ONCOLYTIC ADENOVIRUS ENCODING A B7 PROTEIN OR ACTIVE FRAGMENT

The present disclosure relates to an oncolytic adenovirus comprising a transgene encoding at least a B7 protein such as CD80 or an active fragment thereof, compositions comprising the same and use of the virus and compositions in treatment, particularly in the treatment of cancer.

BACKGROUND

Cancer is still a huge social burden to society in terms of the hardship and suffering of patients and their loved ones, and also in terms of the high financial cost of treating, caring for and supporting patients. It is now thought that the immune system of healthy individuals clears cancerous cells routinely. However, in those patients with cancer one or more of the defense mechanisms involved in this clearance is/are down regulated or turned off completely.

It is now known that tumors change their microenvironment to make it more permissive to their growth. This occurs by the tumor releasing extracellular signals that, for example, promote tumor angiogenesis and/or induce local immune suppression or immune tolerance.

It is clear from many different preclinical and clinical studies that the microenvironment within tumours can suppress the development and activity of anti-tumour immune responses, with a wide variety of mechanisms being shown to potentially play a role. In particular immuno-suppressive mechanisms ultimately prevent T-cell responses from mediating the killing of tumour cells. Suppressive mechanisms may include the exclusion of T-cells from entering tumour tissues, inhibiting activation of T-cells that do enter the tumour and the modulation of tumour cell proteins which reduces the ability of T-cells to recognize or respond to them. The importance of such immunosuppressive pathways in supporting tumour progression has been particularly highlighted by the clinical efficacy shown by antibodies to receptors in two such suppressive pathways, CTLA4 and PD-1/PDL1, which has led to their marketing approval for the treatment of melanoma and other cancers.

B7 is a type of peripheral membrane protein found on activated antigen presenting cells (APC) that, when paired with either a CD28 or CD152 (CTLA-4) surface protein on a T cell, can produce a co-stimulatory signal or a co-inhibitory signal to enhance or decrease the activity of a MHC-TCR signal between the antigen presenting cell (APC) and the T cell, respectively. Besides being present on activated APCs, B7 can also be found on T-cells themselves.

There are several steps to activation of the immune system against an antigen. The T cell receptor must first interact with a complex of its specific peptide antigen (Ag) bound to a major histocompatibility complex (MHC) surface protein. The CD4 or CD8 proteins on the T-cell surface interact with the MHC to help stabilize the MHC/Ag interaction with the T-cell receptor complex, which comprises both the antigen-binding chain dimers (alpha/beta or gamma/delta) and the CD3 signaling complex (comprising

gamma, delta, epsilon and zeta chains). This is also referred to as "Signal 1" and its main purpose is to provide the initial signaling and guarantee antigen specificity of the T cell activation.

However, MHC binding is insufficient by itself for stimulating full effector T cell differentiation and activation. In fact, lack of further stimulatory signals can render the T cell anergic. The co-stimulatory signals necessary to continue the immune response can come from B7-CD28 and CD40-CD40L interactions. There are other activation signals which play a role in immune responses. For example, in the TNF family of molecules, the protein 4-1BB (CD137) on the T cell may bind to 4-1BBL on the APC.

The B7 (CD80/B7-1 and/or CD86/B7-2) protein is present on the APC surface, and it interacts with the CD28 receptor on the T cell surface. This is one source of "Signal 2" (cytokines can also contribute to T-cell activation, which may be referred to as "Signal 3"). This interaction produces a series of downstream signals which promote the target T cell's survival, activation and differentiation into an effector cell that can mediate aspects of the immune response, such as killing of virus infected cells or tumour cells, and the recruitment of inflammatory cells.

Usually for initiating a T-cell response, the stimulatory signal and the co-stimulatory signal are provided by an antigen presenting cell in order to induce both CD4 and CD8 T-cell responses. But effector CD8 T-cells recognize their Ag associated with MHC class I molecules which are present on most nucleated cells, including tumour cells. However, the present inventors have reason to believe that the signals to activate T cells do not need to come from the same cell or cell type. Therefore it would be useful to provide one or more of these signals (i.e. the stimulatory signal and/or the co-stimulatory signal) to the immune system, for example on the surface of a cancer cell.

Currently there is much interest in inhibiting PD-1 (programmed cell death protein 1) and/or its ligand PDL1 (also known as B7-H1) activity because this pathway is thought to play an important role in down-regulating immune responses, for example in cancers.

However, some work done suggests that CD80 (B7-1) not only acts as a T-cell co-stimulator by binding to CD28 on the T-cell, it can also bind to PDL1, for example when expressed in the same cell membrane, and block PDL1-PD1 inhibitory signaling interactions. Thus, by acting in two different ways, CD80 may be a viable and potentially more useful molecule for restoring or enhancing the activation of human T cells. Soluble forms of CD80 also seem to be capable of counteracting PDL1-PD1 mediated T cell inhibition, see for example Haile *et al* Soluble CD80 Restores T Cell Activation and Overcomes Tumor Cell Programmed Death Ligand 1–Mediated Immune Suppression J Immunol 2013; 191:2829-2836. A CD80-Fc fusion protein has been generated and is being tested for safety and efficacy, see the Journal of Immunology, 2014, 193: 3835–3841.

The present inventors believe that the B7 proteins or an active fragment thereof delivered and expressed by an oncolytic virus, for example on the surface of a cancer cell, would be useful in activating the patient's own immune system to fight the cancer.

Furthermore, B7 proteins, such as CD80, if simply administered systemically have the potential to stimulate immune responses systemically in an undesirable way. The present inventors believe that a more sophisticated delivery of these proteins is required to create a suitable therapeutic window where beneficial therapeutic effects are realized and off target effects are minimized.

SUMMARY OF THE DISCLOSURE

Thus there is provided an oncolytic adenovirus with selectivity for cancer cells, wherein the adenovirus comprises a transgene under the control of a promoter endogenous to the virus, wherein the transgene comprises a DNA sequence encoding a B7 protein or an active fragment thereof. This is beneficial because the oncolytic viruses according to the present disclosure preferentially infect cancer cells and thus penetrate the microenvironment created by the cancer. Once in the cancer cells the B7 proteins encoded by the virus can be expressed, for example on the cell surface (i.e. cancer cell surface). This is advantageous because the B7 protein is then in the desired location where it can be biologically active.

In one embodiment the B7protein encoded comprises a sequence capable of anchoring the protein on the surface of a cell, for example a transmembrane domain sequence, GPI anchor or the like.

Thus in one embodiment the cancer cell is infected with a virus of the present disclosure which expresses a B7 protein or molecule, in particular on the surface of the cancer cell, wherein the B7 protein is suitable for providing at least the co-stimulatory signal i.e. signal 2 to activate a T cell, and/or may bind to and inhibit the activity of PD-L1 expressed on the surface of the cancer cell or other cells in the local microenvironment.

In one embodiment the B7 sequence comprises a transmembrane element from a B7 protein, for example a transmembrane element native to the particular B7 protein or a transmembrane domain from a “different” B7 protein to that being particularly expressed.

B7 proteins are surface expressed proteins and can also be employed to carry additional proteins to the cancer cell surface, for example where at least the transmembrane domain of a B7 protein is attached to an additional protein.

Thus in one aspect there is provided a replication competent oncolytic adenovirus with selectivity for cancer cells, wherein the adenovirus comprises a transgene under the control of a promoter endogenous to the virus, wherein the transgene comprises a DNA sequence encoding a B7 protein or an active fragment thereof.

Also provided is a replication competent oncolytic virus according to claim 1, wherein the B7 protein or active fragment thereof is independently selected from the group comprising B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5 and B7-H6, in partcular wherein the B7 protein is B7-1 (CD80) or an active fragment thereof.

In one embodiment the replication competent oncolytic virus is a group B adenovirus.

In one embodiment the replication competent oncolytic virus is a chimeric virus.

In one embodiment the replication competent oncolytic virus has a backbone is enadenotucirev (also referred to as EnAd).

In one embodiment the replication competent oncolytic virus has a formula (I):

5'ITR-B1-BA-B2-BX-BB-BY-B3-3'ITR (I)

B_1 comprises: 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 a transgene encoding a B7 protein or an active fragment thereof; and

B_3 is a bond or comprises E4.

In one embodiment the replication competent oncolytic virus according to any one of claims 1 to 7, wherein the B7 protein or active fragment thereof comprises a transmembrane sequence, for example a transmembrane domain from a PDGF receptor, or GPI anchor suitable for anchoring the protein or fragment in a cell membrane.

In one embodiment the replication competent oncolytic virus further comprises a second transgene, for example encoding a polypeptide selected from the group comprising a cytokine, a chemokine, an antagonistic antibody molecule or fragment thereof, and an agonistic antibody molecule or fragment thereof.

In one embodiment the second and third transgene, for example encoding two different polypeptides selected from the group comprising a cytokine, a chemokine, an antibody, such as an antagonistic antibody molecule or fragment thereof, or an agonistic antibody molecule or fragment thereof.

In one embodiment the second or third transgene encodes a cytokine, selected from the group comprising IL-2, IFN-alpha, IFN-beta, IFN-gamma, Flt3 ligand, GM-CSF, IL-15, and IL-12.

In one embodiment the second or third transgene encodes a chemokine, selected from the group comprising MIP1α, IL-8, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19 and CCL21.

In one embodiment a cytokine and a chemokine combination is encoded by the virus selected from the group comprising Mip1α and Flt3 ligand, and MIP1α and IFNα.

In one embodiment the virus encodes an antibody molecule or fragment thereof for example comprising a transmembrane sequence or GPI anchor such that it is a cell membrane-anchored form or a transmembrane domain, for example from a PDGF receptor .

In one embodiment the antibody molecule or binding fragment thereof comprises an anti-human CD3 antigen binding domain.

In one embodiment the antibody molecule is an inhibitor, for example selected from the group comprising an inhibitor of an angiogenesis factor, such as an anti-VEGF antibody molecule, and inhibitor of T cell deactivation factors, such an anti-CTLA-4 antibody molecule.

In one embodiment the antibody molecule is an agonist, for example of one or more selected from the group comprising CD40, GITR, OX40, CD27 and 4-1BB.

In one embodiment an exogenous protein or proteins encoded by the virus is/are a form suitable for expression on a cancer cell surface.

SUMMARY OF THE FIGURES

Figure 1 shows some of the key molecules involved in T-cell recognition of antigen presenting cells or tumor cells, and some of the signaling events induced in the responding T-cell. The structure of PDL1 and interaction with the IgV domain of PD1 is also illustrated

Figure 2 shows some of the B7 family ligands and binding partners from the CD28 family of receptors

Figure 3 shows schematics of transgene cassettes for viruses expressing human CD80 (Figure 3A), co-expressing human IFNα and human CD80 (Figure 3B), co-expressing OKT3 scFv and human CD80 (Figure 3C), co-expressing human Flt3L, human MIP1α and human IFNα (Figure 3D), co-expressing human Flt3L, human MIP1α and human CD80 (Figure 3E), co-expressing human IFNα, human MIP1α and human CD80 (Figure 3F), and a schematic of the open reading frame (ORF) or the OKT3 scFv (Figure 3G)

Figure 4 shows replication of EnAd (ColoAd1) and human CD80 encoding virus NG-330 in HT-29 (Figure 4A) and A549 (Figure 4B) tumour cells

Figure 5 shows expression of CD80 in the membrane of A549 (Figure 5A) or HT-29 (Figure 5B) tumour cells by fluorescent immunostaining at different times after infection with NG-330. No expression of CD80 on the cell membrane was observed with EnAd or uninfected tumour cells (UIC)

Figure 6 shows comparable oncolytic potencies of EnAd and NG-330 in a HT-29 cytoxicity assay. Thus NG-330 retains its oncolytic properties whilst also carrying a transgene

Figure 7 shows comparable oncolytic potency of EnAd and the CD80 + IFNα expressing NG-343 virus (Figure 7A) and secretion of IFNα by NG-343 infected HT-29 and A549 tumour cells over a period of up to 72 hours

Figure 8 shows expression of CD80 and tumour cell killing at 48 or 72 hours post infection by FACS analysis using anti-CD8 immunostaining together with a cell viability stain. CD80 could be detected at the cell surface of both live and dead NG-343 treated cells but not EnAd or uninfected control (UIC)

A549 tumour cells (Figure 8A-D). Similar CD80 expression was seen with both A549 and HT-29 tumour cells (Figure 8E).

Figure 9 shows comparable virus replication with both EnAd and NG-343 in tumour (HT-29) and non-tumour (MRC5, WI38 and bronchial epthelial cells) cells, with the latter showing much lower levels of replication (Figure 9A), IFNα secretion (Figure 9B) and CD80 expression (Figure 9C) following NG-343 infection only detected in HT-29 tumour cells.

Figure 10 shows that A549 tumour cells infected with NG-343 can induce increased surface levels of both CD80 and PD-L1 on the surface of DCs in PBMC co-cultures when compared to EnAd infected or uninfected tumour cell culture.

Figure 11 shows expression of IFNα and CD80 by HT-29 (Figure 11A&C) and A549 (Figure 11B&C) tumour cells infected with NG-347 virus

Figure 12 shows expression of MIP1α (Figure 12A), IFNα (Figure 12B) and Flt3L (Figure 12C) by A549 tumour cells infected with NG-345 virus

Figure 13 shows comparable oncolytic potency (Figures 13A&B) and infectivity (Figure 13C) of EnAd, NG-347 and NG-348 viruses in an HT-29 cytotoxicity assay

Figure 14 shows high CD80 expression by 48 hours on the cell surface of A549 tumour cells infected with either NG-347 or NG-348 viruses but little or no CD80 expression following EnAd infection

Figure 15 shows high CD80 expression by 48 hours on the cell surface of DLD-1 tumour cells infected with either NG-347 or NG-348 viruses but little or no CD80 expression following EnAd infection

Figure 16 shows CD80 expression on $EpCam^+$ A549 cells infected with NG-348 and co-cultured with human $CD3^+$ T-cells, but not when infection was with EnAd

Figure 17 shows CD25 is upregulated on human $CD3^+$ T-cells following co-culture with NG-348 infected A549 cells, but not when infection was with EnAd (Figure 17A), with both the percentage of $CD25^+$ cells (Figure 17B) and the level of CD25 expression per cell (Figure 17C) was increased.

Figure 18 shows CD25 is upregulated on both $CD4^+$ and $CD4^-$ (primarily CD8) human $CD3^+$ T cell subsets following co-culture with NG-348 infected A549 cells, but not when infection was with EnAd.

Figure 19 shows low level of HLA-DR expression on human $CD3^+$ T cells following co-culture with NG-348 or EnAd infected A549 cells

Figure 20 shows induction of CD107a expression on the surface of live, $CD3^+$ T cells following co-culture with NG-348 infected A549 cells, but not when infection was with EnAd.

Figure 21 shows induction of CD107a expression on the surface of both $CD4^+$ and $CD4^-$ $CD3^+$ T cell subsets following co-culture with NG-348 infected A549 cells, but not when infection was with EnAd.

Figure 22 shows induction of IL-2 (Figure 22A) and IFNγ (Figure 22B) production by $CD3^+$ T cells following co-culture with NG-348 infected A549 cells, but no IL-2 and only low levels of IFNγ when infection was with EnAd.

