

ONCOLYTIC VIRUS AND METHOD

The present disclosure relates to an oncolytic virus, such as an adenovirus, compositions comprising the oncolytic virus of the present disclosure, methods of preparing said oncolytic virus and compositions and use of the oncolytic virus or composition in treatment, in particular in the treatment of cancer. Also provided is the treatment of a patient population characterised as having a cancer expressing CD40, in particular a cancer over expressing CD40, with a therapy according to the present disclosure.

BACKGROUND

CD40 is an activator for B and T cells, for example CD40 on antigen presenting cells binds CD40L (also known as CD154) on T cells to activate the same. CD40 is also present on a number of tumour cells, which use the CD40 to obtain cytokines and growth factors from the surrounding cells to support the growth and expansion of the cancer.

Agonistic anti-CD40 antibodies *in vivo* may be able to stimulate anti-tumor immune responses due to action on immune cells. This may be because the CD40 has the ability to, for example activate macrophages and to “precondition” dendritic cells, allowing them to prime effective cytotoxic T-cell responses.

At the present time there is a significant interest in immune oncology therapies and/or therapies directed to tumor associated macrophages (TAMs). These tumor-associated-macrophages surround the tumor and contribute to creating a microenvironment, which is permissive to tumor growth and development. This microenvironment is often hypoxic and can neutralize immune cells sent to attach and eliminate the tumor. Thus, the microenvironment physically protects the tumor. Furthermore, it supplies energy and nutrients to support tumor growth.

However, to be effective the therapies need to be really targeted to this environment to re-invigorate, liberate, recruit and re-activate immune cells, for example already trapped in the microenvironment. This targeting of the microenvironment is not easy to do because sometimes the tumor has development mechanisms for protection, such as active transport mechanisms which move therapeutics out of the tumor environment. This is, for example one of the mechanisms involved in resistance.

Oncolytic viruses, such as group B oncolytic adenoviruses, which home to the tumor cells and selectively infect the cancer cells may be harnessed to deliver the anti-CD40 antibody to the microenvironment of the tumor. The oncolytic virus has anti-cancer properties, which result in the death of the cancer cell and release of the content of said cell. The contents of the cell include the anti-CD40 antibody made by the oncolytic virus. Thus death of the cancer cell releases the antibody into the microenvironment of the tumor. Surprisingly, the present inventors have found that anti-CD40 antibodies can be efficiently delivered by a variety of different oncolytic viruses by incorporating transgenes encoding anti-CD40 antibodies into such viruses, for example by transducing the viruses with a transgene cassette of the present disclosure.

In addition, because tumors have developed multiple mechanisms for protection, it is thought going forward that the most effective cancer therapies in the future will need to attack the cancer via two or more biological mechanisms.

Employing agonistic anti-CD40 antibodies may have the additional benefit that once the antibody is in the tumor microenvironment at suitable concentrations it can compete with CD40-expressing cancer cells to bind to CD40L. This means ultimately that there will be less opportunity for the cancer cells to bind CD40L on T cell. In turn this may mean that the amount of energy and nutrients available to the tumor may be reduced.

The present inventors have designed a transgene cassette encoding an anti-CD40 antibody or binding fragment thereof that can be used to make stable viruses.

PARAGRAPHS SUMMARISING THE DISCLOSURE

The present disclosure provides:

1. An oncolytic virus (for example a replication competent virus) comprising a transgene cassette encoding an anti-CD40 antibody or binding fragment thereof, wherein the transgene cassette comprises an amino acid sequence given in SEQ ID NO: 12 or a sequence at least 95% identical thereto (such as 96, 97, 98 or 99% identical thereto, especially over the full length of the sequence), in particular a cassette of SEQ ID NO: 12.

(Alternative) paragraph 1: An oncolytic adenovirus (for example a replication competent oncolytic adenovirus) encoding an anti-CD40 antibody or binding fragment thereof, wherein the adenovirus comprises SEQ ID NO: 1 or a sequence at least 95% identical thereto (such as a 96, 97, 98 or 99% identical thereto).

2. An oncolytic virus according to paragraph 1, wherein the virus is selected from an adenovirus, herpes simplex virus, reovirus, measles virus, Newcastle disease virus, Seneca Valley virus, Vesicular stomatitis virus, polio virus, ECHO enterovirus, Coxsackie virus, and vaccinia virus, in particular an adenovirus.
3. An oncolytic virus according to paragraphs 1 or 2, wherein the virus is selected from the group consisting of Enadenotucirev, talimogene laherparepvec, RIGVIR, Ad5-yCD/mutTKSR39rep-hIL12, Cavatak™, CG0070, DNX-2401, G207, HF10, Imlygic®, JX-594, MG1-MA3, MV-NIS, OBP-301, Reolysin®, Toca 511, in particular Enadenotucirev.
4. An oncolytic virus according to any one of paragraphs 1 to 3, wherein the virus comprises SEQ ID NO: 1.
5. An oncolytic virus according to paragraph 4, consists of SEQ ID NO: 1.
6. A pharmaceutical composition comprising a virus according to any one of paragraphs 1 to 5, and a pharmaceutically acceptable excipient, diluent or carrier.
7. An oncolytic virus according to any one of claims 1 to 5 or a pharmaceutical composition according to paragraph 6, for use in treatment.
8. An oncolytic virus according to any one of paragraphs 1 to 5 or a pharmaceutical composition according to paragraph 6, for use in the treatment of cancer, insulin resistance, obesity and/or immune deficiency.

9. A use according to paragraph 6, wherein the virus or composition is for use in the treatment of cancer, for example for the treatment of cancer expressing CD40 (such as cancer with upregulated expression of CD40)
10. A combination therapy (for example for use in the treatment of cancer) comprising a virus according to any one of paragraphs 1 to 5 or a composition according to paragraph 6 and a further anti-cancer therapy.
11. A combination therapy according to paragraph 10, wherein the further anti-cancer therapy is chemotherapy.
12. A combination therapy according to paragraph 10 or 11, wherein the further anti-cancer therapy is a check point inhibitor.
13. A combination therapy according to paragraph 12, wherein the anti-cancer therapy is selected from the group comprising a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, a TIM-3 inhibitor, a LAG-3 inhibitor, a TIGIT inhibitor, a B7-H3 (CD276) inhibitor, a B7-H4 (B7S1) inhibitor, a B7H7 (HHLA2) inhibitor, a CD96 inhibitor, a VISTA inhibitor and a combination of two or more of the same.
14. A combination therapy according to paragraph 13, wherein the inhibitor is an antibody or binding fragment thereof.
15. A combination therapy according to any one of paragraphs 10 to 14, wherein the further anti-cancer therapy is a costimulatory pathway agonist.
16. A combination therapy according to paragraph 15, wherein the further anti-cancer therapy is selected from the group comprising a CD27 agonist, a CD28 agonist, an ICOS agonist, a TMIGD2 (IGPR-1/CD28H) agonist, a CD226 agonist, an OX40 agonist, a 4-1BB agonist, and a combination of two or more of the same.
17. A combination therapy according to paragraph 16, wherein the therapy is an antibody or binding fragment thereof.
18. A combination therapy according to any one of paragraphs 10 to 17, wherein the further anti-cancer therapy activates immune responses or reverse suppression of immune responses, for example selected from IL-10, TGF β , IDO inhibitors, and a combination of two or more of the same.
19. A combination therapy according to any one of paragraphs 10 to 18, wherein the further cancer therapy is an oncolytic virus (further oncolytic virus), for example a replication competent oncolytic virus, such as an adenovirus, in particular a group B adenovirus.
20. A combination therapy according to paragraph 19, wherein the oncolytic virus (further oncolytic virus) encodes therapeutic gene encoding material selected from the group consisting of an RNAi sequence, an antibody or binding fragment thereof, chemokines, cytokines, immunomodulator and enzymes.
21. A combination therapy according to paragraph 20, wherein the antibody or binding fragment thereof is specific to OX40, OX40 ligand, CD27, CD28, CD30, CD40, CD40 ligand, CD70, CD137, GITR, 4-1BB, ICOS, ICOS ligand, CTLA-4, PD-1, PD-L1, PD-L2, VISTA, B7-H3, B7-H4, HVEM, ILT-2, ILT-3, ILT-4, TIM-3, LAG-3, BTLA, LIGHT, CD160,

- CTLA-4, PD-1, PD-L1, PD-L2, CD40, CD40 ligand and combinations of two or more of the same.
22. A combination therapy according to paragraph 20 or 21, wherein the cytokine is independently selected from the group comprising IL-1 α , IL-1 β , IL-6, IL-9, IL-12, IL-13, IL-17, IL-18, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-33, IL-35, IL-2, IL-4, IL-5, IL-7, IL-10, IL-15, IL-21, IL-25, IL-1RA, IFN α , IFN β , IFN γ , TNF α , TGF β , lymphotoxin α (LTA) and GM-CSF, for example IL-12, IL-18, IL-22, IL-7, IL-15, IL-21, IFN γ , TNF α , TGF β and lymphotoxin α (LTA) and combinations of two or more of the same.
 23. A combination therapy according to any one of paragraphs 20 to 22, wherein the chemokine independently selected from the group comprising IL-8, CCL3, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19, CCL21, CXCR2, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, CXCR4, CXCR5 and CRTH2, for example CCL5, CXCL9, CXCL12, CCL2, CCL19, CCL21, CXCR2, CCR2, CCR4 and CXCR4, a receptor of any one of the same, and combinations of two or more of the same.
 24. A combination therapy according to any one of paragraphs 19 to 23, wherein the oncolytic virus (further oncolytic virus) encodes a transmembrane anchored form of a B7 protein, for example B7-1 or B7-2.
 25. A combination therapy according to any one of paragraphs 19 to 24, wherein the oncolytic virus (further oncolytic virus) encodes a checkpoint inhibitor selected from the group comprising a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, a TIM-3 inhibitor, a LAG-3 inhibitor, a TIGIT inhibitor, a B7-H3 (CD276) inhibitor, a B7-H4 (B7S1) inhibitor, a B7H7 (HHLA2) inhibitor, a CD96 inhibitor, a VISTA inhibitor and combinations of two or more of the same.
 26. A combination therapy according to paragraph 25, wherein the inhibitor is an antibody or binding fragment thereof.
 27. A combination therapy according to any one of paragraphs 19 to 26, wherein the oncolytic virus (further oncolytic virus) encodes a costimulatory pathway agonist.
 28. A combination therapy according to paragraph 27, wherein the oncolytic virus (further oncolytic virus) encodes a costimulatory pathway agonist selected from the group comprising a CD27 agonist, a CD28 agonist, an ICOS agonist, a TMIGD2 (IGPR-1/CD28H) agonist, a CD226 agonist, an OX40 agonist, a 4-1BB agonist and a combination of two or more of the same.
 29. A combination therapy according to any one of paragraphs 19 to 28, wherein the oncolytic virus (further oncolytic virus) encodes a molecule which activates immune responses or reverse suppression of immune responses, for example selected from IL-10, TGF β , IDO inhibitors and a combination of two or more of the same.
 30. A pharmaceutical formulation comprising an oncolytic adenovirus as defined in any one of paragraphs 1 to 5 and comprising a further oncolytic virus as defined in any one of claims 19 to 29.
 31. A combination therapy as defined in any one of paragraphs 19 to 29 or a pharmaceutical composition as defined in claim 30, for use in treatment.

32. An oncolytic virus according to any one of paragraphs 1 to 5 or a pharmaceutical composition according to claim 6 or 30, for use in the manufacture of a medicament for the treatment of cancer, insulin resistance, obesity, and/or immune deficiency.
33. A use according to paragraph 32, wherein the adenovirus or composition is for use in the manufacture of a medicament for the treatment of cancer.
34. A use according to paragraph 33, wherein the target patient population for treatment have cancer which expresses CD40, such as a population where CD40 is upregulated in the cancer.
35. A combination therapy for use in the manufacture of a medicament for the treatment of cancer comprising a virus according to any one of paragraphs 1 to 5 or a composition according to paragraph 6 or 30 and a further anti-cancer therapy, for example where the target patient population for treatment have cancer which expresses CD40, such as a population where CD40 is upregulated in the cancer.
36. A combination therapy according to paragraph 35, wherein the further anti-cancer therapy is chemotherapy.
37. A combination therapy according to paragraph 35 or 36, wherein the further anti-cancer therapy is a check point inhibitor.
38. A combination therapy according to paragraph 37, wherein the anti-cancer therapy is selected from the group comprising a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, a TIM-3 inhibitor, a LAG-3 inhibitor, a TIGIT inhibitor, a B7-H3 (CD276) inhibitor, a B7-H4 (B7S1) inhibitor, a B7H7 (HHLA2) inhibitor, a CD96 inhibitor, a VISTA inhibitor and combinations of two or more of the same.
39. A combination therapy according to any one of paragraphs 35 to 38, wherein the further cancer therapy is an oncolytic virus, for example a replication competent oncolytic virus, such as defined in any one of claims 19 to 29.
40. A method of treatment comprising administering a therapeutically effective amount of an oncolytic adenovirus according to any one of paragraphs 1 to 5 or a pharmaceutical composition according to paragraph 6 or 30 to a patient in need thereof.
41. A method according to paragraph 40, for the treatment of cancer, insulin resistance, obesity, and/or immune deficiency.
42. A method according to paragraph 41, for the treatment of cancer, for example a cancer expressing CD40 (such as a cancer with upregulated CD40 expression).
43. A method according to any one of paragraphs 37 to 42, wherein treatment further comprises an additional anti-cancer therapy.
44. A combination therapy according to paragraph 43, wherein the further anti-cancer therapy is chemotherapy.
45. A combination therapy according to paragraph 42 or 44, wherein the further anti-cancer therapy is a check point inhibitor.
46. A combination therapy according to paragraph 45, wherein the anti-cancer therapy is selected from the group comprising a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, a TIM-3 inhibitor, a LAG-3 inhibitor, a TIGIT inhibitor, a B7-H3 (CD276)

inhibitor, a B7-H4 (B7S1) inhibitor, a B7H7 (HHLA2) inhibitor, a CD96 inhibitor, a VISTA inhibitor and combinations of two or more of the same.

47. A combination therapy according to any one of paragraphs 42 to 46, wherein the further cancer therapy is an oncolytic virus, for example a replication competent oncolytic virus, for example a virus defined in any one of claims 19 to 29.

BRIEF DESCRIPTION OF FIGURES

- Figure 1** shows a schematic of the anti-CD40 transgene cassette
- Figure 2A & B** shows NG-350 virus activity in terms of total particle production (A) and virus particle production in the cell supernatant (B)
- Figure 2C** shows concentration of secreted IgG2 anti-CD40 antibody measured using ELISA.
- Figure 3** shows results of restriction digestion of control vs NG-350 DNA using the combination EcoRv/NheI (A) or individual enzymes NcoI or FspI (B)
- Figure 4** shows location in NG-350 transgene cassette where primers bind
- Figure 5** shows separation of PCR products using gel electrophoresis
- Figure 6** shows separation of PCR products using Primer set D (A) and Primer set K (B)
- Figure 7A** shows quantification of the % cell survival at various infection density for EnAd and NG-350A (virus of SEQ ID NO: 1)
- Figure 7 & 8** shows quantification of the number of detected virus genomes per cell for NG-350A and EnAd
- Figure 9A & B** show absorbance at 450nm in each well of the plate measured using a plate reader (BioTek) and the concentrations of secreted IgG2 anti-CD40 antibody
- Figure 9C** shows absorbance at 450nm for EnAd, NG-350A and positive controls (Figure 4C) and specific binding to CD40 by the secreted anti-CD40 antibody present in the supernatant of NG-350A infected cells.
- Figure 10** shows absorbance at 620nm measured for each sample using a plate reader for NG-350A, EnAd or NG-165 infected cells
- Figure 11A & B** shows absorbance at 450nm in each well of the plate was measured using a plate reader (BioTek) and the concentrations of secreted IgG2 anti-CD40 antibody
- Figure 12** shows concentration of secreted IgG2 anti-CD40 antibody measured using ELISA
- Figure 13** shows percentage of moDCs expressing CD86 (A), CD54 (B) and HLA-DR (C) activation markers
- Figure 14** shows secretion of IL12p40 by moDCs cultured with purified anti-CD40 antibody produced by NG-350A infected tumour cells in the presence or absence of EnAd virus, or EnAd virus only

- Figure 15** shows percentage of CD19+ cells expressing CD86 (A), CD54 (B), MFI HLA-DR (C) and CD80 (D)
- Figure 16** shows percentage of dividing B cells after treatment with purified anti-CD40 antibody produced by NG-350A infected tumour cells and EnAd virus together compared with antibody or virus treatment alone
- Figure 17** shows that a virus-depleted antibody preparation produced by NG-350A infected tumor cells binds to CD40 on the surface of human MoDC's
- Figure 18** shows effects of virus-depleted anti-CD40 Ab produced by NG-350A infected tumour cells on cell surface marker upregulation on human MoDCs as an example (A) and dose responses at 24 and 48 hours for 4 different MoDC donors (B-E)
- Figure 19** shows effects of virus-depleted anti-CD40 Ab produced by NG-350A infected tumour cells on cytokine secretions by human MoDCs as dose responses at 24 and 48 hours for 4 different MoDC donors (B-D)
- Figure 20** shows that an antibody preparation produced by NG-350A infected tumor cells, not depleted of virus, binds to CD40 on the surface of human MoDC's
- Figure 21** shows effects of anti-CD40 Ab produced by NG-350A infected tumour cells, not depleted of virus, on cell surface marker upregulation at 24 and 48 hours on human MoDC's from 2 different donors
- Figure 22** shows effects of anti-CD40 Ab produced by NG-350A infected tumour cells, not depleted of virus, on cytokine secretions by human MoDCs as dose responses at 24 and 48 hours for 2 different donors
- Figure 23** shows effects of virus-depleted anti-CD40 Ab produced by NG-350A infected tumour cells on cell surface marker upregulation on human B-cells as dose responses at 24 and 48 hours for 3 different B-cell donors
- Figure 24** shows effects of anti-CD40 Ab produced by NG-350A infected tumour cells, not depleted of virus, on cell surface marker upregulation at 24 and 48 hours on human B-cells from 3 different donors
- Figure 25** shows time courses of viral genome replication (qPCR) by 10 different tumour cell lines infected with NG-350A, compared to those for EnAd
- Figure 26** shows time courses of virus-induced oncolysis (xCELLigence assay) in four example tumour cells lines infected with NG-350A, compared to those for EnAd
- Figure 27** shows time courses of anti-CD40 antibody mRNA expression by 10 different tumour cell lines infected with NG-350A
- Figure 28** shows detection of anti-CD40 transgene protein expression by IgG2 ELISA by different tumour cells lines

- Figure 29** shows a time course of acute plasma cytokine responses following IV dosing with NG-350A particles compared to those of EnAd; MCP-1 (A), IL-6 (B) and TNF α (C)
- Figure 30** shows a time course of alanine transaminase (ALT) concentrations in plasma after a single intravenous dose of EnAd or NG-350A
- Figure 31** shows acute plasma cytokine responses following first and third dose of a repeat IV dosing regimen with NG-350A particles compared to those for EnAd; MCP-1 (A), IL-6 (B)
- Figure 32** shows virus pharmacokinetics in peripheral blood after each of three intravenous doses of EnAd or NG-350A
- Figure 33** shows recovery of live virus from murine tissues after a single intravenous dose of NG-350A virus particles
- Figure 34** shows virus genome replication in subcutaneous A549 (A&B) and HCT116 (C&D) tumours after three IV injections or a single fractionated IT dose of NG-350A or EnAd
- Figure 35** shows virus E3 mRNA expression in subcutaneous A549 (A&B) or HCT-116 (C&D) tumours after three IV injections or a single fractionated IT dose of NG-350A or EnAd
- Figure 36** shows anti-CD40 agonist antibody transgene mRNA expression in subcutaneous A549 (A&B) and HCT-116 (C&D) tumours after three IV injections or a single fractionated IT dose of NG-350A or EnAd
- Figure 37** shows anti-CD40 antibody protein in subcutaneous A549 tumours after three IV injections or a single fractionated IT dose or NG-350A or EnAd

DETAILED DISCLOSURE

CD40 is a co-stimulatory protein found, for example on antigen presenting cells. CD40 is also known as Bp50, CDW40, TNFRSF5, p50, CD40 protein, CD40 molecule. The human protein has the UniProt number P25942. The murine protein has the UniProt number P27512.

