

VIRUS ENCODING TRANSGENES TO COMPLEMENT CELLULAR THERAPY

The present disclosure relates to oncolytic viruses constructed encoding transgenes to specifically “paint” tissue cells with target antigen for antigen specific immune cells, formulations comprising said virus, use of said virus and formulations in therapy in particular in a combination therapy, especially a combination therapy comprising a cell therapy such as a CAR-T.

BACKGROUND

Cell-based immunotherapy, such as chimeric antigen receptor (CAR) T cell therapy has emerged as an important treatment modality for hematologic malignancies.

CAR-T cells directed against B cell antigens have been tested in the clinic often inducing long-term remissions even in heavily pre-treated patients. At the present time about 5 CAR T cell therapies are on the market for hematologic malignancies.

In contrast, CAR-T cell therapy has yet to achieve the same depth and reproducibility of response in patients with solid tumours.

CAR T cells targeting, for example B7-H3, CEACAM5, CD133, CDI 71, Claudin-6, EGFR, EGFRvIII, FRa, GD2, GPC3, HER2, IL-13Ra2, mesothelin, MUC1, PSMA, RORI, and VEGF-R2 have been tested in the clinic but demonstrated limited ability to control disease.

Chimeric Antigen Receptor (CAR) T-cells are T cells expressing a genetically modified receptor able to recognize a surface antigen on a target cell and trigger T cell activation and antigen-specific cytotoxicity. A number of alternative cellular technologies are now being developed which are also suitable for use in combination with the viruses of the present disclosure.

Several elements have been hypothesized to contribute to this underwhelming activity in solid tumours, including a) fewer solid tumour target antigens, b) heterogenous target expression amongst tumour cells, c) poor trafficking of CAR-T cells to the solid tumour and d) the solid tumour microenvironment that is hostile to the function and survival of immune cells (Hou et al. Nat Rev Drug Discov. 2021). In particular infused cell-based immune therapies disperse widely in the blood and only a small subset reaches the tumour microvasculature. Thus, inadequate CAR-T cell trafficking to solid tumours (c, above) may be particularly important.

As mentioned above target antigens expressed exclusively on tumour cells are lacking, but molecules can be designed to bind tumour cell antigens that are not fully tumour-specific (i.e. can be expressed at some level also within healthy tissues) but this can lead to off target effects.

Ambrose et al. (Ambrose, Christine et al. “Anti-CD19 CAR T cells potently redirected to kill solid tumour cells.” *PloS one* vol. 16,3 e0247701. 18 Mar. 2021, doi:10.1371/journal.pone.0247701) developed anti-CD19 CAR-T cells able to secrete an anti-HER2-CD19 bridging protein, which - by binding to HER2 positive tumour cells - allows anti-CD19 CAR-T cells to recognize both CD19 expressing and HER-2 expressing

cells. However, this secreted protein is expressed wherever CAR-T cell are found after intravenous (IV) injection, thus potentially allowing for off-tumour cytotoxicity.

It has been suggested to use a fusion protein comprising an antigen target for a CAR-T therapy and second binding domain that binds some entity in the tumour, see for example WO2018/156791. The fusion protein may, for example be expressed in the tumour and activation of the CAR-T therapy occurs on binding. However, this approach does not fundamentally address the problem of getting the immunotherapy into the tumour.

What is more, stroma around solid tumours acts like a city wall that is difficult to penetrate for immune cells and creates a microenvironment, which is a hypoxic quagmire, that stymies the cytotoxic activity of immune cells. Thus, even after successful trafficking to the local microenvironment the latter may impair the function of and/or promote clearance of cell-based immunotherapy via a variety of different soluble molecules and immunosuppressive cells (such as TGF β , adenosine, regulatory T-cells and myeloid derived suppressor cells).

Some cells engineered for use in cell-based immunotherapy have been engineered to express "exogeneous" cytokines to keep them activated. However, this can cause a problem in that if the cytokine is constitutively expressed then it can cause off-target effects (side effects). In contrast if the cytokine is expressed inducibly, for example after the cell binds its target antigen, then the cell may never reach the target because of the tumour microenvironment.

To realize successful treatment of solid tumours with CAR-T cells, it will be important to find ways to promote cell-based immunotherapy homing to the tumour and/or reprogram the tumour microenvironment, for example from an immunosuppressive to an immunostimulatory microenvironment.

SUMMARY OF THE DISCLOSURE

The present inventors have designed oncolytic viruses, which preferentially infect cancer cells and are highly adapted to survival in the tumour microenvironment. These viruses not only pioneer the route into the cancer by infecting the cancer cells, replicating and lysing cells, but in the present disclosure have also been designed to express transgenes with two independent aspects, namely:

- Express an antigen target (referred to herein as a target sequence) of the cell-based immunotherapy locally in the tumour (for example to increase the local concentration of the target antigen), and
- Promote recruitment to and/or activity of the cell-based immunotherapy from within the tumour.

Thus, the present disclosure works by increasing the local concentration of a target or activator for **antigen specific cells** (in particular engineered antigen specific cells). The virus also supports changing the tumour microenvironment to support influx and/or survival of the antigen-specific cells.

Advantageously the stimulation of antigen-specific cells minimises any off target effects and maximises the therapy by only targeting diseased tissue.

The local increase of target antigen can be increased by one or more (i.e. combinations) of the following:

- cell surface expression of target-sequence on an infected cell;
- secretion of a fusion protein from an infected cell, wherein the fusion protein binds an entity on diseased cells (infected cell and/or surrounding cells) and presents a target-sequence;

This combined approach has never been taken and the present inventors have shown it results in higher numbers of active immunotherapy cells recruited into the tumour.

To assist the recruitment of the cell-based immunotherapy to the tumour, the oncolytic virus can be armed with transgenes that re-program the tumour microenvironment, to facilitate infiltration and activity of cell-based immunotherapy.

Once the tumour is rendered more accessible by the “trafficking transgene”, higher concentrations of target antigen may act like a magnet to recruit and activate the cell-based therapy, thereby increasing the local concentration of cell-based therapy where it is needed.

The cell lysis by the oncolytic virus also results in release of proinflammatory mediators, such as HMGB1, ATP, type I interferons, which are then able to induce anti-tumour immune responses and recruit native immune cells from peripheral lymphoid organs.

Non-specific activation of immune cells may be employed in combination with the technology of the present disclosure, for example a bispecific T cell activator comprising an agonist for CD3 can be encoded in the viruses of the present disclosure.

Depending on the antigen target of the cell-based immunotherapy, for example if the target is a tumour antigen the therapeutic cells recruited to the tumour location are directly active against tumour cells that express the antigen but are not necessarily infected by the oncolytic virus. A fusion protein comprising the tumour antigen targeted by the cellular therapy and a binding domain for a second antigen for example a stromal antigen can be secreted from an infected cell. After binding stroma the tumour antigen will be presented for the cellular therapy, thereby allowing targeting of two different tissue types by a single cellular therapy. Thus, the mechanisms of activity of the virus and the cellular therapy are complementary but also independent.

Thus, in one embodiment virus infected cells secrete fusion proteins that bind an antigen on surrounding cells, such as other tumour cells and/or stroma cells (which may or may not be infected) and present a **target-sequence** for a cellular therapy to bind. Generally this binding will activate the cellular the therapy.

In one embodiment the viruses of the present disclosure encodes 1 or more, 2 or more; 3 or more, 4 or more fusion proteins with binding domains and a target-sequence for a cellular therapy.

Where there are multiple fusion proteins encoded by the virus the binding domains may bind different entities and the target-sequences may be the same, such that one cellular therapy can be employed to target two or more different cells, tissues or therapeutic targets within the cancer tissue.

In some patients different types of cancer cells co-exist and for example express different antigens. It would be useful in these patients to be able to target these different cancer cells with one cellular therapy. This can be achieved by encoding two fusions each with binding domains specific to the different cancer cells and, for example a single target-sequence (or if desired multiple target sequences).

In one embodiment the oncolytic virus encodes a transgene that is expressed, for example as a soluble protein, into the microenvironment to protect/activate the engineered immune cell and/or immune cells in general. For example, IFN α , IL-12 and IL-15 are useful cytokines to protect and/or activate immune cells in the tumour microenvironment, and chemokines such as CXCL9 and CCL21 are useful for guiding immune cells to target cells within the tumor mass.

Thus, there are at least two or three mechanisms by which the oncolytic viruses and the combination therapy of the present disclosure can improve the outcome of treatment of solid tumours.

The present inventors prepared tumour-specific group B adenoviruses, particularly EnAd, encoding a series of different bispecific proteins comprising a portion able to bind an antigen expressed on the surface of a cell, specifically a tumour cell or a tumour-associated fibroblast, and a portion that binds an immune cell, such as a CART or engineered T-cell receptor (TCR)-bearing T cell, by either engaging the engineered CAR or TCR or the endogenous receptor (such as TCR), or other surface molecules on the cell. They found that in many cases, although the tumour-cell binding portion was functional, the immune cell binding portion was dysfunctional. To solve this issue, different protein sequences and designs were screened until functional bispecific proteins were found. However, even when functional bispecific molecules were obtained, expression levels were found to be lower than what desirable.

Surprisingly, when the bispecific protein was encoded sequentially (in tandem) together with one or more distinct immunomodulatory molecules, including chemokines and cytokines, its expression level was consistently increased.

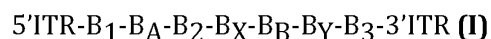
The tumour microenvironment (TME) is permissive to infiltration by adenoviruses according to the present disclosure, thereby allowing generous levels of bispecific protein to be delivered to the desired location. Additionally, simultaneous expression of bispecific proteins and immunomodulatory molecules with ability to recruit T cells or promote direct or indirect T cell activation have the potential to synergistically increase CAR-T cell ability to recognize and kill target cells in the TME.

Surprisingly, we found that addition of two or more transgenes to the same transgene cassette encoding a bispecific protein resulted in a clearly increased bispecific protein expression as compared to the corresponding virus design encoding for the bispecific protein alone.

The viruses of the present disclosure are complementary to and/or improve cellular therapy.