Figure 23 shows induction of IFNγ production by both $CD4^+$ and $CD8^+$ (Figure 22B) $CD3^+$ T cells following co-culture with NG-348 infected A549 cells, but no ($CD4^+$ cells) or low ($CD8^+$ cells) IFNγ when infection was with EnAd.

Figure 24 shows CD69 is upregulated on more human $CD3^+$ T-cells following co-culture with NG-347 infected A549 cells than when infection was with EnAd

Figure 25 shows induction of IFNγ production by human $CD3^+$ T cells following co-culture with NG-347 infected A549 cells, but not when infection was with EnAd

Figure 26 shows schematics of the NG-348A, NG-420 and NG-420A transgene cassettes

Figure 27 shows genome replication and hexon gene expression (mRNA levels) for EnAd, NG-347, and NG-348 in MRC-5 fibroblast cells compared to A549 tumour cells

Figure 28 shows CD80 and anti-CD3-scFv transgene mRNA and CD80 trasngene protein (flow cytometry) expression for virus NG-348 in MRC-5 fibroblast cells compared to A549 tumour cells

Figure 29 shows CD80 transgene mRNA and CD80 transgene protein for virus NG-347 in MRC-5 fibroblast cells compared to A549 tumour cells.

Figure 30 shows mRNA and secreted protein levels of MIP1α and IFNα generated by virus NG-347 in MRC-5 fibroblast cells compared to A549 tumour cells.

Figure 31 shows genome replication and hexon gene expression (mRNA levels) for EnAd, NG-347, and NG-348 in purified human T-cell cultures.

Figure 32 shows CD80 and anti-CD3 scFv transgene mRNA and protein expression (flow cytometry) for virus NG-348 in human T-cells compared to A549 tumour cells

Figure 33 shows CD80 transgene mRNA and CD80 transgene protein for virus NG-347 in purified human T-cells compared to A549 tumour cells

Figure 34 shows IFNα and MIP1α transgene mRNA generated by virus NG-347 in T cells compared to A549 tumour cells

Figure 35 shows NG-347 and NG-348 genome replication and hexon gene expression by human PBMCs compared to A549 tumour cells

Figure 36 shows CD80 and anti-CD3 scFv mRNA generated by virus NG-348 by PBMCs compared to A549 tumour cells

Figure 37 shows CD80, IFNα and MIP1α mRNA generated by virus NG-347 by PBMCs compared to A549 tumour cells

Figure 38 shows the similar activation of human dendritic cells by EnAd, NG-347 and NG-348 virus particles, as measured by down-regulation of CD14 expression and upregulation of CD80 on the cell surface

Figure 39 shows similar particle-mediated MIP1α and IFNα protein secretion from PBMCs cultured with NG-348 (A) or NG-347 (B) compared to EnAd.

Figure 40 shows NG-347 or NG-348 genome replication in co-cultures or T-cells or PBMCs with MRC-5 fibroblast cells compared to co-cultures with A549 tumour cells

Figure 41 shows INFγ secreted by PBMCs or T-cells co-cultured with MRC-5 fibroblast cells compared to A549 tumour cells, and treated with EnAd or virus NG-348.

Figure 42 shows MIP1α and IFNα secreted by human dendritic cells treated with EnAd, NG-347 or NG-348 virus particles

Figure 43 shows NFκB and IFN reporter gene activation in JurkatDual reporter T-cells co-cultured with EnAd, NG-347 or NG-348 infected A549 tumour cells

Figure 44 shows NF-κB-luciferase reporter activity generated by JurkatDual reporter T-cells co-cultured with EnAd, NG-347, NG-348 or NG-420 treated A549 HCT-116, DLD and HT29 tumour cells

Figure 45 shows NF-κB-luciferase reporter activity generated by JurkatDual cells co-cultured with either A549 or HT29 tumour cells infected with virus NG-348 and virus NG-420 as a function of virus particles added

Figure 46 shows the pharmacokinetics of EnAd and virus NG-348 in blood; blood cytokine levels after exposure to EnAd or virus NG-348; tissue biodistribution of EnAd or NG-348 viruses 6 or 24 hours after IV administration to CD1 mice

Figure 47 shows the pharmacokinetics in blood of EnAd, NG-347 and NG-348 viruses following IV administration to CB17-SCID mice bearing a subcutaenous HCT-116 tumour xenograft.

Figure 48 shows the tissue distribution of EnAd, NG-347 and NG-348 viruses 6 hours post intravenous dosing in tumour-bearing CB17-SCID mice, and virus genomes in HCT-116 tumour xenografts at day 7 and day 14-21 following intravenous or intra-tumoral dosing of EnAd, NG-347 and NG-348

Figure 49 shows virus hexon mRNA generated in HCT-116 tumour xenografts by EnAd, NG-347 or NG-348 viruses on day 7 or 14-21 following intravenous or intra-tumoral dosing

Figure 50 shows mRNA levels for hexon and CD80 transgene in HCT-116 tumour xenografts 7 or 21 days following intravenous dosing with virus NG-348

Figure 51 shows mRNA levels for a transgenes encoding anti-CD3 ScFv and CD80 in HCT-116 tumour xenografts 7 or 14-21 days following IV dosing with virus NG-348

Figure 52 shows mRNA levels of MIP1α and IFNα transgenes in HCT-116 tumour xenografts 7 or 14-21 days following intravenous dosing with virus NG-347

Figure 53 shows CD80 protein expression in HCT-116 tumour xenografts 7 and 21 days following an intravenous dose of virus NG-348; and shows MIP1α and

CD80 protein expression in HCT-116 tumours following an intravenous dose of virus NG-347.

SUMMARY OF THE SEQUENCE LISTING

SEQ ID NO: 1	shows BγDNA sequence corresponding to and including bp 29345-29379 of the EnAd genome.
SEQ ID NO: 2	PDGF TM domain
SEQ ID NO: 3	SPLICE ACCEPTOR SEQUENCE
SEQ ID NO: 4	SPLICE ACCEPTOR SEQUENCE
SEQ ID NO: 5	poly adenylation sequence (SV40 late polyA sequence)
SEQ ID NO: 6	Internal Ribosome Entry Sequence (IRES)
SEQ ID NO: 7	High efficiency self-cleavable P2A peptide sequence
SEQ ID NO: 8	High efficiency self-cleavable F2A peptide sequence
SEQ ID NO: 9	High efficiency self-cleavable E2A peptide sequence
SEQ ID NO: 10	High efficiency self-cleavable T2A peptide sequence
SEQ ID NO: 11	Human CD80 amino acid sequence
SEQ ID NO: 12	Human Interferona amino acid sequence
SEQ ID NO: 13	Human soluble Flt3 ligand amino acid sequence
SEQ ID NO: 14	Human Macrophage Inflammatory protein 1a amino acid sequence (LD78b isoform)
SEQ ID NO: 15	Membrane anchored form of the anti-human CD3 single chain Fv
SEQ ID NO: 16	NG-330 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes the T lymphocyte activation antigen, CD80, inserted in the region Bγ. The transgene cassette contains a 5' SSA, human CD80 cDNA sequence and a 3' poly(A)
SEQ ID NO: 17	NG-343 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes IFNa, and CD80, inserted in the region Bγ. The transgene cassette contains a 5' SSA, IFNα cDNA sequence, P2A peptide, CD80 cDNA sequence and a 3' poly(A)
SEQ ID NO: 18	NG-345 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes Flt3 Ligand, MIP1a and IFNα, inserted in the region Bγ. The transgene cassette contains a 5' SSA, Flt3 Ligand cDNA , P2A peptide sequence, MIP1a cDNA sequence
SEQ ID NO: 19	NG-346 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes Flt3 Ligand, MIP1a and CD80,

	inserted in the region B_Y. The transgene cassette contains a 5' SSA, Flt3 Ligand cDNA sequence, P2A peptide sequence, MIP1a cDNA
SEQ ID NO: 20	NG-347 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes IFNα, MIP1α and CD80, inserted in the region B_Y. The transgene cassette contains a 5' SSA, IFNα cDNA sequence, P2A peptide sequence, MIP1α cDNA sequence, T2A
SEQ ID NO: 21	EnAd Genome
SEQ ID NO: 22	E2B region of EnAd genome (BP 10355-5068)
SEQ ID NO: 23	E3 REGION FROM EnAd
SEQ ID NO: 24	A non-coding sequence for inclusion into B_X
SEQ ID NO: 25	A non-coding sequence for inclusion into B_Y
SEQ ID NO: 26-34	Hinge linker sequences
SEQ ID NO: 35-74	Flexible linker sequence
SEQ ID NO: 75 & 76	Rigid linker sequence
SEQ ID NO: 77-90	Linker sequence
SEQ ID NO: 91	PDGFR receptor A
SEQ ID NO: 92	PDGFR receptor B
SEQ ID NO: 93	Insulin like growth factor 1
SEQ ID NO: 94	IL6-R
SEQ ID NO: 95	CD28
SEQ ID NO: 96	NG-348 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes a membrane-anchored chimeric form of the single chain Fv anti-human CD3e and the T lymphocyte activation antigen, CD80 inserted in the region B_Y.
SEQ ID NO: 97	Nucleic acid encoding membrane tethered OKT3-scFv
SEQ ID NO: 98	Transgene Cassette sequence for NG-348
SEQ ID NO: 99	Membrane anchored form of the anti-human CD3 scFv with C-terminal V5 tag
SEQ ID NO: 100	V5 tag (9 amino acid variant)
SEQ ID NO: 101	NG-348A virus genome sequence comprising the EnAd genome with a transgene cassette that encodes a membrane-anchored chimeric form of the single chain Fv anti-human CD3e with C-terminal V5 tag and the T lymphocyte activation antigen, CD80 inserted in the region
SEQ ID NO: 102	NG-420 virus genome sequence comprising the EnAd genome with a transgene cassette that encodes a membrane-anchored chimeric

	form of the single chain Fv anti-human CD3e inserted in the region BY. The transgene cassette contains a 5' SSA
SEQ ID NO: 103	NG-420A virus genome sequence comprising the EnAd genome with a transgene cassette that encodes a membrane-anchored chimeric form of the single chain Fv anti-human CD3e and a C-terminal V5 tag, inserted in the region BY. The transgene cassette contains a
SEQ ID NO: 104	Linker
SEQ ID NO: 105	Sequence comprising a start codon
SEQ ID NO: 106	c-myc tag
SEQ ID NO: 107	c-myc tag with amino acid spacer at the N and C-terminal
SEQ ID NO: 108	spacer - c-myc tag -spacer PDGF TM domain
SEQ ID NO: 109	Fully synthetic EnAd genome with incorporated cloning site for transgene cassette insertion as in plasmid pEnAd2.4

DETAILED DESCRIPTION OF THE DISCLOSURE

B7 is a family of proteins.

A B7 protein encoded in an oncolytic viruses of the present disclosure can be useful because the extracellular domain of the protein family member generally modulates a biological function, for example the B7-1 extracellular domain may be employed to prime or stimulate T cells. The actual biological function is specific to the extracellular domain of each given B7 protein (i.e. generally different proteins members of the B7 family have different functions). Other functions of B7 proteins, such as B7-1 and/or B7-2 may include the ability to bind CD28 and/or CTLA-4, and in particular to signal or activate the relevant signaling cascade or cascades.

In addition or alternatively the transmembrane domain of the B7 proteins can be employed to direct proteins encoded by a virus of the present disclosure to the surface of a cancer cell, for example by fusing the transmembrane domain to the C-terminus of the relevant protein.

B7 protein as employed herein, unless the context indicates otherwise, refers to the full length sequence of a protein from the B7 family or a sequence at least 95% similar or identical thereto (such as 96%, 97%, 98%, 99% or 100% similar or identical thereto along the entirety of the relevant sequence). The B7 family includes B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6, B7-H7. When the full length protein is employed then at least one normal biological function of the protein will generally be present.

Full length, protein as employed in respect of the B7 family, refers to at least the extracellular domain, including chimeric B7 proteins wherein the sequence of the chimaera has the structure and a function of a B7 protein and wherein the sequences that make up the chimaera are selected from proteins in the B7 family. The elements in a

fragment or full length B7 protein may be from the same or different B7 proteins. Thus in one embodiment the B7 fragment or protein is chimeric.

The chimeric B7 proteins as employed herein refer to where substantially all the sequences making up the chimaera are from a B7 protein, for example at least 98% of the sequence of the chimaera is fragments of B7 proteins fused together. Thus a chimeric fragment as employed herein refers a fragment comprising a sequence from two or more different B7 proteins.

In one embodiment the full length B7 protein comprises the extracellular domain, for example from a single B7 protein, such as B7-1 and/or B7-2.

In one embodiment the full length B7 protein comprises the extracellular domain and the transmembrane domain, for example from the same B7 protein or alternatively the extracellular domain from a B7 protein (such as B7-1 and/or B7-2) and a transmembrane domain or equivalent, such as lipid membrane anchor, from a completely different protein.

In one embodiment a full length chimeric B7 protein may comprise an extracellular domain of one B7 protein (such as B7-1 and/or B7-2) and the transmembrane from a different B7 protein.

In one embodiment the full length B7 protein comprises the extracellular domain, the transmembrane domain and intracellular domain, for example all from the same B7 protein or from two or more different B7 proteins.

Active fragment of a B7 protein as employed herein refers to a fragment that has at least one function of a B7 protein, for example to facilitate expression on the cancer cell surface or other biological function of a B7 protein.

In one embodiment the fragment has at least 50% of the activity of the full-length protein, such as 60, 65, 70, 75, 80, 85, 90, 95 or 100% of the activity of the full-length protein.

In one embodiment the active fragment comprises or consists of a B7 extracellular domain or a sequence at least 95% similar or identical thereto, such as 96, 97, 98, 99 or 100% similar or identical.

In one embodiment the B7 fragment comprises or consists of a transmembrane domain from a B7 protein in particular one described herein, such as B7-1. Employing the latter is thought to contribute expression on the cell surface.

In one embodiment the active B7 fragment may be part of an extracellular domain.

An active fragment, for example a transmembrane fragment or a larger fragment comprising more B7 domains may be employed in a fusion protein with an additional protein, for example to facilitate expression of the additional protein on the cancer cell surface.

Larger fragment as employed herein does not refer to size or weight *per se* but to a larger repertoire of sequence information (i.e. the fragment comprises sequences from at least two B7 domains) which in turn may provide more functionality.

In one embodiment the larger fragment comprises some biological activity of the relevant B7 protein. In one embodiment an active B7 fragment is a fragment that retains

the essential biological activity of the full-length protein, for example the ability to prime or activate T cells.

The activity of a given protein fragment may be analysed in a relevant *in vitro* assay, for example using full-length protein as a comparator, for example employing an assay described in the Examples herein. Where the active fragment is a transmembrane domain the activity can be assessed by analysing the surface expression on cells of the relevant protein to which the transmembrane domain is attached, for example using an assay described in the Examples herein.

When the full-length B7 protein is part of a fusion protein then the B7 portion may be linked to the additional protein by an amide bond between the end of one sequence and the beginning of the next protein sequence or connected by a linker. Examples of linkers are given below.

A full length B7 protein comprising a transmembrane domain can be employed to present the extracellular domain of the B7 protein and the protein or fragment fused or linked thereto on the surface of the infected cancer cell. Generally in this embodiment the B7 protein will be attached to the surface of the cancer cell and the "other" protein will be at the N-terminus and on the extracellular side of the cancer cell surface.