Oncolytic virus with selectivity for cancer cells as employed herein refers to a virus that preferentially kills cancer cells, for example because it preferentially infects cancer cells and/or the virus life cycle is dependent on a gene, such as p53 that is dysregulated, for example over-expressed in cancer cells. In one embodiment the oncolytic virus preferentially infects cancer cells and goes on to replicate its genome and produce capsid proteins to generate new virus particles, for example as per EnAd.

The selectivity for cancer cells (therapeutic index) can be tested as described in WO2005/118825 incorporated herein by reference.

In one embodiment, the oncolytic virus is a virus selected from an adenovirus, herpes simplex virus, reovirus, measles virus, Newcastle disease virus, Seneca Valley virus,

Vesicular stomatitis virus, polio virus, ECHO enterovirus, Coxsackie virus, and vaccinia virus, in particular an adenovirus.

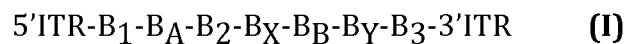
In one embodiment, the adenovirus is selected from the group consisting of Enadenotucirev, talimogene laherparepvec, RIGVIR, Ad5-yCD/mutTKSR39rep-hIL12, Cavatak™, CG0070, DNX-2401, G207, HF10, Imlygic®, JX-594, MG1-MA3, MV-NIS, OBP-301, Reolysin®, Toca 511, in particular Enadenotucirev.

In one embodiment the oncolytic adenovirus employed in the combination therapy of the present disclosure is replication competent.

In one embodiment the oncolytic adenovirus employed in the combination of the present disclosure is replication deficient.

In one embodiment the virus of the present disclosure is employed in a combination therapy.

In one embodiment an oncolytic adenovirus employed in the present disclosure or as a second component in combination therapy of the present disclosure, for example has a formula **(I)**:



wherein:

B_1 is a bond or comprises: E1A, E1B or E1A-E1B (in particular E1A, E1B or E1A-E1B);

B_A is E2B-L1-L2-L3-E2A-L4;

B_2 is a bond or comprises E3 or a transgene, for example under an endogenous or exogenous promoter;

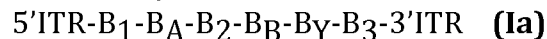
B_X is a bond or a DNA sequence comprising: a restriction site, one or more transgenes or both;

B_B comprises L5;

B_Y comprises the transgene cassette which encodes a therapeutic protein or an active fragment thereof (in particular, comprises SEQ ID NO: 12); and

B_3 is a bond or comprises E4.

In one embodiment the oncolytic adenovirus has a formula **(Ia)**:



wherein:

B_1 is a bond or comprises: E1A, E1B or E1A-E1B (in particular E1A, E1B or E1A-E1B);

B_A is E2B-L1-L2-L3-E2A-L4;

B_2 is a bond or comprises E3;

B_B comprises L5;

B_Y comprises the transgene cassette which encodes a therapeutic protein or an active fragment thereof (in particular comprises SEQ ID NO: 12); and

B_3 is a bond or comprises E4.

In one embodiment the virus genome in constructs of formula **(I)** and/or **(Ia)** is from Ad11 or EnAd, in particular EnAd.

In one embodiment the transgene cassette is under the control of an endogenous promoter, for example the major late promoter.

Therapeutic proteins include an antibody or binding fragment (for example selected from the group comprising antibodies or fragments specific to CTLA-4, PD-1, PD-L1, PD-L2, VISTA, B7-H3, B7-H4, HVEM, ILT-2, ILT-3, ILT-4, TIM-3, LAG-3, BTLA, LIGHT or CD160 for example CTLA-4, PD-1, PD-L1 and/or PD-L2), a B-7 protein (such as B7-1 and/or B7-2), a cytokine (for example selected from IL-1 α , IL-1 β , IL-6, IL-9, IL-12, IL-13, IL-17, IL-18, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-33, IL-35. Interleukin-2 (IL-2), IL-4, IL-5, IL-7, IL-10, IL-15, IL-21, IL-25, IL-1RA, IFN α , IFN β , IFN γ , TNF α , TGF β , lymphotoxin α (LTA) and GM-CSF), and a chemokine (for example IL-8, CCL3, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19, CCL21, CXCR2, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, CXCR4, CXCR5 and/or CRTH2), and combinations of two or more of the same.

The therapy of the present disclosure may comprise two or more oncolytic viruses.

Regulatory Elements

In one embodiment B γ comprises a transgene cassette according to the present disclosure, said cassette further comprises a transgene encoding a checkpoint inhibitor, for example an anti-CTLA-4 antibody, an anti-PD-1, and anti-PD-L1 antibody or a binding fragment of any one of the same and a regulatory element, such as combination of regulatory elements.

In one embodiment the regulatory element is splice acceptor sequence.

In one embodiment the regulatory element is a Kozak sequence.

In one embodiment, for example where the transgene encodes a polycistronic RNA molecule, the regulatory element is an IRES sequence.

In one embodiment the regulatory sequence is a high efficiency self-cleavable peptide sequence such as P2A, T2A, F2A, E2A.

In one embodiment the regulatory sequence is a polyA tail.

In one embodiment there are at least two regulatory sequences, for example a splice acceptor and a Kozak sequence or a splice acceptor and a polyA tail, or a splice acceptor and an IRES sequence, or a splice acceptor and a P2A sequence.

In one embodiment there are at least three regulator sequences, for example a splice acceptor sequence, a Kozak sequence and polyA tail, or a splice acceptor sequence an IRES or 2A sequence and a polyA tail; or a splice acceptor sequence, Kozak sequence and an IRES or 2A sequence.

In one embodiment there are at least four regulatory sequences, for example a splice acceptor sequence, a Kozak sequence, an IRES or 2A sequence and a polyA tail, in particular located between L5 and E4 in the order splice acceptor sequence, Kozak sequence, IRES or 2A sequence and a polyA tail.

In one embodiment the transgene encodes a polycistronic RNA molecule comprising both an IRES and a 2A regulatory sequence.

In one embodiment the protein or proteins encoded in the transgene cassette for cell membrane expression may also comprise a peptide linker or spacer between the transmembrane domain or GPI anchor and the extracellular ligand binding domain. Such linkers or spacers may add flexibility to the cell surface expressed protein that enhances the ability of the protein to interact with its target molecule, for example on an adjacent cell. Such linkers or spacers may also be designed or selected to promote dimerisation or trimerisation of the proteins at the cell surface, via disulphide bond formation or protein-protein interactions. For example, the hinge regions of immunoglobulin molecules or CD8 may be employed to enhance both flexibility and dimerisation.

In one embodiment the protein or proteins encoded in the transgene cassette may also comprise a peptide tag.

The peptide tag may include c-myc, poly-histidine, V5 or FLAG tags and can be located on the N-terminus or C-terminus of the polypeptide, for example intracellularly or extracellularly, or may be encoded within the protein for example in an extracellular loop or between the transmembrane domain and the extracellular domain. Peptide tags can be used as spacers or linkers between different protein domains, for example the transmembrane and the extracellular domain, and can be used for detection or purification or detection of the protein, or cells expressing the protein.

In one embodiment the one or more additional transgenes, for example in the virus of formula (I) or (Ia) is under the control of an exogenous or endogenous promoter, for example an endogenous promoter. In one embodiment a transgene in the E3 region (B₂) is under control of an exogenous promoter.

In one embodiment the one or more additional transgenes genes are between the E3 region and the fibre L5 in the adenovirus genome, for example at a position B_X in the construct of formula (I), in particular under the control of an exogenous promoter. Thus, in one embodiment a transgene in B_X is under the control of an exogenous promoter.

In one embodiment the one or more additional transgenes genes are between the E4 region and the fibre L5 in the adenovirus genome, for example at a position B_Y in the construct of formula (I) or (Ia), in particular under the control of an endogenous promoter, such as the major late promoter. This may be in addition to the therapeutic protein or active fragment thereof encoded in the region B_Y.

Transgene as employed herein refers to a gene that has been inserted into the genome sequence of the adenovirus, wherein the gene is unnatural to the virus (exogenous) or not normally found in that particular location in the virus. Examples of transgenes are given herein. Transgene as employed herein also includes a functional fragment of the gene that is a portion of the gene which when inserted is suitable to perform the function or most of the function of the full-length gene, for example 50% of the function or more.

Transgene and coding sequence are used interchangeably herein in the context of inserts into the viral genome, unless the context indicates otherwise. Coding sequence as

employed herein means, for example a DNA sequence encoding a functional RNA, peptide, polypeptide or protein. Typically, the coding sequence is cDNA for the transgene that encodes the functional RNA, peptide, polypeptide or protein of interest. Functional RNA, peptides, polypeptide and proteins of interest are described below.

5 In one embodiment transgene as employed herein refers to a segment of DNA containing a gene or cDNA sequence that has been isolated from one organism is introduced into a different organism i.e. the virus of the present disclosure. In one embodiment this non-native segment of DNA will generally retain the ability to produce functional RNA, peptide, polypeptide or protein. Transgenes employed may for example encode a single
10 protein or active fragment thereof, chimeric protein or a fusion protein.

Clearly the virus genome contains coding sequences of DNA. Endogenous (naturally occurring genes) in the genomic sequence of the virus are not considered a transgene, within the context of the present specification unless then have been modified by recombinant techniques, such as that they are in a non-natural location or in a non-natural
15 environment.

Thus, in one embodiment the transgene(s) inserted encode(s) a human or humanised protein, polypeptide or peptide. The transgene(s) may be located within a transgene cassette for example.

GPI anchor as employed herein refers to is a glycolipid that can be attached to the C-terminus of a protein during posttranslational modification. It is composed of a
20 phosphatidylinositol group linked through a carbohydrate-containing linker (glucosamine and mannose glycosidically bound to the inositol residue) and via an ethanolamine phosphate (EtNP) bridge to the C-terminal amino acid of a mature protein. The two fatty acids within the hydrophobic phosphatidyl-inositol group anchor the protein to the cell
25 membrane.

Glypiated (GPI-linked) proteins generally contain a signal peptide, thus directing them into the endoplasmic reticulum (ER). The C-terminus is composed of hydrophobic amino acids that stay inserted in the ER membrane. The hydrophobic end is then cleaved and replaced by the GPI-anchor. As the protein progresses through the secretory pathway,
30 it is transferred via vesicles to the Golgi apparatus and finally to the extracellular space where it remains attached to the exterior leaflet of the cell membrane. Since the glypiation is the sole means of attachment of such proteins to the membrane, cleavage of the group by phospholipases will result in controlled release of the protein from the membrane. The latter mechanism is used *in vitro*; i.e., the membrane proteins released from the membranes
35 in the enzymatic assay are glypiated protein.

Phospholipase C (PLC) is an enzyme that is known to cleave the phospho-glycerol bond found in GPI-anchored proteins. Treatment with PLC will cause release of GPI-linked proteins from the outer cell membrane. The T-cell marker Thy-1 and acetylcholinesterase, as well as both intestinal and placental alkaline phosphatases, are known to be GPI-linked
40 and are released by treatment with PLC. GPI-linked proteins are thought to be preferentially

located in lipid rafts, suggesting a high level of organization within plasma membrane microdomains.

A review of GPI anchors written by Ferguson, Kinoshita and Hart is available in Chapter 11 of Essentials of Glycobiology 2nd Edition.

5 **Viruses**

Replication competent in the context of the present specification refers to a virus that possesses all the necessary machinery to replicate in cells *in vitro* and *in vivo*, i.e. without the assistance of a packaging cell line. A viral vector, for example deleted in at least the E1A region, which is capable of replicating in a complementary packaging cell line is not a replication competent virus in the present context.

A viral vector is a replication deficient virus, which requires a packaging cell line (comprising a complementary transgene) to replicate.

A replication capable virus as employed herein refers to a replication competent virus or a virus whose replication is dependent on a factor in the cancer cells, for example an upregulated factor, such as p53 or similar.

In one embodiment the adenovirus is a human adenovirus. "Adenovirus", "serotype" or adenoviral serotype" as employed herein refers to any adenovirus that can be assigned to any of the over 50 currently known adenoviral serotypes, which are classified into subgroups A-F, and further extends to any, as yet, unidentified or unclassified adenoviral serotypes. See, for example, Strauss, "Adenovirus infections in humans," in The Adenoviruses, Ginsberg, ea., Plenum Press, New York, NY, pp. 451-596 (1984) and Shenk, "Adenoviridae: The Viruses and Their Replication," in Fields Virology, Vol.2, Fourth Edition, Knipe, 35ea., Lippincott Williams & Wilkins, pp. 2265-2267 (2001), as shown in below:

SubGroup	Adenoviral Serotype
A	12, 18, 31
B	3, 7, 11, 14, 16, 21, 34, 35, 51
C	1, 2, 5, 6
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-
E	4
F	40, 41

Adenoviruses are grouped based on their capsid.

In one embodiment the adenovirus is a subgroup B, for example independently selected from the group comprising or consisting of: Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34 and Ad51, such as Ad11, in particular Ad11p (the Slobitski strain). In one embodiment the adenovirus of the invention has the capsid, such as the hexon and/or fibre of a subgroup B adenovirus, such as Ad11, in particular Ad11p. In one embodiment the adenovirus is Ad11 or has the fibre and/or hexon and/or penton of Ad11, such as Ad11p.

In one embodiment the virus of the present disclosure is not a group A virus.

In one embodiment the virus of the present disclosure does not comprise an adeno death protein (ADP).

In one embodiment the virus of the present disclosure is not a group C virus.

5 In one embodiment the virus of the present disclosure does not comprise one more fragments of an Ad5 virus.

In one embodiment the virus of the present disclosure is not Ad5.

Enadenotucirev (EnAd) is a chimeric oncolytic adenovirus, formerly known as ColoAd1 (WO2005/118825), with fibre, penton and hexon from Ad11p, hence it is a subgroup B virus. It has a chimeric E2B region, which comprises DNA from Ad11p and Ad3.
10 Almost all of the E3 region and part of the E4 region is deleted in EnAd. Therefore, it has significant space in the genome to accommodate additional genetic material whilst remaining viable. Furthermore, because EnAd is a subgroup B adenovirus, pre-existing immunity in humans is less common than, for example, Ad5. Other examples of chimeric oncolytic viruses with Ad11 fibre, penton and hexon include OvAd1 and OvAd2 (see
15 WO2006/060314).

EnAd seems to preferentially infect tumour cells, replicates rapidly in these cells and causes cell lysis. This, in turn, can generate inflammatory immune responses thereby stimulating the body to also fight the cancer. Part of the success of EnAd is hypothesised to be related to the fast replication of the virus *in vivo*.

20 Importantly, it has been demonstrated clinically that EnAd can be administered systemically (e.g. by intravenous or intraperitoneal injection or infusion) and then subsequently selectively infect and express proteins within tumour cells. This property of EnAd, which may be shared by Ad11p and other group B adenoviruses in particular those expressing the capsid proteins of Ad11p (such as those described herein), makes it possible
25 to express the encoded proteins in the tumor and/or tumor microenvironment.

Whilst EnAd selectively lyses tumour cells, it may be possible to introduce further beneficial properties, for example increasing the therapeutic activity of the virus or reducing side-effects of the virus by arming it with transgenes, such as a transgene which encodes a cell signalling protein or an antibody, or a transgene which encodes an entity which
30 stimulates a cell signalling protein(s).

Advantageously arming a virus, with DNA encoding certain proteins that can be expressed inside the cancer cell, may enable the body's own defences to be employed to combat tumour cells more effectively, for example by making the cells more visible to the immune system or by delivering a therapeutic gene/protein preferentially to target tumour
35 cells.

In one embodiment the oncolytic adenovirus of the present disclosure stimulates the patient's immune system to fight the tumor, for example by reducing the cancers ability to suppress immune responses.

In one embodiment the anti-CD40 antibody or binding fragment encoded by the virus of the present disclosure has the ability to activate immune cells, for example T cells, in the tumor microenvironment and/or in the vicinity of the tumor.

In one embodiment the oncolytic virus has a fibre, hexon and penton proteins from the same serotype, for example Ad11, in particular Ad11p, for example found at positions 30812-31789, 18254-21100 and 13682-15367 of the genomic sequence of the latter wherein the nucleotide positions are relative to Genbank ID 217307399 (accession number: GC689208).

In one embodiment the adenovirus is enadenotucirev (also known as EnAd and formerly as ColoAd1). Enadenotucirev as employed herein refers the chimeric adenovirus of SEQ ID NO: 21 disclosed in WO2016/174200 and incorporated by reference. It is a replication competent oncolytic chimeric adenovirus which has enhanced therapeutic properties compared to wild type adenoviruses (see WO2005/118825). EnAd has a chimeric E2B region, which features DNA from Ad11p and Ad3, and deletions in E3/E4. The structural changes in enadenotucirev result in a genome that is approximately 3.5kb smaller than Ad11p thereby providing additional "space" for the insertion of transgenes.

In one embodiment the cassette according to the present disclosure is located between L5 and the E4 region in an adenovirus such as a group B adenovirus, in particular under the control of the major late promoter.

In one embodiment the virus employed is not EnAd.

Other viruses that may be employed in the present invention include herpes simplex virus, reovirus, measles virus, Newcastle disease virus, Seneca Valley virus, Vesicular stomatitis virus, polio virus, ECHO enterovirus, Coxsackie virus, and vaccinia virus, in particular an adenovirus, for example selected from the group consisting of talimogene laherparepvec, RIGVIR, Ad5-yCD/mutTKSR39rep-hIL12, Cavatak™, CG0070, DNX-2401, G207, HF10, Imlygic®, JX-594, MG1-MA3, MV-NIS, OBP-301, Reolysin®, Toca 511.

Antibody or Antibody fragment

In one embodiment the virus of the present disclosure encodes a full-length anti-CD40 antibody.