The disclosure is summarised in the following paragraphs:

1. An oncolytic group B adenovirus suitable for treating a solid tumour (for example sarcoma, carcinoma and/or lymphoma) comprising a sequence of formula **(I)**:



wherein:

B_1 is bond or comprises: E1A, E1B or E1A-E1B;

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

B_2 is a bond or comprises: E3;

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 is a DNA sequence encoding at least two transgenes namely a first transgene and a second transgene, for example under the control of the major late promoter; and

B_3 is a bond or comprises: E4

wherein:

- the first transgene encodes a polypeptide comprising a **target-sequence** specific for a binding domain on a cell-based immunotherapy for example bearing a (exogenous) recombinant surface expressed protein, such as a chimeric antigen receptor an NKG2D receptor, in particular wherein the target-sequence specifically binds to said surface expressed protein (more especially the chimeric antigen receptor) on the immunotherapy cell, and
 - a second transgene encodes a polypeptide comprising a molecule that promotes trafficking of the cell-based immunotherapy into and within the tumour.
2. An oncolytic group B adenovirus according to paragraph 1, expression of said first transgene increases the local concentration of the **target-sequence** within the tumour.
 3. An oncolytic group B adenovirus according to paragraph 1 or 2, wherein the **target-sequence** is specific to a recombinant receptor on the immunotherapy cell
 4. An oncolytic group B adenovirus according to any one of paragraphs 1 to 3, wherein the **target-sequence** is a tumour antigen.
 5. An oncolytic group B adenovirus according to any one of paragraphs 1 to 4, wherein the **target-sequence** is one expressed on a cancer cell (including, CD20, CD19, CD22, CD33, CD34, CD37, CD38, CD47, CD52, CD56, CD70, CD74, CD133, CD138, CD147, CD152, CD221, CD254, CD261, CD262, CD309, CD340, BCMA, C-MYC, CAIX, Claudins [such as claudin 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and/or 24, in particular claudin-6 or claudin-18.2], EGFRvIII, EPHA3, Folate Receptor alpha [FR α] GPC3, WT1, CEA, MUC-1, EpCAM, MAGE, Mesothelin, PRAME, NYESO AFP, CA-125, ETA, tyrosinase, RAS, p53, HER

receptors HER1 [EGFR], HER2, HER3, HER4, MCAM, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3, episialin, FOLR-1, 5T4, GPNMB, integrin α V β 3, integrin α 5 β 1, Lewis-Y antigen, MET [HGFR], mucin, PMSA, TAG-72, VEGFR, PDL1 [or an antigenic fragment of any one of the same] for example a tumour antigen, such as CD19, BCMA, CEA, Claudin-6, Claudin-18.2, EGFRvIII, FR α , GPC3, MCAM, Mesothelin, MUC-1, EpCAM, MAGE, PRAME, AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1, HER2, HER3, HER4, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3 or an antigenic fragment of any one of the same..

6. An oncolytic group B adenovirus according to any one of paragraphs 1 to 5, wherein the **target-sequence** is CD19 or an antigen fragment thereof.
7. An oncolytic group B adenovirus according to any one of paragraphs 1 to 6, wherein the target ligand is a non-human sequence, for example from a yeast (beneficial in that it reduces off-target effects).
8. An oncolytic virus according to any one of paragraphs 1 to 7, wherein the **target-sequence** is a ligand (including an antibody or an antigen binding fragment thereof, such as an idiotypic antibody).
9. An oncolytic group B adenovirus according to any one of paragraphs 1 to 8, wherein the target-sequence is a ligand (including an antibody binding domain) which activates signalling through a "native" receptor on the immunotherapy cell, such as NKG2D ligands (the latter interacting with native NKG2D on NK and CD8 T cells stimulates cytotoxic activity) or CD40L, OX40L, CD80, CD86, 4-1BBL (TNFSF9), CD70, LIGHT (TNFSF14), GITRL (TNFSF18), CD258 (HVEM, TNFRSF14), ICOSL (B7-H2) interacting with their "native" receptors on immune cells.
10. An oncolytic group B adenovirus according to any one of paragraphs 1 to 8, wherein the target-sequence is a ligand (including an antibody binding domain) which inhibits signalling through a "native" receptor on the immunotherapy cell, such as PD1, TIM3, LAG3, VISTA, TIGIT, B7-H3, B7-H4, HVEM, ILT-2, ILT-3, ILT-4, BTLA, CD160 on the immunotherapy cell, for example PD1, TIM3, LAG3, VISTA, TIGIT on T-cells.
11. An oncolytic group B adenovirus according to any one of paragraphs 1 to 10, wherein the first transgene encodes a membrane anchored form of the **target-sequence** suitable for expression on the surface of an infected cancer cell, in particular allowing the cell-based therapy to bind directly to the cancer cell (i.e. not via a fusion protein).
12. An oncolytic group B adenovirus according to paragraph 11, wherein the membrane anchored form comprises a transmembrane domain or GPI anchor.

13. An oncolytic group B adenovirus according to any one of paragraphs 1 to 12, wherein the **target-sequence** is a non-human for example a mouse or yeast antigen, such as GCN4.
14. An oncolytic group B adenovirus according to any one of paragraphs 1 to 13, wherein the **target-sequence** is a label, for example a HA-Tag (amino acids 98 to 106 of human influenza hemagglutinin), His-tag (for example comprising at least 6 histidine residues), FLAG-tag or a 2A peptide tag (such as P2A, T2A, E2A and/or F2A).
15. An oncolytic group B adenovirus according to any one of paragraphs 1 to 13, wherein the **target-sequence** is the epitope E5B9 (from the La-SS/B antigen). For example as disclosed in WO2015/181282, incorporated herein by reference.
16. An oncolytic group B adenovirus according to any one of paragraphs 1 to 15, wherein the first transgene encodes a fusion protein comprising:
 - a. the **target-sequence** which binds and activates the cell-based immunotherapy, and
 - b. a first binding protein specific to a protein expressed on a cancer cell, on a stromal cell, or in stromal tissue (such as non-cellular stroma matrix, in particular collagen), in particular allowing the cell-based immunotherapy to indirectly bind the cancer cell and/ or stromal cells or stromal tissue through said fusion protein
17. An oncolytic group B adenovirus according to paragraph 16, wherein the virus encodes at least two fusion proteins, wherein the first binding protein in part b) is different in each fusion protein, for example where one fusion protein is encoded by the first transgene and the second fusion protein is encoded by the second transgene.
18. An oncolytic group B adenovirus according to paragraph 16 wherein the **target-sequence** of part a) is the same for said at least two fusion proteins i.e. both fusion proteins bind the same entity on the immunotherapy cells.
19. An oncolytic group B adenovirus according to paragraphs 17, wherein the **target-sequence** of part a) is different in said two fusion proteins (i.e. said fusion proteins bind different entities on the same or different immune cells).
20. An oncolytic group B adenovirus according to any one of paragraphs 16 to 19, wherein the binding protein of part b) is a ligand, for example to a protein or receptor found on the cancer and/or stromal cells (for example where there are multiple fusion proteins the binding domains are specific for different antigen/marker/target).
21. An oncolytic group B adenovirus according to paragraph 20 wherein the ligand is antibody or an antigen binding fragment thereof.
22. An oncolytic group B adenovirus according to any one of paragraphs 1 to 21, wherein **target-sequence** is one expressed in stroma or on a stromal cell.

23. An oncolytic group B adenovirus according to paragraph 22, where stromal cells are independently selected from the group comprising or consisting of cancer associated fibroblasts, tumour-associated macrophages or other suppressive cells, for example where the target-sequence is CD163, CD206, CD68, CD11c, CD11b, CD14, CSF1 receptor, CD15, CD33 and CD66b, fibroblast activation protein (FAP), TREM1, IGFBP7, FSP-1, platelet-derived growth factor- α receptor (PDGFR- α), platelet-derived growth factor- β receptor (PDGFR- β) and vimentin or an antigenic fragment thereof.
24. An oncolytic group B adenovirus according to any one of paragraphs 1 to 23, wherein encoding the second transgene increases the effectiveness (or broadens the spectrum of targeted tissue) of the cell-based immunotherapy
25. An oncolytic group B adenovirus according to paragraph 24 wherein a further transgene (for example the second transgene or a third transgene) increases the effectiveness of the of the cell-based immunotherapy by modulating the tumour microenvironment (for example blocking inhibitory features of the tumour microenvironment).
26. An oncolytic group B adenovirus according to paragraph 25, wherein the microenvironment is modulated to render it more pervasive to the cell-based therapy and/or to render the microenvironment more inflammatory.
27. An oncolytic group B adenovirus according to paragraph 25 or 26, wherein the further transgene modulates the tumour microenvironment to render it more pervasive (permissive) to infiltration of immunotherapy cells and/or more promoting/supporting of an innate immune response, in particular where said immune cells remain activated in the microenvironment, for example matrix degrading or loosening agents to aid cell migration within the tumour
28. An oncolytic group B adenovirus according to paragraph 27, wherein the microenvironment is rendered more pervasive(permissive) by targeting a stromal antigen, for example fibroblast activation protein (FAP), LRRC15, CD10, GPR77, TREM1, IGFBP7, FSP-1, platelet-derived growth factor- α receptor (PDGFR- α), platelet-derived growth factor- β receptor (PDGFR- β) and vimentin, for example the second gene may encode a bispecific T cell activator for example as disclosed in WO2018/041838 and WO2018/041827 both incorporated by reference.
29. An oncolytic group B adenovirus according to any one of paragraphs 25 to 28, wherein the microenvironment is modulated by assisting immune cells, including the immunotherapy cells and native immune cells to overcome suppression.
30. An oncolytic group B adenovirus according to claim 29, wherein the ability to overcome suppression is provided by an immune checkpoint inhibitor, for example an anti-CTLA-4 inhibitor, an anti-PD-1 inhibitor, and anti-PD-L1 inhibitor in particular selected from ipilimumab, tremelimumab, nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab and cemiplimab.