Having said that the proteins can be arrange as desired, for example with the B7 extracellular domain at the N-terminal, fused or linked at its C-terminal to the next protein or fragment, which in turn is fused or linked at the C-terminal to the transmembrane domain, for example a transmembrane domain from a B7 protein.

Generally when a full-length B7 protein is employed in a fusion protein then both the B7 protein and the additional protein will have a biological function.

Fusion protein as employed herein refers to at least two proteins or fragments or a combination of at least one protein and at least one fragment fused directly or connected to each other, for example by a linker.

Fused as employed herein generally refers to an amide bond between the end of one polypeptide (or protein/fragment) and the beginning of the next polypeptide (or protein/fragment).

Linked, unless the context indicates otherwise, refers to wherein two entities, such as two polypeptide sequences are connected via a linker. A linker is a sequence which is not naturally present in either polypeptide or a sequence, which is not present in that particular position relative to both polypeptides.

In one embodiment the fusion protein comprises a B7 protein or an active fragment thereof. Fusion proteins comprising B7 fragments or protein and additional proteins are not referred to as chimeric proteins herein. Generally fusion protein as employed herein refers to a combination of a B7 protein or fragment thereof and another non-B7-protein/fragment.

Only proteins containing fragments from different B7 proteins are referred to as chimeric herein, as described *supra*.

In one embodiment fusion proteins of the present disclosure do not comprise a B7 protein or active fragment thereof and are encoded by a virus of the present disclosure in addition to the B7 protein or fragment thereof.

Thus viruses of the present disclosure may encode entities in addition to the B7 protein or active fragment thereof, such entities include further proteins.

B7 Family

In one embodiment the B7 is independently selected from B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6, B7-H7, active fragments of the same, and combinations thereof. In one embodiment the B7 protein is B7-1 (CD80), B7-2 (CD86) or an active fragment of any of the same and combinations thereof, in particular B7-1 or an active fragment thereof.

B7 proteins include B7-1(also known as CD80 uniprot number P33681), B7-2 (also known as CD86 uniprot number P42081). These proteins bind CD28 and CTLA-4.

In one embodiment CD80 has the following sequence:
MGHTRRQGTSPSKCPYLNFFQLLVLAGLSHFCSGVIHVTKEVKEVATLSCGHNVSVEELAQTRIY
WQKEKKMVLTMMSGDMNIWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVLKYEKDAFKRE
HLAEVTLSVKADFPTPSISDFEIPTSNIRRIICSTSGGFPEPHLSWLENGEELNAINTTVSQDPETE
LYAVSSKLDFNMTTNHSFMCLIKYGHLRVNQTFNWNTTKQEHFPDNLLPSWAITLISVNGIFVIC
CLTYCFAPRCRERRRNERLRRESVRPV **SEQ ID NO: 11**

Other B7 proteins include B7-DC (also known as PDCD1LG2 and PD-L2 uniprot number Q9BQ51), B7-H1 (also known as PD-L1 and CD274: Uniprot number Q9NZQ7). Both these proteins bind PD-1.

Programmed death-ligand 1 (PD-L1) is a 40kDa type 1 transmembrane protein that has been speculated to play a major role in suppressing the immune system. It appears that upregulation of PD-L1 may allow cancers to evade the host immune system. An analysis of 196 tumor specimens from patients with renal cell carcinoma found that high tumor expression of PD-L1 was associated with increased tumor aggressiveness and a 4.5-fold increased risk of death. Ovarian cancer patients with higher expression of PD-L1 had a significantly poorer prognosis than those with lower expression. PD-L1 expression correlated inversely with intraepithelial CD8+ T-lymphocyte count, suggesting that PD-L1 on tumor cells may suppress antitumor CD8+ T cells. The effect might be tumor type dependent; a study on patients with non-small cell lung cancer showed that greater PD-L1 protein and mRNA expression is associated with increased local lymphocytic infiltrate and longer survival. A number of anti-PDL1 antibodies have been shown to be of interest for treating several cancers in clinical trials.

In one embodiment the B7-DC and/or B7-H1 protein or fragment thereof employed in the virus of the present disclosure does not stimulate immune suppression, for example is mutated to remove the immune suppressive function.

Alternatively, a virus encoding B7-H1 extracellular domain in an unmutated form may be employed to treat appropriate cancers, where upregulation of PD-L1 is associated with a good/improved prognosis, such as lung cancer.

In one embodiment at least the cytoplasmic (intracellular domain) of B7-DC and/or B7-H1 is deleted or non-functional. Whilst not wishing to be bound by theory there is evidence to suggest that removal of the intracellular domain reduces the cancer cells resistance to lysis Blood 2008, April 1; 111(7) 3635-3643.

In one embodiment only the transmembrane domain fragment of B7-DC and/or B7-H1 is employed. In one embodiment the following proteins are not provided as full-length proteins B7-DC and B7-H1 with a relevant biological activity.

Other B7 proteins include B7-H2 (also known as ICOSLG, B7RP1, CD275: Uniprot number O75144) which binds ICOS, B7-H3 (also known as CD276: Uniprot number Q5ZPR3), B7-H4 (also known as VTCN1: Uniprot number Q727D3), B7-H5 (also known as VISTA, Platelet receptor Gi24, SISP1), B7-H6 (also known as NCR3LG1, NR3L1) which binds NKp30, B7-H7 (also known as HHLA2) which binds CD28H.

In one embodiment the fragment only comprises the transmembrane domain of any one B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7.

Individual proteins include single proteins, that is proteins or active fragments thereof that are not part of a fusion protein (including chimeric proteins), and also fusion proteins. In one embodiment the individual proteins are single proteins (including active fragments thereof).

In one embodiment the cytoplasmic domain of the B7 protein is present. In one embodiment the cytoplasmic domain is absent. The absence of the cytoplasmic domain may reduce or eliminate intracellular signaling to the cancer cell, which is relevant to one or more embodiments discussed below.

"Transmembrane Domains"

In one embodiment a transmembrane domain other than one derived from a B7 protein is employed to express a protein (including a fusion protein) encoded by a virus of the present disclosure on the surface of an infected cancer cell, for example the transmembrane domain can be employed to present an active B7 protein fragment or another protein of interest on the surface of the infected cancer cell. Alternatively it can be employed to present a fusion protein, for example comprising a B7 protein or active fragment thereof on said surface. In one embodiment the transmembrane domain from a PDGF receptor or fragment thereof is employed to express a B7 and/or another protein on the cancer cell surface.

In one embodiment a transmembrane tether or anchor sequence employed in the present disclosure comprises a PDGFR TM domain (e.g.ala513-arg561), such as AVGQDTQEVIVVPHSLPFKVVVISAILALVVLTIISLIILIMLWQKPR (**SEQ ID NO: 2**).

In one embodiment a tether or anchor sequence employed in the present disclosure comprises a tag attached, for example to a PDGF receptor or fragment thereof, such as PDGFR TM domain, in particular SEQ ID NO: 2.

Suitable tags include His-tags, Flag-tags, c-myc tag and the like. More specifically the tether or anchor may comprise a c-myc tag eg. of SEQ ID NO: 106 EQKLISEEDL

followed by a PDGFR TM domain is employed, (for example ala513-arg561), such as shown in SEQ ID NO: 2 AVGQDTQEVIVVPHSLPFKVVVISAILALVVLTIISLIILIMLWQKKPR.

In one embodiment the c-myc tag comprises a spacer or spacer amino acids at the 3' and/or 5' end, for example gsEQKLISEEDLn (**SEQ ID NO: 107** wherein the lower case letters represent the amino acids which are added to the tag as spacers).

In one embodiment the tether or anchor sequence employed is gsEQKLISEEDLnAVGQDTQEVIVVPHSLPFKVVVISAILALVVLTIISLIILIMLWQKKPR (**SEQ ID NO: 108**) wherein the lower case letter represent amino acid spacers).

Generally the protein/polypeptide to which the tether or anchor is attached does not comprise a stop codon.

An exogenous protein or proteins encoded by the virus according to the present disclosure will generally comprise a leader sequence (also referred to as a signal peptide). A leader sequence is, for example a sequence about 5 to 30 amino acids long located at the N-terminal of the protein or polypeptide.

In one embodiment the leader sequence for the protein to be expressed on the cancer cell surface is human, for example HuVHSS.

In one embodiment the structure of the ORF cassette is as follows:

LS-POLY-TAG-TM_D

wherein

LS is a leader sequence, for example a human leader sequence;

POLY is a polynucleotide encoding polypeptide or proteins of interest, in particular one disclosed herein;

TAG is a tag for example one disclosed herein, such as c-myc, in particular SEQ ID NO: 100 or 106;

TM_D is a TM domain for example a PDGFR TM domain, for example SEQ ID NO: 2.

When the polypeptide is a scFv then the ORF may be as follows:

LS-VAR_1-LINK-VAR_2-TAG-TM_D

wherein

LS is a leader sequence, for example a human leader sequence;

VAR_1 is a polynucleotide encoding a variable region such as VH region;

LINK is a linker, for example as disclosed herein, such as a linker based on the units of G_4S, in particular **SEQ ID NO: 104** GGGGSGGGGSGGGGS;

VAR_2 is a polynucleotide encoding a variable region, such as a VL region;

TAG is a tag, for example one disclosed herein, such as c-myc, in particular **SEQ ID NO: 100** or **106**;

TM_D is a TM domain for example a PDGFR TM domain, for example **SEQ ID NO: 2**.

The disclosure also extends to embodiments, in particular those described specifically herein, which comprise a tag at the N- or C-termini of the polypeptide chains, such that it resides inside or on the outside of the membrane. Thus a C-termini tag located inside the membrane is advantageous because it is not likely to interfere with the binding or function of the polypeptide.

Having said this expressing the tag on the N-terminal of a surface expressed protein may be useful in some situations because may facilitate isolation, identification and purification of cells expressing the protein.

In one embodiment a combination of a transmembrane domain and a secretory signal sequence is employed to express a protein encoded by the virus (for example as described herein) on the surface of an infected cancer cell. The present inventors have shown that the proteins encoded are expressed only on cells which are permissive to infection by the oncolytic virus, i.e. cancer cells.

In one embodiment the fragment employed to express the protein on the surface of the infected cancer cell (such as the transmembrane fragment) is selected from about 20 to 25 hydrophobic amino acids which form a transmembrane alpha helix, for example from the proteins including PDGF receptor, insulin-like growth factor receptor, IL-6 receptor, CD28, glycophorin, LDL receptor, influenza HA protein, insulin receptor, Asialoglycoprotein receptor, Transferrin receptor.

In one embodiment the fragment employed to express the protein on the surface of the infected cancer cell (such as the transmembrane fragment) is selected from the group comprising TM domain sequences (minimal portions) given in **SEQ ID NO: 91, 92, 93, 94** or **95**:

SEQ ID NO:	**Name**	**SEQUENCE**
91	PDGFR Receptor A	AVLVLLVIVIISLIVLVVIW
92	PDGFR Receptor B	VVISAILALVVLTIISLIILI
93	INSULIN-LIKE GROWTH FACTOR 1	IIIGPPLIFVFLFSVVIGSIYLFL
94	IL6-R	SSSVPLPTFLVAGGSLAFGTLLCIAIVL
95	CD28	FWVLVVVGGVLACYSLLVTVAFIIFWV

In one embodiment the transmembrane domain employed is derived from a G protein-coupled receptor or S antigen from hepatitis B.

In one embodiment a fusion protein comprising a full length extracellular domain of a B7 protein or fragment and also a transmembrane domain derived from a protein other than B7 is arranged such that the B7 protein is located at the terminal end of the fusion protein distal from the cancer cell surface, that is on the outside of the cancer cell facing the extracellular space.

VIRUSES

Having the DNA sequence encoding a B7 protein or an active fragment under the control of an endogenous promoter is also advantageous because the protein is expressed in accordance with the virus life cycle as opposed to being constitutively expressed. In the present situation continuous expression under an exogenous promoter, for example a

strong promoter like the CMV promoter, may produce more B7 protein than is necessary for a therapeutic effect and may result in off-target effects.

Alternatives to transmembrane domains for expressing proteins on the surface of the infected cancer cell include approaches employing glycophospholipid anchor (also referred to as a GPI anchor) attached to the C-terminal amino acid of the extracellular protein or fragment (Low *et al* 1986, Cross 1987, Low and Saltiel 1988, Ferguson and William 1988). Suitable glycophospholipid anchors, for use in the present disclosure include those from Thy-1, N-CAM and DAF.

In one embodiment oncolytic virus according to present disclosure is an adenovirus, for example a group B adenovirus. In one embodiment the virus according to the present disclosure is a chimeric virus, for example EnAd. In one embodiment the adenovirus is replication competent.

In one embodiment the virus is replication deficient and provided as a viral vector.

In one embodiment the sequence encoding the B7 protein or active fragment thereof is located between the stop codon and polyA recognition site of the adenoviral gene L5 and the stop codon and polyA recognition site of the gene E4.

In one embodiment the sequence encoding the B7 protein or active fragment thereof is located between about bp 29356 and about 29357 of the EnAd genome, for example as shown in SEQ ID NO: 21, or a position equivalent thereto. The skilled person will understand that the absolute numerical value of the location can change based on how the numbering is allocated. However, the relative position of the inserted gene remains the same irrespective of the absolute numerical values employed.

In one embodiment the oncolytic adenovirus according to the present disclosure has a formula **(I)**:

$$5'ITR\text{-}B_1\text{-}B_A\text{-}B_2\text{-}B_X\text{-}B_B\text{-}B_Y\text{-}B_3\text{-}3'ITR \qquad \textbf{(I)}$$

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 a transgene encoding a B7 protein or an active fragment thereof; and

B_3 is a bond or comprises E4.

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

$$5'ITR\text{-}B_1\text{-}B_A\text{-}B_2\text{-}B_B\text{-}B_Y\text{-}B_3\text{-}3'ITR \qquad \textbf{(Ia)}$$

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 a transgene encoding a B7 protein or an active fragment thereof; 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 encoding the B7 protein or active fragment thereof, is under the control of an endogenous promoter, for example the major late promoter.

Regulatory Elements

In one embodiment B_Y comprises a transgene cassette, said cassette comprising a transgene encoding a B7 protein or fragment thereof 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.

Proteins Encoded By the Virus

In one embodiment the virus of the present disclosure encodes multiple proteins for expression on the surface of the infected cancer cell wherein at least one is a B7 protein or an active fragment thereof, for example two, three, four or more different

proteins are encoded, in particular two or three proteins are encoded by the virus for expression on the cancer cell surface or secretion into the extracellular space. Protein in this context includes a fusion protein. In one embodiment the virus of the present disclosure encodes two different B7 proteins, active fragments thereof or combinations of the same, for example both for expression on a cancer cell surface.

In one embodiment the virus according to the present disclosure encodes one or two protein for cell surface expression and one or two proteins which are not capable of being anchored on the cell surface, for example are intended to act with the cancer cell or are for secretion/release from the cells.

In one embodiment a B7 protein or active fragment is encoded by the virus of the present disclosure for expression on the surface of the cancer cell and a soluble form, which is released or secreted from the cell, of the same B7 protein or a different B7 protein (including active fragments) is also encoded by the virus.

In one embodiment at least two different B7 proteins or active fragments are encoded by a virus of the present disclosure.

In one embodiment at least one protein expressed on the cell surface is a B7 protein and at least one non-cell-anchored (e.g .secreted) proteins is a non-B7 protein.