The term antibody as used herein refers to an immunoglobulin molecule capable of specific binding to a target antigen, such as a carbohydrate, polynucleotide, lipid, polypeptide, peptide etc., via at least one antigen recognition site (also referred to as a binding site herein), located in the variable region of the immunoglobulin molecule.

As used herein antibody molecule includes antibodies and binding fragments thereof and molecules comprising one or more of the same.

Antigen binding site as employed herein refers to a portion of the molecule, which comprises a pair of variable regions, in particular a cognate pair that interact specifically with the target antigen.

Antibody binding fragment as employed herein refers to less than the whole antibody, which is still capable of specifically binding to a target antigen.

Specifically, as employed herein, is intended to refer to a binding site that only recognises the antigen to which it is specific or a binding site that has significantly higher binding affinity to the antigen to which is specific compared to affinity to antigens to which it is non-specific, for example 5, 6, 7, 8, 9, 10 times higher binding affinity.

5 Antibody molecules as employed may comprise a complete antibody molecule having full length heavy and light chains, bispecific antibody format comprising full length antibodies or a fragment of any one of the same including, but are not limited to Fab, modified Fab, Fab', modified Fab', F(ab')₂, Fv, single domain antibodies (e.g. VH or VL or VHH), scFv, bi, tri or tetra-valent antibodies, Bis-scFv, diabodies, triabodies, tetrabodies and
10 epitope-binding fragments of any of the above (see for example Holliger and Hudson, 2005, Nature Biotech. 23(9):1126-1136; Adair and Lawson, 2005, Drug Design Reviews - Online 2(3), 209-217). The methods for creating and manufacturing these antibody fragments are well known in the art (see for example Verma et al., 1998, Journal of Immunological Methods, 216, 165-181). Other antibody fragments for use in the present invention include
15 the Fab and Fab' fragments described in International patent applications WO2005/003169, WO2005/003170 and WO2005/003171. Multi-valent antibodies may comprise multiple specificities e.g bispecific or may be monospecific (see for example WO 92/22853, WO05/113605, WO2009/040562 and WO2010/035012).

20 In one embodiment an antibody molecule employed in the virus of the present disclosure is humanised, chimeric or non-human.

Humanised (which include CDR-grafted antibodies) as employed herein refers to molecules having one or more complementarity determining regions (CDRs) from a non-human species and a framework region from a human immunoglobulin molecule (see, e.g. US 5,585,089; WO91/09967). It will be appreciated that it may only be necessary to transfer
25 the specificity determining residues of the CDRs rather than the entire CDR (see for example, Kashmiri et al., 2005, Methods, 36, 25-34). Humanised antibodies may optionally further comprise one or more framework residues derived from the non-human species from which the CDRs were derived.

When the CDRs or specificity determining residues are grafted, any appropriate
30 acceptor variable region framework sequence may be used having regard to the class/type of the donor antibody from which the CDRs are derived, including mouse, primate and human framework regions. Suitably, the humanised antibody according to the present disclosure has a variable domain comprising human acceptor framework regions as well as one or more of the CDRs provided herein.

35 Examples of human frameworks which can be used in the present disclosure are KOL, NEWM, REI, EU, TUR, TEI, LAY and POM (Kabat et al., supra). For example, KOL and NEWM can be used for the heavy chain, REI can be used for the light chain and EU, LAY and POM can be used for both the heavy chain and the light chain. Alternatively, human germline sequences may be used; these are available at: <http://vbase.mrc-cpe.cam.ac.uk/>

In a humanised antibody of the present disclosure, the acceptor heavy and light chains do not necessarily need to be derived from the same antibody and may, if desired, comprise composite chains having framework regions derived from different chains.

5 The framework regions need not have exactly the same sequence as those of the acceptor antibody. For instance, unusual residues may be changed to more frequently-occurring residues for that acceptor chain class or type. Alternatively, selected residues in the acceptor framework regions may be changed so that they correspond to the residue found at the same position in the donor antibody (see Reichmann et al., 1998, Nature, 332, 323-324). Such changes should be kept to the minimum necessary to recover the affinity of
10 the donor antibody. A protocol for selecting residues in the acceptor framework regions which may need to be changed is set forth in WO91/09967.

Chimeric antibodies generally contain non-human variable regions and human constant regions.

15 In one embodiment the antibody molecules of the present disclosure are fully human, in particular one or more of the variable domains are fully human.

Fully human molecules are those in which the variable regions and the constant regions (where present) of both the heavy and the light chains are all of human origin, or substantially identical to sequences of human origin, not necessarily from the same antibody. Examples of fully human antibodies may include antibodies produced, for
20 example by the phage display methods described above and antibodies produced by mice in which the murine immunoglobulin variable and optionally the constant region genes have been replaced by their human counterparts eg. as described in general terms in EP0546073, US5,545,806, US5,569,825, US5,625,126, US5,633,425, US5,661,016, US5,770,429, EP0438474 and EP0463151.

25 **Definitions Relevant to Formula (I) and (Ia)**

A bond refers to a covalent bond connecting one DNA sequence to another DNA sequence, for example connecting one section of the virus genome to another. Thus when a variable in formula (I) and (Ia) herein represents a bond the feature or element represented by the bond is absent i.e. deleted.

30 As the structure of adenoviruses is, in general, similar the elements below are discussed in terms of the structural elements and the commonly used nomenclature referring thereto, which are known to the skilled person. When an element is referred to herein then we refer to the DNA sequence encoding the element or a DNA sequence encoding the same structural protein of the element in an adenovirus. The latter is relevant
35 because of the redundancy of the DNA code. The viruses' preference for codon usage may need to be considered for optimised results.

Any structural element from an adenovirus employed in the viruses of the present disclosure may comprise or consist of the natural sequence or may have similarity over the given length of at least 95%, such as 96%, 97%, 98%, 99% or 100%. The original sequence
40 may be changed or modified to omit 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the

genetic material. However, in one embodiment the DNA sequence which is at least 95% similar or identical encodes the same gene product i.e. RNA and/or protein. The skilled person is aware that when making changes the reading frames of the virus must be not disrupted such that the expression of structural proteins is disrupted. The present disclosure also extends to a polynucleotide sequence that hybridises to a sequence disclosed herein under stringent conditions.

In one embodiment the given element is a full-length sequence i.e. the full-length gene. Full-length gene as employed herein refers to at least the entirety of the coding sequence of a gene, but may include any associated non-coding regions, especially if they are relevant to the function of the gene.

In one embodiment the given element is less than a full-length sequence and retains the same or corresponding function as the full-length sequence.

In one embodiment for a given element which is optional in the constructs of the present disclosure, the DNA sequence may be less than a full-length and have no functionality, for example the E3 region may be totally or partly deleted. However, it may be useful to delete essentially all the E3 region as this optimises the space available for inserting transgenes.

The structural genes encoding structural or functional proteins of the adenovirus are generally linked by non-coding regions of DNA. Thus, there is some flexibility about where to “cut” the genomic sequence of the structural element of interest (especially in non-coding regions thereof) for the purpose of inserting a transgene into the viruses of the present disclosure. Thus, for the purposes of the present specification, the element will be considered a structural element of reference to the extent that it is fit for purpose and does not encode extraneous material. Thus, if appropriate the gene will be associated with suitable non-coding regions, for example as found in the natural structure of the virus.

Thus, in one embodiment an insert, such as DNA encoding a transgene, is inserted into a non-coding region of genomic virus DNA, such as an intron or intergenic sequence. Having said this some non-coding regions of adenovirus may have a function, for example in alternative splicing, transcription regulation or translation regulation, and this may need to be taken into consideration.

The sites identified herein, that are associated with the L5 region, are suitable for accommodating a variety of DNA sequences encoding complex entities such as RNAi, cytokines, single chain or multimeric proteins, such as antibodies, in particular SEQ ID NO: 12.

Gene as employed herein refers to coding and any non-coding sequences associated therewith, for example introns and associated exons. In one embodiment a gene comprises or consists of only essential structural components, for example coding region.

Below follows a discussion relating to specific structural elements of adenoviruses.

The Inverted Terminal Repeat (ITR) sequences are common to all known adenoviruses (so named because of their symmetry) and are the viral chromosome origins of replication. Another property of these sequences is their ability to form a hairpin.

5 The 5'ITR as employed herein refers to part or all of an ITR from the 5' end of an adenovirus, which retains the function of the ITR when incorporated into an adenovirus in an appropriate location. In one embodiment the 5'ITR comprises or consists of the sequence from about 1bp to 138bp of SEQ ID NO: 21 of WO2016/174200 (said sequence is incorporated herein by reference) or a sequence 90, 95, 96, 97, 98 or 99% identical thereto along the whole length, in particular the sequence consisting of from about 1bp to 138bp of
10 SEQ ID NO: 17 disclosed in WO2016/174200 (said sequence is incorporated herein by reference).

The 3'ITR as employed herein refers to part or all of an ITR from 3' end of an adenovirus which retains the function of the ITR when incorporated into an adenovirus in an appropriate location. In one embodiment the 3'ITR comprises or consists of the sequence
15 from about 32189bp to 32326bp of SEQ ID NO: 17 disclosed in WO2016/174200 or a sequence 90, 95, 96, 97, 98 or 99% identical thereto along the whole length, in particular the sequence consisting of from about 32189bp to 32326bp of SEQ ID NO: 17 disclosed in WO2016/174200.

B1 as employed herein refers to the DNA sequence encoding: part or all of an E1A
20 from an adenovirus, part or all of the E1B region of an adenovirus, and independently part or all of E1A and E1B region of an adenovirus.

When B1 is a bond then E1A and E1B sequences will be omitted from the virus. In one embodiment B1 is a bond and thus the virus is a vector.

In one embodiment B1 further comprises a transgene. It is known in the art that the
25 E1 region can accommodate a transgene which may be inserted in a disruptive way into the E1 region (i.e. in the "middle" of the sequence) or part or all of the E1 region may be deleted to provide more room to accommodate genetic material.

E1A as employed herein refers to the DNA sequence encoding part or all of an adenovirus E1A region. The latter here is referring to the polypeptide/protein E1A. It may
30 be mutated such that the protein encoded by the E1A gene has conservative or non-conservative amino acid changes (e.g. 1, 2, 3, 4 or 5 amino acid changes, additions and/or deletions over the whole length) such that it has: the same function as wild-type (i.e. the corresponding non-mutated protein); increased function in comparison to wild-type protein; decreased function, such as no function in comparison to wild-type protein; or has
35 a new function in comparison to wild-type protein or a combination of the same as appropriate.

E1B as employed herein refers to the DNA sequence encoding part or all of an adenovirus E1B region (i.e. polypeptide or protein), it may be mutated such that the protein encoded by the E1B gene/region has conservative or non-conservative amino acid changes
40 (e.g. 1, 2, 3, 4 or 5 amino acid changes, additions and/or deletions over the whole length)

such that it has: the same function as wild-type (i.e. the corresponding non-mutated protein); increased function in comparison to wild-type protein; decreased function, such as no function in comparison to wild-type protein; or has a new function in comparison to wild-type protein or a combination of the same, as appropriate.

Thus, B1 can be modified or unmodified relative to a wild-type E1 region, such as a wild-type E1A and/or E1B. The skilled person can easily identify whether E1A and/or E1B are present or (part) deleted or mutated.

Wild-type as employed herein refers to a known adenovirus or a sequence from a known adenovirus. A known adenovirus is one that has been identified and named, regardless of whether the sequence information is available.

In one embodiment B1 has the sequence from 139bp to 3932bp of SEQ ID NO: 17 disclosed in WO2016/174200.

B_A as employed herein refers to the DNA sequence encoding the E2B-L1-L2-L3-E2A-L4 regions including any non-coding sequences, as appropriate (in particular corresponding to the natural sequence from an adenovirus). Generally, this sequence will not comprise a transgene. In one embodiment the sequence is substantially similar or identical to a contiguous sequence from a known adenovirus, for example a serotype shown in Table 1, in particular a group B virus, for example Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34, Ad35, Ad51 or a combination thereof, such as Ad3, Ad11 or a combination thereof. In one embodiment is E2B-L1-L2-L3-E2A-L4 refers to comprising these elements and other structural elements associated with the region, for example B_A will generally include the sequence encoding the protein IV2a, for example as follows: IV2A IV2a-E2B-L1-L2-L3-E2A-L4.

In one embodiment the E2B region is chimeric. That is, comprises DNA sequences from two or more different adenoviral serotypes, for example from Ad3 and Ad11, such as Ad11p. In one embodiment the E2B region has the sequence from 5068bp to 10355bp of SEQ ID NO: 17 disclosed in WO2016/174200 or a sequence 95%, 96%, 97%, 98% or 99% identical thereto over the whole length.

In one embodiment the E2B in component B_A comprises the sequences shown in SEQ ID NO: 18 disclosed in WO2016/174200 (said sequence is incorporated herein by reference).

In one embodiment B_A has the sequence from 3933bp to 27184bp of SEQ ID NO: 18 disclosed in WO2016/174200.

E3 as employed herein refers to the DNA sequence encoding part or all of an adenovirus E3 region (i.e. protein/polypeptide), it may be mutated such that the protein encoded by the E3 gene has conservative or non-conservative amino acid changes (e.g. 1, 2, 3, 4 or 5 amino acid changes, additions and/or deletions over the whole length), such that it has the same function as wild-type (the corresponding unmutated protein); increased function in comparison to wild-type protein; decreased function, such as no function in comparison to wild-type protein or has a new function in comparison to wild-type protein or a combination of the same, as appropriate.

In one embodiment the E3 region is form an adenovirus serotype given in Table 1 or a combination thereof, in particular a group B serotype, for example Ad3, Ad7, Ad11 (in particular Ad11p), Ad14, Ad16, Ad21, Ad34, Ad35, Ad51 or a combination thereof, such as Ad3, Ad11 (in particular Ad11p) or a combination thereof.

5 In one embodiment the E3 region has a sequence shown in SEQ ID NO: 19 disclosed in WO2016/174200.

In one embodiment the E3 region is partially deleted, for example is 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% deleted.

10 In one embodiment B₂ is a bond, wherein the DNA encoding the E3 region is absent.

In one embodiment the DNA encoding the E3 region can be replaced or interrupted by a transgene. As employed herein "E3 region replaced by a transgene as employed herein includes part or all of the E3 region is replaced with a transgene.

15 In one embodiment the B₂ region comprises the sequence from 27185bp to 28165bp of SEQ ID NO: 98 disclosed in WO2016/174200 (said sequence is incorporated herein by reference).

In one embodiment B₂ consists of the sequence from 27185bp to 28165bp of SEQ ID NO: 98 disclosed in WO2016/174200.

20 B_X as employed herein refers to the DNA sequence in the vicinity of the 5' end of the L5 gene in BB. In the vicinity of or proximal to the 5' end of the L5 gene as employed herein refers to: adjacent (contiguous) to the 5' end of the L5 gene or a non-coding region inherently associated herewith i.e. abutting or contiguous to the 5' prime end of the L5 gene or a non-coding region inherently associated therewith. Alternatively, in the vicinity of or proximal to may refer to being close the L5 gene, such that there are no coding sequences
25 between the B_X region and the 5' end of L5 gene.

Thus, in one embodiment B_X is joined directly to a base of L5 which represents, for example the start of a coding sequence of the L5 gene.

30 Thus, in one embodiment B_X is joined directly to a base of L5 which represents, for example the start of a non-coding sequence, or joined directly to a non-coding region naturally associated with L5. A non-coding region naturally associated L5 as employed herein refers to part of all of a non-coding regions which is part of the L5 gene or contiguous therewith but not part of another gene.

35 In one embodiment B_X comprises the sequence of SEQ ID NO: 98 disclosed in WO2016/174200. This sequence is an artificial non-coding sequence wherein a DNA sequence, for example comprising a transgene (or transgene cassette), a restriction site or a combination thereof may be inserted therein. This sequence is advantageous because it acts as a buffer in that allows some flexibility on the exact location of the transgene whilst minimising the disruptive effects on virus stability and viability.

40 The insert(s) can occur anywhere within SEQ ID NO: 98 disclosed in WO2016/174200 from the 5' end, the 3' end or at any point between bp 1 to 201, for

example between base pairs 1/2, 2/3, 3/4, 4/5, 5/6, 6/7, 7/8, 8/9, 9/10, 10/11, 11/12, 12/13, 13/14, 14/15, 15/16, 16/17, 17/18, 18/19, 19/20, 20/21, 21/22, 22/23, 23/24, 24/25, 25/26, 26/27, 27/28, 28/29, 29/30, 30/31, 31/32, 32/33, 33/34, 34/35, 35/36, 36/37, 37/38, 38/39, 39/40, 40/41, 41/42, 42/43, 43/44, 44/45, 45/46, 46/47, 47/48, 48/49, 49/50, 50/51, 51/52, 52/53, 53/54, 54/55, 55/56, 56/57, 57/58, 58/59, 59/60, 60/61, 61/62, 62/63, 63/64, 64/65, 65/66, 66/67, 67/68, 68/69, 69/70, 70/71, 71/72, 72/73, 73/74, 74/75, 75/76, 76/77, 77/78, 78/79, 79/80, 80/81, 81/82, 82/83, 83/84, 84/85, 85/86, 86/87, 87/88, 88/89, 89/90, 90/91, 91/92, 92/93, 93/94, 94/95, 95/96, 96/97, 97/98, 98/99, 99/100, 100/101, 101/102, 102/103, 103/104, 104/105, 105/106, 106/107, 107/108, 108/109, 109/110, 110/111, 111/112, 112/113, 113/114, 114/115, 115/116, 116/117, 117/118, 118/119, 119/120, 120/121, 121/122, 122/123, 123/124, 124/125, 125/126, 126/127, 127/128, 128/129, 129/130, 130/131, 131/132, 132/133, 133/134, 134/135, 135/136, 136/137, 137/138, 138/139, 139/140, 140/141, 141/142, 142/143, 143/144, 144/145, 145/146, 146/147, 147/148, 148/149, 150/151, 151/152, 152/153, 153/154, 154/155, 155/156, 156/157, 157/158, 158/159, 159/160, 160/161, 161/162, 162/163, 163/164, 164/165, 165/166, 166/167, 167/168, 168/169, 169/170, 170/171, 171/172, 172/173, 173/174, 174/175, 175/176, 176/177, 177/178, 178/179, 179/180, 180/181, 181/182, 182/183, 183/184, 184/185, 185/186, 186/187, 187/188, 188/189, 189/190, 190/191, 191/192, 192/193, 193/194, 194/195, 195/196, 196/197, 197/198, 198/199, 199/200 or 200/201.

In one embodiment B_X comprises SEQ ID NO: 98 disclosed in WO2016/174200 with a DNA sequence inserted between bp 27 and bp 28 or a place corresponding to between positions 28192bp and 28193bp of SEQ ID NO: 98 disclosed in WO2016/174200.

In one embodiment B_X has the sequence from 28166bp to 28366bp of SEQ ID NO: 21 disclosed in WO2016/174200 (said sequence is incorporated herein by reference). In one embodiment B_X is a bond.

B_B as employed herein refers to the DNA sequence encoding the L5 region. As employed herein the L5 region refers to the DNA sequence containing the gene encoding the fibre polypeptide/protein, as appropriate in the context. The fibre gene/region encodes the fibre protein which is a major capsid component of adenoviruses. The fibre functions in receptor recognition and contributes to the adenovirus' ability to selectively bind and infect cells.