31. An oncolytic group B adenovirus according to any one of paragraphs 25 to 30, wherein the microenvironment is modulated to render it more inflammatory, for example through the production of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF α , IFN γ) and/or chemokines (e.g. CXCL9, CCL3, CCL5) – encoded as transgenes or production induced by an encoded transgene (e.g. IL-12, IL-15).
32. An oncolytic group B adenovirus according to any one of paragraphs 24 to 31, wherein the microenvironment is modulated by enhancing the activation of immune cells (such as immunotherapy cells and/or native immune cells), for example the second gene or a further transgene encodes a cytokine.
33. An oncolytic group B adenovirus according to paragraph 32, wherein enhanced activation of immune cells is provided by a cytokine, for example selected from: IFN α , IFN β , IFN γ , TNF α , TNF β (LT α), IL-2, IL-7, IL-9, IL-12, IL-15, IL-17, IL-18, IL-21, IL-22, IL-33, IL-35, VEGF-C, VEGF-D IL-1 α , IL-1 β , IL-6, IL-9, IL-12, IL-13, IL-17, IL-18, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-33, IL-35, IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-15, IL-21, IL-25, IL-1RA, IFN α , IFN β , IFN γ , TNF α , VEGF-A, VEGF-C, VEGF-D, TGF β , lymphotoxin α (LTA) and GM-CSF.
34. An oncolytic group B adenovirus according to paragraph 32 or 33, wherein enhanced activation of immune cells is provided by a membrane or soluble ligand comprising an extracellular domain of a protein involved in cell to cell interactions between immune cells, such as costimulatory molecules expressed by antigen presenting cells, for example selected from CD40L, OX40L, CD80, CD86, 4-1BBL, TNFSF14 (LIGHT), GITRL (TNFSF18), CD70, CD258 (HVEM), ICOSL.
35. An oncolytic group B adenovirus according to paragraph 32, wherein the enhanced activity is increased cytotoxic activity, for example by increasing levels of IFN α .
36. An oncolytic group B adenovirus according to any one of paragraphs 32 to 34, wherein enhanced activity is increased longevity of the immune cells (survival).
37. An oncolytic group B adenovirus according to paragraph 36, wherein increased longevity is provided by a cytokine, such as IL-2, IL-7, IL-15, and/or IL-21.
38. An oncolytic group B adenovirus according to any one of paragraphs 1 to 37, wherein the second transgene or a further transgene encodes a chemokine to aid recruitment of immunotherapy cells into the tumour.
39. An oncolytic according to any one of claims 1 to 38, wherein a further transgene encodes a synthetic protein designed to engage with additional recombinant receptors expressed by the cells of the therapy to enhance their ability to enter and act within the tumour and/or their survival within the patient, for example Ortho IL-2 as synthetic ligand for ortho IL-2R β (synthetic receptor – e.g. Zhang et al 2021 Sci. Transl. Med. 13 (625) eabg6986 incorporated herein by reference; TIM3/CD28 switch receptors in CAR-T interacting with secreted TIM3 ligands e.g. galectin 9, HMGB1) which may be engineered as fusions with antibody fragments so they bind tumour cells (Zhao et al 2021 J. Immunotherapy Cancer 9, e003176

incorporated herein by reference); synNotch fusion receptors on CAR-T (e.g. the CAR is an anti-CD19 Scfv coupled to IC domain of Notch to signal responses in engineered CAR when they recognize CD19 (including wherein the CD19 is in a fusion protein, such as anti-HER2 ScFv-CD19 fusion protein) – Roybal et al 2016 Cell 167(2), 419-432 incorporated herein by reference.

40. An oncolytic according to any one of paragraphs 1 to 39, wherein a further transgene encodes a polypeptide that enhances the recruitment and anti-tumour activity of endogenous immune cells of the patient
41. An oncolytic group B adenovirus according to any one of paragraphs 1 to 38, where the cells expressing the (exogenous) recombinant antigen receptor are selected from the group comprising or consisting of: T-cells (T), macrophages (Mac), natural killer cells (NK), natural killer T-cells (NKT) or innate lymphoid cells (ILC)
42. An oncolytic group B adenovirus according to any one of paragraphs 1 to 41, where the exogenous recombinant antigen receptor (or a synthetic receptor) is a chimeric antigen receptor (CAR) or a T-cell receptor (TCR).
43. An oncolytic group B adenovirus according to any one of paragraphs 1 to 41, for use in treatment, in particular for use the treatment of cancer, such as solid tumours.
44. Use of an oncolytic group B adenovirus according to paragraph 43, where cancer is transformed epithelial cancer cells.
45. An oncolytic group B adenovirus according to any one of paragraphs 1 to 42, for use in the manufacture of a medicament for the treatment of cancer, such as solid tumours, in particular transformed epithelial cancer cells.
46. A method of treating a patient comprising administering a therapeutically effective amount of an oncolytic group B adenovirus as defined in any one of paragraph 1 to 42, in particular for the treatment of cancer, more specifically a solid tumour, such as transformed epithelial cancer cells.
47. A combination therapy comprising an oncolytic group B adenovirus according to any one of claims 1 to 42 and an engineered immunotherapy cell, for example for the treatment of cancer.
48. A method of producing a virus according to any one of paragraphs 1 to 42, wherein the virus is replicated in a host cell, for example a mammalian cell, such as a HEK cell.
49. A virus obtainable from paragraph 48.

Also provided is an oncolytic group B adenovirus according to the present disclosure, wherein the second transgene encodes a further fusion protein, for example comprising a second binding protein which is different to said first binding protein and an antigen target-sequence to allow binding of same or a different cell-based immunotherapy via said further fusion protein to a second protein expressed on a cancer cell, on stromal cell, or in stromal tissue which is a different entity to that bound by the

first binding protein, in particular allowing the cell-based immunotherapy indirectly bind the cancer cell and/ or stroma through said fusion protein.

Where technically feasible combinations of embodiments may be employed.

In one embodiment the proviso is that the fusion protein does not comprise (or
5 consist of) i) a tumour associated antigen or tumour specific antigen and ii) an anti-idiotypic antibody or fragment or an anti-idiotypic peptide.

In one embodiment the virus of the present disclosure does not encode a bispecific T cell activator.

In one embodiment a second or further transgene encodes IL-15 or an active
10 fragment thereof, optionally co-expressed i.e. encoded next to or linked to (for example via a linker or amide bond) at least the sushi domain of IL-15R alpha, such as full length 15R alpha extracellular domain.

Thus, in one embodiment the presently disclosed viral constructs are designed to provide antigen in the target tissue for activating an increase in a class of antigen
15 specific immune cells (such as a local increase in the number of specific immune cells [in particular antigen specific T cells] including exogenously engineered immune cells, especially CAR-Ts).

By increasing the local concentration of antigen in the target tissue it is hypothesised that the threshold levels of antigen required to activate the immune cells
20 is exceeded and a large activating signal is sent to the requisite cell population.

In one embodiment the disclosure is not about non-specific activation of immune cells, such as T cells. Bispecific T cell activators are non-specific activators of T cells by engaging CD3 with an agonist. This type of technology can be included in as
25 complementary to the present technology but functions by different mechanisms to the present technology because the latter concerns trafficking and activation of antigen-specific cells.

DETAILED DISCLOSURE

Target-sequence, as employed herein refers to a target antigen sequence for an immune cell, for example a specific immune cell, such as an antigen specific immune cell,
30 in particular an engineered immune cell (including a sequence that stimulates (in particular activates and/or protects) existing immune cells or stimulates production of said immune cells in a specific manner). In one embodiment it does NOT refer to a binding domain of an antibody that bypasses specificity and non-specifically stimulates immune cells, such as T cells. An anti-CD3 agonistic antibody non-specifically stimulates
35 to T cells is therefore NOT within the definition of target-sequence.

The target-sequence may bind a recombinant (engineered receptor) on the immune cell and/or may bind a native receptor on the immune cell, in particular a recombinant receptor on the immune cell, provided antigen specific cells are functionally effected, for example activated, protected, proliferated.

40 In one embodiment the target-sequence is not an antibody sequence (such as an idiopathic antibody sequence) that non-specifically stimulates immune cells.

Antigen fragment as employed herein refers to a fragment of antigen that is still capable of binding specifically to a binding domain, for example an epitope which may be at least 5 amino acids.

Cell-based immunotherapy as employed herein refers to a cell-based therapy prepared or created *ex-vivo* and then administered to the patient, for example prepared using recombinant techniques, such as vectors (like viral vectors). In one embodiment the cells in the therapy encode a transgene, in particular encoding a non-native protein. Cells employed in cellular therapies include but are not limited to T cells (including subclasses thereof), NK cells (including subclasses thereof, such as memory NK cells), NKT cells, macrophages and the like.

Engineered cell as employed herein is a cell, which has been modified using recombinant techniques including vectors such as viral, in particular manipulated *ex vivo*. Having said that, technology, including vectors can be used to target cells *in vivo* and modify them. Provided the cell is modified at a genetic level, including transiently, by the manipulation then will be considered an engineered cell in the context of the present specification.

Recombinant receptor or protein as employed herein refers to a receptor or protein made or introduced by recombinant techniques.

In one embodiment one or more constructs encoded by a virus according to the present disclosure are designed to engage an engineered cell.

In one embodiment one or more constructs encoded by a virus according to the present disclosure are designed to be specific to a moiety (for example an epitope or binding domain/ligand) introduced into a cell (an engineered cell) by recombinant techniques. This mean the original cell was created by recombinant techniques even if the created cell can then be replicated to produce multiple cells containing the modification.

Thus, reference to a cell-based immunotherapy does not simply refer to engaging with native cell, such as native T-cells, for example which have not been modified by recombinant techniques *ex-vivo*.

Recombinant techniques as employed herein refer to modifications to cells generated by human design and/or intervention, for example essentially arising from a laboratory i.e. is not naturally occurring in nature.

In one embodiment the viruses disclosed according to the present invention are isolated, that is contained outside a body, in particular in a purified form.

In one embodiment the cellular therapy employed in the present disclosure is isolated. That is contained outside a body, in particular in a purified form.

In one embodiment the constructs of the present disclosure do not solely engage a native TCR. In one embodiment the constructs of the present disclosure do not engage with a native TCR. Having said the construct or further elements encoded in the virus may additionally engage a native TCR.

In one embodiment the target sequence is not a binding domain of an antigen specific T cell activator molecule. Thus, the present disclosure does not relate to bispecific T cell activator and disclosed in WO2018/041827 & WO2018/041838, incorporated herein by reference. However, a bispecific T cell activator may be included as a "further" or additional transgene in the viruses of the present disclosure.