In one embodiment the multiple proteins may be encoded to be expressed as separate proteins which are independently processed and expressed in the cancer cell membrane. The independence of the proteins on the surface of the cancer cell may make a positive contribution to the immune activation. Whilst not wishing to be bound by theory, lipid packing can influence the fluidity (i.e. the viscosity) of the lipid bilayer in the membrane of the cancer cell. Viscosity of the membrane can affect the rotation and orientation of proteins and other bio-molecules within the membrane, thereby affecting the functions of these molecules. Thus when the proteins encoded by the virus are located as individual and separate proteins within the membrane of the infected cancer cell, the fluidity of the lipid bilayer allows independent movement of the molecules which may be a particularly suitable format, for example similar to a natural format that is conducive to biological function.

In one embodiment the independently processed and expressed proteins are located (anchored) in different locations, such as physically separate locations, in the cancer cell membrane.

In one embodiment one or more proteins (for example all the proteins) encoded by the virus and expressed on the surface of the infected cancer cell are not fusion proteins.

As described *supra* in some embodiment the proteins are expressed as a fusion protein.

In one embodiment the virus of the present disclosure provides one or more separate independent proteins for cell surface expression and one or more fusion proteins for cell surface expression.

Thus in one embodiment the virus according to the present disclosure comprises DNA sequences encoding said multiple proteins for expression, for example on the surface or the infected cancer cell.

Thus in one embodiment the virus according to the present disclosure comprises two or more transgenes, in the same or different locations in the virus genome. When located at the same position in the virus genome the multiple proteins will still be expressed independently at the surface of the cancer cell.

In one embodiment the multiple proteins (including fusion proteins) are encoded in different locations in the virus genome, for example in E3, B_X and/or B_Y and are expressed separately on the surface of the infected cancer cell.

In one embodiment the multiple proteins (including fusion proteins) are encoded in the same location in the virus genome and expressed together on the infected cancer cell surface, for example where the proteins encoded are provided as a fusion protein, in particular wherein the fusion protein comprises a B7 protein or an active fragment thereof.

In one embodiment the B7 protein in the fusion protein is a full length protein, in particular a protein described herein, such as B7-1 and/or B7-2, fused or linked to another protein of interest or an active fragment thereof. In one embodiment, the fusion protein comprises a transmembrane from a B7 protein. In one embodiment the B7 is an active fragment excluding the transmembrane domain. In the latter embodiment a transmembrane other than one derived from a B7 protein may be employed to ensure the fusion protein is presented on the surface of the infected cancer cell.

In one embodiment the multiple proteins are encoded in the same location in the virus and are expressed as one or more fusion proteins together on the surface of the infected cancer cell.

When the location of the gene(s) encoding a protein or protein(s) of interest in the virus is the same then the genes may, for example be linked by an IRES sequence or a 2A peptide.

In one embodiment the virus according to the present disclosure comprises a “second” transgene and optionally a third transgene (i.e. one or more of said multiple proteins, for example encoding a polypeptide selected from the group comprising a cytokine, a chemokine, a ligand, and an antibody molecule, such as an antagonistic antibody molecule, and an agonistic antibody molecule.

In one embodiment the additional protein or proteins is/are independently selected from the group comprising an antibody, antibody fragment or protein ligand that binds CD3, CD28, CD80, CD86, 4-1BB, GITR, OX40, CD27, CD40 and combinations, for example in forms suitable for expression on the surface of a cancer cell.

In one embodiment the additional protein is an anti-CD3 antibody, for example independently selected from a Muromonab-CD3 (also known as OKT3), otelixizumab (also known as TRX4), teplizumab (also known as hOKT3γ1(Ala-Ala)), or visilizumab.

In one embodiment the anti-CD3 antibody is in the form of an antibody fragment, for example an scFv that is part of a fusion protein with the transmembrane region of another protein, for example the transmembrane domain from the PDGF receptor or from the cell surface form of IgG

In one embodiment an antibody molecule is an inhibitor (antagonistic antibody) is independently selected from the group comprising an inhibitor of an angiogenesis factor, such as an anti-VEGF antibody molecule, and inhibitor of T cell deactivation factors, such as an anti-CTLA-4, anti-PD1 or anti-PDL1 antibody molecule. In one embodiment antibody molecule is an agonist independently selected from the group comprising antibodies to CD40, GITR, OX40, CD27 and 4-1BB.

In one embodiment an additional transgene encodes a cytokine, or soluble variant thereof selected from the group comprising IL-2, IFNα, IFNβ, IFNγ, GM-CSF, IL-15, IL-12 and fms-related tyrosine kinase 3 ligand (FLT3L). Advantageously, one or more of this group of proteins expressed by the virus, in particular as a free protein secreted from the cancer cell, may be particularly suitable for stimulating an immune response *in vivo* to the cancer cell.

In one embodiment an additional transgene encodes a chemokine, selected from the group comprising MIP1-alpha, IL-8, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19 and CCL21. Advantageously, one or more of this group of proteins is expressed by the virus as a free protein which may be secreted from the cancer cell may be particularly suitable for attracting immune cells and stimulating an immune response to the cancer cell *in vivo.*

In one embodiment in addition to at least the B7 protein or active fragment thereof expressed on the surface of the infected cancer cell, one or more molecules are also expressed on the surface and/or secreted.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an anti-CD3 (agonist) antibody or antibody binding fragment (such as a scFv) also for expression on the cancer cell surface, in particular where the proteins are expressed as individual proteins on the cell surface.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an anti-VEGF (antagonist) antibody or a binding fragment thereof also for expression on the cancer cell surface or for release from the cancer cell, for example by secretion or after lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an antibody, antibody fragment or protein ligand that binds CD3, CD28, CD80, CD86, 4-1BB, GITR, OX40, CD27, CD40 also for expression on the cancer cell surface or for release from the cancer cell, for example by secretion or release after lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and a cytokine selected from IL-2, IFN-alpha, IFN-beta, IFN-gamma, GM-CSF,

IL-15, IL-12, and FLT3L, for example for release from the cancer cell, in particular by secretion or release after cell lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and a chemokine selected from MIP1-alpha, IL-8, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19, CCL21, for example for release from the cancer cell, in particular by secretion or release after cell lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an anti-CD3 (agonist) antibody or antibody binding fragment (such as a scFv) also for expression on the cancer cell surface (in particular where the proteins are expressed as individual proteins on the cell surface) and further encodes a cytokine or chemokine selected from IL-2, IFN-alpha, IFN-gamma, GM-CSF, IL-15, IL-12, FLT3L, MIP1-alpha, IL-8, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19, CCL21 for example for release from the cancer cell, in particular by secretion or after cell lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an anti-CD3 (agonist) antibody or antibody fragment (such as a scFv) also for expression on the cancer cell surface (in particular where the proteins are expressed as individual proteins on the cell surface) and further encodes an antibody, antibody fragment or protein ligand that binds CD28, CD80, CD86, 4-1BB, GITR, OX40, CD27, CD40 or an anti-VEGF (antagonist) antibody also for expression on the cancer cell surface or for release from the cancer cell, for example by secretion or release after lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and two different cytokines or chemokines selected from IL-2, IFNα, IFNβ, IFNγ, GM-CSF, IL-15, and IL-12, FLT3L, MIP1α, IL-8, CCL5, CCL17, CCL20, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, CXCL12, CCL2, CCL19, CCL21, for example for release from the cancer cell, in particular by secretion of after cell lysis/death of the infected cancer cell.

Thus in one embodiment the virus encodes B7-1, B7-2 or an active fragment of any one of the same or a combination thereof for expression on the surface of the infected cancer cell and an anti-CD3 (agonist) antibody or antibody binding fragment (such as a scFv) also for expression on the cancer cell surface (in particular where the proteins are expressed as individual proteins on the cell surface) and further encodes a cytokine independently selected from IL-2, IFNα, IFNγ, GM-CSF, IL-15, and IL-12, and or a chemokine selected from RANTES (CCL5), MIP1α (LD78α (CCL3) or LD78β (CCL3L1) isoforms), MIP1β which can be released from the cancer cell, in particular by secretion before and release after cell lysis/death of the infected cancer cell.

In one embodiment which in particular may be combined with any of the embodiments above the virus further encodes an anti-PD-1 antibody (an antagonist).

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 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, either 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 (other than the gene encoding the B7 protein or fragment thereof) is under the control of an exogenous or endogenous promoter, for example an endogenous promoter. In one embodiment a transgene in the E3 region (B_2) 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.

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 B7 protein or active fragment thereof encoded in the region B_Y.

In one embodiment there is provided a composition comprising an oncolytic adenovirus according to the present disclosure, for example a pharmaceutical composition, in particular comprising a pharmaceutically acceptable excipient, such as a diluent or carrier.

In one embodiment there is provided an oncolytic adenovirus according to the present disclosure or a composition comprising the same, for use in treatment, in particular for use in the treatment of cancer.

In one embodiment there is provided a method of treating a patient in need thereof comprising administering a therapeutically effective amount of an oncolytic virus according to the present disclosure or a composition, such as a pharmaceutical composition comprising the same.

In one embodiment there is provided use of an oncolytic adenovirus according to the present disclosure or a composition comprising the same for the manufacture of a medicament for the treatment of cancer, in particular carcinomas, for example colorectal, lung, bladder, renal, pancreatic, hepatic, head and neck, breast or ovarian cancer.

In one embodiment there is provided a polynucleotide comprising a genomic sequence of at least 50% of a virus according to the present disclosure (for example 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) and comprising a sequence encoding a B7 protein or an active fragment thereof, for example a B7 protein disclosed herein, such as B7-1 or an active fragment thereof. In one embodiment the polynucleotide sequence is in the form of a plasmid.

In one embodiment there is provided a host cell, for example a mammalian cell, such as a HEK293 cell or a derivative thereof, comprising an oncolytic virus according to the present disclosure or a polynucleotide sequence according to the present disclosure.

In one embodiment there is provided a process for preparing an oncolytic adenovirus according to the present disclosure comprising a step of inserting a polynucleotide encoding B7 protein or an active fragment thereof into an oncolytic adenovirus.

In one embodiment there is provided a process of replicating a virus according to the present disclosure comprising the step of culture host cells in the presence of the virus under conditions suitable for replication. Generally the method will comprise a further step of harvesting the virus, for example from the supernatant or after lysis of the host cells.

Definitions

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 disregulated, 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.

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.

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 and 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 proteins 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 environment.

Thus in one embodiment the transgene inserted encodes a human or humanised protein, polypeptide or peptide.

In one embodiment the transgene comprises a DNA sequence encoding a B7 protein or an active fragment thereof. The present disclosure provides that the B7 protein or activate fragment thereof may be provided in one or more formats independently selected from a fusion protein, a simple B7 protein or an active fragment thereof.

Simple B7 protein or an active fragment thereof as employed herein refers to proteins which are essentially wild-type proteins, for example which are not part of a fusion protein and which has a sequence identical or similar to the relevant known protein, in particular the known human protein. Simple gene also includes wherein 10% of the amino acids are substituted or deleted over the whole length of the relevant protein.

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 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 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 off and replaced by the GPI-anchor. As the protein progresses through the secretory pathway, 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 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 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.

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, 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 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 **Table 1.**

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-
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 disclsoure does not comprise an adeno death protein (ADP).

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

In one embodiment the virus of the present disclosure does not comprise more and a fragment of part of an Ad5 virus.

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. 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 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*.

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 to express proteins on the surface of cancer cells without having to directly inject the transgenes into the tumour, which is not feasible for many cancers.

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 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 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 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. 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.

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 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 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).

Antibody as employed herein, unless the context indicated otherwise refers to a full length antibody.

Antibody binding fragments refers to a fragment comprising a binding domains which, such as a VH and/or VL which retains specificity for the target antigen to which it binds and for example 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 epitope-binding fragments of any of the same.

Linkers

Linkers suitable for use in fusion proteins of the present disclosure include:

Table 2. Hinge linker sequences

SEQ ID NO:	SEQUENCE
26	DKTHTCAA
27	DKTHTCPPCPA
28	DKTHTCPPCPATCPPCPA
29	DKTHTCPPCPATCPPCPATCPPCPA
30	DKTHTCPPCPAGKPTLYNSLVMSDTAGTCY
31	DKTHTCPPCPAGKPTHVNVSVVMAEVDGTCY
32	DKTHTCCVECPPCPA
33	DKTHTCPRCPEPKSCDTPPPCPRCPA
34	DKTHTCPSCPA

Table 3. Flexible linker sequences

5

SEQ ID NO:	SEQUENCE
35	SGGGGSE
36	DKTHTS
37	(S)GGGGS
38	(S)GGGGSGGGGS
39	(S)GGGGSGGGGSGGGGS
40	(S)GGGGSGGGGSGGGGSGGGGS
41	(S)GGGGSGGGGSGGGGSGGGGSGGGGS
42	AAAGSG-GASAS
43	AAAGSG-XGGGS-GASAS
44	AAAGSG-XGGGSXGGGS –GASAS
45	AAAGSG- XGGGSXGGGSXGGGS –GASAS
46	AAAGSG- XGGGSXGGGSXGGGSXGGGS-GASAS
47	AAAGSG-XS-GASAS
48	PGGNRGTTTTRRPATTTGSSPGPTQSHY
49	ATTTGSSPGPT
50	ATTTGS
-	GS
51	EPSGPISTINSPPSKESHKSP
52	GTVAAPSVFIFPPSD
53	GGGGIAPSMVGGGGS
54	GGGGKVEGAGGGGGS
55	GGGGSMKSHDGGGGS
56	GGGGNLITIVGGGGS
57	GGGGVVPSLPGGGGS
58	GGEKSIPGGGGS

59	RPLSYRPPFPFGFPSVRP
60	YPRSIYIRRRHPSPSLTT
61	TPSHLSHILPSFGLPTFN
62	RPVSPFTFPRLSNSWLPA
63	SPAAHFPRSIPRPGPIRT
64	APGPSAPSHRSLPSRAFG
65	PRNSIHFLHPLLVAPLGA
66	MPSLSGVLQVRYLSPPDL
67	SPQYPSPLTLTLPPHPSL
68	NPSLNPPSYLHRAPSRIS
69	LPWRTSLLPSLPLRRRP
70	PPLFAKGPVGLLSRSFPP
71	VPPAPVVSLRSAHARPPY
72	LRPTPPRVRSYTCCPTP-
73	PNVAHVLPLLTVPWDNLR
74	CNPLLPLCARSPAVRTFP

(S) is optional in sequences 37 to 41,

Examples of rigid linkers include the peptide sequences GAPAPAAPAPA (SEQ ID NO: 75), PPPP (SEQ ID NO: 76) and PPP.

Other linkers are shown in **Table 4:**

SEQ ID NO:	**SEQUENCE**
77	DLCLRDWGCLW
78	DICLPRWGCLW
79	MEDICLPRWGCLWGD
80	QRLMEDICLPRWGCLWEDDE
81	QGLIGDICLPRWGCLWGRSV
82	QGLIGDICLPRWGCLWGRSVK
83	EDICLPRWGCLWEDD
84	RLMEDICLPRWGCLWEDD
85	MEDICLPRWGCLWEDD
86	MEDICLPRWGCLWED
87	RLMEDICLARWGCLWEDD
88	EVRSFCTRWPAEKSCKPLRG
89	RAPESFVCYWETICFERSEQ
90	EMCYFPGICWM

Definitions Relevant to Formula (I) and (Ia)

A bond refers to a covalent bond connecting the 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.