In viruses of the present disclosure the fibre can be from any adenovirus serotype and adenoviruses which are chimeric as result of changing the fibre for one of a different serotype are also envisaged with the present disclosure. In one embodiment the fibre is from a group B virus, in particular Ad11, such as Ad11p.

In one embodiment B_B has the sequence from 28367bp to 29344bp of SEQ ID NO: 17 disclosed in WO2016/174200 (said sequence is incorporated herein by reference).

DNA sequence in relation to B_Y as employed herein refers to the DNA sequence in the vicinity of the 3' end of the L5 gene of B_B. In the vicinity of or proximal to the 3' end of

the L5 gene as employed herein refers to: adjacent (contiguous) to the 3' end of the L5 gene or a non-coding region inherently associated therewith i.e. abutting or contiguous to the 3' prime end of the L5 gene or a non-coding region inherently associated therewith (i.e. all or part of a non-coding sequence endogenous to L5). Alternatively, in the vicinity of or proximal to may refer to being close the L5 gene, such that there are no coding sequences between the B_Y region and the 3' end of the L5 gene.

Thus, in one embodiment B_Y is joined directly to a base of L5 which represents the "end" of a coding sequence.

Thus, in one embodiment B_Y is joined directly to a base of L5 which represents the "end" of a non-coding sequence, or joined directly to a non-coding region naturally associated with L5.

Inherently and naturally are used interchangeably herein. In one embodiment B_Y comprises the sequence of SEQ ID NO: 99 disclosed in WO2016/174200 (said sequence is incorporated herein by reference). This sequence is a non-coding sequence wherein a DNA sequence, for example comprising a transgene (or transgene cassette), a restriction site or a combination thereof may be inserted. This sequence is advantageous because it acts a buffer in that allows some flexibility on the exact location of the transgene whilst minimising the disruptive effects on virus stability and viability.

The insert(s) can occur anywhere within SEQ ID NO: 18 disclosed in WO2016/174200 (said sequence is incorporated herein by reference) from the 5' end, the 3' end or at any point between bp 1 to 35, for example between base pairs 1/2, 2/3, 3/4, 4/5, 5/6, 6/7, 7/8, 8/9, 9/10, 10/11, 11/12, 12/13, 13/14, 14/15, 15/16, 16/17, 17/18, 18/19, 19/20, 20/21, 21/22, 22/23, 23/24, 24/25, 25/26, 26/27, 27/28, 28/29, 29/30, 30/31, 31/32, 32/33, 33/34, or 34/35.

In one embodiment B_Y comprises SEQ ID NO: 99 disclosed in WO2016/174200 (said sequence is incorporated herein by reference) with a DNA sequence inserted between positions bp 12 and 13 or a place corresponding to 29356bp and 29357bp in SEQ ID NO: 17 disclosed in WO2016/174200 (said sequence is incorporated herein by reference). In one embodiment the insert is a restriction site insert. In one embodiment the restriction site insert comprises one or two restriction sites. In one embodiment the restriction site is a 19bp restriction site insert comprising 2 restriction sites. In one embodiment the restriction site insert is a 9bp restriction site insert comprising 1 restriction site. In one embodiment the restriction site insert comprises one or two restriction sites and at least one transgene, for example one or two or three transgenes, such as one or two transgenes. In one embodiment the restriction site is a 19bp restriction site insert comprising 2 restriction sites and at least one transgene, for example one or two transgenes. In one embodiment the restriction site insert is a 9bp restriction site insert comprising 1 restriction site and at least one transgene, for example one or two transgenes. In one embodiment two restriction sites sandwich one or more, such as two transgenes (for example in a transgene cassette). In one embodiment when B_Y comprises two restrictions

sites the said restriction sites are different from each other. In one embodiment said one or more restrictions sites in B_Y are non-naturally occurring (such as unique) in the particular adenovirus genome into which they have been inserted. In one embodiment said one or more restrictions sites in B_Y are different to other restrictions sites located elsewhere in the adenovirus genome, for example different to naturally occurring restrictions sites or restriction sites introduced into other parts of the genome, such as B_X. Thus in one embodiment the restriction site or sites allow the DNA in the section to be cut specifically.

In one embodiment B_Y has the sequence from 29345bp to 29379bp of SEQ ID NO: 17 disclosed in WO2016/174200. In one embodiment B_Y is a bond.

In one embodiment the insert is after bp 12 in SEQ ID NO: 99 disclosed in WO2016/174200.

In one embodiment the insert is at about position 29356bp of SEQ ID NO: 17 disclosed in WO2016/174200.

In one embodiment the insert is a transgene cassette comprising one or more transgenes, for example 1, 2 or 3, such as 1 or 2.

E4 as employed herein refers to the DNA sequence encoding part or all of an adenovirus E4 region (i.e. polypeptide/protein region), which may be mutated such that the protein encoded by the E4 gene has conservative or non-conservative amino acid changes (e.g. 1, 2, 3, 4 or 5 amino acid changes, additions and/or deletions), and has the same function as wild-type (the corresponding non-mutated protein); increased function in comparison to wild-type protein; decreased function, such as no function in comparison to wild-type protein or has a new function in comparison to wild-type protein or a combination of the same as appropriate.

In one embodiment the E4 region is partially deleted, for example is 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% or 5% deleted. In one embodiment the E4 region has the sequence from 32188bp to 29380bp of SEQ ID NO: 17 disclosed in WO2016/174200.

In one embodiment E4 is present except for the E4orf4 region which is deleted.

In one embodiment B₃ is a bond, i.e. wherein E4 is absent.

In one embodiment B₃ has the sequence consisting of from 32188bp to 29380bp of SEQ ID NO: 17 disclosed in WO2016/174200.

As employed herein number ranges are inclusive of the end points.

The skilled person will appreciate that the elements in the formulas herein, such as formula (I), (Ia) are contiguous and may embody non-coding DNA sequences as well as the genes and coding DNA sequences (structural features) mentioned herein. In one or more embodiments the formulas of the present disclosure are attempting to describe a naturally occurring sequence in the adenovirus genome. In this context it will be clear to the skilled person that the formula is referring to the major elements characterising the relevant section of genome and is not intended to be an exhaustive description of the genomic stretch of DNA.

E1A, E1B, E3 and E4 as employed herein each independently refer to the wild-type and equivalents thereof, mutated or partially deleted forms of each region as described herein, in particular a wild-type sequence from a known adenovirus.

“Insert” as employed herein refers to a DNA sequence that is incorporated either at the 5’ end, the 3’ end or within a given DNA sequence reference segment such that it interrupts the reference sequence. A reference sequence employed as a reference point relative to which the insert is located. In the context of the present disclosure inserts generally occur within either SEQ ID NO: 98 or SEQ ID NO: 99 both disclosed in WO2016/174200. An insert will generally contain a transgene cassette. When the sequence is interrupted the virus will still comprise the original sequence, but generally it will be as two fragments sandwiching the insert.

In one embodiment the transgene or transgene cassette does not comprise a non-biased inserting transposon, such as a TN7 transposon or part thereof. Tn7 transposon as employed herein refers to a non-biased insertion transposon as described in WO2008/080003.

In one embodiment the transgene or transgene cassette further comprises a regulatory element or sequence.

Promoters

Promoter as employed herein means a region of DNA that initiates transcription of a particular gene or genes. Promoters are generally located proximal to the genes they transcribe, on the same strand and upstream (i.e. 5’) on the DNA. Proximal as employed in this context means sufficiently close to function as a promoter. In one embodiment the promoter is within 100 bp of the transcription start site. Thus endogenous promoter as employed herein refers to a promoter that naturally occurs in (i.e. is native to) the adenovirus (or construct) into which the transgene, is being inserted. In one or more embodiments the endogenous promoter employed is the naturally occurring promoter in the virus in its original location in the virus genome, in particular this is the primary or only promoter employed in the expression of the transgene or transgenes. In one embodiment the endogenous promoter used to promote the translation and optionally the transcription of the transgene is one resident, i.e. is one integrated in the genome of the adenovirus and not previously introduced by recombinant techniques.

Under the control of an endogenous promoter as employed herein refers to where the transgene/transgene cassette is inserted in the appropriate orientation to be under the control of said endogenous promoter. That is, where the promoter is generally on the antisense strand, the cassette is inserted, for example in the antisense orientation.

Having said this, genes can be expressed in one of two orientations. However, generally one orientation provides increased levels of expression over the other orientation, for a given (particular) transgene.

In one embodiment the cassette is in the sense orientation. That is transcribed in a 5' to 3' direction. In one embodiment the cassette is in the antisense orientation. That is, transcribed in the 3' to 5' orientation.

The endogenous promoters in the virus can, for example, be utilised by employing a gene encoding a transgene and a splice acceptor sequence. Thus in one embodiment the cassette will comprise a splice acceptor sequence which facilitates the transgene utilising an endogenous promoter. Thus in one embodiment the coding sequence, for example the sequence encoding the antibody or antibody binding fragment further comprises a splice acceptor sequence.

In one embodiment the transgene, transgenes, or transgene cassette are under the control of an E4 promoter or a major late promoter, such as the major late promoter (ML promoter).

Under the control of as employed herein means that the transgene is activated, i.e. transcribed, when a particular promoter dictates.

The Major Late Promoter (ML promoter or MLP) as employed herein refers to the adenovirus promoter that controls expression of the "late expressed" genes, such as the L5 gene. The MLP is a "sense strand" promoter. That is, the promoter influences genes that are downstream of the promoter in the 5'-3' direction. The major late promoter as employed herein refers the original major late promoter located in the virus genome.

E4 promoter as employed herein refers to the adenovirus promoter of the E4 region. The E4 region is an antisense region; therefore the promoter is an antisense promoter. That is, the promoter is upstream of the E4 region in the 3'-5' direction. Therefore any transgene cassette under control of the E4 promoter may need to be oriented appropriately. In one embodiment the cassette under the control of the E4 promoter is in the antisense orientation. In one embodiment the cassette is under the control of the E4 promoter in the sense orientation. The E4 promoter as employed herein refers to the original E4 promoter located in the virus genome.

Thus in one embodiment there is provided a replication competent oncolytic adenovirus serotype 11 (such as Ad11p) or virus-derivative thereof wherein the fibre, hexon and capsid are serotype 11 (such as Ad11p), wherein the virus genome comprises a DNA sequence encoding a therapeutic antibody or antibody-binding fragment, wherein said DNA sequence under the control of a promoter endogenous to the adenovirus selected from consisting of E4 and the major late promoter (i.e. the E4 promoter or the major late promoter), such that the transgene does not interfere with virus replication, for example is associated with the L5 region (i.e. before or after said region), such as located after L5 in the virus genome.

In one embodiment an endogenous promoter is introduced into the viral genome at a desired location by recombinant techniques, for example is introduced in the transgene cassette. However, in the context of the present specification this arrangement will generally be referred to as an exogenous promoter.

In one embodiment the transgene cassette comprises an exogenous promoter. Exogenous promoter as employed herein refers to a promoter that is not naturally occurring in the adenovirus into which the transgene is being inserted. Typically exogenous promoters are from other viruses or are mammalian promoters. Exogenous promoter as employed herein means a DNA element, usually located upstream of the gene of interest, that regulates the transcription of the gene.

In one embodiment the regulator of gene expression is an exogenous promoter, for example CMV (cytomegalovirus promoter), CBA (chicken beta actin promoter) or PGK (phosphoglycerate kinase 1 promoter), such as CMV promoter.

In one embodiment the exogenous promoter is inducible.

In one embodiment there is provided a replication competent oncolytic adenovirus serotype 11 (such as Ad11p) or virus-derivative thereof wherein the fibre, hexon and capsid are serotype 11 (such as Ad11p), wherein the virus genome comprises a DNA sequence encoding a therapeutic antibody or antibody-binding fragment located in a part of the virus genome which is expressed late in the virus replication cycle and such that the transgene does not interfere with virus replication, wherein said DNA sequence under the control of a promoter exogenous to the adenovirus (for example the CMV promoter). In one embodiment the DNA sequence encoding an antibody or fragment is associated with the L5 region as described elsewhere herein.

Other Regulatory Sequences

“Regulator of gene expression” (or regulator/regulatory element) as employed herein refers to a genetic element, such as a promoter, enhancer or a splice acceptor sequence that plays a role in gene expression, typically by initiating or enhancing transcription or translation.

“Splice acceptor sequence”, “splice acceptor” or “splice site” as employed herein refers to a regulatory sequence determining when an mRNA molecule will be recognised by small nuclear ribonucleoproteins of the spliceosome complex. Once assembled the spliceosome catalyses splicing between the splice acceptor site of the mRNA molecule to an upstream splice donor site producing a mature mRNA molecule that can be translated to produce a single polypeptide or protein.

Different sized splice acceptor sequences may be employed in the present invention and these can be described as short splice acceptor (small), splice acceptor (medium) and branched splice acceptor (large).

SSA as employed herein refers to a short splice acceptor, typically comprising just the splice site, for example 4 bp. SA as employed herein refers to a splice acceptor, typically comprising the short splice acceptor and the polypyrimidine tract, for example 16 bp. bSA as employed herein refers to a branched splice acceptor, typically comprising the short splice acceptor, polypyrimidine tract and the branch point, for example 26 bp.

In one embodiment the splice acceptor employed in the constructs of the disclosure are CAGG or SEQ ID NO: 15 or 16 (both disclosed in WO2016/174200 as SEQ ID NO: 10 AND

11 therein said sequences incorporated herein by reference). In one embodiment the SSA has the nucleotide sequence of CAGG. In one embodiment the SA has the nucleotide sequence of SEQ ID NO: 15. In one embodiment the bSA has the nucleotide sequence of cagg. In one embodiment the splice acceptor sequence is independently selected from the group comprising: tgctaattctt cctttctctc ttcagg (SEQ ID NO: 15), tttctctctt cagg (SEQ ID NO: 16), and cagg.

In one embodiment the splice site is immediately proceeded (i.e. followed in a 5' to 3' direction) by a consensus Kozak sequence comprising CCACC. In one embodiment the splice site and the Kozak sequence are interspersed (separated) by up to 100 or less bp. In one embodiment the Kozak sequence has the nucleotide sequence of CCACC.

Typically, when under the control of an endogenous or exogenous promoter (such as an endogenous promoter), the coding sequence will be immediately preceded by a Kozak sequence. The start of the coding region is indicated by the initiation codon (AUG), for example is in the context of the sequence (gcc)gccRcc**AUG**g (SEQ ID NO: 8) the start of the start of the coding sequences is indicated by the bases in bold. A lowercase letter denotes common bases at this position (which can nevertheless vary) and uppercase letters indicate highly-conserved bases, *i.e.* the 'AUGG' sequence is constant or rarely, if ever, changes; 'R' indicates that a purine (adenine or guanine) is usually observed at this position and the sequence in brackets (gcc) is of uncertain significance. Thus, in one embodiment the initiation codon AUG is incorporated into a Kozak sequence.

Internal Ribosome Entry DNA Sequence as employed herein refers to a DNA sequence encoding an Internal Ribosome Entry Sequence (IRES). IRES as employed herein means a nucleotide sequence that allows for initiation of translation a messenger RNA (mRNA) sequence, including initiation starting within an mRNA sequence. This is particularly useful when the cassette encodes polycistronic mRNA. Using an IRES results in a polycistronic mRNA that is translated into multiple individual proteins or peptides. In one embodiment the Internal Ribosome Entry DNA sequence has the nucleotide sequence disclosed in WO2016/174200 as SEQ ID NO: 6 therein (said sequence is incorporated herein by reference). In one embodiment a particular IRES is only used once in the genome. This may have benefits with respect to stability of the genome.

"High self-cleavage efficiency 2A peptide" or "2A peptide" as employed herein refers to a peptide which is efficiently cleaved following translation. Suitable 2A peptides include P2A, F2A, E2A and T2A. The present inventors have noted that once a specific DNA sequence encoding a given 2A peptide is used once, the same specific DNA sequence may not be used a second time. However, redundancy in the DNA code may be utilised to generate a DNA sequence that is translated into the same 2A peptide. Using 2A peptides is particularly useful when the cassette encodes polycistronic mRNA. Using 2A peptides results in a single polypeptide chain being translated which is modified post-translation to generate multiple individual proteins or peptides.

In one embodiment the encoded P2A peptide employed has the amino acid sequence of SEQ ID NO: 24. In one embodiment the encoded F2A peptide employed has the amino acid sequence of SEQ ID NO: 25. In one embodiment the encoded E2A peptide employed has the amino acid sequence of SEQ ID NO: 26. In one embodiment the encoded T2A peptide employed has the amino acid sequence of SEQ ID NO: 27.

In one embodiment an mRNA or each mRNA encoded by transgene is/are comprise a polyadenylation signal sequence, such as typically at the end of an mRNA sequence, for example as shown in SEQ ID NO: 10 is employed. Thus, in one embodiment the transgene or the transgene cassette comprises at least one sequence encoding a polyadenylation signal sequence.

"PolyA", "Polyadenylation signal" or "polyadenylation sequence" as employed herein means a DNA sequence, usually containing an AATAAA site, that once transcribed can be recognised by a multiprotein complex that cleaves and polyadenylates the nascent mRNA molecule.

In one embodiment the polyadenylation sequence has the nucleotide sequence of SEQ ID NO: 6. disclosed in WO2016/174200 (said sequence is incorporated herein by reference).

In one embodiment the construct does not include a polyadenylation sequence. In one embodiment the regulator of gene expression is a splice acceptor sequence.

In one embodiment the sequence encoding a protein/polypeptide/peptide, such as an antibody or antibody binding fragment further comprises a polyadenylation signal.

Formulations

The present disclosure relates also extends to a pharmaceutical formulation of a virus as described herein.

In one embodiment there is provided a liquid parenteral formulation, for example for infusion or injection, of a replication capable oncolytic according to the present disclosure wherein the formulation provides a dose in the range of 1×10^{10} to 1×10^{14} viral particles per volume of dose.

Parenteral formulation means a formulation designed not to be delivered through the GI tract. Typical parenteral delivery routes include injection, implantation or infusion. In one embodiment the formulation is provided in a form for bolus delivery.

In one embodiment the parenteral formulation is in the form of an injection. Injection includes intravenous, subcutaneous, intra-tumoral or intramuscular injection. Injection as employed herein means the insertion of liquid into the body via a syringe. In one embodiment the method of the present disclosure does not involve intra-tumoral injection.

In one embodiment the parenteral formulation is in the form of an infusion.

Infusion as employed herein means the administration of fluids at a slower rate by drip, infusion pump, syringe driver or equivalent device. In one embodiment the infusion is administered over a period in the range of 1.5 minutes to 120 minutes, such as about 3, 4, 5,

6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 65, 80, 85, 90, 95, 100, 105, 110 or 115 minutes.

In one embodiment one dose of the formulation less than 100mls, for example 30mls, such as administered by a syringe driver.

5 In one embodiment the injection is administered as a slow injection, for example over a period of 1.5 to 30 minutes.

In one embodiment the formulation is for intravenous (i.v.) administration. This route is particularly effective for delivery of oncolytic virus because it allows rapid access to the majority of the organs and tissue and is particular useful for the treatment of
10 metastases, for example established metastases especially those located in highly vascularised regions such as the liver and lungs.