"Trafficking in the tumour" as employed herein refers to where the gene product increases the number of active immunotherapy cells within the tumour. Thus "trafficking" in the context of the present disclosure is associated with the concept of movement and/or activation of immunotherapy cells to reach the site of action in the tumour in an operative form, i.e. such that they arrive at the intended location without being neutralised. Thus, trafficking agents includes one or more of the following: an agents that attracts cells to a tumour (for example a chemokine) an agents that breaks down the matrix/stromal barrier around the tumour (such as an enzyme or stromal antigen); an agent that changes the microenvironment to be more permissive (or less hostile) to immune cells (such as the immunotherapy cells and/or native immune cells), for example a checkpoint inhibitor; an agent that modulates the activity of an immune cells (such as the immunotherapy cells and/or native immune cells); an agent that recruits native immune cells; a more inflammatory environment, less hypoxic and combinations of two or more of the same.

In one or more embodiments the "second transgene" or trafficking component also assists trafficking of native immune cells.

Modulation of immune cell activity as employed herein refers to "activating an immune cell in some form, for example activating, including directing the activity of an immune cells to a target, increasing the cytotoxic activity of an immune cell, increasing the proliferation of an immune cell, enhancing the production of soluble mediators (for example, cytokines and chemokines) by an immune cell, increasing the survival and/or longevity of the immune cell; and protecting the immune cells from inhibition and/or anergy.

Native immune cell as employed herein is intended to refer to an immune cell found in the body i.e. not a modified cell introduced or designed as a therapy.

"Activation" of immune cells as employed herein refers to triggering or enhancing one or more functions of the immune cell and/or providing signal(s) that enable the cell to functionally respond to other signals.

For example, IFN α can directly trigger the production of cytokines and chemokines (e.g. IL-6, BAFF, APRIL) and can enhance the killing function of a T-cell once that is triggered through ligand interaction with the T-cell receptor or the CAR on a CAR-T cell

"Increases the local concentration of a target-sequence" as employed herein includes an increase from where the starting concentration is zero or undetectable and includes increase in activity not necessarily or not only the number of immune cells.

Changes in the tumour microenvironment as employed herein refer to change that allow trafficking of immune cells in or into said environment, including changes in the vasculature, hypoxia, inflammatory state, and/or immune suppressive state, etc.

“Specific” as employed herein refers to the fact a binding domain recognises a target antigen with greater affinity and/avidity than other antigens to which it is not specific (for example 10, 20, 50, 10 or 1000 greater). It does not necessarily imply that the specific binding region does not bind any non-target antigens but rather the interaction with the target is such that it can be used to purify the target antigen (to which it is specific) from a complex mixture of antigens, including antigens in the same family of proteins.

Bispecific protein as employed herein refers to a native or synthetic protein, such as a fusion protein, that comprises at least two different binding domains.

Binding protein as employed herein refers to a polypeptide comprising a binding domain.

Binding domain as employed herein refers to a protein domain which binds to a specific atom or molecule (generally a molecule, in particular a particular epitope -linear or conformational, often a particular amino acid sequence), and includes, for example a ligand, receptor or antibody or binding fragment of any one of the same.

Binding fragment or antigen binding fragment as employed herein refers to the ability of the entity to bind the target antigen specifically. Generally, it does not include the ability of regions like the Fc to bind to the receptor.

Antibody binding domain (or antibody fragment) as employed herein refers to a variable region with hypervariable domains, for example a molecule comprising a VH, VHH, VL, or a VH & VL, including sc-Fv, 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 including disulfide linked forms thereof (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).

Ligand as employed herein generally a substance that forms a complex with a biological molecule (including one presented on a cell surface) and serves a biological purpose, for example signalling, blocking, occupying, neutralising, activation, or the like. In one embodiment a ligand is binding domain from an antibody.

A soluble ligand involved in cell to cell interaction is basically any cell surface molecule that T-cells or other immune cells can bind to that can signal to the T-cell or other immune cell when employed as soluble EC domains (with or without fusion to a second molecule). In one embodiment an anti-FAP or anti-collagen antibody fragment is to fused to the signalling cytokine or other protein so that it stays in the local microenvironment where it can concentrate to better activate the immune cells).

Soluble in the context of the present specification generally means not membrane anchored.

Stromal antigen as employed herein is an “antigen” only found in stromal tissue or on stromal cells i.e. it does not include an “antigen” also present on cancer cells. Thus, antigens expressed on cancer cells and stromal cells are considered to be cancer antigens in the context of the present specification. Thus, stromal antigens may be presented on the surface of stromal cells (a cell located in the stroma) and/or on soluble molecules located in the stromal matrix, in particular molecules that can be targeted.

Examples of stromal antigens include CD163, CD206, CD68, CD11c, CD11b, CD14, CSF1 receptor, CD15, CD33 and CD66b, CD10, fibroblast activation protein (FAP), GPR77, LRR15, TREM1, IGFBP7, FSP-1, platelet-derived growth factor- α receptor (PDGFR- α), platelet-derived growth factor- β receptor (PDGFR- β) and vimentin, such as CD163, CD206, CD68, CD11c, CD11b, CD14, CSF1 receptor, CD15, CD33 and CD66b, fibroblast activation protein (FAP), TREM1, IGFBP7, FSP-1, platelet-derived growth factor- α receptor (PDGFR- α), platelet-derived growth factor- β receptor (PDGFR- β) and vimentin..

Examples of cancer targets include: PARP, CD20, CD19, CD22, CD33, CD34, CD37, CD38, CD47, CD52, CD56, CD70, CD74, CD133, CD138, CD147, CD152, CD221, CD254, CD261, CD262, CD309, CD340, BCMA, C-MYC, CAIX, Claudins [such as claudin 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and/or 24], EGFR (such as EGFRvIII) VEGFR (such as VEGFR-1, VEGFR-2), EPHA3, Folate Receptor alpha [FR α] GPC3, WT1, CEA, MUC-1, EpCAM, MAGE, Mesothelin, PRAME, NYESO AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1 [EGFR], HER2, HER3, HER4, MCAM, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3, episialin, FOLR-1, 5T4, GPNMB, integrin α V β 3, integrin α 5 β 1, Lewis-Y antigen, MET [HGFR], mucin, PMSA, TAG-72, PDL1, mTOR, BRAF, VISTA, PI3K γ , Bcr-AbL, ROS1, ALK, PDGF, PDGFR, RAF, p38 MAPK, Hsp90, MEK, MET, MKK1, calcineurin, [or an antigenic fragment of any one of the same] and a tumour antigen, such as CD19, BCMA, CEA, Claudin-6, Claudin-18.2, EGFRvIII, FR α , GPC3, MCAM, Mesothelin, MUC-1, EpCAM, MAGE, PRAME, AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1, HER2, HER3, HER4, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3 or an antigenic fragment of any one of the same

In one embodiment the cancer target is selected from PARP, WT1, VEGF, EGFR, mTOR, BRAF, VEGFR-1, VISTA, PI3K γ , Bcr-AbL, ROS1, ALK, PDGF, PDGFR, RAF, p38 MAPK, Hsp90, MEK, MET, MKK1, calcineurin, CD33, CD19, CD52, and a tumour antigen (for example CEA, AFP, CA-125, ETA, Tyrosinase, MAGE, PRAME, ras, p53,, MUC-1, EpCAM, HER receptors HER1, HER2, HER3, HER4, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3).

Cancer antigen (also referred to herein as tumour antigen) as employed herein includes tumour specific antigens (TSA) and cancer associated antigens (TAA). Tumor-Specific Antigens are present only on tumor cells and not on any other cell and Tumor-

Associated Antigens are present on some tumor cells and also some normal cells. Tumour antigens include:

- Products of Mutated Oncogenes and Tumor Suppressor Genes
- Products of Other Mutated Genes
 - Overexpressed or Aberrantly Expressed Cellular Proteins
 - Tumor Antigens Produced by Oncogenic Viruses
 - Oncofetal Antigens
 - Altered Cell Surface Glycolipids and Glycoproteins, and
 - Cell Type-Specific Differentiation Antigens.

5 Cancer antigens (a tumour antigens) are antigens found specifically on cancer cells (i.e. generally not found on healthy cells or highly upregulated on cancer cells) including for example CEA, MUC-1, EpCAM, HER receptors HER1, HER2, HER3, HER4, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2, ErbB3, WT1, MUC1, LMP2, idiotype, HPV E6&E7, EGFRvIII, 10 HER-2/neu, MAGE A3, NY-ESO-1, p53 nonmutant, p53 mutant, NY-ESO-1, GD2, PSMA, PCSA, PSA, MelanA/MART1, Ras mutant, proteinase3 (PR1), bcr-abl, tyrosinase, survivin, 15 PSA, hTERT, particularly WT1, MUC1, HER-2/neu, NY-ESO-1, survivin and hTERT. Cancer antigen as employed herein includes tumour specific antigens and cancer associated antigens. In one embodiment the CAR binding domain is specific to a cancer antigen (including any 20 specific target sequence disclosed herein).

Other cancer antigens include epithelial tumor antigens, CA-125, and alpha-fetoprotein.

Antibodies that target cancer include: avelumab, bevacizumab, brentuximab, cemiplimab, cetuximab, daratumumab, dinutuximab, elotuzumab, enfortumab, 25 gemtuzumab, ibritumomab, inotuzumab, ipilimumab, isatuximab, mogamulizumab, moxetumomab, necitumumab, nivolumab, obinutuzumab, ofatumumab, olaratumab, panitumumab, pembrolizumab, pertuzumab, polatuzumab, ramucirumab, rituximab, Sacituzumab, tositumomab, trastuzumab, Fab-G8 & Fab-Hyb3 (which targets EADPTGHSY in MAGE A1); G2D12, & G3G4 (which target KTWGQYWQV in GP100); 1A9, 1C8, 1A11, 30 1A7 & G1 (which target IMDQVPFSV in GP100); 2F1, 2B2, 2C5 & 2D1 (which target YLEPGPVTVA in GP100); GPA7 (which targets ITDQVPFSV); 4A9 & 4G9 (which targets ILAFLHWL in hTERT) 3H2 & 3G3 (which targets RLVDDFLLV in hTERT); 3M4E5 (which targets SLIMWITQC in NY-ESO-1); 7D4, 8A11, 2G12 & 9E6 (which target FLWGPRALV in MAGE3); RL4B/3.2G1 & 1B10 (which target GVLPALPQV in hCG β); 3F9 (which target 35 TMTRVLQGV in hCG β); 1B8 (which targets KIFGSLAFL in Her2/Neu); CAG10 & CLA12 (which EAAGIGILTV in Melan-A/MARR-1); Fab-D2 (which targets FLRNFSML in TARP); I3.M3-2A6 (which targets LLGRNSFEV in p53); T1-116C, T1-29D & T1-84C (which targets RMPEAAPPV in p53); T2-108A & T2-2A, T2-116A (which target GLAPPQHILRV in p53); T2A (which is specific to YMDGTMSQV in tyrosinase); RL6A (which is specific to 40 YLLPAIVHI in p68); RL21A (which is specific to FLSELTQQL in MIF); 8FA (which is specific to VLQELNVTV in Proteinase 3); ESK1 F2, F3 & Clone45 (which are specific to

RMFPNAPYL in WT1); #131 (which is specific to VLHDDLLEA in HA-1H) and Pr20 (which is specific to ALYVDSLFFL).