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 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 may be modified to omit 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the genetic material. 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.

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 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 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 restriction site and/or 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.

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.

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 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 SEQ ID NO: 21.

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 from about 32189bp to 32326bp of SEQ ID NO: 21 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: 21.

B1 as employed herein refers to the DNA sequence encoding: part or all of an E1A 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 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 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 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(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: 21.

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 BA 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: 21 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: 22 (which corresponds to SEQ ID NO: 3 disclosed in WO2005/118825).
In one embodiment B_A has the sequence from 3933bp to 27184bp of SEQ ID NO: 21.

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. In one embodiment the E3 region has a sequence shown in SEQ ID NO: 23.

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.

In one embodiment B_2 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.

In one embodiment the B_2 region comprises the sequence from 27185bp to 28165bp of SEQ ID NO: 24.

In one embodiment B_2 consists of the sequence from 27185bp to 28165bp of SEQ ID NO: 24.

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 between the BX 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.

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.

In one embodiment B_X comprises the sequence of SEQ ID NO: 24. 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.

The insert(s) can occur anywhere within SEQ ID NO: 24 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, 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: 24 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: 24.

In one embodiment B_X has the sequence from 28166bp to 28366bp of SEQ ID NO: 21. 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: 21.

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 an 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: 25. 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: 22from 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: 25 with a DNA sequence inserted between positions bp 12 and 13 or a place corresponding to 29356bp and 29357bp in SEQ ID NO: 21. 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: 21. In one embodiment B_Y is a bond.

In one embodiment the insert is after bp 12 in SEQ ID NO: 25.

In one embodiment the insert is at about position 29356bp of SEQ ID NO: 21.

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: 21.

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

In one embodiment B_3 is a bond, i.e. wherein E4 is absent.

In one embodiment B_3 has the sequence consisting of from 32188bp to 29380bp of SEQ ID NO: 21.

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: 24 or SEQ ID NO: 25. An insert can be either a restriction site insert, a transgene cassette or both. 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.

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: 3 or 4. In one embodiment the SSA has the nucleotide sequence of SEQ ID NO: CAGG. In one embodiment the SA has the nucleotide sequence of SEQ ID NO: 23. 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: tgctaatctt cctttctctc ttcagg (SEQ ID NO: 4), tttctctctt cagg (SEQ ID NO: 3), 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: 105] the start of the start of the coding sequences is indicated by the bases in bold. A lower case letter denotes common bases at this position (which can nevertheless vary) and upper case 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 of SEQ ID NO: 6. 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: 7. In one embodiment the encoded F2A peptide employed has the amino acid sequence of SEQ ID NO: 8. In one embodiment the encoded E2A peptide employed has the amino acid sequence of SEQ ID NO: 9. In one embodiment the encoded T2A peptide employed has the amino acid sequence of SEQ ID NO: 10.

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: 5. 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: 5**.

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.

In one embodiment there is provided a virus or construct with a sequence disclosed herein, for example a virus selected NG-330 (**SEQ ID NO: 16**); NG-334 (**SEQ ID NO: 17**); NG-345 (**SEQ ID NO: 18**); NG-346 (**SEQ ID NO: 19**); NG-347 (**SEQ ID NO: 20**) and NG-348 (**SEQ ID NO: 96**).

In one embodiment the virus is NG-347 (**SEQ ID NO: 20**) or NG-348 (**SEQ ID NO: 96**).

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 $1x10^{10}$ to $1x10^{14}$ 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.

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

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 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, 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, 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 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 to the present disclosure, for example $1x10^{10}$ to $1x10^{14}$ viral particles per dose, such as $1 x10^{10}$ to $1x10^{12}$ viral particles per dose. In one embodiment the concentration of virus in the formulation is in the range $2x10^{8}$ to $2x10^{14}$ vp/ml, such as $2 x 10^{12}$ vp/ml.

In one embodiment the parenteral formulation comprises glycerol.

In one embodiment the formulation comprises oncolytic adenovirus as described 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.

In one embodiment 0.7 mL of virus of the disclosure at a concentration of $2 x 10^{12}$ 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.

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.

These inhalable powders may include monosaccharides (e.g. glucose or arabinose), disaccharides (e.g. lactose, saccharose, maltose), oligo- and polysaccharides (e.g. dextranes), 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.

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.

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.

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.

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 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

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.

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 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 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.

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.

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.

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.

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 thera

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

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.

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 the therapy, such as a surgery (neoadjuvant therapy), to shrink the tumour, 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), 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.

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.

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 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.

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 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 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 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 $1x10^{10}$ to $1x10^{14}$ 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 $2x10^{10}$ particles per minute to $2x10^{12}$ 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, for example as per SEQ ID NO: 109, which was employed with transgene cassettes in SEQ ID Nos: 18, 20, 96, 101, 102, 103.

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.

Heading 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 invention is further described by way of illustration only in the following examples.

EXAMPLES

Example 1: Production of EnAd virus expressing the T cell activating antigen CD80

The plasmid pEnAd2.4 was used to generate the plasmid pNG-330 by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO: 11). The pNG-330 cassette contains a 5' short splice acceptor sequence CAGG, human CD80 cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the inserted transgene cassette is shown in Figure 3A. Construction of the plasmid was confirmed by DNA sequencing.

Virus Production and characterisation

References herein to viruses such as NG-330-00 are simply references to particular batched "00" of the virus NG-330. Similar nomenclature may be used for other viruses.

The plasmid pNG-330 was linearised by restriction digest with the enzyme AscI to produce the virus genome NG-330 (SEQ ID NO: 16). Digested DNA was purified by phenol/chloroform extraction and precipitated for 16hrs, -20^{0}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 to a T-25 flask containing 293 cells grown to 70% confluency. After incubation of the cells with the transfection mix for 2hrs at 37^0C, 5% CO_2 4mls of cell media (DMEM high glucose with glutamine supplemented with 2% FBS) was added to the cells and the flasks was incubated 37^0C, 5% CO_2.

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-330 virus stock.

Example 2: Characterisation of NG-330 virus activity compared to EnAd in carcinoma cell lines

NG-330 or EnAd virus replication (assessed by qPCR), and CD80 membrane expression (assessed by flow cytometry (Figures 4 and 5) was compared in the colon carcinoma cell line HT-29 and lung carcinoma cell line A549. NG-330 is a virus derived from EnAd that contains a transgene cassette encoding the human T cell activating antigen, CD80 after the EnAd late gene, L5 (Fibre). A schematic of the inserted cassette is shown in Figure 3A. Production of NG-330 virus is detailed in Example 1. A549 or HT-29 carcinoma cell lines were seeded in 6 well plates at cell densities of 7.5e5 cells/well for A549 cells or 2.e6 cells/well for HT-29 cells. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with, 100 EnAd or NG-330 virus particles per cell (ppc) or were left uninfected. Assays were carried out 24, 48 or 72 hrs post infection.

Virus Replication assessed by qPCR

HT-29 and A549 cells lines either infected for 24, 48 or 72 hrs with 100ppc EnAd or NG-330 or left uninfected were used for quantification of viral DNA by qPCR. Cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. DNA was extracted from 10µl 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 EnAd E3 gene specific primer-probe set.

Quantification of the number of detected virus genomes per cell demonstrated that NG-330 and EnAd virus replication was comparable in both HT-29 (Figure 4A) and A549

cell lines (Figure 4B). No virus genomes could be detected in uninfected cells (data not shown).

CD80 cell surface expression assessed by Flow Cytometry

HT-29 and A549 cells lines either infected for 24, 48 or 72 hrs with 100ppc EnAd or NG-330 or left uninfected were used for analysis of CD80 transgene expression on the cell surface. The tumour cells were removed from the plate surface by treatment with trypsin, centrifuged and then resuspended in 1% BSA/PBS. Samples were then either incubated at 5^0C for 1hr with buffer, mouse isotype control antibody conjugated to Cy5 or anti-human CD80 antibody conjugated to Cy5 (clone 2D10). All samples were also co-stained with Zombie Aqua live/dead to differentiate viable cells. Samples were washed 3 times with 1% BSA/PBS before analysis by flow cytometry (FACS, Attune) for cell viability and CD80 protein expression on the cell surface. Analysis showed that CD80 could be detected at the cell surface in both A549 (Figure 5A) or HT-29 (Figure 5B) cells treated with NG-330 but not those treated with EnAd or left untreated.

Comparison of 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, 37^0C, 5% CO_2, before cells were either infected with EnAd or NG-330 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 NG-330 oncolytic potency was comparable to EnAd in HT29 cells (Figure 6).

Example 3: Production of EnAd viruses expressing the T cell activating antigen CD80 and the cytokine IFNα

The plasmid pEnAd2.4 was used to generate the plasmid pNG-343 by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO 11) and the human cytokine interferon α (IFNα, SEQ ID NO: 12). The pNG-343 cassette contains; a 5' short splice acceptor sequence CAGG; human IFNα cDNA; a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human CD80 cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the inserted transgene cassette is shown in Figure 3B. Construction of the plasmid was confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-343 was linearised by restriction digest with the enzyme AscI to produce the virus genome NG-343 (SEQ ID NO: 17)). The virus NG-343 is amplified and purified according to methods detailed in Example 1.

Example 4: Production of EnAd viruses expressing the extracellular domain of FMS-Like tyrosine kinase-3 ligand, the chemokine MIP1α and the cytokine IFNα

The plasmid pEnAd2.4 is used to generate the plasmid pNG-345 by direct insertion of a cassette encoding a soluble variant of the FMS-like tyrosine kinase-3 ligand (Flt3L, SEQ ID

NO: 13), MIP1α (isoform LD78β, SEQ ID NO: 14) and IFNα (SEQ ID NO: 12). The pNG-345 cassette contains; a 5' short splice acceptor sequence CAGG; human Flt3L cDNA; a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human MIP1α cDNA; a high efficiency self-cleavable T2A peptide sequence (SEQ ID NO: 10); human IFNα cDNA and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the inserted transgene cassette is shown in Figure 3D. Construction of the plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-345 is linearised by restriction digest with the enzyme AscI to produce the virus genome NG-345 (SEQ ID NO: 18)). The virus NG-345 is amplified and purified according to methods detailed in Example 1.

Example 5: Production of EnAd viruses expressing the T cell activating antigen CD80, the chemokine MIP1α and Flt3 Ligand

The plasmid pEnAd2.4 was used to generate the plasmids pNG-346 by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO 11), the human macrophage Inflammatory Protein 1α (MIP1α, SEQ ID NO. 14) and the human Flt3 Ligand (SEQ ID NO: 13). The pNG-346 cassette contains; a 5' short splice acceptor sequence CAGG; human IFNα cDNA; a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human MIP1α cDNA (isoform LD78β); a high efficiency self-cleavable T2A peptide sequence (SEQ ID NO: 10); human Flt3 Ligand cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A schematic of the inserted transgene cassette is shown in Figure 3E. Construction of the plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-346 is linearised by restriction digest with the enzyme AscI to produce the virus genome NG-346 (SEQ ID NO: 19). The virus NG-346 is amplified and purified according to methods detailed in Example 1

Example 6: Production of EnAd viruses expressing the T cell activating antigen CD80, the chemokine MIP1α and the cytokine IFNα

The plasmid pEnAd2.4 was used to generate the plasmids pNG-347 by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO: 11), the human macrophage Inflammatory Protein 1α (MIP1α, SEQ ID NO. 14) and the human cytokine interferon α (IFNα, SEQ ID NO: 12). The pNG-347 cassette contains; a 5' short splice acceptor sequence CAGG; human IFNα cDNA; a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human MIP1α cDNA (isoform LD78β); a high efficiency self-cleavable T2A peptide sequence (SEQ ID NO: 10); human CD80 cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the inserted transgene cassette is shown in Figure 3F. Construction of the plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-347 is linearised by restriction digest with the enzyme AscI to produce the virus genome NG-347 (SEQ ID NO: 20). The virus NG-347 is amplified and purified according to methods detailed in Example 1.

Example 7: Production of EnAd viruses expressing the T cell activating antigen CD80 and a membrane-anchored single chain Fv fragment antibody to the ε chain of the human CD3 complex (CD3ε)

The plasmid pEnAd2.4 was used to generate the plasmids pNG-348 by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO: 11) and a membrane-anchored chimeric form of the single chain Fv anti-human CD3e (SEQ ID NO: 15). The pNG-348 cassette contains; a 5' short splice acceptor sequence CAGG; membrane-anchored anti-human CD3e scFv cDNA; a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human CD80 cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the inserted transgene cassette is shown in Figure 3C. Construction of the plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-348 is linearised by restriction digest with the enzyme AscI to produce the virus genome NG-348 (SEQ ID NO: 96). The virus NG-348 is amplified and purified according to methods detailed in Example 1.

Example 8: Activity of EnAd virus, NG-343, expressing two transgenes; the T cell activating antigen CD80 and the cytokine IFNα

Characterisation of NG-343 virus activity compared to EnAd in carcinoma cell lines

NG-343 or EnAd virus replication (assessed by qPCR), CD80 transgene expression (assessed by flow cytometry) or IFNα transgene expression (assessed by ELISA) was compared in the colon carcinoma cell line, HT-29 or the lung carcinoma cell line, A549. NG-343 is a virus derived from EnAd that contains a transgene cassette encoding the human T cell activating antigen, CD80 as well as the human cytokine Interferon alpha 2b located after the EnAd late gene, L5 (Fibre). A schematic of the inserted cassette is shown in Figure 3B. Production of NG-343 virus is detailed in Example 3. A549 or HT-29 carcinoma cell lines were seeded in 12 well plates at cell densities of $7.5x10^5$ cells/well for A549 cells or $1.4x10^6$ cells/well for HT-29 cells. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with EnAd or NG-343 at 100 virus particles per cell (ppc) or were left uninfected. Assays were carried out 24, 48 or 72 hrs post infection.

Virus Replication assessed by qPCR

HT-29 cells infected for 24, 48 or 72 hrs with 100ppc EnAd or NG-343 or left uninfected were used for quantification of viral DNA by qPCR. Cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. DNA was extracted from 10µl of supernatant using the Sigma Genelute DNA extraction Kit, according to the manufacturer's protocol. A standard curve using EnAd virus particles ($2.5x10^{10}$ to $2.5\ x10^5$ vp) was also

prepared and extracted using the Sigma Genelute Kit. Each extracted sample or standard was analysed by qPCR using an EnAd E3 gene specific primer-probe set.

Quantification of the number of detected virus genomes per cell demonstrated that NG-343 and EnAd virus replication was comparable at all time points analysed (Figure 7A). No virus genomes could be detected in uninfected cells (data not shown).

Analysis of IFNα expression by ELISA

Supernatants of HT-29 or A549 cell lines infected for 24, 48 or 72 hrs with 10ppc of EnAd or NG-343 or left uninfected were analysed for expression of secreted IFNα by ELISA.

Culture supernatants were removed from each well and centrifuged for 5 mins, 1200rpm to remove cell debris. Supernatants were diluted into 5% BSA/PBS assay buffer (1:2 or 1:50 or 1:100) and ELISA was carried out using the Verikine Human IFN alpha Kit (Pbl assay science) according to the manufacturer's protocol.