Therapeutic formulations typically will be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other parenteral formulation suitable for administration to a human and may
15 be formulated as a pre-filled device such as a syringe or vial, particular as a single dose.

The formulation will generally comprise a pharmaceutically acceptable diluent or carrier, for example a non-toxic, isotonic carrier that is compatible with the virus, and in which the virus is stable for the requisite period of time.

The carrier can be a solvent or dispersion medium containing, for example, water,
20 ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a dispersant or surfactant such as lecithin or a non-ionic surfactant such as polysorbate 80 or 40. In dispersions the maintenance of the required particle size may be assisted by the presence of a surfactant. Examples of isotonic agents include sugars,
25 polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.

In one embodiment parenteral formulations employed may comprise one or more of the following a buffer, for example 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a phosphate buffer and/or a Tris buffer, a sugar for example dextrose, mannose, sucrose or similar, a salt such as sodium chloride, magnesium chloride or potassium chloride, a
30 detergent such as a non-ionic surfactant such as brij, PS-80, PS-40 or similar. The formulation may also comprise a preservative such as EDTA or ethanol or a combination of EDTA and ethanol, which are thought to prevent one or more pathways of possible degradation.

In one embodiment the formulation will comprise purified oncolytic virus according
35 to the present disclosure, for example 1×10^{10} to 1×10^{14} viral particles per dose, such as 1×10^{10} to 1×10^{12} viral particles per dose. In one embodiment the concentration of virus in the formulation is in the range 2×10^8 to 2×10^{14} vp/ml, such as 2×10^{12} vp/ml.

In one embodiment the parenteral formulation comprises glycerol.

In one embodiment the formulation comprises oncolytic adenovirus as described
40 herein, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), glycerol and buffer.

In one embodiment the parenteral formulation consists of virus of the disclosure, HEPES for example 5mM, glycerol for example 5-20% (v/v), hydrochloric acid, for example to adjust the pH into the range 7-8 and water for injection.

5 In one embodiment 0.7 mL of virus of the disclosure at a concentration of 2×10^{12} vp/mL is formulated in 5 mM HEPES, 20% glycerol with a final pH of 7.8.

A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

In one embodiment the formulation is provided as a formulation for topical administrations including inhalation.

10 Suitable inhalable preparations include inhalable powders, metering aerosols containing propellant gases or inhalable solutions free from propellant gases. Inhalable powders according to the disclosure will generally contain a virus as described herein with a physiologically acceptable excipient.

15 These inhalable powders may include monosaccharides (e.g. glucose or arabinose), disaccharides (e.g. lactose, saccharose, maltose), oligo- and polysaccharides (e.g. dextrans), polyalcohols (e.g. sorbitol, mannitol, xylitol), salts (e.g. sodium chloride, calcium carbonate) or mixtures of these with one another. Mono- or disaccharides are suitably used, such as lactose or glucose, particularly but not exclusively in the form of their hydrates.

20 Particles for deposition in the lung require a particle size less than 10 microns, such as 1-9 microns for example from 0.1 to 5 μm , in particular from 1 to 5 μm . The size of the particle carrying the virus is of primary importance and thus in one embodiment the virus according to the present disclosure may be adsorbed or absorbed onto a particle, such as a lactose particle of the given size.

25 The propellant gases which can be used to prepare the inhalable aerosols are known in the art. Suitable propellant gases are selected from among hydrocarbons such as n-propane, n-butane or isobutane and halohydrocarbons such as chlorinated and/or fluorinated derivatives of methane, ethane, propane, butane, cyclopropane or cyclobutane. The above-mentioned propellant gases may be used on their own or in mixtures thereof.

30 Particularly suitable propellant gases are halogenated alkane derivatives selected from among TG 11, TG 12, TG 134a and TG227. Of the abovementioned halogenated hydrocarbons, TG134a (1,1,1,2-tetrafluoroethane) and TG227 (1,1,1,2,3,3,3-heptafluoropropane) and mixtures thereof are particularly suitable.

35 The propellant gas-containing inhalable aerosols may also contain other ingredients, such as co-solvents, stabilisers, surface-active agents (surfactants), antioxidants, lubricants and means for adjusting the pH. All these ingredients are known in the art.

The propellant gas-containing inhalable aerosols according to the invention may contain up to 5 % by weight of active substance. Aerosols according to the invention contain, for example, 0.002 to 5 % by weight, 0.01 to 3 % by weight, 0.015 to 2 % by weight, 0.1 to 2 % by weight, 0.5 to 2 % by weight or 0.5 to 1 % by weight of active ingredient.

Alternatively, topical administrations to the lung may also be by administration of a liquid solution or suspension formulation, for example employing a device such as a nebulizer, for example, a nebulizer connected to a compressor (e.g., the Pari LC-Jet Plus(R) nebulizer connected to a Pari Master(R) compressor manufactured by Pari Respiratory
5 Equipment, Inc., Richmond, Va.).

The virus of the invention can be delivered dispersed in a solvent, e.g. in the form of a solution or a suspension, for example as already described above for parenteral formulations. It can be suspended in an appropriate physiological solution, e.g., saline or other pharmacologically acceptable solvent or a buffered solution. Buffered solutions
10 known in the art may contain 0.05 mg to 0.15 mg disodium edetate, 8.0 mg to 9.0 mg NaCl, 0.15 mg to 0.25 mg polysorbate, 0.25 mg to 0.30 mg anhydrous citric acid, and 0.45 mg to 0.55 mg sodium citrate per 1 ml of water so as to achieve a pH of about 4.0 to 5.0.

The therapeutic suspensions or solution formulations can also contain one or more excipients. Excipients are well known in the art and include buffers (e.g., citrate buffer, phosphate buffer, acetate buffer and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (e.g., serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. Solutions or suspensions can be encapsulated in liposomes or biodegradable microspheres. The formulation will generally be provided in a substantially sterile form employing sterile manufacture processes.

This may include production and sterilization by filtration of the buffered solvent/solution used for the formulation, aseptic suspension of the antibody in the sterile buffered solvent solution and dispensing of the formulation into sterile receptacles by methods familiar to those of ordinary skill in the art.

Nebulisable formulation according to the present disclosure may be provided, for example, as single dose units (e.g., sealed plastic containers or vials) packed in foil envelopes. Each vial contains a unit dose in a volume, e.g., 2 mL, of solvent/solution buffer.

The present disclosure also extends to liquid solutions or suspensions delivered intra-nasally, for example employing a device as disclosed in WO2009/068877 and US2004/0153033 both incorporated herein by reference.

30 **Treatment**

In a further aspect the present disclosure extends to a virus or a formulation thereof as described herein for use in treatment, in particular for the treatment of cancer.

In one embodiment the method of treatment is for use in the treatment of a tumour.

Tumour as employed herein is intended to refer to an abnormal mass of tissue that
35 results from excessive cell division that is uncontrolled and progressive, also called a neoplasm. Tumours may be either benign (not cancerous) or malignant. Tumour encompasses all forms of cancer and metastases. In one embodiment the tumour is cancerous.

In one embodiment the tumour is a solid tumour. The solid tumour may be localised
40 or metastasised.

In one embodiment the tumour is of epithelial origin.

In one embodiment the tumour is a malignancy, such as colorectal cancer, hepatoma, prostate cancer, pancreatic cancer, breast cancer, ovarian cancer, thyroid cancer, renal cancer, bladder cancer, head and neck cancer or lung cancer.

5 In one embodiment the tumour is a colorectal malignancy.

Malignancy as employed herein refers to cancerous cells.

In one embodiment the oncolytic adenovirus is employed in the treatment or prevention of metastasis.

10 In one embodiment the method or formulation herein is employed in the treatment of drug resistant cancers.

In one embodiment the virus is administered in combination with the administration of a further cancer treatment or therapy.

15 In one embodiment there is provided a virus or formulation according to the present disclosure for use in the manufacture of a medicament for the treatment of cancer, for example a cancer described above.

In a further aspect there is provide a method of treating cancer comprising administering a therapeutically effective amount of a virus or formulation according to the present disclosure to a patient in need thereof, for example a human patient.

20 In one embodiment the oncolytic virus or formulation herein is administered in combination with another therapy.

“In combination” as employed herein is intended to encompass where the oncolytic virus is administered before, concurrently and/or post cancer treatment or therapy. However, generally the treatment regimens for the combination therapy will generally overlap.

25 A “combination therapy” as employed herein refers to the two drug products together, for example as a kit, or co-formulated, in particular for use in the treatment of cancer.

Cancer therapy includes surgery, radiation therapy, targeted therapy and/or chemotherapy.

30 Cancer treatment as employed herein refers to treatment with a therapeutic compound or biological agent, for example an antibody intended to treat the cancer and/or maintenance therapy thereof.

35 In one embodiment the cancer treatment is selected from any other anti-cancer therapy including a chemotherapeutic agent; a targeted anticancer agent, such as an antibody drug conjugate; radiotherapy, radio-isotope therapy or any combination thereof.

In one embodiment the virus of the present disclosure such as an oncolytic adenovirus may be used as a pre-treatment to a therapy, such as a surgery (neoadjuvant therapy), for example to shrink the tumour, for example to treat metastasis and/or prevent metastasis or further metastasis. The oncolytic adenovirus may be used after the therapy,

such as a surgery (adjuvant therapy), for example to keep cancer in remission, to treat metastasis and/or prevent metastasis or further metastasis.

In one embodiment a virus or formulation of the present disclosure is employed in maintenance therapy.

5 Concurrently as employed herein is the administration of the additional cancer treatment at the same time or approximately the same time as the oncolytic adenovirus formulation. The treatment may be contained within the same formulation or administered as a separate formulation.

10 In one embodiment the virus is administered in combination with the administration of a chemotherapeutic agent.

Chemotherapeutic agent as employed herein is intended to refer to specific antineoplastic chemical agents or drugs that are selectively destructive to malignant cells and tissues. For example, alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. Examples of specific
15 chemotherapeutic agents include doxorubicin, 5-fluorouracil (5-FU), paclitaxel, capecitabine, irinotecan, and platins such as cisplatin and oxaliplatin. The dose may be chosen by the practitioner based on the nature of the cancer being treated.

In one embodiment the therapeutic agent is ganciclovir, which may assist in controlling immune responses and/or tumour vascularisation.

20 In one embodiment one or more therapies employed in the method herein are metronomic, that is a continuous or frequent treatment with low doses of anticancer drugs, often given concomitant with other methods of therapy.

Subgroup B oncolytic adenoviruses, in particular Ad11 and those derived therefrom such as EnAd may be particularly synergistic with chemotherapeutics because they seem to
25 have a mechanism of action that is largely independent of apoptosis, killing cancer cells by a predominantly necrolytic mechanism. Moreover, the immunosuppression that occurs during chemotherapy may allow the oncolytic virus to function with greater efficiency.

Therapeutic dose as employed herein refers to the amount of virus, such as oncolytic adenovirus that is suitable for achieving the intended therapeutic effect when employed in
30 a suitable treatment regimen, for example ameliorates symptoms or conditions of a disease, in particular without eliciting dose limiting side effects. A dose may be considered a therapeutic dose in the treatment of cancer or metastases when the number of viral particles may be sufficient to result in the following: tumour or metastatic growth is slowed or stopped, or the tumour or metastasis is found to shrink in size, and/or the life span of the
35 patient is extended. Suitable therapeutic doses are generally a balance between therapeutic effect and tolerable toxicity, for example where the side-effect and toxicity are tolerable given the benefit achieved by the therapy.

In one embodiment there is provided systemically administering multiple doses of a parenteral formulation of an oncolytic adenovirus according to the present disclosure in a

single treatment cycle, for example wherein the total dose given in each administration is in the range of 1×10^{10} to 1×10^{14} viral particles per dose.

In one embodiment one or more doses (for example each dose) of virus or composition comprising the same is administered such that the rate of viral particle delivery is in the range of 2×10^{10} particles per minute to 2×10^{12} particles per minute.

In one embodiment a virus or therapeutic construct according to the present disclosure (including a formulation comprising same) is administered weekly, for example one week 1 the dose is administered on day 1, 3, 5, followed by one dose each subsequent week.

In one embodiment a virus or therapeutic construct according to the present disclosure (including a formulation comprising same) is administered bi-weekly or tri-weekly, for example is administered in week 1 one on days 1, 3 and 5, and on week 2 or 3 is also administered on days 1, 3 and 5 thereof. This dosing regimen may be repeated as many times as appropriate.

In one embodiment a virus or therapeutic construct according to the present disclosure (including a formulation comprising same) is administered monthly, for example in a treatment cycle or as maintenance therapy.

In one embodiment the viruses and constructs of the present disclosure are prepared by recombinant techniques. The skilled person will appreciate that the armed adenovirus genome can be manufactured by other technical means, including entirely synthesising the genome or a plasmid comprising part of all of the genome. The skilled person will appreciate that in the event of synthesising the genome the region of insertion may not comprise the restriction site nucleotides as the latter are artefacts following insertion of genes using cloning methods.

In one embodiment the armed adenovirus genome is entirely synthetically manufactured.

The disclosure herein further extends to an adenovirus of formula (I) or a sub-formula thereof, obtained or obtainable from inserting a transgene or transgene cassette.

"Is" as employed herein means comprising.

In the context of this specification "comprising" is to be interpreted as "including".

Embodiments of the invention comprising certain features/elements are also intended to extend to alternative embodiments "consisting" or "consisting essentially" of the relevant elements/features.

Where technically appropriate, embodiments of the invention may be combined.

Technical references such as patents and applications are incorporated herein by reference.

Any embodiments specifically and explicitly recited herein may form the basis of a disclaimer either alone or in combination with one or more further embodiments.

Headings herein are employed to divide the document into sections and are not intended to be used to construe the meaning of the disclosure provided herein.

The present disclosure also extends to any virus sequence or cassette sequence specifically disclosed herein, compositions comprising said viruses, use of the said cassettes to generate viruses, and use of viruses according to the disclosure in therapy, in particular cancer therapy.

5 The present application claims priority for GB1708779.2 and GB1708778.4 both filed 1 June 2017. The disclosure of each is incorporated herein by reference, in particular incorporated are the amino acid and polynucleotide sequences disclosed therein. The priority documents may be employed as basis for a correction to the present specification.

10 The present invention is further described by way of illustration only in the following examples.

EXAMPLES

Example 1: Production of EnAd viruses expressing anti-CD40 monoclonal antibodies (NG-350).

15 The first enadenotucirev virus genome generated encoding an anti-CD40 monoclonal antibody was designated NG-350 (SEQ ID NO. 13).

To produce the NG-350 genome a plasmid, pNG-350, was generated by direct insertion of a cassette encoding; a 5' short splice acceptor sequence (CAGG, SEQ ID NO.2); a heavy chain leader sequence (SEQ ID NO. 3), the anti-CD40 VH chain (SEQ ID NO. 4), antibody heavy chain constant region (SEQ ID NO. 5), a P2A high efficiency self-cleavable peptide (SEQ ID
20 NO. 6), a light chain leader sequence (SEQ ID NO. 7), the anti-CD40 VL chain (SEQ ID NO. 8), an antibody light chain constant region (SEQ ID NO. 9) and a SV40 poly(A) tail (SEQ ID NO.10), into the plasmid pEnAd2.4. A schematic of the transgene cassette (SEQ ID NO. 11) is shown in Figure 1.

Virus Production and characterisation

25 The plasmid pNG-350 was linearised by restriction digest with the enzyme *AscI* to produce the virus genome NG-350 (SEQ ID NO. 13). The virus NG-350 was amplified and purified according to methods given below.

Digested DNA was purified by phenol/chloroform extraction and precipitated for 16hrs, -
30 20°C in 300µl >95% molecular biology grade ethanol and 10µl 3M Sodium Acetate. The precipitated DNA was pelleted by centrifuging at 14000rpm, 5 mins and was washed in 500µl 70% ethanol, before centrifuging again, 14000rpm, 5mins. The clean DNA pellet was air dried, resuspended in 500µl OptiMEM containing 15µl lipofectamine transfection reagent and incubated for 30 mins, RT. The transfection mixture was then added drop wise
35 to a T-25 flask containing 293 cells grown to 70% confluency. After incubation of the cells with the transfection mix for 2hrs at 37°C, 5% CO₂ 4mls of cell media (DMEM high glucose with glutamine supplemented with 2% FBS) was added to the cells and the flasks was incubated 37°C, 5% CO₂.

40 The transfected 293 cells were monitored every 24hrs and were supplemented with additional media every 48-72hrs. The production of virus was monitored by observation of

a significant cytopathic effect (CPE) in the cell monolayer. Once extensive CPE was observed the virus was harvested from 293 cells by three freeze-thaw cycles. The harvested viruses were used to re-infect 293 cells in order to amplify the virus stocks. Viable virus production during amplification was confirmed by observation of significant CPE in the cell monolayer.

Once CPE was observed the virus was harvested from 293 cells by three freeze-thaw cycles. The amplified stock was used for further amplification before the virus was purified by double caesium chloride banding to produce a NG-350 virus stock.

Example 2: NG-350 virus activity and anti-CD40 antibody production

NG-350 virus activity in terms of particle yield and secreted anti-CD40 antibody production was assessed in a HEK293 suspension culture cell line. HEK293 cells were seeded in shake flasks at a density of 1×10^6 vp/mL and infected with 50 NG-350 virus particles per cell (ppc). HEK293 cells were also infected with the NG-350 parental virus, enadenotucirev (EnAd) as a control (50ppc). At 48 and 72hrs post-infection cellular supernatants were collected by centrifuging for 5 mins, 1200rpm. Samples of the clarified supernatant were used for assessing virus particle concentration by HPLC or anti-CD40 antibody concentration by human IgG2 ELISA.

HPLC Analysis

Virus particle concentration in the supernatants was quantified by High Performance Liquid Chromatography (HPLC) using a Resource Q (anion exchange) column. Virus elution was detected at 260nm and virus concentration was determined by integrating the 260nm signal peak and calculating the concentration from an enadenotucirev standard curve. Comparison of virus production from NG-350 and enadenotucirev cells showed that NG-350 virus activity in terms of total particle production and virus particle production in the cell supernatant was significantly lower than enadenotucirev (Figure 2A and 2B).

IgG2 ELISA

The clarified supernatants were diluted 1 in 2 into PBS 10% BSA. A standard curve and negative control samples were prepared according to the manufacturer's protocol (IgG2 Human SimpleStep ELISA kit, ab202402, Abcam). Samples and standards were added to the ELISA plates and the assay performed according to the manufacturer's protocol. The absorbance at 450nm in each well of the plate was measured using a plate reader (BioTek) and the concentrations of secreted IgG2 anti-CD40 antibody were determined by interpolating from the standard curve (Figure 2C). Anti-CD40 was produced and secreted from NG-350 but not enadenotucirev infected cells.

Taken together these data indicated that although NG-350 was able to produce anti-CD40 antibody the virus activity of this modified virus was significantly compromised. Further characterisation of the NG-350 virus particle was therefore carried out (Example 3).