In one embodiment the construct encoded by the virus according to the present disclosure, such as an antibody or binding fragment thereof is human or humanised

5 In one embodiment "immune checkpoint inhibitor antibodies" or fragments are encoded within a virus of the present disclosure, for example. anti-PD1, PDL1, CTLA4, LAG3, GITR, TIGIT, CD40.

10 In one embodiment the immune suppression of the microenvironment is inhibited by intrinsic checkpoint blockade, for example inhibiting an E3 ubiquitin-protein ligase (such as CBL-B) and/or a cytokine-inducible SH2-containing protein (such as CISH).

Thus, in one embodiment the virus according to the present disclosure encodes a checkpoint kinase inhibitor, for example selected from checkpoint kinase inhibitor 1 (CHEK1/CHK1), checkpoint kinase inhibitor 2 (CHEK2/CHK2), Ataxia telangiectasia and Rad3 related (ATR) inhibitor, ataxia-telangiectasia mutated (ATM) inhibitor, Wee1 dual
15 specificity protein kinase (Wee1) inhibitor, Poly ADP Ribose polymerase (PARP) inhibitor and Myt1 inhibitor, and combinations of two or more of the same, such as a checkpoint kinase inhibitor, in particular a CHK1 inhibitor.

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

25 Glypiated (GPI-linked) proteins 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 processes through the secretory pathway, it is transferred via vesicles to the Golgi apparatus and finally to the extracellular space
30 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.

35 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
40 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.

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 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 the group comprising TM Domain Sequences (Minimal portions) given in table below.

SEQ ID NO:	Name	SEQUENCE
100	PDGFR Receptor A	AVLVLLVIVIISLIVLVVIW
101	PDGFR Receptor B	VVISAILALVVLTIISLIILI
102	INSULIN-LIKE GROWTH FACTOR 1	IIIGPPLIFVFLFSVVIGSIYFL
103	IL6-R	SSSVPLPTFLVAGGSLAFGTLLCIAIVL
104	CD28	FWVLVVGGVLACYSLLVTVAFIIFWV

Using a non-human sequence may be advantageous because it may increase specificity for the cancer, thereby increasing the therapeutic window, and effectively reducing the off-target effects.

In one embodiment the cell immunotherapy is a transgenic cell, in particular a CAR-T, engineered NK cell, and/or engineered NKT cell, more specifically a CAR-T.

Transgenic cells as employed herein refer to engineered cells, for example engineered using recombinant techniques to include non-native polynucleotide(s) that modify the function of the cell i.e the cell is modified to express a synthetic receptor on its surface.

CAR as employed herein refers to chimeric antigen receptor i.e a synthetic receptor, such as an antibody binding domain coupled to signalling function, such as an intracellular signalling function. CARs are most commonly created by joining heavy and light chain variable regions from a monoclonal antibody, but may also be created with other antibody forms (e.g. camelid single chain VHH antibodies) or other antigen/ligand binding proteins. The receptors bind antigen or ligand to which they are specific and stimulate signalling pathways in the transgenic cell.

In CARs the signalling is intrinsic or integrated with the receptor. However alternative technologies are being developed where the signalling is not physically linked with the synthetic receptor. All these types are technologies are suitable for use with the viruses of the present disclosure.

Examples given below for CAR-T cells may be applied to other immunotherapy cells including NK and NKT cells, as technically appropriate.

First generation CAR-T cells often had intracellular signalling unit based on CD3-zeta. However, second generation CARs generally have a costimulatory element, such as CD28, 4-1BB, CD136, CD137 or CD27 and ICOS built into the intracellular signalling domain, while third generation CARs can incorporate more than one costimulatory element, such as CD28 plus 4-1BB (for example see US7,446,190, Dotti *et al* 2009 Human Gene Therapy 20: 1229-1239; Finney et al J Immuno. 1998, 161(6): 2791-2797; Finney et al 2004 J Immunol 172(1) 104-113; Milone et al Mol Ther. 2009 17(8): 1453-1464).

In one embodiment there is provided engineered cells, such as T-cells, engineered to express a recombinant (also referred to herein as synthetic) TCR specific for a tumour or other target antigen. In this case the TCR recognizes MHC/antigen peptide complexes. These cells can be engineered in similar ways to those described herein for CAR-Ts.

The immunotherapy cells may also be engineered by technology, such as CRISPR/Cas9, for example to remove expression of inhibitory proteins (e.g. PD1) and/or to remove endogenous receptors such as the endogenous TCR (to enhance target specificity).

Companies such as ProMab Biotechnologies, Inc make these products commercially available.

Thus, in one embodiment the CAR comprises a CD3 zeta signalling unit. The following disclose first generation CARs: Irving and Weiss, Cell 1991 Mar 8; 64(5): 891-901. Letourmeur 1991 Oct 15; 88(20) 8905-8909. Romeo, Cell, Vol 68, issue 5, p889-897, March 06, 1992.*

In one embodiment the CAR comprises a CD28 signalling unit, see for example Maher *et al*, Nat Biotechnol 2002 Jan; 20(1); 70-75 and Carpenito *et al* PNAS Mar 3, 2009 106(9) 3360-3365.* In one embodiment the CAR comprises a CD27 signalling unit. The later makes an essential contribution to mature CD4+ and CD8+ T cell function. In one embodiment the CAR comprises an ICOS signalling unit, wherein ICOS stands for inducible T-cell co-stimulator. In one embodiment the CAR comprises 4 1BB, see for example Imai 2004, Leukemia 18, 676-684.* In one embodiment the CAR therapy comprises one co-stimulatory factor. In one embodiment the CAR therapy comprises a combination of co-stimulatory factors, for example 2, 3, or 4, such as CD28 and 4-1BB, CD28 and ICOS, CD27 and 4-1BB or CD27 and ICOS. Guedan *et al* Blood 2014, 124(7): 1070-1080, incorporated herein by reference, discloses ICOS based chimeric antigen receptors.* Duong PLoS 2013 May 7;8(5) discloses engineering T cell function using chimeric antigen receptors.* Signalling unit as employed herein is element that contribute to the cellular signalling of the CAR. Chimeric T cell receptors are disclosed in US2004043401.*

* The construction features (signalling aspects not specificity) of the CAR disclosed here are incorporated by reference and may be used as the basis for an amendment to the claims.

The binding domain of the CAR is similar to an antibody and may, for example comprise an scFv, see for example Kuwana et al Biochem Biophys Res Commun. 1987 Dec

31, 149(3); and Eshhar et al Proc Natl Acad Sci USA 1993 Jan 15; 90(2):270-724.* Second generation CARs.

In one embodiment the binding domain of the CAR is specific to a blood cell antigen, for example CD19, CD30, CD123, FLT, (including combinations such as CD19 and CD20 or CD22) in particular useful in the treatment of a hematological cancer, such as ALL, AML, CLL, DLBCL, BCMA, leukemia and multiple myeloma. Porter et al N Engl J Med 2011; 365: 1937-1939 discloses CAR modified T cells in CLL. Grupp et al N Engl J Med 2013 April 18; 368 (16) 1509-1518 discloses CAR modified T cells for ALL. Maude et al N Engl J Med 2014, 371: 1507-1517 disclosed CAR-T cells for sustained remission of Leukemia. Garfall et al N Engl J Med 2015; 373: 1040-1047 disclosed CAR T cells against CD19 for multiple myeloma.

In one embodiment the recombinant receptor, such as a CAR, is specific to a solid tumour and/or supporting tissue.

In one embodiment the recombinant receptor, such as a CAR, is specific to a cancer antigen. Cancer antigens are described above.

In one embodiment the recombinant receptor, such as a CAR, binding domain targets aberrant sugars on the surface of cancer cells.

In one embodiment the recombinant receptor, such as a CAR, is specific to a stromal antigen, for example as defined herein.

In one embodiment the recombinant receptor, such as a CAR, binding domain is specific to an antigen selected from the group comprising CD19, HER-3, HER-4, CEA, EGFR, EpCAM, EGFRvIII, PSMA, CD20, VEGFR-1, VEGFR-3, c-Met, Lewis A, ROR-1, CD326, CD133, NKG2d, MUC-1, PSCA, PSA, CA-125, Notch FLT-3 CD20, CD22, CD33, CD34, CD37, CD38, CD47, CD52, CD56, CD70, CD74, CD133, CD138, CD147, CD152, CD221, CD254, CD261, CD262, CD309, CD340, BCMA, C-MYC, CAIX, Claudins [such as claudin 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and/or 24], EGFRvIII, EPHA3, Folate Receptor alpha [FR α] GPC3, WT1, CEA, MUC-1, EpCAM, MAGE, Mesothelin, PRAME, NYESO AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1 [EGFR], HER2, HER3, HER4, MCAM, PEM, A33, G250, carbohydrate antigens Ley, Lex, Leb, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3, episialin, FOLR-1, 5T4, GPNMB, integrin α V β 3, integrin α 5 β 1, Lewis-Y antigen, MET [HGFR], mucin, PMSA, TAG-72, VEGFR, and PDL1.

In one embodiment the engineered cell encodes at least two entities, for example CD19 CAR and PD-1 siRNA, CD19 TIGIT siRNA, BCMA-CS1, or BCMA-CD33.