The concentrations of secreted IFNα were determined by interpolating from the standard curves. IFNα expression which increased in the cellular supernatants over the course of infection was detected in both HT-29 and A549 cells lines (Figure 7B)

CD80 cell surface expression assessed by Flow Cytometry

A549 cells lines infected for 48 or 72 hrs with 10ppc EnAd or NG-343 or left uninfected were used for analysis of CD80 transgene expression on the cell surface. At the appropriate time point post-infection A549 cells were removed from the plate surface by treatment with trypsin, centrifuged and then resuspended in 1% BSA/PBS. Samples were then either incubated at 5^0C for 1hr with buffer, mouse isotype control antibody conjugated to Cy5 or anti-human CD80 antibody conjugated to Cy5 (clone 2D10). All samples were also co-stained with Zombie Aqua live/dead to differentiate viable cells. Samples were washed 3 times with 1% BSA/PBS before analysis by flow cytometry (FACS, Attune) for cell viability and CD80 protein expression on the cell surface. Analysis of CD80 expression vs Live/dead staining showed that at both 48 and 72hrs post infection CD80 could be detected at the cell surface of NG-343 treated cells but not EnAd or uninfected control (UIC) cells (Figure 8). Cell viability at 72hrs post virus treatment was not sufficient to carry out comprehensive CD80 expression analysis, however high levels of CD80 could be detected on both live and dying cells treated with NG-343 at this time point (Figure 8D, lower panel).

CD80 protein expression was then compared in HT-29 and A549 cells at 48hrs post-infection with 100ppc. Samples were harvested and stained as above before analysis of cell viability and CD80 protein expression on the cell surface. Analysis of CD80 expression at this time point on only cells stained as live cells showed CD80 could be detected on the surface of ~91% of NG-343 treated HT-29 cells and ~98% of NG-343 treated A549 cells but not on EnAd treated controls.

Example 9: Selectivity of NG-343 virus activity and transgene expression in carcinoma, stromal fibroblast and epithelial cell lines.

To show that the IFNα and CD80 transgenes encoded in the NG-343 virus are selectively expressed only in cells permissive to NG-343 or EnAd infection, virus replication (assessed by qPCR), IFNα expression (assessed by ELISA) and CD80 expression (assessed by flow cytometry) were measured in cancer cells (HT-29) known to be permissive to EnAd infection, fibroblast cell lines (WI-38 and MRC-5) previously characterised to be non-permissive and a bronchial epithelial cell line (BE) which shows only limited permissivity to EnAd infection. Briefly, cells were seeded in 12 well plates and infected 18hrs post-seeding with 100ppc NG-343 or EnAd virus for WI38, MRC5 or BE cells or 10ppc NG-343 or EnAd virus for HT-29 cells. Cells were incubated with virus particles for 4 hrs before the infection media was removed from the cells and replaced with fresh culture media. At 1hr or 72hrs post the 4hr infection period, cell supernatants were harvested for qPCR or ELISA analysis and the cells were treated with trypsin to remove them from the plates for analysis by flow cytometry.

NG-343 and EnAd selective virus replication

For qPCR, cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. DNA was extracted from 10µl of supernatant using the Sigma Genelute DNA extraction Kit, according to the manufacturer's protocol. A standard curve using EnAd virus particles ($2.5x10^{10}$ to $2.5x10^{5}$ vp) was also prepared and extracted using the Sigma Genelute Kit. Each extracted sample or standard was analysed by qPCR using an EnAd E3 gene specific primer-probe set.

Quantification of the number of detected virus genomes per cell demonstrated that NG-343 and EnAd virus replication was comparable in all cell lines analysed (Figure 9A).

NG-343 selective transgene expression

For detection of IFNα expression, cell supernatants were collected and clarified by centrifuging for 5 mins, 1200rpm. Supernatants were diluted into 5% BSA/PBS assay buffer (1:2 or 1:50 or 1:100) and ELISA was carried out using the Verikine Human IFN alpha Kit (Pbl assay science) according to the manufacturer's protocol.

The concentrations of secreted IFNα were determined by interpolating from the standard curve. IFNα expression could only be detected in the supernatants of NG-343 infected HT-29 cells and was not detectable (less than the lower limit of quantitation [<LLOQ]) in either of the fibroblast cell lines, or the bronchial epithelial cell line (Figure 9B).

For CD80 cell surface expression, cells were then either incubated at $5^{0}C$ for 1hr with buffer, mouse isotype control antibody conjugated to Cy5 or anti-human CD80 antibody conjugated to Cy5 (clone 2D10). All samples were also co-stained with Zombie Aqua live/dead to differentiate viable cells. Samples were washed 3 times with 1% BSA/PBS before analysis by flow cytometry (FACS, Attune) for cell viability and CD80 protein expression on the cell surface. In keeping with the IFNα expression data, CD80 expression could only be detected on HT-29 cells, with no detectable expression on either the fibroblast or bronchial epithelial cell lines (Figure 9C).

Taken together these data demonstrated that both IFNα and CD80 transgenes are selectively expressed in cells permissive to EnAd virus infection i.e. carcinoma cells, and the encoding of transgenes does not alter the selectivity of the NG-343 virus when compared to the parental EnAd virus.

Example 10: Activity of NG-343 transgene expression on immune cell activation

To determine if treatment of tumour cells with NG-343 virus could lead to enhanced innate immune cell responses compared to no treatment or to EnAd treatment, freshly isolated peripheral blood mononuclear cells (PBMCs) were co-cultured with tumour cells either infected with NG-343 or EnAd or left uninfected. Immune cell activation was assessed by flow cytometry analysis of innate immune cell populations or ELISA analysis of co-culture supernatants. Briefly, A549 lung carcinoma cells were seeded in 12 well plates at a density of $4x10^5$ cells/well. After 20 hrs cells were infected with 10ppc of EnAd or NG-343 virus or left uninfected and then incubated for 24hrs, 37 degrees, 5% CO_2. PBMCs isolated from a healthy human donor by density gradient centrifugation were then added to the A549 culture wells at a ratio of 5 PBMCs to 1 A549 cell. At 48 hrs post addition of PBMCs co-culture supernatants were harvested from the plates. To analyse dendritic cell activation at this point, cells were incubated at 5^0C for 1hr with buffer, mouse isotype control antibodies conjugated to Alexa Fluor 488, PE, PerCP/Cy5.5, BV605 or BV412, anti-CD14 antibody conjugated to Alexa Fluor 488, anti-CD80 antibody conjugated to PE, anti-HLA-DR conjugated to PerCP.Cy5.5, anti-CD3 conjugated to BV605 or anti-PD-L1 antibody conjugated to BV421. All samples were also co-stained with Zombie Aqua live/dead to differentiate viable cells. Samples were washed 3 times with 1% BSA/PBS before analysis by flow cytometry (FACS, Attune). Viable cells that stained negative for both CD14 and CD3 but positive for HLA-DR were defined as the dendritic cell population. Expression of the DC activation marker, CD80 and PD-L1 was compared on this population (Figure 10). These analyses revealed that tumour cells infected with NG-343 could induce increased surface levels of both CD80 and PD-L1 on the surface of DCs when compared to EnAd infected or uninfected tumour cell culture.

Example 11: Activity of EnAd virus, NG-347, expressing three transgenes; the T cell activating antigen CD80, the chemokine MIP1α and the cytokine IFNα

Characterisation of NG-347 virus activity compared to EnAd in carcinoma cell lines

CD80 transgene expression (assessed by flow cytometry) and IFNα or MIP1α (CCL3) transgene expression (assessed by ELISA) was compared in NG-347 and EnAd treated colon carcinoma cell line, HT-29 or lung carcinoma cell line, A549. NG-347 is a virus derived from EnAd that contains a transgene cassette encoding the human T cell activating antigen, CD80, the human cytokine Interferon alpha 2b and the human chemokine MIP1α (LD78β isoform). Transgene expression is under the control of the virus endogenous major late promoter. A schematic of the inserted cassette is shown in Figure 3F. Production of NG-347 virus is detailed in Example 6. A549 or HT-29

carcinoma cell lines were seeded in 12 well plates at cell densities of $7.5x10^5$ cells/well for A549 cells or $1.4x10^6$ cells/well for HT-29 cells. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with 100 EnAd or NG-347 virus particles per cell (ppc) or were left uninfected. Assays were carried out 24, 48 or 72 hrs post infection.

Analysis of IFNα or MIP1α expression by ELISA

Supernatants of HT-29 or A549 cells lines infected for 24, 48 or 72 hrs with 100ppc of EnAd or NG-347 or left uninfected were analysed for expression of secreted IFNα or secreted MIP1α by ELISA.

Culture supernatants were prepared according to the methods detailed in Example 9. IFNα ELISA was carried out using the Verikine Human IFN alpha Kit (Pbl assay science) and MIP1α ELISA was carried out using the Human CCL3 Quantikine ELISA kit (R & D systems). Both assays were carried out according to the manufacturers' protocol.

The concentrations of secreted IFNα or MIPα were determined by interpolating from the standard curves. IFNα and MIP1α expression increased in the cellular supernatants over the course of infection and was detected for both HT-29 and A549 cells lines (Figure 11A and Figure 11B).

Analysis of CD80 expression by flow cytometry

CD80 protein expression was compared on the surface of HT-29 and A549 cells at 48hrs post-infection. Cells were harvested and stained according to methods detailed in example 9. Cells were analysed for cell viability and CD80 protein expression on the cell surface by flow cytometry. Analysis of CD80 expression at this time point on live cells showed CD80 could be detected on the surface of ~96% of NG-347 treated HT-29 cells and ~99% of NG-347 treated A549 cells but no staining was detected on EnAd treated controls (Fig. 11C).

Example 12: Activity of EnAd virus, NG-345, expressing three transgenes; the cytokine Flt3 Ligand, the chemokine Mip1α and the cytokine IFNα.

Characterisation of NG-345 virus activity compared to EnAd in carcinoma cell lines

Flt3 Ligand, IFNα and MIP1α transgene expression (assessed by ELISA) was compared in NG-345 and EnAd treated colon carcinoma cell line, HT-29 or lung carcinoma cell line, A549. NG-345 is a virus derived from EnAd that contains a transgene cassette encoding a soluble variant of human Flt-3 ligand, the human cytokine Interferon alpha 2b and the human chemokine MIP1α (LD78β isoform). Transgene expression is under the control of the virus endogenous major late promoter. A schematic of the inserted cassette is shown in Figure 3D. Production of NG-345 virus is detailed in Example 4. A549 or HT-29 carcinoma cell lines were seeded in 12 well plates at cell densities of $7.5x10^5$ cells/well for A549 cells or $1.4x10^6$ cells/well for HT-29 cells. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with 100 EnAd or NG-345 virus particles per cell (ppc) or were left uninfected. Assays were carried out 24, 48 or 72 hrs post infection.

Analysis of FLt-3 Ligand, IFNα or MIP1α expression by ELISA

Supernatants of HT-29 or A549 cells lines infected for 24, 48 or 72 hrs with 100ppc of EnAd or NG-345 or left uninfected were analysed for expression of secreted Flt3-Ligand, secreted IFNα or secreted MIP1α by ELISA.

Culture supernatants were prepared according to the methods detailed in Example 9. IFNα ELISA was carried out using the Verikine Human IFN alpha Kit (Pbl assay science), MIP1α ELISA was carried out using the Human CCL3 Quantikine ELISA kit (R & D systems) and Flt3L ELISA was carried out using the Flt3L human ELISA kit (Abcam). All assays were carried out according to the manufacturers' protocol.

The concentrations of secreted IFNα, MIPα or FLt3L were determined by interpolating from the standard curves. IFNα, MIP1α and Flt3 L expression increased in the cellular supernatants over the course of infection and was detected in both HT-29 and A549 cells lines (Figure 12A - C).

Example 13. Oncolytic activity and infectivity of NG-347 and NG-348 viruses 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^{0}C, 5% CO_2, before cells were either infected with EnAd, NG-347 or NG-348 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 NG-347 and NG-348 oncolytic potency was comparable to EnAd (Figure 13A and 13B).

Viral particle infectivity

HT-29 colon carcinoma cells were seeded in 12 well plates at a cell density of 4e5 cells/well. Plates were incubated for 24 hrs, 37^{0}C, 5% CO_2, before cells were either infected with EnAd, NG-347 or NG-348 virus particles at an infection density range of 1.6e7-2e6 vp/mL. Infection of HT-29 cells was detected by antibody staining of the virus protein hexon. Stained cells were quantified by manual counting of 6 fields of view per well, across 6 replicate wells for each dilution tested. The particle to infectivity ratio (P:I) was calculated for each virus from the viral titre and demonstrated both NG-347 and NG-348 have similar infectivity ratios to EnAd reference controls (Figure 13C).

Example 14. Cell surface expression of the T cell activating antigen, CD80, in NG-347 and NG-348 infected carcinoma cell lines

CD80 transgene expression (assessed by flow cytometry) was compared in NG-347, NG-348 and EnAd treated colon carcinoma cell line, DLD-1 or lung carcinoma cell line, A549. A549 or DLD-1 carcinoma cell lines were seeded in 12 well plates at cell densities of 7.5e5 cells/well. Plates were incubated for 18 hrs, 37^{0}C, 5% CO_2, before cells were either infected with, 10 EnAd, NG-348 or NG-347 virus particles per cell (ppc) or were left uninfected. CD80 protein expression was compared on the surface of A549 or DLD-1 cells

at 24, 48, 72 or 96hrs post-infection. At each time point cells were harvested and stained according to methods detailed in example 9. Cells were analysed for cell viability and CD80 protein expression at the cell surface by flow cytometry. Analysis of CD80 expression at 72hrs post infection in A549 cells showed CD80 could be detected on the surface of >95% of NG-347 or NG-348 treated cells (Figure 14A and 14B). At 96hrs post infection the virus treatments had lysed the majority of A549 cells therefore FACs analysis was not carried out. For DLD-1 cells expression could be detected on >50% of cells by 96hrs post-treatment with NG-348 and >70% of cells following NG-347 treatment (Figure 15A and 15B). Staining was not detected on EnAd or untreated controls.

Example 15. T cell activation and degranulation mediated by NG-348 infected carcinoma cell lines

A549 lung carcinoma cells, either infected with NG-348 or EnAd virus particles or left uninfected, were co-cultured with T cells isolated from human PBMC donors. The selectivity of expression of NG-348 virus encoded CD80 was assessed on the surface of both A549 and T cells by flow cytometry. T cell activation was assessed by analysing cell surface activation markers (by Flow cytometry), CD107a cell surface expression as a marker for degranulation (by Flow cytometry) and secretion of stimulatory cytokines, IL-2 and IFNγ (by ELISA).

A549 cells were seeded into 12 well plates at a density of 5e5 cells/well. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with 10 EnAd or NG-348 virus particles per cell (ppc) or were left uninfected. At 48hrs post-infection $CD3^+$ T cells, isolated by negative selection from PBMCs (MACs) were added to the A549 cell monolayers at a ratio of 8 T cells: 1 tumour cell. The co-culture was carried out for 16hrs, after which point cellular supernatants were collected for ELISA analysis and tumour cells and T cells harvested for Flow cytometry analysis.