Example 3: NG-350 virus identity testing and genome analysis

Restriction Enzyme analysis

The identity of the NG-350 virus stock material was initially investigated by analysing genome identity via restriction enzyme analysis. NG-350 or enadenotucirev virus particles

(3.5×10^{11} vp) were diluted in PBS to a final volume of 200 μ L. DNA was extracted from the virus using the QIAgen Minelute virus spin kit according to the manufacturer's protocol. Control DNA was prepared for the assay by linearizing the pNG-350 plasmid with the enzyme *AscI* for 2 hrs, 37°C and then purifying the DNA by agarose gel electrophoresis and gel extraction with a QiaQuick Gel extraction Kit (Qiagen). Purified control or NG-350 DNA were restriction digested using a combination of restriction enzymes, *EcoRV*/*NheI* (Figure 3A) or individual restriction enzymes, *NcoI* or *FspI* (Figure 3B). Digested DNA was separated by agarose gel electrophoresis and visualised using a UV transilluminator. Digestion with *FspI* or *EcoRV*/*NheI* showed an additional band in the NG-350 virus genome material, that was not predicted (Figure 3, red arrows).

PCR Analysis

The genome identity was further assessed by PCR analysis of the virus genome using 8 different primer probe sets, shown in **Table 1**:

Primer Set	Forward Primer	Reverse Primer
D	ACGGAAC TTGTTACTACACAGC	CTTTCACAGTCCA ACTGCTGC
1	AGCCGGAGAACA ACTACAAGAC	CTTTCACAGTCCA ACTGCTGC
2	CATCCAGATGACCCAGTCTCC	GGACAAACCACA ACTAGAA TGCAG
3	CATCCAGATGACCCAGTCTCC	CTTTCACAGTCCA ACTGCTGC
4	AGCCGGAGAACA ACTACAAGAC	GGACAAACCACA ACTAGAA TGCAG
5	CCTCAGTGAAGGTCTCCTGC	CTTTCACAGTCCA ACTGCTGC
6	CCTCAGTGAAGGTCTCCTGC	GGACAAACCACA ACTAGAA TGCAG

These PCRs were designed to determine whether more than one genome species was present in the NG-350 viral stock and if this species contained the anti-CD40 antibody transgene cassette. NG-350 or enadenotucirev virus particles (2×10^{10} vp) were diluted in PBS to a final volume of 200 μ L. DNA was extracted from the virus using the QIAgen Minelute virus spin kit according to the manufacturer's protocol. Control DNA was prepared as for the restriction enzyme analysis. PCR reactions were set up using 1 μ L DNA (100ng/ μ L) in a 50 μ L reaction volume containing forward and reverse primer (2 μ M) and Phusion high fidelity master mix (NEB). PCR products were separated by agarose gel electrophoresis and visualised using a UV transilluminator (Figure 5A and 5B). Analysis with Primer set D revealed the expected band size of 2920bp in the positive control DNA and NG-350 test sample. However, the NG-350 test sample additionally contained a second PCR product of ~800bp, which was not seen with the pNG-350-PSI-01 plasmid DNA used to generate the NG-350 virus. Analysis with Primer sets 1-6 also showed additional contaminant bands when primer sets 5 and 6 were used. These data indicated that two virus species were present in the NG-350 virus stock, one containing the full length anti-CD40 transgene

cassette and one containing a truncated version of the cassette. This truncation was confirmed by sequencing of the contaminant PCR product.

Transgene cassette optimisation

An explanation for the truncation occurring during virus amplification was an unexpected instability in the transgene cassette resulting in recombination between the VH region and the SV40 polyA and therefore loss of most of the antibody coding region. The transgene cassette DNA sequence therefore needed to be modified to overcome this issue. Therefore, the DNA sequence was changed to reduce homology with other cassette and virus sequences and to remove minor direct and inverted repeats (carried out by Oxford Genetics, UK). The optimised cassette sequence was used to generate a new plasmid pNG-350A according to Example 4.

Example 4: Production of EnAd viruses expressing an anti-CD40 monoclonal antibody (NG-350A)

To produce the NG-350A genome a plasmid, pNG-350A, was generated by direct insertion of a cassette encoding; a 5' short splice acceptor sequence (CAGG, SEQ ID NO.2); a heavy chain leader sequence (SEQ ID NO. 3), the anti-CD40 VH chain (SEQ ID NO. 4), antibody constant heavy chain (SEQ ID NO. 5), a P2A high efficiency self-cleavable peptide (SEQ ID NO. 6), a light chain leader sequence (SEQ ID NO. 7), the anti-CD40 VL chain (SEQ ID NO. 8), an antibody constant light chain (SEQ ID NO. 9) and a SV40 poly(A) tail (SEQ ID NO.10), into the plasmid pEnAd2.4. The amino acid sequence of the NG-350A encoded anti-CD40 antibody were identical to those encoded in the NG-350 virus and the cassette structure was the same (Figure 1). However, the nucleic acid sequence of the transgene cassette was modified and significantly different to that of NG-350 (SEQ ID NO.11).

Virus Production and characterisation

The plasmid pNG-350A was linearised by restriction digest with the enzyme *Ascl* to produce the virus genome NG-350A (SEQ ID NO. 1). The virus NG-350A was amplified and purified according to methods described in Example 1.

Example 5: NG-350A virus identity testing by PCR

NG-350A genome identity was confirmed by PCR analysis using 2 primer probe sets (Table 2; D and K), which generate products spanning the transgene cassette. NG-350A DNA and control DNA was prepared according to the methods detailed in Example 3. PCR analysis was carried out using Primer Sets D and K according to the methods detailed in Example 3. Visualisation of the PCR products showed single products of the predicted size (Figure 6). No contaminating products were detected with either primer set.

Table 2 Identity PCR Primer Sets

Primer Set	Fwd Primer	Rev Primer
D	ACGGAAGTTGTTACTACACAGC	CTTTCACAGTCCAACTGCTGC
K	AGCCGGAGAACAACTACAAGAC	CTTTCACAGTCCAACTGCTGC

Example 6: Replication and oncolytic activity of the NG-350A virus in colon carcinoma cells

Virus oncolytic potency

HT-29 colon carcinoma cells were seeded in 96 well plates at a cell density of 2.5e4 cells/well. Plates were incubated for 4 hrs, 37°C, 5% CO₂, before cells were either infected with EnAd or NG-350A virus particles at an infection density range of 100-0.39 particles per cell (ppc). HT-29 cell viability was assessed using Cell Titre 96 MTS Reagent (Promega: G3581) 72 hrs post infection. Quantification of the % cell survival at each infection density demonstrated that similar to EnAd, NG-350A shows strong oncolytic activity against HT-29 cells (Figure 7A).

Virus replication

Lung carcinoma cells (A549) or colon carcinoma cells (HCT-116) were infected for 24, 48, 72 or 96 hrs with 100ppc NG-350A or the NG-350A parental virus, enadenotucirev, or were left uninfected. Colon carcinoma cells (HT-29) or bladder carcinoma cells (HTB-5, HT-1197 and HT-1376) were infected for 24, 48, 72, 144 and 168hrs with 100ppc NG-350A or enadenotucirev or were left uninfected. At each time point, cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. DNA was extracted from 10µL (HT-29, A549 or HCT-116) or 50µL (HTB-5, HT-1197 and HT-1376) of supernatant using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. A standard curve using EnAd virus particles (2.5e10-2.5e5vp) was also prepared and extracted using the DNeasy Blood and Tissue Kit (Qiagen). Each extracted sample or standard was analysed by qPCR using an enadenotucirev E3 gene specific primer-probe set.

Quantification of the number of detected virus genomes per cell demonstrated that NG-350A and enadenotucirev kinetics of virus replication was comparable in all cell lines tested and at all time points analysed (Figure 7 and Figure 8). No virus genomes could be detected in uninfected cells (data not shown).

Example 7: Anti-CD40 Antibody expression in NG-350A infected colon carcinoma cell lines

IgG2 ELISA

A549 cells either infected for 24, 48 or 72 hrs with 100ppc EnAd, two different batches of NG-350A (NG-350A B1 or NG-350A B2) or left uninfected were used for analysis of anti-CD40 antibody expression by IgG2 ELISA (IgG2 Human SimpleStep ELISA kit, ab202402, Abcam). Culture supernatants were removed from each well and centrifuged for 5 mins, 1200rpm to remove cell debris. The clarified supernatants were diluted 1 in 2 into PBS 10% BSA. A standard curve and negative control samples were prepared according to the manufacturer's protocol. Samples and standards were added to the ELISA plates and the assay performed according to the manufacturer's protocol. The absorbance at 450nm in each well of the plate was measured using a plate reader (BioTek) and the concentrations of secreted IgG2 anti-CD40 antibody were determined by interpolating from the standard curve (Figure 9A & 9B).

CD40 binding ELISA

A549 cells either infected for 24, 48 or 72 hrs with 100ppc EnAd, two different batches of NG-350A (NG-350A B1 or NG-350A B2) or left uninfected were used for analysis of anti-CD40 antibody expression by CD40 binding ELISA.

Culture supernatants were removed from each well and centrifuged for 5 mins, 1200rpm to remove cell debris. ELISA plates (A Nunc Immuno MaxiSorp 96 well microplate) were prepared by coating overnight at 4°C with human CD40 (100µg/mL, R and D Systems, 1493-CD) in carbonate/bicarbonate buffer. Plates were washed between all subsequent binding steps with PBS 0.05% Tween 20. The plates were blocked for 1 hour at room temperature with PBS 5% BSA.

Clarified infection supernatants were diluted into PBS 5% BSA (1 in 2, 1 in 10 and 1 in 100). In this assay, anti-CD40 antibody (BioLegend, 334308) was used as a positive control for human CD40 binding to the ELISA plate. It was prepared in PBS 5% BSA at a concentration of 30ng/mL. All samples were added to the CD40 coated plates and incubated for 1 hr at room temperature. The detection antibodies, HRP conjugated anti-human IgG2-Fc (Abcam, ab97225) for virus supernatants or anti- mouse IgG Abs (HRP) (Abcam, ab6728), for the positive control antibody were then applied for 1 hr at room temperature to all wells. HRP detection was performed with HRP substrate solution 3.3.5.5'-teramethylethylenediamine (TMB, Thermo-Fisher). 1M HCl was used for stopping the reaction and the developed colour was measured at 450nm on a plate reader. Absorbance at 450nm was plotted for the EnAd, NG-350A and positive controls (Figure 9C) and demonstrated specific binding to CD40 by the secreted anti-CD40 antibody present in the supernatant of NG-350A infected cells.

Example 8: CD40 functional signalling reporter assay

A549 cells either infected for 24 or 48 hrs with 10ppc EnAd, NG-350A or for 48 hrs with NG-165 (a virus expressing a control antibody [anti-VEGF]) or left uninfected were used for analysis of anti-CD40 antibody functional activity using CD40+ HEK-Blue reporter cells that secrete alkaline phosphatase (Invivogen) in response to activation via their membrane expressed CD40 molecules. Post infection, culture supernatants from A549 cells were removed from each well and centrifuged for 5 mins, 1500rpm to remove cell debris.

20µL of culture supernatants were diluted in 180µL of culture media and applied to HEK-Blue CD40 cells for 20hrs. CD40L at a concentration of 10ng/mL was prepared in culture media as a positive control. The supernatants were then collected from the HEK-Blue CD40 cells and clarified by centrifuging. 40µL of the clarified supernatant was assayed for alkaline phosphatase activity by incubating for 1hr at 37 degrees with 160µL Quanti-Blue reagent.

Absorbance at 620nm was measured for each sample using a plate reader and demonstrated that supernatants from NG-350A but not EnAd or NG-165 infected cells triggered SEAP production from the CD40 expressing HEK-Blue reporter cells (Fig 10).

Example 9: Selective activity of the NG-350A virus in carcinoma cell lines

Lung carcinoma cells (A549), colon carcinoma cells (HCT-116) or lung fibroblast cells (MRC-5), which are semi-permissive to EnAd virus activity, were used to demonstrate the

selectivity of NG-350A virus for cancer cells. The cell lines were infected for 72hrs with 100ppc NG-350A or the NG-350A parental virus, enadenotucirev or were left uninfected.

At each time point culture supernatant was removed from each well and used for analysis of virus genome replication by qPCR or anti-CD40 antibody expression by ELISA. The cells remaining in the well were analysed for expression of the viral gene E3 and transgene by RT-qPCR.

Virus Replication

Cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. DNA was extracted from 10 μ L (A549, HCT-116) or 100 μ L (MRC-5) of supernatant using the Sigma Genelute DNA extraction Kit, according to the manufacturer's protocol. A standard curve using EnAd virus particles (2.5e10-2.5e5vp) was also prepared and extracted using the Sigma Genelute Kit. Each extracted sample or standard was analysed by qPCR using an enadenotucirev E3 gene specific primer-probe set.

Quantification of the number of detected virus genomes per cell demonstrated that NG-350A and enadenotucirev virus replication was detectable at comparable levels in A549 and HCT-116 cells but expression in MRC-5 was significantly lower than in the carcinoma cell lines (Figure 11A). No virus genomes could be detected in uninfected cells (data not shown).

IgG2 antibody expression

Antibody expression in cell supernatants was assessed by IgG2 ELISA (IgG2 Human SimpleStep ELISA kit, ab202402, Abcam). Culture supernatants were removed from each well and centrifuged for 5 mins, 1200rpm to remove cell debris. The clarified supernatants were diluted 1 in 2 into PBS 10% BSA. A standard curve and negative control samples were prepared according to the manufacturer's protocol. Samples and standards were added to the ELISA plates and the assay performed according to the manufacturer's protocol. The absorbance at 450nm in each well of the plate was measured using a plate reader (BioTek) and the concentrations of secreted IgG2 anti-CD40 antibody were determined by interpolating from the standard curve (Figure 11B).

Example 10: Purification of anti-CD40 antibody from NG-350A infected cells

Suspension HEK293 cells were seeded at 1x10⁶ cells/mL in Erlenmeyer flasks and infected with 100 ppc NG-350A. After 72hrs, 5% FBS and protease inhibitor cocktail (1:2000) were added to the cells and the suspension was centrifuged for 15 minutes, 4600rpm. The supernatant was carefully removed and filtered through a 500kDa molecular weight cut off hollow fibre membrane to separate the NG-350A virus particles from the anti-CD40 antibody. The flow through from the filtration step, which contained the anti-CD40 antibody was passed through a second hollow fibre membrane with a 30kDa molecular weight cut off. The anti-CD40 antibody was purified from the retentate from the second filtration step on a protein A column using an AKTA. The purified antibody was filter sterilised and stored at -80°C. The concentration of purified antibody was determined by IgG2 ELISA using the IgG2 Human SimpleStep ELISA kit, ab202402, Abcam according to the manufacturer's protocol (Figure 12).

Example 11: NG-350A derived anti-CD40 antibody activity and synergy with virus activity in primary human monocyte derived DCs

PBMCs were isolated by Ficoll-Paque gradient centrifugation from a NC24 leucocyte cone sourced from NHS Blood and Transplant unit in Oxford, UK. CD14⁺ monocytes were isolated using the CD14 MicroBeads kit (MiltenyiBiotec). Monocytes were then counted, centrifuged (300xg) and resuspended at 5×10^5 cells/mL in 10% RPMI culture media supplemented with GM-CSF (800 U/mL) and IL-4 (500 U/mL). 40 mL of monocyte suspension were transferred into one T175 flask.

After 72hrs culture, monocyte derived DCs (moDCs) were seeded at a density of 1×10^6 cells per well in 24 well plates in 10% RPMI culture media. They were treated with 0.5 µg/mL of anti-CD40 antibody (purified from virus infected cells according to Example 10), EnAd (100ppc), human CD40L or were left untreated. In parallel, moDCs were treated with both the purified anti-CD40 antibody (0.5 µg/mL) and EnAd (100ppc). The plates were then incubated for 48hrs before supernatants and cells were harvested.

Supernatants and cells were removed from culture wells and centrifuged (300xg). The supernatant was diluted 1 in 2 with PBS 5% BSA and stored for ELISA analysis. Cell pellets were washed in 200 µL of PBS, centrifuged, then resuspended in 50 µL of PBS containing LIVE/DEAD® Fixable Near-IR (Life tech) for 15 minutes at RT. The cells were washed once in FACs buffer (1% FBS/PBS) before staining with panels of directly conjugated antibodies: anti-CD86 conjugated to BV421; anti-CD54 conjugated to AF647 and anti-HLA-DR conjugated to PeCy5. A sample of cells from each co-culture condition was also stained with relevant isotype control antibodies. All staining was carried out in FACs buffer in a total volume of 50 µL/well for 15 minutes, 4°C. Cells were then washed twice with FACs buffer (200 µL) before resuspension in 200 µL of FACs buffer and analysis by Flow cytometry (Attune).

Supernatant samples were thawed and analysed by ELISA (IL-12 Quantikine ELISA, DP400, R&D systems) by diluting in and carrying out the assay according to the manufacturer's protocol.

Treatment with anti-CD40 antibody purified from virus infected cells led to an increase in the percentage of moDCs expressing CD86, CD54 and HLA-DR activation markers and to the secretion of IL12p40 (Figure 13 and Figure 14). Significantly, combination treatment of moDCs with both the anti-CD40 antibody and EnAd virus resulted in a stronger moDC activation compared to treatment with anti-CD40 Ab or EnAd virus alone.

Example 12: NG-350A derived anti-CD40 antibody activity and synergy with virus activity in primary human B cells

PBMCs were isolated by Ficoll-Paque gradient centrifugation from a NC24 leucocyte cone sourced from NHS Blood and Transplant unit in Oxford, UK. CD19⁺ B cells were isolated using the Pan B Cell Isolation Kit (MiltenyiBiotec). B cells were then seeded at a density of 1×10^6 cells per well in 24 well plates in 10% RPMI culture media. They were treated with increasing concentration of purified anti-CD40 Transgene Abs, human CD40L or were left

untreated. The plates were then incubated for 48hrs before supernatants and cells were harvested.

Supernatants and cells were removed from culture wells and centrifuged (300xg). The supernatant was carefully removed, diluted 1 in 2 with PBS 5% BSA and stored for ELISA analysis. Cell pellets were washed in 200µL of PBS, centrifuged then resuspended in 50µL of PBS containing LIVE/DEAD® Fixable Near-IR (Life tech) for 15 minutes at RT. The cells were washed once in FACS buffer before staining with panels of directly conjugated antibodies: anti-CD86 conjugated to BV421; anti-CD54 conjugated to AF647; anti-HLA-DR conjugated to PeCy5 and anti-CD80 conjugated to BV605. A sample of cells from each co-culture condition was also stained with relevant isotype control antibodies. All staining was carried out in FACS buffer in a total volume of 50µL/well for 15 minutes, 4°C. Cells were then washed twice with FACS buffer (200µL) before resuspension in 200µL of FACS buffer and analysis by Flow cytometry (Attune).