In one embodiment the recombinant receptor, such as a CAR, is specific to:
CD19 (for example CD19-CD28, CD19scFv-CD28-CD3 ζ , CD19scFv-4-1BB-CD3 ζ , CD19scFv-CD28-4-1BB, CD19scFv-CD28-4-1BB-CD3 ζ , or iCas9-T2A-antiCD19scFv-CD28-CD3 ζ , CD19FLAG-CD28-CD3 ζ or iCas9 HA-T2A-antiCD19scFv-CD28-CD3 ζ -GGGS-FLAG, humanised CD19 scFv-TM28-CD28-CD3 ζ , CD19 scFv-Beam-TM28-CD28-CD3 ζ or humanised CD19 scFv-Beam-TM28-CD28-CD3 ζ , CD19 scFv-CD22 scFv-4-1BB-CD3-T2A-tEGFR or, CD19 scFv-TM28-GITR-CD3 ζ or CD19 scFv-TM8-GITR-CD3 ζ); **Mesothelin** (for

example mesothelin scFv- CD28-CD3ζ, mesothelin scFv- 4-1BB-CD3ζ, mesothelin scFv- CD28-4-1BB-CD3ζ, mesothelin scFv FLAG- 4-1BB-CD3ζ, mesothelin scFv-TM28-CD28-4-1BB-CD3ζ, mesothelin scFv-Beam-TM28-4-1BB-CD3ζ, mesothelin scFv-Beam-CD28-CD3ζ, mesothelin scFv-TM8-4-1BB-CD3ζ, mesothelin scFv-TM28-CD28-CD3ζ); **VGFR2** (for example VGFR2 scFv-CD28 CD3ζ); **GPC3** (for example GPC3 scFv-CD28- CD3ζ); **CD133** (CD133 scFv-CD28- CD3ζ); **EpCAM** (for example EpCAM scFv-CD28- CD3ζ such as a version where NheI restriction site introduced, N-terminal of scFv amino acid); **EGFR** (for example EGFR scFv-CD28- CD3ζ, EGFR scFv-4-1BB- CD3ζ, EGFR scFv-TM28-GITR-CD3ζ, scFv-TM28-CD3ζ-GITR; **CD33** (for example CD33 scFv-TM28- CD28- CD3ζ or CD33 scFv-Beam 2-TM28-CD28-CD3ζ); **CD38** (for example CD38 scFv-TM28- CD28- CD3ζ); **CD138** (for example CD138 scFv- Beam- TM28-CD28- CD3ζ); **CD22** (for example CD22 scFv-TM28-CD28 CD3ζ, -CD22 scFv-TM28-4-1BB CD3ζ or CD22 scFv-Beam-TM28-CD28-CD3ζ); **BCMA** (for example BCMA-4-CD28 CD3ζ or humanized, BCMA-4 scFv-TM8-4-1BB-CD3ζ or BCMA-2 scFv-Tm-CD28- CD3ζ); **HER2** (for example HER2 scFv-CD28- CD3ζ, HER2 scFv-4-1-BB-CD3ζ-EGFRt or HER scFv-4-1BB-CD3ζ -GFP); **CD4** (for example CD4 scFv-Beam-TM28-CD28- CD3ζ); **ROR-1** (for example ROR-1 scFv TM28-CD28- CD3ζ, ROR-1 scFv TM28-4-1BB- CD3ζ or humanised ROR-1 scFv TM28-4-1BB- CD3ζ); **CD19&CD22** (for example CD19 scFv CD22 scFv-4-1BB- CD3ζ or CD19 scFv-CD22 scFv-4-1BB-CD3-T2A-RQR8); **CEA** (for example CEA scFv -TM28-CD28 CD3ζ or humanised CEA scFv -TM28-CD28 CD3ζ; **NGFR** (for example NGFR scFv-TM28-CD28-CD3ζ); **MCAM** (for example MCAM scFv-TM28-CD28-CD3ζ); **CD47** (for example CD47 scFv-TM28-CD28- CD3ζ or humanised CD47 scFv-TM28-CD28- CD3ζ); **PDL-1**(for example PDL-1 scFv-TM28-CD28-CD3ζ); **CD123** (for example CD123 scFv-TM28-CD28-CD3ζ); **CD37** (for example CD37 scFv-TM28-CD28-CD3ζ, CD37 scFv-TM28-4-1-BB-CD3ζ; **CS1** (for example CS1 scFv-TM28-CD28-CD3ζ); **B7H4** (for example B7H4 scFv-TM28-CD28-CD3ζ); **CD24** (for example CD24 scFv-TM28-CD28-CD3ζ), and **CD20** (for example CD20 scFv-TM28-CD28-CD3ζ); **NKG2D** such as CYAD-01; **FLT3**, such as AMG 553; **DLL3**.

In one embodiment the recombinant receptor, such as a CAR, is specific to HER-2, for example with a specificity of the CAR employed in the Examples disclosed herein.

In one embodiment the CAR is provided in a T cell (such as autologous T cells or allogenic T cells, more specifically HLA matched T cells). In one embodiment the CAR-T cell is selected from tisagenlecleucel, axicabtagene ciloleucel, lisocabtagene maraleucel, idecabtagene vicleucel, brexucabtagene autoleucel, JCAR015 (CD19 CAR T from Juno), Descartes-08 (BCMA) and AMG119.

In one embodiment the immune cells is a phagocytic cell, for example encoding a recombinant receptor (such as a CAR) listed herein, such as CD19 scFv -CAR or mesothelin scFv CAR. In one embodiment the phagocytic cell is a macrophage),

In one embodiment the recombinant receptor, such as a CAR, is provided in an NKT cell. The benefit of NKT cell cars, is that they do not require HLA matching with the patient. Thus, they can be employed to provide an “off the shelf product”, for example with the specificity listed herein. WO2013/040371 discloses NKT engineered with a CAR

and incorporated herein by reference. In one embodiment the NKT cell encodes a cytokine.

In one embodiment the immune cell is an NK cell, see for example Tran et al, J Immunol 1995 Jul, 155(2); 1000-1009 incorporated herein by reference.

5 In one embodiment the engineered cell is derived from a pluripotent stem cell, for example iPSCs.

Thus, in one embodiment the immune cell therapy further comprises a transgene (i.e. an engineered gene) encoding a cytokine, for example selected from IL-2, IL-5, IL-7, IL-12 and IL-15.

10 In one embodiment the engineered cell does not comprise a transgene encoding a cytokine.

In one embodiment the engineered cell only comprises the transgene that express the recombinant receptor (such as a CAR), although this does not preclude deleting certain wild-type genes, if this is beneficial (for example improves specificity).

15 In one embodiment the immune cell therapy does NOT comprises a transgene (i.e. an engineered gene) encoding a cytokine, for example selected from IL-2, IL-5, IL-7, IL-12 and IL-15

Immune cells such as T cells, NKT cells may be activated or have activity sustained by IL-15 expressed by a virus of the present invention. This may help counteract the anergic/hypoxic microenvironment of tumour. The hypoxic microenvironment may have the ability to neutralise the killing power of the native cells and even the engineered cellular therapy employed in combination with the present invention. Thus, use of the virus of the present disclosure may trigger several mechanisms for killing cancer, especially when used in combination with cellular therapy.

25 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 dose limiting toxicities. 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. Infection of cancer cells after systemic delivery of the viruses of the present disclosure is an indication of a therapeutic dose i.e. it has been delivered to the target cells. Suitable therapeutic doses are generally a balance between
30 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, a virus or therapeutic construct according to the present disclosure (including a formulation comprising same) is administered every 3-4 weeks, for example on week 1 the dose is administered on day 1, 3, 5, for example followed by
40 further sets of multiple doses 3-4 weeks later.

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 3 or 4 is also administered on days 1, 3 and 5 thereof. This dosing regimen may be repeated as many times as appropriate.

In one embodiment the first dose is lower than the subsequent doses, for example the first dose is in the range 1×10^{11} to 1×10^{12} viral particles and the subsequent doses are in the range 1×10^{12} to 1×10^{13} viral particles.

In one embodiment 6 doses are given over a two week period, for example day 1, 3, 5, 8, 10 and 12, such as where each dose may be given +/- 1 day, including where the dose on day 1 is lower than the other doses.

In one embodiment, a virus or therapeutic construct according to the present disclosure (including a formulation comprising same) is administered monthly.

In one embodiment the first doses of virus are given before treatment with an immune cell therapy, for example 7 to 28 days prior to the cell 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.

The disclosure herein further extends to an adenovirus of formula (I) or a subformula 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.

The background section contains technically relevant details may be used as basis for an amendment.

DESCRIPTION OF THE FIGURES

Figure 1 Schematic representation of transgene cassettes encoding a range of bispecific proteins and membrane anchored antigens with or without additional transgenes

- Figure 2** Diagram illustrating the design of bispecific proteins and how they enable tumour cell recognition by cell therapeutics (CAR-T cells as example) and enhancement of cell therapy activity by trafficking agents
- Figure 3** Screening of NG-1100 -1126 designs for ability to bind to A549 tumour cells and present cell therapy ligands *in vitro*. A. Expression of transmembrane CD19 on A549 cells following transfection with the pUC57 plasmid for NG-1108; B. Supernatants from A549 cells transfected with pUC57 plasmids for NG-1100, NG-1101, NG-1102, NG-1106, NG-1107 or control pUC57 vector were incubated with fresh A549 cells and binding of bispecific proteins was measured by flow cytometry to detect the C-terminal 2A peptide tag; C-E. Supernatants from A549 cells transfected with pUC57 plasmids for NG-1109 to NG-1118 or control plasmid were incubated with fresh A549 cells and binding of bispecific proteins to EpCAM was measured as a decrease in the binding of AF647-labelled anti-EpCAM antibody using flow cytometry; F. Supernatants from A549 cells transfected with pUC57 plasmids for NG-1106, NG-1107, NG-1109, NG-1110, NG-1113 to NG-1121 or NG-1123 to NG-1126 or control plasmid were incubated with fresh A549 cells and binding of bispecific proteins was measured by detecting CD19 by flow cytometry
- Figure 4** A&B. Binding of anti-HER2ScFv-CD19 fusion protein to A549 and SKOV3 tumour cells following incubation with infection supernatants. A549 tumour cells were infected with EnAd or NG-1124 and infection supernatants were collected and incubated with uninfected A549 (A) or SKOV3 (B) before cell were analysed for CD19 expression by flow cytometry. C. Transmembrane CD19 expression following infection with NG-1108. A549 tumour cells were infected with EnAd or NG-1108 viruses and culture supernatants added to uninfected A549 cells, then both sets of cells were analysed for CD19 expression by flow cytometry
- Figure 5** Binding of virus-encoded bi-specific proteins secreted by infected A549 tumour cells to non-infected SKOV3 cells in a co-culture system. A&B. A549 cell cultures, SKOV3 cell cultures and A549+SKOV3 cell co-cultures were infected with either EnAd or NG-1100 and binding of the anti-EpCAM – RAE1TG3 fusion protein to SKOV3 (A) or A549 (B) cell surfaces was assessed by flow cytometry detection of the C-terminal 2A peptide tag peptide. C&D. A549 cell cultures, SKOV3 cell cultures and A549+SKOV3 cell co-cultures were infected with EnAd or NG-1124 and binding of the anti-HER2-CD19 fusion protein to SKOV3 (C) or A549 (D) cell surfaces was assessed by flow cytometry detection of CD19 antigen.
- Figure 6** Anti-CD19 CAR-T cell-mediated cytotoxicity against SKOV3 tumour cells in the presence of cell culture supernatants (SN) from NG-1124 infected A549 cells. SKOV3 cells were incubated in presence of cell culture supernatant

from A549 cells infected either with EnAd (A) or NG-1124 (B) and then anti-CD19 CAR or control (Ctrl) T cells were added and tumour cell death was monitored in real time

Figure 7 Anti-CD19 CAR-T-mediated cytotoxicity against A549 tumour cells in the presence of cell culture supernatants from NG-1124-infected cells. A549 cells were infected with either NG-1124 or EnAd and virus particles removed from the culture supernatants by exclusion size column filtration before testing. A. Levels of anti-HER2-CD19 bispecific protein were shown to be similar before and after removal of virus particles. B-D. A549 cells were incubated in presence of these virus-depleted culture supernatants (VR SN) and then anti-CD19 CAR or control (Ctrl) T cells were added and tumour cell death was monitored in real time.