Culture media containing non-adherent cells was removed from co-culture wells and centrifuged (300xg). The supernatant was carefully removed, diluted 1 in 2 with PBS 5% BSA and stored for ELISA analysis. The adherent cell monolayers were washed once with PBS and then detached using trypsin. The trypsin was inactivated using complete media and the cells were added to the cell pellets that had been collected from the culture supernatants. The cells were centrifuged (300xg), the supernatant discarded and the cell pellet washed in 200µL of PBS. The cells were centrifuged again then resuspended in 50µL of FACs buffer (5% BSA PBS) containing Live/Dead Aqua (Life tech) for 15 minutes at RT. The cells were washed once in FACs buffer before staining with panels of directly conjugated antibodies: anti-CD3 conjugated to BV605; anti-CD25 conjugated to BV421; anti-CD107a conjugated to FITC; anti-EpCam conjugated to PE or anti-CD4 conjugated to PE; and either anti-CD80 conjugated to PE/Cy5 or anti-HLA-DR conjugated to PE/Cy5. 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 with FACs buffer (200µL) before resuspension in 200µL of FACs buffer and analysis by Flow cytometry (Attune).

Selective expression of CD80

Similar to results shown in example 14, CD80 expression was detectable at the surface of >80% of NG-348 infected EpCam$^+$ A549 cells but not EnAd infected or uninfected control cells (Figure 16). In contrast CD3$^+$ T cells showed no detectable expression of CD80 at the cell surface indicating, at least under these experimental conditions, transgene expression is selective for tumour cells in the co-culture.

Upregulation of T cell activation markers

Flow cytometry analysis of T cell activation was assessed by expression of the T cell activation markers CD25 and HLA-DR on live, CD3$^+$, single cells. These data showed that both the number of T cells expressing CD25 (Figure 17A and 17B) and the average level of CD25 expression on the T cell surface (Figure 17C) were significantly higher for T cells cultured with NG-348 infected A549 cells than EnAd or uninfected controls. Specifically, there was no difference in T cell activation status when comparing untreated controls to EnAd (26.9 % ± 3.4% and 25.3 ± 3.5% of T cells expressing CD25, respectively) whereas CD25 was upregulated on the majority of cells co-cultured with NG-348 (83.2 ± 1.5%). CD25 expression was also analysed on CD4 and CD8 T cell subsets by gating the CD3$^+$ T cells based on their expression of CD4. These analyses showed that CD25 expression is significantly upregulated on both CD4$^+$ and CD4$^-$ T cell subsets in NG-348 treated co-cultures compared to EnAd and uninfected controls (Figure 18).

In contrast to CD25 the number of cells expressing HLA-DR was low, <5%, for all conditions tested (Figure 19A). This is likely due to the early time point after co-culture at which flow cytometry analysis was carried out. However, there was a slight but significant increase in the mean fluorescence intensity of HLA-DR staining CD3$^+$HLA-DR$^+$ cells from NG-348 treated co-cultures compared to controls (Figure 19B).

Stimulation of T cell degranulation

Analysis of CD107a expression on the surface of live, CD3$^+$ T cells showed a significant increase in the number of T cells which had degranulated and were therefore stained with CD107a, when A549 cells were infected with NG-348 (8.3% ± 1.7% of cells) compared to either EnAd (0.6% ± 0.2% of cells) or untreated controls (0.1% ± 0.02% of cells) (Figure 20). Similar to CD25 upregulation, both CD4$^+$ and CD4$^-$ T cell subsets showed significantly increased CD107a expression compared to EnAd or A549 controls (Figure 21).

Secretion of the stimulatory cytokines IL-2 and IFNγ

For detection of IL-2 or IFNγ expression, co-culture supernatants were diluted into 5% BSA/PBS assay buffer (in a range of 1:100 to 1:1000) and ELISA was carried out using the Human IL-2 Ready Set go Kit (Affymetrix) or Human IFN gamma Ready set go kit (Affymetrix) according to the manufacturer's protocol.

The concentrations of secreted IL-2 or IFNγ were determined by interpolating from the standard curves. Expression of IL-2 could only be detected in the supernatants of co-cultures using NG-348 infected A549 cells and was not detectable in either the EnAd, or untreated controls (Figure 22A). Expression of IFNγ could also be detected, at very high levels (>300ng/mL) in supernatants of co-cultures from NG-348 infected A549 cells, which was significantly higher that either EnAd or untreated controls (Figure 22B).

Example 16. T cell activation of CD4 and CD8 T cells can be independently mediated by NG-348 infected carcinoma cell lines

A549 lung carcinoma cells infected with NG-348 or EnAd virus particles or left uninfected, were co-cultured with either $CD4^+$ T cells or $CD8^+$ T cells isolated from human PBMC donors. T cell activation was assessed by the secretion of the stimulatory cytokine IFNγ into culture supernatants.

A549 cells were seeded and infected with NG-348 or EnAd virus particles or left uninfected according to the methods detailed in Example 14. 48hrs post infection $CD4^+$ T cells or $CD8^+$ T cells isolated by negative selection from a PBMC donor were added to the A549 cell monolayer at a ratio of 8 T cells to 1 tumour cells. After 16hrs of co-culture supernatants were harvested and assessed for IFNγ according to the methods detailed in Example 14.

For $CD4^+$ T cells Expression of IFNγ was only detected in supernatants of co-cultures from NG-348 infected A549 cells and was not detectable in either the EnAd or untreated controls (Figure 23A). For $CD8^+$ T cells expression of IFNγ was detected at significantly higher levels for NG-348 infected A549 cells than for EnAd or untreated controls (Figure 23B), demonstrating that both CD8 and CD4 cells can be activated to secret IFNγ by NG-348 virus activity in tumour cell lines.

Example 17. T cell activation mediated by NG-347 infected carcinoma cell lines

A549 lung carcinoma cells, either infected with NG-347 or EnAd virus particles or left uninfected, were co-cultured with T cells isolated from human PBMC donors. T cell activation was assessed by analysing cell surface activation markers (by Flow cytometry) and secretion of the stimulatory cytokine, IFNγ (by ELISA analysis of cellular supernatants).

A549 cells were seeded into 12 well plates at a density of 5e5cells/well. Plates were incubated for 18 hrs, 37^0C, 5% CO_2, before cells were either infected with 10 EnAd or NG-347 virus particles per cell (ppc) or were left uninfected. At 24hrs post-infection $CD3^+$ T cells, isolated by negative selection from PBMCs (MACs) were added to the A549 cell monolayers at a ratio of 5 T cells: 1 tumour cell. The co-culture was carried out for 48hrs, before cellular supernatants were collected for ELISA analysis and tumour cells and T cells harvested for Flow cytometry analysis according to the methods detailed in EG 15. The harvested cells were stained with directly conjugated antibodies: anti-CD3 conjugated to BV605 and anti-CD69 conjugated to BV421. 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 with FACs buffer (200µL) before resuspension in 200µL of FACs buffer and analysis by Flow cytometry (Attune).

Upregulation of T cell activation marker, CD69

Flow cytometry analysis of T cell activation was assessed by expression of the T cell activation marker CD69 on live, $CD3^+$, single cells. These data showed that the number of T cells expressing CD69 was significantly higher for T cells cultured with NG-347 infected A549 cells than EnAd or uninfected controls (Figure 24).

Secretion of the stimulatory cytokine IFNγ

For detection of IL-2 or IFNγ expression, co-culture supernatants were diluted into 5% BSA/PBS assay buffer (in a range of 1:100 to 1:1000) and ELISA was carried out using the Human IFN gamma Ready set go kit (Affymetrix) according to the manufacturer's protocol.

The concentration of secreted IFNγ was determined by interpolating from the standard curve. Expression of IFNγ could only be detected in the supernatants of co-cultures using NG-347 infected A549 cells and was not detectable in either the EnAd, or untreated controls (Figure 25).

Example 18: Production of EnAd viruses expressing the T cell activating antigen CD80 and a membrane-anchored single chain Fv fragment antibody to the ε chain of the human CD3 complex (CD3ε)

The plasmid pEnAd2.4 was used to generate the plasmids pNG-348A by direct insertion of a cassette encoding the human T cell activating antigen CD80 (SEQ ID NO 11) and a membrane-anchored chimeric form of the single chain Fv anti-human CD3e with a C-terminal V5 tag (SEQ ID NO: 99). The pNG-348 cassette contains; a 5' short splice acceptor sequence (SEQ ID NO. 2); membrane-anchored anti-human CD3e ScFv cDNA; a C-terminal V5 tag (SEQ ID NO: 100); a high efficiency self-cleavable P2A peptide sequence (SEQ ID NO: 7); human CD80 cDNA sequence and a 3' polyadenylation sequence (SEQ ID NO: 5). A Schematic of the NG-348A transgene cassettes is shown in Figure 26A. Construction of the plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmid pNG-348A is linearised by restriction digest with the enzyme AscI to produce the virus genome NG-348A (SEQ ID NO: 101). The virus NG-348A is amplified and purified according to methods detailed in Example 1.

Example 19: Production of EnAd viruses a membrane-anchored single chain Fv fragment antibody to the ε chain of the human CD3 complex (CD3ε)

The plasmid pEnAd2.4 was used to generate the plasmids pNG-420 and pNG-420A by direct insertion of a cassettes encoding a membrane-anchored chimeric form of the single chain Fv anti-human CD3e with a C-terminal V5 tag (SEQ ID NO: 99) or without a V5 tag

(SEQ ID NO: 15). The pNG-420 cassette contains; a 5' short splice acceptor sequence CAGG; membrane-anchored anti-human CD3e scFv cDNA and a 3' polyadenylation sequence (SEQ ID NO: 5). The pNG-420A cassette contains; a 5' short splice acceptor sequence cagg; membrane-anchored anti-human CD3e ScFv cDNA; a C-terminal V5 tag (SEQ ID NO: 100) and a 3' polyadenylation sequence (SEQ ID NO: 5). Schematics of the NG-420 and NG-420A transgene cassettes are shown in Figure 26B and 26C. Construction of each plasmid is confirmed by DNA sequencing.

Virus Production and characterisation

The plasmids pNG-420 and pNG-420A are linearised by restriction digest with the enzyme AscI to produce the virus genomes NG-420 (SEQ ID NO: 102) and NG-420A (SEQ ID NO: 103). The viruses NG-420 and NG-420A are amplified and purified according to methods detailed in Example 1.

Example 20

A549 human lung carcinoma cells and MRC5 human fibroblast cells were cultured with EnAd, NG-347 or NG-348 viruses (at 10ppc) to compare virus genome replication, virus hexon and transgene expression by these cell types. After 72 hours culture, cells were either stained for FACS analyses of surface markers or supernatants and cell lysates prepared for virus genome replication (qPCR) or mRNA (RT-qPCR) analyses of hexon or transgene expression.

Virus genome replication and hexon mRNA expression for the two transgene bearing viruses, NG-347 and NG-348 were equivalent to those for the parental virus, EnAd (Figure 27). For NG-348 (Figure 28), CD80 and anti-human CD3-scFv transgene mRNA expression levels were high with A549 tumour cells, with only a low level signal for the non-tumour MRC5 cells. CD80 protein expression on the surface of cells assessed by FACS was detected on the majority of NG-348 treated A549 cells but was not detectable on MRC5 cells, with no CD80 detected on either cell type left untreated or treated with EnAd. Similarly, CD80 transgene mRNA and protein expression following NG-347 treatment was selectively detected in A549 tumour cells not MRC5 cells (Figure 29).

For EnAd and NG-347 treated cell cultures, levels of MIP1α and IFNα mRNA in cell lysates and secreted proteins in supernatants were measured by RT-qPCR and specific ELISAs, respectively. Data (Figure 30) show selective expression of both transgenes by A549 tumour cells, with no detectable MIP1α chemokine or IFNα cytokine in MRC5 supernatants.

Example 21

The selectivity/activity of EnAd, NG-347 and NG-348 viruses with human T-cells was evaluated by culturing isolated $CD3^+$ T cells for 3 days with either 500 ppc or 5000 ppc of each virus. Selectivity/activity was assessed by a) flow cytometry analysis of T cells stained with antibodies targeting CD69, CD4, CD80, CD25 and CD3, b) ELISA analysis of

human MIP1α, IFNα and IFNγ protein secretion, c) qPCR analysis of virus replication and d) RT-qPCR analysis of gene expression.

As shown in Figure 31, T-cells were not supportive of virus genome replication for any of the viruses tested with only background signals in the virus hexon RT-qPCR assay. A549 tumour cells supported high levels of hexon mRNA expression. RT-qPCR analyses for transgene mRNA expression by T-cells showed only background signals (<1 copy/cell) for CD80 by both NG-347 and NG-348, and a similar lack of significant expression of anti-CD3-ScFv mRNA by NG-348, despite the high virus exposure (5000ppc). High levels of expression of both transgenes were detected with treated (10ppc) A549 tumour cells (Figures 32 & 33). Expression of IFNα and MIP1α transgene mRNA was also selectively detected by NG-347 (not EnAd) treated A549 tumour cells (at 10ppc) and not by T-cells treated with 5000ppc (Figure 34). In addition, CD80 cell surface protein expression was only detectable with A549 cells not T-cells for both NG-347 and NG-348 (Figures 32 & 33). EnAd treatment did not lead to CD80 expression by either cell type, and A549 cell death (as assessed by dye uptake) was similarly high for all three viruses; a low level of non-specific T-cell death was induced by all viruses due to the very high levels of virus particles used in the experiment (Figures 32 & 33). Similar transgene mRNA and protein expression data were obtained when viruses were used at 500ppc (data not shown).

In the absence of tumour cells, purified human T-cells were not activated to upregulate activation markers CD25 or CD69 when cultured with any of the viruses (Table 5).

Table 5. Lack of expression of activation markers CD25 and CD69 by purified human CD3+ T-cells treated with 5000 ppc of different viruses

	Untreated	**EnAd**	**NG-347**	**NG-348**
CD25+ CD4 T-cells	30.7	24.6	23.4	23.3
CD69+ CD4 T-cells	0.1	0.4	0.3	0.7
CD25+ CD8 T-cells	5.9	4.7	4.1	4.1
CD69+ CD8 T-cells	0.5	1.0	0.9	1.3

Example 22

A similar virus selectivity experiment to that described in Example 21 was carried out using unseparated human PBMCs rather than purified T-cells, including making the same activity assessments. As with human T-cells in example 21, the data from this study collectively demonstrate lack of virus replication and transgene expression by human PBMCs. Figures 35-37 show data using 5000 ppc of EnAd, NG-347 or NG-348, but similar data was generated using 500 ppc (not shown). Figure 35 shows virus genome replication and hexon mRNA expression and Figures 36 & 37 show transgene mRNA expression. Assay backgrounds were set according to signals generated in the assay with the respective virus spiked into culture media and then processed in the same way as for the cell lysate samples. There was no detectable expression of CD80 transgene on CD3+

T-cells or CD40+ cells (primarily B-cells) in these PBMC cultures with any of the viruses (not shown).

NG-347 and NG-348 virus particle-mediated activation of innate immune cells (monocytes, DCs) in the PBMC cultures were similar to those of EnAd, as shown in Figures 38 and 39 for downregulation of CD14 expression and upregulation of HLA-DR and endogenous cell surface CD80, as well as secretion of MIP1α and IFNα (note that despite NG-347 encoding both of these molecules in its genome there was no increase in production levels over those for EnAd and NG-348 which do not encode the transgenes).