Treatment of B cells with anti-CD40 antibody at all concentrations tested resulted in B cell activation in terms of an increase in the percentage of B cells expressing CD86, CD19 and CD80 and an increase in the HLA-DR MFI on CD19+ cells, compared to untreated or isotype control treated B cells (Figure 15). Treatment with anti-CD40 antibody also resulted in B cell activation in terms of increasing the percentage of proliferating cells (Figure 16). Significantly, combination treatment of B cells with anti-CD40 antibody and EnAd virus resulted in an enhancement in the % of proliferating B cells compared to antibody or virus treatment alone (Figure 16).

Example 13: NG-350A derived anti-CD40 antibody activity on primary human monocyte derived DCs

A549 tumor cells were seeded in T175 flasks or Hyperflasks at a density of, respectively, 10x10⁶ or 50x10⁶ cells per flask. After 4hrs, cells were infected with 10 EnAd or NG-350A virus particles per cell. After 72hrs, supernatants were harvested and virus depleted using 300 kDa cut-off size exclusion columns. Virus-depleted supernatants were subsequently enriched for antibodies using 50 kDa cut-off size exclusion columns. These virus-depleted Ab-enriched fractions were stored at -80°C. Anti-CD40 Ab titer was determined using IgG2 ELISA and functionality confirmed using the HEK Blue reporter cell assay described in Example 8.

Monocyte-derived dendritic cells (MoDCs) were prepared essentially as outlined in Example 11 for a further set of studies. PBMCs (donor 177) were isolated by Ficoll-Paque gradient centrifugation from a NC24 leucocyte cone sourced from NHS Blood and Transplant unit in Oxford, UK. CD14⁺ monocytes were isolated using the CD14 MicroBeads kit (MiltenyiBiotec). After 72hrs culture with GM-CSF and IL-4, MoDCs were seeded at a density of 1.25x10⁵ cells per well in 48 well plates or 2.5x10⁵ cells per well in 24 well plates in 10% RPMI culture media and incubated with the virus-depleted NG-350A or EnAd culture supernatants described in paragraph above. The cells were stained for flow cytometry

analysis. Supernatants were used for cytokine analysis by cytokine bead arrays (Legendplex, BioLegend).

To demonstrate anti-CD40 Ab binding to CD40 on the surface of MoDCs, cells were treated with increasing concentrations of the virus-depleted, anti-CD40 Ab enriched supernatant from NG-350A infected cells (different dilutions used to provide different amounts of antibody) or were left untreated (media). As a control, they were also treated with virus-depleted EnAd supernatant (dEnAd), with volumes matching those of the NG-350A anti-CD40 Ab containing samples. After 24hrs and 48hrs, cells were harvested and stained with fluorescent labelled antibodies to CD40 before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). **Figure 17A** shows a representative flow cytometric result obtained 24hrs post-treatment with 1000 ng/mL Ab, with **Figure 17B** showing CD40 expression on MoDCs 24hrs and 48hrs post-treatment with different concentrations of virus-depleted supernatants. At the higher antibody concentrations, CD40 FACS staining is reduced or absent, reflecting blockade by binding of the anti-CD40 antibody in the NG-350A virus-depleted culture supernatants.

The effects of anti-CD40 Tg Ab treatment (using the virus-depleted supernatants) on cell surface marker upregulation on MoDCs was then evaluated. MoDCs were treated with increasing concentrations of purified anti-CD40 Tg Ab or were left untreated (media). They were also treated with virus-depleted EnAd supernatant (dEnAd), with volumes matching those of anti-CD40 Ab. After 24hrs and 48hrs, cells were harvested and stained with antibodies to CD54, CD83 and CD86 surface markers before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). A representative flow cytometric result obtained 24hrs post-treatment with 1000 ng/mL Ab is shown in **Figure 18A** for MoDCs prepared with PBMCs from donor 177. **Figure 18B** shows activation marker expression on MoDCs from 4 different donors 24hrs and 48hrs post-treatment.

Effects of anti-CD40 Tg Ab on MoDC cytokine production were then evaluated. MoDCs from four different donors were treated with increasing concentrations of anti-CD40 Ab containing virus-depleted supernatant or were left untreated (media). They were also treated with virus-depleted EnAd supernatant (dEnAd), with volumes matching those of anti-CD40 Ab. After 24hrs and 48hrs, supernatants were collected and analysed for inflammatory cytokine secretion using a LEGENDplex™ bead-based immunoassays (BioLegend). **Figure 19** shows selective induction of TNF α , IL-6 and IL-8.

Example 14: Activity of NG-350A virus and derived anti-CD40 antibody containing virus infected tumour cell supernatants on activity of primary human monocyte derived DCs

In a similar study to that described in Example 13, A549 cells were seeded in T25 flasks at a density of 4×10^6 cells. After 4hrs, cells were infected with 10 EnAd or NG-350A virus particles per cell. After 72hrs, supernatants were harvested and centrifuged at 1600 rpm for

5 minutes. In this study, these clarified supernatants were kept at 37°C until use (within 1h) rather than removing the virus. Thus, supernatants contain the products of NG-350A (or EnAd control) infected tumor cells, including both virus and anti-CD40 antibody transgene product.

5 PBMCs were isolated by Ficoll-Paque gradient centrifugation from a NC24 leucocyte cone sourced from NHS Blood and Transplant unit in Oxford, UK. CD14⁺ monocytes were isolated using the CD14 MicroBeads kit (MiltenyiBiotec). After 72hrs culture with GM-CSF and IL-4, MoDCs were seeded at a density of 1.25×10^5 cells per well in 48 well plates and treated with EnAd and NG-350A virus supernatants at different dilutions. The plates were then
10 incubated for 24hrs and 48hrs before supernatants and cells were harvested. Supernatants were centrifuged and removed from cell pellets and stored at -80°C. The cells were stained for flow cytometry analysis. Supernatants were used for cytokine analysis by CBA.

To demonstrate anti-CD40 Ab binding to CD40 on the surface of MoDCs, cells were treated with increasing concentrations of the supernatants from NG-350A or EnAd infected cells
15 (different dilutions used to provide different amounts of antibody) or were left untreated (media). After 24hrs and 48hrs, cells were harvested and stained with fluorescent labelled antibodies to CD40 before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). **Figure 20** shows CD40 expression on MoDCs 24hrs and 48hrs post-treatment with different
20 concentrations of supernatants. At the 1:2 supernatant dilution from NG-350A (but not EnAd) infected tumour cells, CD40 FACS staining is reduced or absent, reflecting blockade by binding of the anti-CD40 antibody in the NG-350A virus-treated culture supernatants.

The effects of NG-350A virus treated tumour cell supernatant on cell surface marker upregulation on MoDCs from two donors (177 & 179) was then evaluated. MoDCs were
25 treated with diluted EnAd or NG-350A virus supernatants or were left untreated (media). After 24hrs and 48hrs, cells were harvested and stained with antibodies to CD86, CD54 and CD83 before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). Results in **Figure 21** show NG-350A selectively upregulated all three MoDC activation markers.

30 Effects of NG-350A virus supernatant on MoDC cytokine production was then evaluated. MoDCs from two donors (177 & 179) were treated with diluted EnAd or NG-350A virus-treated tumour cell supernatants or were left untreated (media). After 24hrs and 48hrs, supernatants were collected and analysed for inflammatory cytokine secretion using a LEGENDplex™ bead-based immunoassays (BioLegend). The results in **Figure 22** show
35 selective upregulation of TNF α , IL-6, IL-8, IL-28 and IL-29 by NG-350A treated cell supernatants.

Example 15: NG-350A derived anti-CD40 antibody activity on primary human B-cells

PBMCs were isolated by Ficoll-Paque gradient centrifugation from NC24 leucocyte cones
40 sourced from NHS Blood and Transplant unit in Oxford, UK. B cells were isolated using the

Pan B Cell Isolation Kit (MiltenyiBiotec). Cells were seeded at a density of 1.25×10^5 cells per well in 48 well plates in 10% RPMI culture media. They were treated with the different concentrations of the virus-depleted, anti-CD40 Ab enriched supernatant from NG-350A infected cells described in Example 13 or were left untreated. B cells were also treated with the virus-depleted EnAd virus supernatant as a control. The plates were then incubated for 24hrs and 48hrs before cells were harvested. The cells were stained with antibodies to CD23, CD54, CD86 and HLA-DR before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). **Figure 23** shows selective upregulation of all four surface markers (CD23, CD54, CD86 & HLA-DR) on B-cells from 3 different donors (177, 178, 179) by the virus-depleted NG-350A anti-CD40 antibody containing supernatants.

Example 16: Activity of NG-350A virus and derived anti-CD40 antibody containing virus infected tumour cell supernatants on activity of primary human B-cells

PBMCs were isolated by Ficoll-Paque gradient centrifugation from NC24 leucocyte cones sourced from NHS Blood and Transplant unit in Oxford, UK. B cells were isolated using the Pan B Cell Isolation Kit (MiltenyiBiotec). Cells were seeded at a density of 1.25×10^5 cells per well in 48 well plates and treated with different dilutions of samples of the same EnAd and NG-350A virus supernatants described in Example 14. The plates were then incubated for 24hrs and 48hrs before cells were harvested and stained with antibodies to CD23, CD54, CD86 and HLA-DR before flow cytometry analysis was carried out. Cells were gated on single (FSC-H versus FSC-A) live cells (LIVE/DEAD® Fixable Aqua negative). **Figure 24** shows selective upregulation of all four markers (CD23, CD54, CD86 & HLA-DR) on B-cells from 3 different donors (177, 178, 179) by the virus and anti-CD40 antibody containing supernatants from NG-350A infected tumour cells.

Example 17:

In another study, the activity of NG-350A was evaluated against a range of different tumour cell lines across six cancer indications (colorectal, prostate, pancreatic, breast, ovarian and bladder). Cells were infected with 1 or 100 particles per cell (ppc) of enadenotucirev (EnAd) or NG-350A and cultured for 3-11 days, evaluating viral genome replication, virus mediated oncolysis, viral transgene expression (at the mRNA and protein level) and functional viral transgene expression in NG-350A-infected tumour cells. In all experiments, the activity of NG-350A was compared to that of EnAd. A549 (non-small cell lung carcinoma) cells were used as a positive control, and CT26 (mouse colon tumour) cells as a negative control.

Virus genome replication

Tumour cell lines were cultured in 175cm² flasks in high glucose DMEM supplemented with L-glutamine, non-essential amino acids, sodium pyruvate and 10% foetal bovine serum (FBS) (growth media). Before use, cells were inspected using a microscope to ensure 60-80% confluency. The cells were then washed with PBS and 0.25% Trypsin-EDTA added to detach the cells from the bottom of the flask. The cells were incubated for 2-10 minutes at

37°C, 5% CO₂ until the cells detached, after which the trypsin was deactivated using growth media. The cell suspension was mixed and spun at 300g for 5 minutes. The supernatant was discarded, and the cell pellet resuspended in 10 mL of assay media; DMEM media as described above, supplemented with 2% (instead of 10%) FBS. Cells were counted, and the cell suspension diluted using assay media to achieve a concentration of 5x10⁵ cells/mL, 100 µL of which was seeded into flat bottom 96 well plates (each cell line was seeded into two wells per virus and 2 wells per uninfected control (UIC) per plate- with one plate per timepoint). Samples for EnAd and UIC were seeded (and infected) on one set of plates, and samples for NG-350A and UIC were seeded (and infected) on another set of plates. The plates were incubated at 37°C, 5% CO₂ before inoculation 4-6 hours post-cell seeding.

Following this time, EnAd and NG-350A viruses were diluted in assay media to achieve a concentration of 5x10⁵ virus particles/mL, 100 µL of which was added to each relevant well of cells (each virus was added to each cell line in duplicate on each plate). This resulted in an inoculation of 1ppc of EnAd or 1ppc of NG-350A. Instead of the addition of virus, 100 µL of assay media was also added to duplicate wells of each cell line (UIC) on each plate. 20 hours post-inoculation, media was removed from the cells and replaced with 200 µL of fresh assay media. The plates were then incubated at 37°C, 5% CO₂ for 3, 4, 8 or 11 days post inoculation. If the cells were to be incubated for 8 or more days, they were fed with 50 µL of assay media 4 days post-inoculation.

DNA Harvest: Post incubation, media was removed from the cells and centrifuged in 96 well V-bottom plates. The supernatant was then transferred to fresh 96 well flat bottom plates and stored at -80°C. 200 µL of 1 x Reporter Lysis Buffer (RLB) was then added to the original plates of cells, and stored at -80°C.

Standard curve preparation: Stock EnAd was diluted to between 1.25x10¹¹ and 1.25x10⁶ virus particles/mL before DNA extraction with the Qiagen DNeasy 96 kit according to the manufacturer's protocol (200 µL of each standard curve sample was extracted, and the purified DNA eluted in a volume of 200 µL). The purified DNA was then aliquoted and stored at -20°C before use on each qPCR plate. 2 µL of these standard curve samples were used in each qPCR reaction which equates to 2.5x10³ - 2.5x10⁸ genomes per qPCR well.

DNA extraction: Supernatant and lysate test samples were thawed before DNA extraction with the Qiagen DNeasy 96 kit according to the manufacturer's protocol (50 µL of the test samples was extracted and the purified DNA eluted in a volume of 200 µL). An extraction control of 1 x RLB was extracted on each plate.

Virus genome replication was assessed by qPCR of cell lysates and supernatants (S/N) using an E3 primer-probe set as outlined in Example 6. Time courses for both cell lysate and supernatant samples from 10 test tumour cell lines, plus positive (A549) cells are plotted according to tumour type (Colorectal, HT-29 & HCT-116; Prostate DU145; Pancreatic BxPC-3; Breast MBA-MB-453; Ovarian PA-1 & Caov-3; Bladder RT24, T24 & UM-UC-3) in **Figure 25 A-F**. Negative control cells (mouse CT26) were negative and not plotted. Viral genomes detected in EnAd infected cells are shown with dotted lines and viral genomes detected in

NG-350A infected cells are shown with solid lines. The data shows that NG-350A replicates its genomic DNA comparably to the EnAd parental virus across a range of different carcinoma cell types,

To evaluate the oncolytic effects of NG-350A, four tumour cell lines were inoculated with 100ppc of EnAd, 100ppc of NG-350A, assay media alone (uninfected control) or 4% tween (lysed). The growth and viability of the cells were monitored by the xCELLigence Real Time Cell Analyzer (RTCA) until over 50% lysis of cells inoculated with virus had occurred. The Cell Index (CI) across all timepoints was calculated by the xIMT software, and mean and SD of the uninfected, EnAd, NG-350A and lysed triplicates was determined; the mean was plotted in the graphs, and the SD represented by error bars. xCELLigence traces for uninfected controls, EnAd and NG-350A inoculated cells, and lysed cells are shown in **Figure 26**. Cells were seeded at 0 hours, and infected 24 hours post seeding (at the timepoint indicated by the arrow on each graph). Tumour cell lines are shown as one cell line per graph: colorectal (HT-29 & HCT-116), prostate (BxPC-3) and breast (MDA-MB-453). NG-350A showed comparable oncolytic activity to the EnAd parental virus.

Anti-CD40 transgene mRNA expression was also evaluated in lysates taken at different times from tumour cell lines infected with NG-350A. The same 10 test tumour cell lines as used for the genome replication part of this study, plus positive (A549) and negative (CT26) control cell lines, were inoculated with 1ppc of EnAd, 1ppc of NG-350A or assay media alone (uninfected control). At different times (3, 4, 8 and 11 days) post-inoculation, cell lysates were harvested before RNA extraction and DNase clean-up was carried out. One-Step RT-qPCR was then run using an anti-CD40 specific primer/probe set. A synthetic RNA oligonucleotide corresponding to the sense strand of anti-CD40 was used to create a standard curve from which RNA quantity of the test samples was calculated. Mean RNA quantity for each uninfected control triplicate was background subtracted from the corresponding individual EnAd and NG-350A values. Anti-CD40 mRNA copies per cell was then calculated and the mean of the two EnAd and the two NG-350A RT-qPCR triplicates determined. The mean and SD of these duplicate values was then calculated for each cell line, at each time point (n=2 except for BxPC-3 day 8 sample where n=1). The mean was plotted and the graphs presented in **Figure 27**, with the SD represented by error bars. EnAd inoculated cells gave negligible response whereby the mean genomes per cell calculated from biological duplicates ranged from 0×10^0 to 6.26×10^0 across all timepoints and cell lines. As such, EnAd data was not plotted. For the uninfected control samples, mean anti-CD40 copies per cell calculated from biological duplicates ranged from 0.00×10^0 to 9.09×10^0 . The graphs depict anti-CD40 transgene expression in colorectal (A), prostate, pancreatic and breast (B), ovarian (C) and bladder (D) tumour cell lines. NG-350A infection led to anti-CD40 transgene mRNA expression in all human tumour cell lines, with differences in kinetics observed for different cells.

To evaluate transgene protein IgG2 expression, production of human IgG2 (the isotype of the anti-CD40 antibody heavy chain encoded by NG-350A) by different tumour cell lines

infected with NG-350A was measured. Six test tumour cell lines, plus positive (A549) and negative (CT26) control cell lines, were inoculated with 1ppc of NG-350A, 1ppc of EnAd or assay media alone (uninfected control). At different times (3, 4, 8 and 11 days) post-inoculation, supernatant was harvested and stored at -80°C before use. Supernatant from NG-350A-inoculated test cell lines and A549 cells was diluted 1:2, 1:5, 1:10, 1:20, 1:40 and 1:80. Supernatant from all uninfected control samples, EnAd infected cells, and NG-350A infected CT26 cells was diluted 1:2 only. The human IgG2 *in vitro* SimpleStep ELISA (Abcam) was then carried out according to the manufacture's protocol. Human IgG2 purified protein (supplied with the ELISA kit) was used to create a standard curve from which the concentration of IgG2 protein in the samples was determined (ng/mL). The quantity of IgG2 protein per 1×10^6 cells (ng/ 1×10^6 cells) was then calculated. Mean and SD was determined from the means of the ELISA duplicates that sat within the standard curve. The mean was plotted and the graphs presented in **Figure 28**, with the SD represented by error bars. Background subtraction using uninfected control samples was not carried out as for each cell line and timepoint these samples gave no response (0×10^0 ng/ 1×10^6 cells). EnAd inoculated cells also gave no response therefore EnAd data was not plotted. IgG2 antibody production was detected with all cell lines infected with NG-350A.

The HEK-Blue CD40 signaling reporter assay described in Example 8 was used to test the functionality of the antibody produced by different tumour cell lines infected with NG-350A. Six test tumour cell lines plus positive (A549) and negative (CT26) control cell lines were inoculated with 1ppc of EnAd, 1ppc of NG-348 or assay media alone (UIC). 8 or 11 days post-inoculation, supernatant was harvested. Supernatant was then incubated with HEK-Blue cells for 20-24 hours. Supernatant was then removed from the treated HEK-Blue cells, added to Quanti-Blue and incubated for 1 hour before reading the optical density (OD) on a SpectraMax i3x set to read an absorbance of 620 nm. Mean optical density for each UIC assay duplicate was subtracted from the corresponding individual EnAd and NG-350A values, giving an assessment of CD40 signaling activity of the antibodies in the supernatants of infected tumour cells. The mean of the UIC background-subtracted EnAd and NG-350A assay duplicates was then taken, giving 2 values for each cell line (per condition), corresponding to each seeding and inoculation replicate. Mean and standard deviation (SD) of these replicates was then determined, and shown in **Table 3**.