Figure 8 A. Effect of encoding multiple transgenes on the level of bi-specific protein expression and binding to tumour cells. B. Expression of CXCL9, CCL21, IFN α & IL-15 transgene proteins by NG-1101, NG-1104 and NG-1125 infected A549 tumour cells.

Figure 9 *In vivo* expression and tumour cell binding of NG-1125-encoded anti-HER2-CD19 fusion protein. A. Flow cytometry gating following intracellular staining for virus, separating cells into those that are negative (Uninfected) or with low (low VP) or high (high VP) levels of infection. B. Frequency of tumour cells remaining uninfected or having low VP or high VP following IV dosing with either EnAd or NG-1125. C. Levels of cell surface-bound anti-HER2-CD19 on uninfected, low VP or high VP subsets of A549 tumour cells in the tumour mass following IV dosing with EnAd or NG-1125.

Figure 10 *In vivo* demonstration of enhanced CAR-T cell activity in A549 xenograft tumours following IV dosing with NG-1125, which expresses anti-HER2-CD19 bispecific protein plus two further transgenes compared to that seen with NG-1124, which expresses only the anti-HER2-CD19 protein, or with EnAd. Excised tumours were processed into single cell suspension and analysed by flow cytometry for the presence of total human CD45⁺ cells (A), activated cytotoxic human CD8⁺ CD107a⁺ cells (B) or activated cytotoxic human CD4⁺ CD107a⁺ cells (C).

Figure 11 Shows the generic structure of chimeric antigen receptors

Figure 12 *In vivo* demonstration of enhanced CAR-T cell recruitment and activation in A549 xenograft tumors following IV dosing with NG-1125 (expressing anti-HER2-CD19 bispecific protein plus CXCL9 and IFN α), NG-641 (expressing CXCL9, CXCL10, IFN α and an irrelevant bispecific protein) or with EnAd. Excised tumors were processed into single cell suspension and analysed by flow cytometry for the presence of total human CD45⁺ cells (corresponding to transferred T cells) (A), activated cytotoxic human CD45⁺CD107a⁺ cells (B) or activated human CD45⁺CD25⁺ cells (C). Blood

was also processed and assessed for the presence of circulating human CD45⁺ cells (D).

SEQUENCE LISTING

SEQ ID NO: 1	Short Splice Acceptor (SSA) Sequence (CAGG)
SEQ ID NO: 2	Splice Acceptor Sequence
SEQ ID NO: 3	Splice Acceptor Sequence
SEQ ID NO: 4	Poly adenylation (PA) sequence (SV40 late polyA sequence)
SEQ ID NO: 5	High efficiency self-cleavable T2A peptide sequence
SEQ ID NO: 6	High efficiency self-cleavable E2A peptide sequence
SEQ ID NO: 7	High efficiency self-cleavable F2A peptide sequence
SEQ ID NO: 8	High efficiency self-cleavable P2A peptide sequence
SEQ ID NO: 9	Flexible linker (FL)
SEQ ID NO: 10	Rigid linker (RL)
SEQ ID NO: 11	Myc peptide tag
SEQ ID NO: 12	His Tag
SEQ ID NO: 13	Human CXCL9
SEQ ID NO: 14	Human CXCL9-T2A
SEQ ID NO: 15	Human CXCL9-P2A
SEQ ID NO: 16	Human CXCL9-E2A
SEQ ID NO: 17	Human CCL21 with N-terminal signal peptide
SEQ ID NO: 18	Human CCL21 with N-terminal signal peptide and C-terminal E2A peptide
SEQ ID NO: 19	Human IL-15Ra sushi domain with signal sequence
SEQ ID NO: 20	Human IL-15Ra sushi domain with signal sequence with a C-terminal P2A peptide
SEQ ID NO: 21	Human IL-15 with immunoglobulin leader sequence
SEQ ID NO: 22	(Gly ₄ Ser) ₃ Linker (G4S)
SEQ ID NO: 23	Short Gly ₄ Ser liner (sG4S)
SEQ ID NO: 24	Long (Gly ₄ Ser) ₄ Linker (G4S4)
SEQ ID NO: 25	Human CD19 (extracellular domain)
SEQ ID NO: 26	Human CD19m1 (extracellular domain, mutated)
SEQ ID NO: 27	Human CD19m2 (extracellular domain, mutated)
SEQ ID NO: 28	Protein sequence of anti-EpCAM-ScFv-OKT3-ScFv fusion protein (without C-terminal tag), including leader sequence, encoded in NG-611
SEQ ID NO: 29	Protein sequence of anti-EpCAM-ScFv-RAET1G3 fusion protein with F2A C-terminal tag, including leader sequence, encoded within NG-1100, NG-1101
SEQ ID NO: 30	Protein sequence of RAET1G3-anti-EpCAM -ScFv fusion protein with F2A C-terminal tag, including leader sequence, encoded within NG-1102

SEQ ID NO: 31	Protein sequence of anti-EpCAM-ScFv-OKT3-ScFv fusion protein with an F2A C-terminal tag, including leader sequence, encoded in NG-1104
SEQ ID NO: 32	Protein sequence of anti-EpCAM-ScFv-human CD19 EC domain fusion protein with a P2A C-terminal tag, including leader sequence, encoded in NG-1106, NG-1119
SEQ ID NO: 33	Protein sequence of human CD19 EC domain-anti-EpCAM-ScFv fusion protein with a P2A C-terminal tag, including leader sequence, encoded in NG-1107
SEQ ID NO: 34	Protein sequence of transmembrane human CD19 with short cytoplasmic tail and P2A C-terminal tag, including leader sequence, encoded in NG-1108
SEQ ID NO: 35	Protein sequence of anti-EpCAM-ScFv-human CD19 EC domain fusion protein (without a C-terminal tag), including leader sequence, encoded in NG-1109
SEQ ID NO: 36	Protein sequence of human CD19 EC domain-anti-EpCAM-ScFv fusion protein (without a C-terminal tag), including leader sequence, encoded in NG-1110
SEQ ID NO: 37	Protein sequence of anti-EpCAM-ScFv-RAET1G3 fusion protein (without a C-terminal tag), including leader sequence, encoded within NG-1111
SEQ ID NO: 38	Protein sequence of RAET1G3-anti-EpCAM -ScFv fusion protein (without a C-terminal tag), including leader sequence, encoded within NG-1112
SEQ ID NO: 39	Protein sequence of anti-EpCAM-ScFv -flexible linker-human CD19 EC domain fusion protein (without a C-terminal tag), including leader sequence, encoded in NG-1113, NG-1115
SEQ ID NO: 40	Protein sequence of anti-EpCAM-ScFv -rigid linker-human CD19 EC domain fusion protein (without a C-terminal tag), including leader sequence, encoded in NG-1114, NG-1116
SEQ ID NO: 41	Protein sequence of anti-EpCAM-ScFv-flexible linker-RAET1G3 fusion protein (without a C-terminal tag), including leader sequence, encoded within NG-1117
SEQ ID NO: 42	Protein sequence of anti-EpCAM-ScFv-rigid linker-RAET1G3 fusion protein (without a C-terminal tag), including leader sequence, encoded within NG-1118
SEQ ID NO: 43	Protein sequence of anti-EpCAM-ScFv-human CD19m1 EC domain fusion protein with a P2A C-terminal tag, including leader sequence, encoded in NG-1120