Example 23

This example is similar in design to experiments in examples 15-17, 21 and 22 but in these studies, the human PBMCs or purified T-cells were co-cultured with virus pre-treated (48 hours) A549 tumour cells or MRC5 fibroblasts. A549 or MRC5 cells were treated with 10ppc of EnAd, NG-347, NG-348 or left untreated (UTC) and cultured for 48 hours to allow sufficient time for virus replication and any transgene expression. PBMCs or T-cells were then added to the cultures and left for 24 or 48 hours to evaluate the ability of virus treated cells to activate T-cells.

Figure 40 shows virus genome replication data showing comparable replication of the three viruses in PBMC or T-cell co-cultures with both cell types, replication levels being high with A549 tumour cells and low with MRC5 fibroblasts.

T-cell activation as measured by upregulation of CD25 surface expression and CD8 effector T-cell degranulation, as measured by upregulation of CD107a on the cell surface, and IFNγ production measured by intracellular cytokine staining were all selectively stimulated by NG-348 treated A549 cells compared to EnAd, with no stimulation mediated with MRC co-cultures (Table 6).

Table 6. Flow cytometry analyses of activation of human CD3+ T-cells in T-cell and PBMC co-cultures with viruses

Cells	Treatment	%CD25$^+$	%CD8$^+$CD107a$^+$	%IFNγ$^+$
A549 + T-cells	Untreated	37.5	0.1	0.1
A549 + T-cells	EnAd	38.4	0.1	0.2
A549 + T-cells	NG-348	**88.2**	**17.9**	**12.0**
MRC5 + T-cells	Untreated	38.8	0.3	0.4
MRC5 + T-cells	EnAd	38.9	0.2	0.4
MRC5 + T-cells	NG-348	39.1	0.3	0.3
A549 + PBMCs	Untreated	28.3	ND	ND
A549 + PBMCs	EnAd	29.4	ND	ND
A549 + PBMCs	NG-348	73.7	ND	ND
MRC5 + PBMCs	Untreated	23.0	ND	ND
MRC5 + PBMCs	EnAd	23.3	ND	ND
MRC5 + PBMCs	NG-348	21.7	ND	ND

ND = Not determined

IFNγ secretion into co-culture supernatants was also quantified by ELISA. The data (Figure 41) similarly demonstrate selective activation of T-cells co-cultured with NG-348 treated A549 tumour cells not MRC5 fibroblasts, with either purified T-cells or PBMCs used in the assays.

Ability of NG-347 to activate T-cells was also assessed by measuring CD69 levels on T-cells from co-cultures of either purified T-cells or PBMCs with A549 tumour cells or MRC5 fibroblasts. As shown in Table Z, a small enhancement in CD69 positive T-cells was seen with NG-347 treatment of A549 tumour cells compared to EnAd, which itself leads to upregulation of this early activation marker. These effects were not seen in MRC5 co-cultures. No CD80 expression was detected on the T-cells (not shown).

Table 7. CD69 expression on T-cells from NG-347 or EnAd treated co-cultures

Cells	Treatment	%CD69+
A549 + T-cells	Untreated	2.1
A549 + T-cells	EnAd	18.7
A549 + T-cells	NG-348	**35.0**
MRC5 + T-cells	Untreated	3.8
MRC5 + T-cells	EnAd	3.6
MRC5 + T-cells	NG-348	4.4
A549 + PBMCs	Untreated	1.2
A549 + PBMCs	EnAd	19.1
A549 + PBMCs	NG-348	**28.7**
MRC5 + PBMCs	Untreated	2.6
MRC5 + PBMCs	EnAd	2.7
MRC5 + PBMCs	NG-348	3.9

In a separate experiment, A549 cells treated with NG-347 and co-cultured with human CD3+ T-cells led to upregulation of CD69 activation marker on the T-cells and secretion of IFNγ (see Figures 24 & 25).

Example 24

CD14+ monocytic cells were isolated from PBMCs by antibody coated magnetic bead separation and cultured with human IL-4 and GM-CSF to differentiate them into dendritic cells. After 3 days of culture, the cells were treated with EnAd, NG-347 or NG-348 at 5000 ppc or left untreated. As a positive activation control, some cells were stimulated with LPS. Two days later supernatants were taken for cytokine ELISAs and cells were stained for surface activation marker expression and analysed by flow cytometry. As shown in table 8 all viruses induced upregulation of the costimulatory molecules CD80 and CD86, indicating that this previously identified particle-mediated innate immune cell activation effect was not altered by the transgene incorporation into the genomes of NG-347 and NG-348. All viruses also stimulated secretion of similar levels of MIP1α and IFNα (Figure 42).

Table 8. Particle-mediated activation of human dendritic cells by EnAd, NG-347 and NG-348

DC treatment	% CD80+	% CD86+
Untreated	3.0	10.4
EnAd	81.6	99.3
NG-347	82.1	99.4
NG-348	62.5	99.5
LPS positive control	97.5	98.5

Example 25

In a set of experiments, JurkatDual cells were used in co-cultures with tumour cells as a T-cell activation reporter assay for assessing functionality of transgene expression by NG-347, NG-348 and NG-420 viruses, with EnAd serving as a negative control. JurkatDual cells stably express two different reporter genes: an NFκB reporter gene producing a secreted form of luciferase which is responsive to signalling via the T-cell receptor complex and an IFNα-responsive secreted alkaline phosphatase (SEAP) reporter gene. A549 cells were pre-cultured with viruses at `10 ppc for two days, and then JurkatDual cells were added for overnight co-culture (18-24h) and then supernatants collected for assay of luciferase and SEAP activities. As shown in Figure 43, NG-347 infected A549 cells selectively induced SEAP production, which aligns with their production of IFNα (see Figure 11) but did not induce luciferase activity. In contrast, NG-348 which expresses the membrane anti-CD3-ScFv to activate the T-cell receptor complex induced luciferase but not SEAP.

In another experiment A549 lung carcinoma cells and HCT-116, HT-29 & DLD colon carcinoma cells were pre-cultured for 48 hours with 10 ppc of EnAd, NG-347, NG-348 or NG-420 viruses before co-culturing with JurkatDual cells overnight, with supernatants tested for levels of luciferase to indicate level of activation induced. As shown in Figure 44, all four tumour cell types cultured with NG-348 or NG-420 viruses, which encode cell surface anti-CD3-ScFv, activated the JurkatDual cells whereas EnAd and NG-347 did not, with levels of luciferase similar to that of uninfected tumour cell controls (UIC).

In another experiment, A549 or HT-29 tumour cells were pre-cultured with different amounts of either NG-348 or NG-420 before adding the JurkatDual cells and measuring their luciferase secretion. The data in Figure 45 show that activation of the NFκB activity in JurkatDual cells is dependent on the dose of virus used to treat the tumour cells with.

Example 26

The *in vivo* pharmacokinetic, biodistribution and particle-mediated systemic cytokine induction activities of EnAd and NG-348 following IV dosing in immunocompetent CD1 mice were compared, Mice were dosed intravenously with $5x10^9$ particles of either EnAd or NG-348 and bled 2, 10, 30, 60 and 120 minutes post dosing. Whole blood was DNA

extracted and analysed by qPCR for levels of virus genome (Figure 46). Clearance of both viruses from the blood followed similar kinetics. Similarly, the induction of MCP-1 cytokine response (a measure of particle-mediated activation of innate immune such as liver Kupffer cells) was also similar for both viruses, as were the tissue biodistribution patterns (Figure 46).

Example 27

CB17 SCID mice were implanted subcutaneously with HCT116 cells and injected intratumourally (IT) or intravenously (IV) with EnAd, NG-347 or NG-348 viruses ($5x10^9$ virus particles), or control, once tumours were greater than $70mm^3$. For the IV dosed mice, blood samples were taken from three mice from each group 3, 15 and 30 minutes after IV dosing, DNA extracted and the level of virus genomes in the blood assessed by qPCR (pharmacokinetics [PK] analysis). Data (Figure 47) show that NG-347 and NG-348 have similar PK to EnAd (and to each other). After 6 hours, tumours, livers, lungs and spleens were resected from 3 mice from each group. Homogenised tissues were DNA extracted and analysed for level of virus genomes by qPCR (biodistribution analysis). Data (Figure 48A) show similar tissue biodistribution for the three viruses. After 7 days or 14-21 days, tumours were excised from three mice from each group and homogenized to produce a tumour lysate which was used to prepare both DNA and RNA. Level of virus genomes in the tumours at the two time points were measured by qPCR analyses of the extracted DNA. Data (Figure 48B) show that tumours from both IV and IT dosed mice have levels of virus genomes higher than the amount of virus dosed, indicating virus replication in the tissue, with IT dosing giving higher genome levels than IV at day 7, but both being similarly high at the 14-21 day timeframe. All three viruses replicated to similar levels.

Similarly, levels of virus hexon mRNA in tumour lysates detected by RT-qPCR were comparable between EnAd, NG-347 and NG-348 at both time points tested (Figures 49 and 50). Similar levels of anti-CD3-ScFv and CD80 mRNA were detected at both time points and both dosing routes for NG-348 treatment, with only assay background readings with EnAd dosing (Figure 50 & 51). MIP1α and IFNα mRNA levels were also selectively detected following NG-347 dosing, either IT or IV (Figure 52).

Levels of CD80 protein encoded by both NG-347 and NG-348, and MIP1a protein encoded by NG-347 were measured in tumour lysates using specific ELISAs. The data in Figure 53 show that following the single IV virus dose, both proteins could also be detected selectively in tumour extracts. Neither protein was detected in blood samples from the same mice.

Example 28

To evaluate the activity and tumour cell dependency of NG-348 virus in vivo, different combination of human PBMCs ($5x10^7$ cells), A549 human tumour cells ($5x10^6$) and either EnAd or NG-348 (at $5x10^9$ ppc) were injected into the peritoneum of immune-deficient

SCID-beige mice, with viruses or control (saline) being dosed within 15 minutes after injection of the cells. After 3 days, the peritoneal cavity was lavaged with 5mL of saline and recovered cells were analysed by flow cytometric analyses with a panel of T-cell activation markers (CD25, CD69 and HLA-DR) to assess levels of T-cell activation, following gating on the CD3+ T-cell population. Data from two separate experiments (Table 9) demonstrate that NG-348 selectively leads to human T-cell activation *in vivo* in a tumour cell dependent manner.

Table 9.

***In vivo* activation of human T-cells in A549 tumour bearing mice by NG-348**

Group	Virus	Tumour	N	% $CD25^+$	% $CD69^+$	$\%DR^+$	% $CD25^+,CD69^+$	% $CD25^+,DR^+$
Experiment 1								
1	EnAd	Saline	2	1.9, 2.3	1.6, 3.0	7.7, 9.1	0.2, 0.5	0.3, 0.6
2	EnAd	$5x10^6$ A549 cells	2	4.2, 2.9	6.2, 5.5	8.4, 8.4	0.8, 0.3	1.4, 0.4
3	NG-348	Saline	1	3.4	2.6	9.2	0.5	0.8
4	NG-348	$5x10^6$ A549 cells	2	35.8, 36.6	50.4, 42.2	26.3, 19.2	22.4, 18.0	16.4, 12.2
Experiment 2								
1	Saline	Saline	1	25.6	37.3	14.8	14.1	7.08
2	EnAd	Saline	2	6.5, 7.3	17.8, 18.2	5.50, 6.1	3.58, 3.46	1.01, 1.49
3	NG-348	Saline	2	10.2, 6.5	26.7, 18.3	7.7, 6.0	6.73, 3.61	2.16, 1.44
4	Saline	$5x10^6$ A549 cells	2	28.4, 22.7	54.4, 51.1	13.3 15.0	22.3 17.5	8.54, 7.72
5	EnAd	$5x10^6$ A549 cells	1	13.2	29.4	5.1	7.84	1.62
6	NG-348	$5x10^6$ A549 cells	3	34.4, 29.6, 56.4	58.9, 59.2, 85.0	12.5 9.8, 17.0	27.2, 23.3, 52.7	9.07 7.5, 14.2

Claims

1. A replication competent oncolytic group B adenovirus enadenotucirev (EnAd) comprising:
   two or more transgenes,
   wherein a first transgene comprises a DNA sequence encoding B7-1 protein,
   wherein an additional transgene encodes an anti-CD3 antibody or binding fragment thereof, and
   wherein said transgenes are located between the stop codon and polyA recognition site of the adenoviral gene L5 and the stop codon and polyA recognition site of gene E4, under the control of a major late promoter endogenous to the adenovirus.
2. The replication competent oncolytic virus according to claim 1, wherein the virus has a formula (I):

$$5'ITR\text{-}B_1\text{-}B_A\text{-}B_2\text{-}B_X\text{-}B_B\text{-}B_Y\text{-}B_3\text{-}3'ITR \qquad (I)$$

   B_1 is a bond or comprises: 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;
   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 a transgene cassette comprising a transgene encoding a B7-1 protein , a transgene encoding said anti-CD3 antibody or binding fragment thereof, and a regulatory element or a combination of regulatory elements; and
   B_3 comprises said E4.
3. The replication competent oncolytic virus according to claim 2, wherein the regulatory element is a high efficiency self-cleavable peptide.
4. The replication competent oncolytic virus according to claim 3, wherein the high efficiency self-cleavable peptide is selected from P2A, T2A, F2A and E2A.
5. The replication competent oncolytic virus according to claim 1, wherein one or more proteins encoded by the virus are suitable for expression on the surface of an infected cancer cell, and wherein said proteins are not fusion proteins.
6. The replication competent oncolytic virus according to claim 1, wherein the virus encodes multiple proteins for surface expression on an infected cancer cell.
7. The replication competent oncolytic virus according to claim 6, wherein the proteins are independently processed and expressed in physically separate locations on the cancer cell membrane.
8. The replication competent oncolytic virus according to claim 1, wherein the antibody or binding fragment further comprises a transmembrane sequence or GPI anchor such that it is a cell membrane-anchored form.
9. The replication competent oncolytic virus according to claim 1, wherein the anti-CD3 is a scFv antibody fragment.

10. The replication competent oncolytic virus according to claim 1, wherein the B7-1 protein is a full-length protein.
11. The replication competent oncolytic virus according to claim 1, wherein the B7-1 protein comprises a transmembrane domain or GPI anchor.
12. The replication competent oncolytic virus according to claim 1, wherein the B7-1 protein comprises a transmembrane domain from a B7 protein.
13. The replication competent oncolytic virus according to claim 1, wherein the virus comprises a third transgene encoding a polypeptide selected from the group comprising a cytokine, a chemokine, a ligand, an antagonistic antibody molecule, and an agonistic antibody molecule.
14. The replication competent oncolytic virus according to claim 1, wherein the B7-1 protein is human.
15. The replication competent oncolytic virus according to claim 14, wherein the B7-1 protein has the sequence shown in SEQ ID NO: 11 or a sequence at least 95% identical thereto.
16. The replication competent oncolytic virus according to claim 1, wherein the antibody molecule or binding fragment thereof comprises an anti-human CD3 antigen binding domain.
17. The replication competent oncolytic virus according to claim 16, wherein the binding fragment has a sequence shown in SEQ ID NOs: 15 or 99.
18. The replication competent oncolytic virus according to claim 1, comprising SEQ ID NO: 96.
19. A pharmaceutical composition comprising the replication competent oncolytic virus according to claim 1, and a pharmaceutically acceptable excipient.
20. The replication competent oncolytic virus according to claim 1, wherein E4 open reading frame 4 is deleted.