Table 3 Functional anti-CD40 antibody activity in supernatants from NG-350A infected tumour cells (HEK-Blue reporter assay)

Summary of NG-350A transgene (anti-CD40 antibody) function, detected by the HEK-Blue assay					
Cancer Indication	Cell line	SEAP activity (Optical Density at 620 nm)			
		NG-350A		EnAd	
		Mean	SD	Mean	SD
Positive control	A549	1.0600	0.0933	0.1488	0.0124
Negative control	CT26	0.0000	0.0103	0.0017	0.0088
Colorectal	HT-29	0.7175	0.0212	0.0650	0.0021

Prostate	DU145	0.5555	0.0276	0.0910	0.0120
Pancreatic	BxPC-3	0.7118	0.2054	0.0428	0.0088
Breast	MDA-MB-453	0.8578	0.1439	0.1033	0.0011
Ovarian	Caov-3	0.0273	0.0293	0.0198	0.0124
Bladder	RT4	0.3653	0.0209	0.0470	0.0085

Example 18

In a further series of experiments, the *in vivo* virus particle-mediated effects of NG-350A virus were monitored and compared to those of EnAd following single or multiple intravenous doses in mice. Female CD-1 mice were injected IV with vehicle control or 2.2×10^{10} particles of either EnAd or NG-350A. Mice were cardiac bled 6 hours, 24 hours, 48 hours, or 7 days post injection and blood was collected into anticoagulant tubes and processed to recover plasma. Plasma samples were tested by ELISA assays to detect acute cytokine responses to the virus particles. Data shown in **Figure 29** represents 3-6 mice per group. MCP-1 (**Figure 29A**), IL-6 (**Figure 29B**) and TNF α (**Figure 29C**) levels stimulated by NG-350A dosing were similar to those for EnAd. Solid black lines represent the mean. Baseline represents the mean of vehicle control group + 3 x SD.

Other aliquots of the plasma samples were tested for alanine amino transferase (ALT) levels as a measure of acute liver toxicity. Plasma samples were analysed using an ALT colorimetric endpoint enzymatic assay kit. Data shown in **Figure 30** represents 3-6 mice per group. Solid black lines represent the mean. Baseline represents the mean of vehicle control group + 3 x SD. ALT levels were variable between different mice but NG-350A and EnAd induced similar response profiles.

Further groups of female CD-1 mice were injected IV with PBS on day 1, or 2.2×10^{10} viral particles of either EnAd or NG-350A on days 1, 3 and 5. PBS treated mice were cardiac bled 6 hours after dosing. Virus treated mice were bled via a lateral tail vein and cardiac puncture 6 hours and 24 hours (respectively) after the first dose, or via cardiac puncture 6 hours, 24 hours or 7 days after the third dose. Plasma samples were tested by ELISA assays to detect acute cytokine responses to the virus particles. Data shown in **Figure 31** represents 3-6 mice per group. MCP-1 (**Figure 31A**) and IL-6 (**Figure 31B**) levels stimulated by NG-350A dosing were similar to those for EnAd. Solid black lines represent the mean. Baseline represents the mean of vehicle control group + 3 x SD.

Example 19

Blood pharmacokinetics of NG-350A were compared to enadenotucirev (EnAd) after the administration of each of three intravenous (IV) doses of 2.2×10^{10} viral particles on days 1, 3 and 5 in immunocompetent CD-1 mice. Female CD-1 mice were injected IV with 2.2×10^{10} viral particles of either EnAd or NG-350A on days 1, 3 and 5. After each dose, a group of 4 mice treated with each virus were bled at 1, 2, 3, 5, 7, 10 and 60 minutes post-dosing. DNA was extracted and analysed by qPCR targeting the viral E3 gene common to both EnAd and NG-350A. Data shown in **Figure 32** represent 4 mice per group + SD. Where values fell

below the limit of quantitation for the assay they were excluded. Data beyond 7 minutes was not plotted as they fell consistently below the limit of quantitation for the assay. NG-350A showed a comparable pharmacokinetic profile to that of EnAd.

Example 20

5 Virus biodistribution was evaluated following dosing of mice with NG-350A by measuring the recovery of live virus from tissues after a single intravenous dose. Female CD-1 mice were injected IV with vehicle control or 2.2×10^{10} particles of NG-350A. Groups of mice were euthanised at either 6 hours, 24 hours, 8 days or 28 days post dosing and their liver, lungs and spleens were resected and immediately frozen on dry ice. Samples were later thawed,
10 homogenised in a protein-preserving lysis buffer and the lysate was diluted and added to confluent A549 monolayers, with NG-350A-spiked tissue homogenate for each organ as a positive control and negative controls. Monolayers were cultured for 96 hours before being fixed and subjected to an immunostaining assay utilising an anti-adenovirus hexon antibody. The data are represented by sample photos of each well in **Figure 33**. Hexon
15 positive cells stained brown (shown as dark grey in the figure). No live virus could be detected in liver, lung or spleen of mice (the primary sites of virus biodistribution in mice) later than 24 hours.

Example 21

In this series of studies, NG-350A virus activity was assessed *in vivo* in human tumor
20 xenograft bearing immunodeficient mice.

Virus replication was evaluated in subcutaneous A549 lung cell line tumours after three IV injections or a single fractionated intratumoral (IT) dose. Female SCID mice were implanted subcutaneously with A549 tumour cells on their flank and injected either IT or IV with virus or control once tumours reached at least 50mm^3 . IT dosed mice were injected with two $10\mu\text{L}$
25 injections of PBS, or 2.2×10^9 viral particles of either EnAd or NG-350A into spatially separate regions of the tumour for a total dose of $20\mu\text{L}$ / 4.4×10^9 viral particles. IV dosed mice were injected with $100\mu\text{L}$ of PBS, or 2.2×10^9 viral particles of either EnAd or NG-350A on days 1, 3 and 5 for a total dose of $300\mu\text{L}$ / 6.6×10^9 viral particles. Tumours were resected from mice after euthanasia, either 7 or 21 days post-dosing, and frozen. Tumours were later
30 homogenised, DNA extracted and analysed by qPCR using primers and probe targeting the viral E3 region common to both EnAd and NG-350A. Data shown in **Figure 34 A&B** represent 2-4 mice per group +/- SD. NG-350A showed comparable genome replication in the A549 tumour xenografts to that of EnAd following either IT (**Figure 34A**) or IV (**Figure 34B**) dosing.

35 This experiment was also repeated using a second tumour xenograft model using the HCT-116 colorectal cancer cell line. Female SCID mice were implanted subcutaneously with HCT116 tumour cells on their flank and injected either IT or IV with virus or control once tumours reached at least 50mm^3 . IT dosed micewere injected with two $10\mu\text{L}$ injections of PBS, or 2.2×10^9 viral particles of either EnAd or NG-350A into spatially separate regions of
40 the tumour for a total dose of $20\mu\text{L}$ / 4.4×10^9 viral particles. IV dosed mice were injected

with 100µL of PBS, or 2.2x10⁹ viral particles of either EnAd or NG-350A on days 1, 3 and 5 for a total dose of 300µL / 6.6x10⁹ viral particles. Tumours were resected from mice after euthanasia, either 7 or 21 days post-dosing, and frozen. Tumours were later homogenised, DNA extracted and analysed by qPCR using primers and probe targeting the viral E3 region common to both EnAd and NG-350A. Data shown in Figure 34 C&D represent 2-4 mice per group +/- SD. NG-350A showed comparable genome replication in the HCT-116 tumour xenografts to that of EnAd following either IT (**Figure 34C**) or IV (**Figure 34D**) dosing.

In similar experiments with both A549 and HCT-116 subcutaneous xenograft tumours in SCID mice, virus RNA expression was measured in the tumours. Female SCID mice were implanted subcutaneously with A549 or HCT-116 tumour cells on their flank and injected either IT or IV with virus or control once tumours reached at least 50mm³. IT dosed mice were injected with two 10µL injections of PBS, or 2.2x10⁹ viral particles of either EnAd or NG-350A into spatially separate regions of the tumour for a total dose of 20µL / 4.4x10⁹ viral particles. IV dosed mice were injected with 100µL of PBS, or 2.2x10⁹ viral particles of either EnAd or NG-350A on days 1, 3 and 5 for a total dose of 300µL / 6.6x10⁹ viral particles. Tumours were resected from mice after euthanasia, 7 days post-dosing, and frozen. Tumours were later homogenised, RNA extracted and analysed by RT-qPCR using primers and probe targeting viral E3 mRNA common to both EnAd and NG-350A. Data shown in **Figure 35** represent 3-4 mice per group. Black lines represent the mean. A549 IT (**Figure 35A**), IV (**Figure 35B**), HCT-116 IT (**Figure 35C**) and HCT-116 IV (**Figure 35D**) show comparable virus E3 mRNA expression following either NG-350A or EnAd dosing.

Levels of anti-**CD40 antibody transgene mRNA expression were also measured in the same subcutaneous A549 and HCT-116 xenograft tumour RNA samples.** RNA was analysed by RT-qPCR using primers and probe targeting αCD40 antibody transgene mRNA. Data shown in Figure 36 represent 3-4 mice per group. Black lines represent the mean. Anti-CD40 antibody transgene mRNA expression was readily detected only in NG-350A treated tumours. A549 IT (**Figure 36A**), IV (**Figure 36B**), HCT-116 IT (**Figure 36C**) and HCT-116 IV (**Figure 36D**)

Levels of anti-CD40 antibody protein were also measured in both tumour lysates and sera of mice bearing A549 tumours using an IgG2 ELISA. The data shown in **Figure 37** show selective detection of antibody following NG-350A administration, either IT (A) or IV (B), with higher levels in tumours than in the blood.

SEQUENCES

SEQ ID NO. 4 – anti-CD40 VH chain amino acid sequence

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWRQAPGQGLEWMGWINPDSGGTNYAQKFQGRVTMTRDTSISTAYMELNRLRSDDTAVYYCARDQPLGYCTNGVCSYFDYWGQGTLTIVSS

SEQ ID NO. 5 – antibody constant heavy chain amino acid sequence

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDPKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVSVSLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPMLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO. 8 – anti-CD40 VL chain amino acid sequence

DIQMTQSPSSVSASVGDRTITCRASQGIYSWLAWYQQKPGKAPNLLIYTASTLQSGVPSRFSG
SGSGTDFTLTITSSLPEDFATYYCQQANIFPLTFGGGTKEIK

SEQ ID NO. 9 – constant light chain amino acid sequence

5 RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 12 – NG-350A transgene cassette nucleic acid sequence

GCGATCGCCAGGCCACCATTGGACTGGACCTGGCGCATCCTGTTCTTGGTGGCAGCTGCTACGG
GAGCTCATTTCCAGGTGCAGCTGGTGCAATCCGGCGCTGAGGTGAAGAAACCCGGGGCTTCAGT
10 CAAAGTCAGCTGCAAGGCTAGCGGCTACACCTTTACTGGCTATTACATGCACTGGGTGAGGCAG
GCTCCGGGACAGGGTCTGGAATGGATGGGATGGATCAATCCGGACAGCGGCGGGACCAATTACG
CACAAAAGTTCCAAGGCCGCGTGACGATGACCCGGGACACTTCGATCTCAACCGCCTACATGGA
GCTGAACCGCCTGAGGTGCGATGACACCGCTGTGTACTACTGCGCTCGCGACCAACCCCTGGGG
TACTGCACCAACGGAGTGTGTTTACTACTTTCGACTACTGGGGCCAAGGCACGCTGGTCACTGTGT
15 CATCGGCGTCCACTAAGGGCCCCGTCGGTCTTCCCACTAGCTCCGTGCTCGCGGTGCACTTCGGA
ATCAACTGCGGCACTCGGATGCCTTGTCAAGGACTACTTCCCAGAACCCGTGACCGTCTCGTGG
AACTCAGGCGCCCTGACGAGCGGTGTCCACACTTTCCCGGCGGTGCTGCAGTCATCGGGGCTAT
ACAGCCTGAGCAGCGTGGTTACTGTGCCGTCATCAAACCTTCGGGACCCAGACTTACACTTGCAA
TGTGGACCACAAGCCGTCAAATACCAAAGTGGACAAGACTGTGGAACGCAAATGTTGCGTGGA
20 TGCCCTCCGTGCCCCGGCCCCCCCCAGTCGCTGGCCCATCCGTGTTTCTTCCCTCCGAAGCCAA
AAGACACTCTGATGATTTTCGAGAACTCCGGAGGTCACTTGCCTGGTGGTGCAGCTGTGCACGA
GGATCCAGAGGTGCAGTTCAACTGGTACGTGGATGGAGTGGAGGTGCACAATGCCAAGACCAAG
CCGCGCGAAGAACAATTCAACTCCACCTTTTCGGGTGCTGTCCGTGCTGACCGTGGTACACCAAG
ACTGGCTGAACGGAAAGGAGTACAAATGCAAGGTGAGCAACAAGGGGCTGCCGGCTCCAATCGA
25 AAAGACCATCTCAAAGACTAAGGGGCAACCTCGCGAGCCACAGGTGTATACCCTGCCTCCAAGC
AGGGAGGAAATGACCAAAAACAGGTGAGCCTGACCTGTCTGGTGAAGGGCTTTTACCCCAGCG
ACATCGCCGTCGAGTGGGAAAGCAACGGACAACCCGAGAACAACCTACAAGACCACTCCGCCC
GCTGGACTCCGACGGGTCAATTTTCTTCTGACTCAAAGCTGACTGTGGACAAGTCCCGGTGGCAG
CAAGGTAACGTGTTCTCCTGCTCGGTGATGCACGAAGCTTTGCACAACCACTACACTCAAAGT
30 CACTTTCTTGTACCCGGGCAAGGGGTCGGGCGCCACTAACTTTTCTTGTCTCAAGCAGGCGGG
CGATGTGGAGGAGAATCCGGGCCCCGCGCCTCCCGGCGCAACTGCTGGGCTCCTCCTCCTCTGG
TTTCCCGGCTCCCGCTGTGACATCCAGATGACTCAGTCGCCCAGCTCCGTGTCCGCATCGGTGG
GGGACAGAGTCACCATCACCTGCAGAGCTTCACAAGGGATCTATTCTTGGCTGGCGTGGTATCA
GCAGAAGCCTGGAAAGGCCCCCAACCTCCTGATTTACACCGCATCGACTCTCCAGTCAGGCGTG
35 CCATCCCGGTTCTCAGGGTCCGGTCCGGAACCGACTTCACTCTGACTATCAGCTCCCTGCAAC
CAGAAGATTTTCGCTACCTACTACTGCCAGCAGGCAAACATCTTTCCGCTAACTTTTCGGCGGAGG
CACGAAGGTGGAGATCAAGAGAACCGTGGCGGGCCCCCTTCCGTCTTCATCTTCCACCGTCAGAC
GAACAACCTCAAATCCGGTACCGCCTCCGTGCTGTGCCTGCTCAATAACTTCTATCCACGCGAGG
CCAAGGTCCAGTGGAAAGTGGATAACGCCCTGCAGTCCGGAACAGCCAGGAGTCAGTGACCGA
40 ACAGGATTCCAAGGACAGCACTTACTCGCTCTCAAGCACCCCTCACCTGTGCAAGGCGGATTAC
GAGAAGCACAAAGTCTACGCCTGCGAAGTGACTCATCAAGGACTCTCATCACCGGTAACCTAAGA
GCTTCAATCGCGGAGAATGCTAGGCTAGCTTACTGACTGAGATACAGCGTACCTTCAGCTCAC
AGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACCTAGAATGCAGTGAAAAAATGC
TTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTTATAAGCTGCAATAACAAG
45 TTAACAACAACAATTGCATTCATTTTATGTTTTCAGGTTTCAGGGGAGGTGTGGGAGGTTTTTTA
AAGCAAGTAAACCTCTACAAATGTGGTCCTGCAGG

CLAIMS

1. A replication competent oncolytic group B adenovirus comprising a transgene cassette encoding an anti-CD40 antibody, wherein the transgene cassette comprises a nucleic acid sequence given in SEQ ID NO: 12.
2. An oncolytic virus according to claim 1, wherein the virus is Enadenotucirev.
3. An oncolytic virus according to any one of claims 1 to 2, wherein the virus comprises SEQ ID NO: 1.
4. An oncolytic virus according to claim 3, which consists of SEQ ID NO: 1.
5. A pharmaceutical composition comprising a virus according to any one of claims 1 to 4, and a pharmaceutically acceptable excipient, diluent or carrier.
6. An oncolytic virus according to any one of claims 1 to 4 or a pharmaceutical composition according to claim 5, for use in treatment.
7. Use of an oncolytic virus according to any one of claims 1 to 4 or a pharmaceutical composition according to claim 5 in the manufacture of a medicament for the treatment of cancer, insulin resistance, obesity and/or immune deficiency.
8. The use according to claim 7, wherein the virus or composition is for use in the treatment of cancer.
9. An oncolytic virus according to any one of claims 1 to 4 or a pharmaceutical composition according to claim 5 for use in treatment, wherein the oncolytic virus or the pharmaceutical composition is to be administered in combination with a further anti-cancer therapy.
10. An oncolytic virus or a composition for use according to claim 9, wherein the further anti-cancer therapy is chemotherapy.
11. An oncolytic virus or a composition for use according to claim 9 or 10, wherein the further anti-cancer therapy is a check point inhibitor.

12. An oncolytic virus or a composition for use according to claim 11, wherein the anti-cancer therapy is selected from the group comprising a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, a TIM-3 inhibitor, a LAG-3 inhibitor, a TIGIT inhibitor, a B7-H3 (CD276) inhibitor, a B7-H4 (B7S1) inhibitor, a B7H7 (HHLA2) inhibitor, a CD96 inhibitor, a VISTA inhibitor and a combination of two or more of the same.
13. An oncolytic virus or a composition for use according to claim 12, wherein the inhibitor is an antibody or antigen binding fragment thereof.
14. An oncolytic virus or a composition for use according to any one of claims 9 to 10, wherein the further anti-cancer therapy is a co-stimulatory pathway agonist.
15. An oncolytic virus or a composition for use according to claim 14, wherein the further anti-cancer therapy is selected from the group comprising a CD27 agonist, a CD28 agonist, an ICOS agonist, a TMIGD2 (IGPR-1/CD28H) agonist, a CD226 agonist, an OX40 agonist, a 4-1BB agonist, and a combination of two or more of the same.
16. An oncolytic virus or a composition for use according to claim 15, wherein the further anti-cancer therapy is an antibody or antigen binding fragment thereof.
17. An oncolytic virus or a composition for use according to any one of claims 9 to 16, wherein the further anti-cancer therapy activates immune responses or reverses suppression of immune responses.
18. An oncolytic virus or a composition for use according to claim 9, wherein the further anti-cancer therapy is an oncolytic virus.
19. An oncolytic virus or a composition for use according to claim 18, wherein the oncolytic virus encodes therapeutic gene encoding material selected from the group consisting of an RNAi sequence, an antibody or antigen binding fragment thereof, chemokines, cytokines, immunomodulator and enzymes.