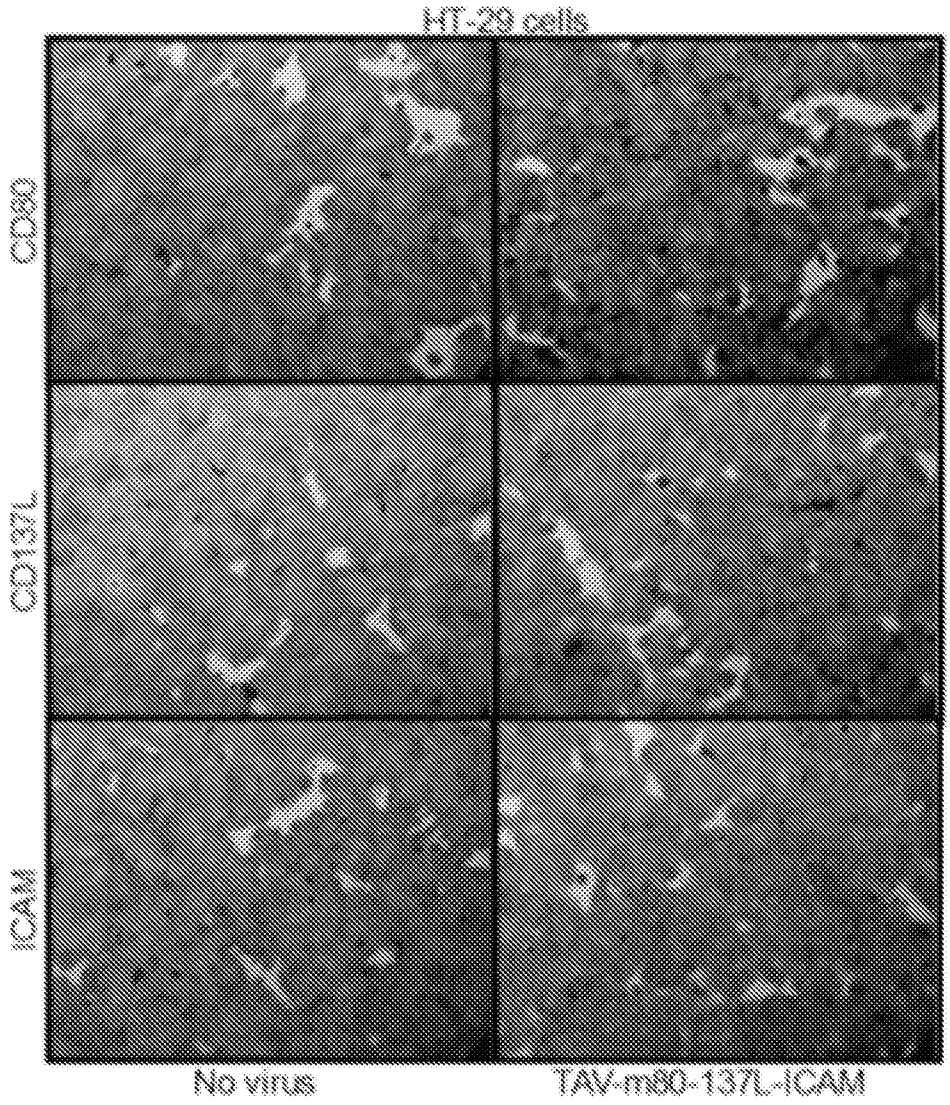


FIGURE 16A

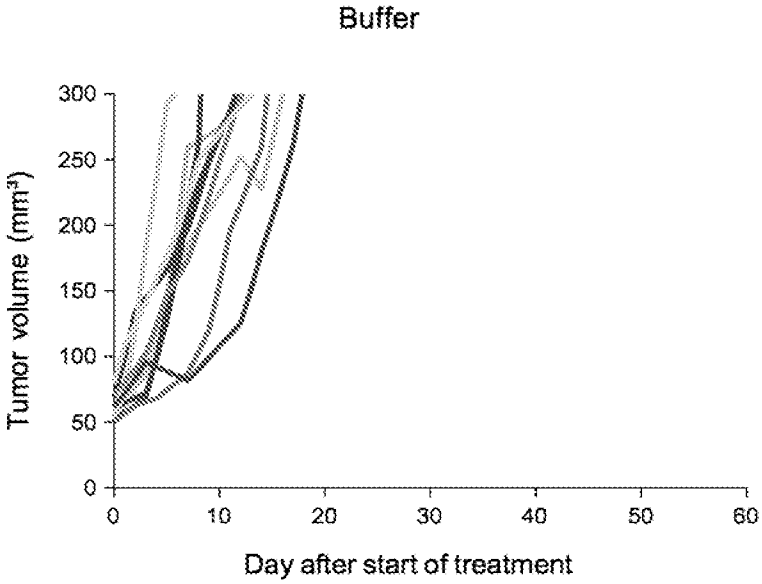


FIGURE 16B

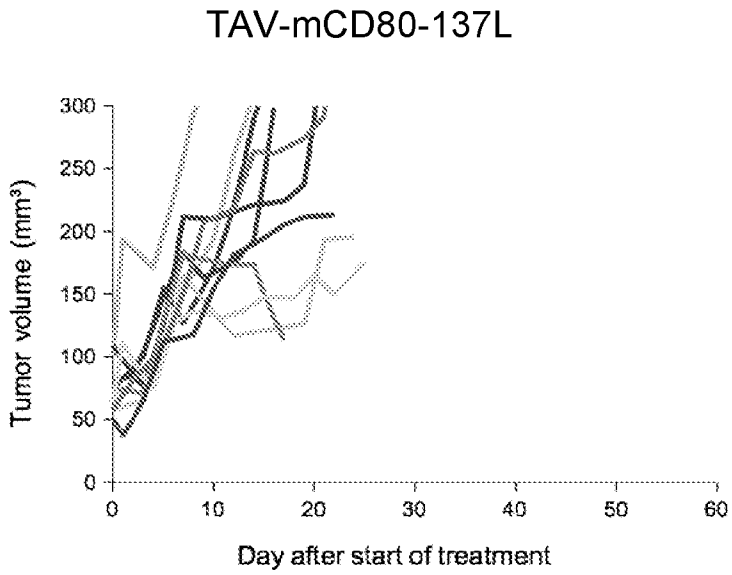
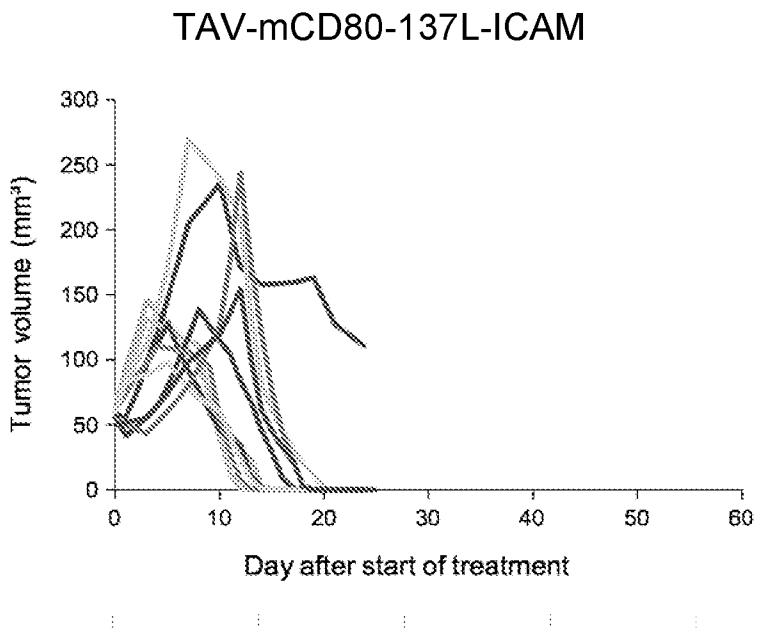


FIGURE 16C


TAV-mCD80-137L-ICAM
Tumor volume (mm³)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Day after start of treatment

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/016032

A. CLASSIFICATION OF SUBJECT MATTER

C12N 7/01 (2006.01) C12N 15/861 (2006.01) A61K 35/761 (2015.01) A61P 35/00 (2006.01)

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)

PATENW, WPI, CAPLUS, BIOSIS, EMBASE, MEDLINE & keywords: E1B, E1B-19K, E3, insertion, IRES and like terms; CPC and IPC symbols: A61K35/761, C12N2710/10011, C12N15/861, C12N2840/203

GENOMEQUEST: SEQ ID NO: 14

ESPACENET, PUBMED, Internal Databases: Applicant and Inventor names

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:

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"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
11 April 2018	11 April 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	Richard Filmer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832735

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/016032

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 filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/016032

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2018/016032**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/038606 A1 (ONCOS THERAPEUTICS OY) 29 March 2012 Abstract; page 17 lines 4-35; page 22 line 31 – page 23 line 29, page 27 lines 11-23, page 30 line 30 – page 31 line 7	155-174
X Y	WO 2016/049201 A1 (SALK INSTITUTE FOR BIOLOGICAL STUDIES) 31 March 2016 Abstract; page 2 lines 6-13, page 43 line 31- page 44 line 10, page 47 line 11 – page 52 line 19; SEQ ID NOs: 1-31, 62, 63, 69-73, 82-84, 86-91 and 98 Abstract; page 2 lines 6-17	155, 156, 159-161, 164-174 84-90
Y	WO 2010/101921 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 September 2010 Abstract; [0006], [0011]-[0013], [0059], [0063], [0125], [0142]-[0144], [0169]-[0173], [0176]	1-117, 151-154, 156-174
Y	SMALL, J. C. et al., "Construction and Characterization of E1- and E3-Deleted Adenovirus Vectors Expressing Two Antigens from Two Separate Expression Cassettes", Human Gene Therapy, 2014, vol. 25, pages 328-338 Abstract; Introduction: 1st paragraph; Materials and Methods	1-32, 63-82, 84-117, 151-154, 156-174
Y	KR 10-0896483 B1 (INDUSTRY-ACADEMIC COOPERATION FOUNDATION, YONSEI UNIVERSITY) 08 May 2009 Abstract; paragraphs <30> to <32>	1-32, 63-82, 84-117, 151-154, 156-174
Y	WEIPING, Z. et al., "Construction of the Dicistronic Adenovirus Vector Expressing Bioactive Human Interleukin-12", Chinese Journal of Cancer Research, 1997, vol. 9, no. 4, pages 299-303 Abstract; page 299 column 2	33-117, 151-154, 156-174

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

• Invention 1 is defined by claims 1-32 (completely) and claims 63-82, 84-136, 138, 139, 142-148, 151-154 and 156-174 (in part). The feature of a recombinant adenovirus comprising a first therapeutic transgene inserted into an E1b-19K insertion site and a second therapeutic transgene inserted into an E3 insertion site is specific to this group of claims.

• Invention 2 is defined by claims 33-62, 83, 137, 140, 141, 149 and 150 (completely) and claims 63-82, 84-136, 138, 139, 142-148, 151-154, and 156-174 (in part). The feature of a recombinant adenovirus comprising a first and second therapeutic transgene inserted into an E1b-19K insertion site and separated by an IRES sequence is specific to this group of claims.

• Invention 3 is defined by claim 155 (completely) and claims 156-174 (in part). The feature of a recombinant adenovirus comprising a sequence that at least 80% identical to SEQ ID NO: 14 is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a recombinant adenovirus comprising a first therapeutic transgene inserted into an E1b-19K insertion site or a recombinant adenovirus comprising multiple transgenes. However these features do not make a contribution over the prior art because they are disclosed in:

WO2010/101921 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 September 2010

SMALL, J. C. et al., "Construction and Characterization of E1- and E3-Deleted Adenovirus Vectors Expressing Two Antigens from Two Separate Expression Cassettes", Human Gene Therapy, 2014, vol. 25, pages 328-338

Therefore in the light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2018/016032**

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 2012/038606 A1	29 March 2012	WO 2012038606 A1	29 Mar 2012
		AU 2011306845 A1	02 May 2013
		AU 2011306845 B2	20 Nov 2014
		BR 112013006699 A2	14 Jun 2016
		CA 2812093 A1	29 Mar 2012
		CN 103221544 A	24 Jul 2013
		EP 2619312 A1	31 Jul 2013
		FI 20105991 A	25 Mar 2012
		FI 124927 B	31 Mar 2015
		JP 2014500004 A	09 Jan 2014
		KR 20130108371 A	02 Oct 2013
		RU 2013118723 A	27 Oct 2014
		SG 189001 A1	31 May 2013
		US 2013243731 A1	19 Sep 2013
		ZA 201302429 B	30 Jul 2014
WO 2016/049201 A1	31 March 2016	WO 2016049201 A1	31 Mar 2016
		AU 2015320665 A1	06 Apr 2017
		CA 2961748 A1	31 Mar 2016
		EP 3198009 A1	02 Aug 2017
		JP 2017534263 A	24 Nov 2017
		KR 20170063801 A	08 Jun 2017
		US 2017202893 A1	20 Jul 2017
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
		SM T201500329 B	25 Feb 2016
		US 2011318311 A1	29 Dec 2011
		US 9073980 B2	07 Jul 2015

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/US2018/016032**

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 2016017294 A1	21 Jan 2016
KR 10-0896483 B1	08 May 2009	KR 20090007067 A	16 Jan 2009
		KR 100896483 B1	08 May 2009

End of Annex

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