SEQ ID NO: 44	Protein sequence of human CD19 EC domain-anti-HER2-ScFv fusion protein, with a C-terminal P2A tag, including leader sequence, encoded in NG-1121
SEQ ID NO: 45	Protein sequence of human CD19 EC domain-anti-HER2-ScFv fusion protein, with a C-terminal Myc tag, including leader sequence, encoded in NG-1122
SEQ ID NO: 46	Protein sequence of human CD19m2 EC domain-anti-HER2-ScFv fusion protein, with a C-terminal P2A tag, including leader sequence, encoded in NG-1123
SEQ ID NO: 47	Protein sequence of anti-HER2-ScFv-CD19m1 fusion protein with C-terminal P2A tag, including leader sequence, encoded in NG-1124, NG-1125
SEQ ID NO: 48	Protein sequence of anti-HER2-ScFv-CD19 fusion protein with C-terminal P2A tag, including leader sequence, encoded in NG-1126
SEQ ID NO: 49	Protein sequence encoded by NG-611 transgene cassette
SEQ ID NO: 50	Protein sequence encoded by NG-1100 transgene cassette
SEQ ID NO: 51	Protein sequence encoded by NG-1101 transgene cassette
SEQ ID NO: 52	Protein sequence encoded by NG-1102 transgene cassette
SEQ ID NO: 53	Protein sequence encoded by NG-1104 transgene cassette
SEQ ID NO: 54	Protein sequence encoded by NG-1106 transgene cassette
SEQ ID NO: 55	Protein sequence encoded by NG-1107 transgene cassette
SEQ ID NO: 56	Protein sequence encoded by NG-1108 transgene cassette
SEQ ID NO: 57	Protein sequence encoded by NG-1109 transgene cassette
SEQ ID NO: 58	Protein sequence encoded by NG-1110 transgene cassette
SEQ ID NO: 59	Protein sequence encoded by NG-1111 transgene cassette
SEQ ID NO: 60	Protein sequence encoded by NG-1112 transgene cassette
SEQ ID NO: 61	Protein sequence encoded by NG-1113 transgene cassette
SEQ ID NO: 62	Protein sequence encoded by NG-1114 transgene cassette
SEQ ID NO: 63	Protein sequence encoded by NG-1115 transgene cassette
SEQ ID NO: 64	Protein sequence encoded by NG-1116 transgene cassette
SEQ ID NO: 65	Protein sequence encoded by NG-1117 transgene cassette
SEQ ID NO: 66	Protein sequence encoded by NG-1118 transgene cassette
SEQ ID NO: 67	Protein sequence encoded by NG-1119 transgene cassette
SEQ ID NO: 68	Protein sequence encoded by NG-1120 transgene cassette
SEQ ID NO: 69	Protein sequence encoded by NG-1121 transgene cassette
SEQ ID NO: 70	Protein sequence encoded by NG-1122 transgene cassette
SEQ ID NO: 71	Protein sequence encoded by NG-1123 transgene cassette
SEQ ID NO: 72	Protein sequence encoded by NG-1124 transgene cassette
SEQ ID NO: 73	Protein sequence encoded by NG-1125 transgene cassette
SEQ ID NO: 74	Protein sequence encoded by NG-1126 transgene cassette
SEQ ID NO: 75	Genome sequence of NG-1100 virus

SEQ ID NO: 76	Genome sequence of NG-1101 virus
SEQ ID NO: 77	Genome sequence of NG-1102 virus
SEQ ID NO: 78	Genome sequence of NG-1104 virus
SEQ ID NO: 79	Genome sequence of NG-1106 virus
SEQ ID NO: 80	Genome sequence of NG-1107 virus
SEQ ID NO: 81	Genome sequence of NG-1108 virus
SEQ ID NO: 82	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1109
SEQ ID NO: 83	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1110
SEQ ID NO: 84	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1111
SEQ ID NO: 85	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1112
SEQ ID NO: 86	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1113
SEQ ID NO: 87	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1114
SEQ ID NO: 88	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1115
SEQ ID NO: 89	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1116
SEQ ID NO: 90	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1117
SEQ ID NO: 91	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1118
SEQ ID NO: 92	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1119
SEQ ID NO: 93	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1120
SEQ ID NO: 94	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1121
SEQ ID NO: 95	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1122
SEQ ID NO: 96	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1123
SEQ ID NO: 97	Genome sequence of NG-1124 virus
SEQ ID NO: 98	Genome sequence of NG-1125 virus
SEQ ID NO: 99	DNA sequence of pUC57 CMV expression plasmid comprising the transgene cassette insert for NG-1126
SEQ ID NO: 100	Transmembrane protein sequence from human PDGFR Receptor A

SEQ ID NO: 101	Transmembrane protein sequence from human PDGFR Receptor B
SEQ ID NO: 102	Transmembrane protein sequence from human INSULIN-LIKE GROWTH FACTOR 1
SEQ ID NO: 103	Transmembrane protein sequence from human IL-6R
SEQ ID NO: 104	Transmembrane protein sequence from human CD28

EXAMPLES

EXAMPLE 1: Production of expression plasmids and viruses encoding bispecific proteins comprising a tumour cell binding antibody fused to a target ligand for CAR/TCR or other cell therapies

A series of transgene cassette designs were initially synthesized as pUC57 plasmids where expression of the transgene proteins is under the control of a CMV promoter, enabling initial studies of different bispecific proteins and transgene combinations to be evaluated by transient transfection experiments. In each transgene cassette, the cDNA encoding the protein sequences was flanked at the 5' end with a short splice acceptor sequence (SSA, SEQUENCE ID NO: 1). At the 3' end of the protein sequences, a SV40 late poly(A) sequence (PA, SEQUENCE ID NO: 4) was encoded. In viruses with more than one encoded transgene, the individual sequences were linked with a 2A ribosome skipping sequence (T2A, E2A, F2A or P2A,) (SEQ ID NOs: 5-8) to enable each individual protein to be translated and produced as separate chains. In some designs, a 2A sequence was also added to the final transgene in the cassette to serve as an epitope tag for analytical purposes. Schematics of the different transgene cassettes are shown in **Figure 1** and listed in **Table 1**.

Table 1

pUC57 Plasmid or Virus ID	Transgene Cassette
NG-611 virus (SEQ ID NO: 77 in WO2019/043020)	SSA ¹ -anti-EpCAMScFv-GS_linker-anti-CD3ScFv ² -HisTag ³ -PA ⁴ (SEQ ID 49)
NG-1100 (SEQ ID NO: 75)	SSA ¹ -anti-EpCAMScFv-GS_linker-RAET1G3 ⁵ -F2A ⁶ -PA ⁴ (SEQ ID 50)
NG-1101 (SEQ ID NO: 76)	SSA ¹ -anti-EpCAMScFv-GS_linker-RAET1G3 ⁵ -F2A ⁶ -CXCL9 ⁷ -T2A ⁸ -CCL21 ⁹ -E2A ¹⁰ -Sushi ¹¹ -P2A ¹² -IL-15 ¹³ -PA ⁴ (SEQ ID 51)
NG-1102 (SEQ ID NO: 77)	SSA ¹ -RAET1G3-GS_linker-anti-EpCAMScFv ¹⁴ -F2A ⁶ -PA ⁴ (SEQ ID 52)

NG-1104 (SEQ ID NO: 78)	SSA ¹ -anti-EpCAMScFv-GS_linker-anti-CD3ScFv ² -F2A ⁶ -CXCL9 ⁷ -T2A ⁸ -CCL21 ⁹ -E2A ¹⁰ -Sushi ¹¹ -P2A ¹² -IL-15 ¹³ -PA ⁴ (SEQ ID 53)
NG-1106 (SEQ ID NO: 79)	SSA ¹ -anti-EpCAMScFv-GS_linker-CD19 ¹⁵ -P2A ¹² -PA ⁴ (SEQ ID 54)
NG-1107 (SEQ ID NO: 80)	SSA ¹ -CD19-GS-linker-anti-EpCAMScFv ¹⁶ -P2A ¹² -PA ⁴ (SEQ ID 55)
NG-1108 (SEQ ID NO: 81)	SSA ¹ - CD19-TM ¹⁷ -P2A ¹² -PA ⁴ (SEQ ID 56)
NG-1109 (SEQ ID NO: 82)	SSA ¹ -CXCL9 ⁷ -T2A ⁸ -anti-EpCAMScFv-GS_linker-CD19 ¹⁵ -PA ⁴ (SEQ ID 57)
NG-1110 (SEQ ID NO: 83)	SSA ¹ -CXCL9 ⁷ -T2A ⁸ - CD19-GS-linker-anti-EpCAMScFv ¹⁶ -PA ⁴ (SEQ ID 58)
NG-1111 (SEQ ID NO: 84)	SSA ¹ -CXCL9 ⁷ -T2A ⁸ -anti-EpCAMScFv-GS_linker-RAET1G3 ⁵ -PA ⁴ (SEQ ID 59)
NG-1112 (SEQ ID NO: 85)	SSA ¹ -CXCL9 ⁷ -T2A ⁸ - RAET1G3-GS_linker-anti-EpCAMScFv ¹⁴ -PA ⁴ (SEQ ID 60)
NG-1113 (SEQ ID NO: 86)	SSA ¹ -anti-EpCAMScFv-FL_linker-CD19 ¹⁸ -PA ⁴ (SEQ ID 61)
NG-1114 (SEQ ID NO: 87)	SSA ¹ -anti-EpCAMScFv-RL_linker-CD19 ¹⁹ -PA ⁴ (SEQ ID 62)
NG-1115 (SEQ ID NO: 88)	SSA ¹ -CXCL9 ⁷ -P2A ¹² -anti-EpCAMScFv-FL_linker-CD19 ¹⁸ -PA ⁴ (SEQ ID 63)
NG-1116 (SEQ ID NO: 89)	SSA ¹ -CXCL9 ⁷ -P2A ¹² -anti-EpCAMScFv-RL_linker-CD19 ¹⁹ -PA ⁴ (SEQ ID 64)
NG-1117 (SEQ ID NO: 90)	SSA ¹ -CXCL9 ⁷ -P2A ¹² -anti-EpCAMScFv-FL_linker-RAET1G3 ²⁰ -PA ⁴ (SEQ ID 65)
NG-1118 (SEQ ID NO: 91)	SSA ¹ -CXCL9 ⁷ -P2A ¹² -anti-EpCAMScFv-RL_linker-RAET1G3 ²¹ -PA ⁴ (SEQ ID 66)
NG-1119 (SEQ ID NO: 92)	SSA ¹ -anti-EpCAMScFv-GS_linker-CD19 ¹⁵ -P2A ¹² -PA ⁴ (SEQ ID 67)

NG-1120 (SEQ ID NO: 93)	SSA ¹ -anti-EpCAMScFv-GS_linker-CD19m1 ²² -P2A ¹² -PA ⁴ (SEQ ID 68)
NG-1121 (SEQ ID NO: 94)	SSA ¹ -CD19-GS-linker-anti-HER2ScFv ²³ -P2A ¹² -PA ⁴ (SEQ ID 69)
NG-1122 (SEQ ID NO: 95)	SSA ¹ -CD19-GS-linker-anti-HER2ScFv ²³ -Myc ²³ -PA ⁴ (SEQ ID 70)
NG-1123 (SEQ ID NO: 96)	SSA ¹ -CD19m2-GS-linker-anti-HER2ScFv ²⁴ -P2A ¹² -PA ⁴ (SEQ ID 71)
NG-1124 (SEQ ID NO: 97)	SSA ¹ -anti-HER2ScFv-GS_linker-CD19m1 ²⁵ -P2A ¹² -PA ⁴ (SEQ ID 72)
NG-1125 (SEQ ID NO: 98)	SSA ¹ - CXCL9 ⁷ -E2A ¹⁰ -IFN α -T2A ⁸ -anti-HER2ScFv- GS_linker-CD19m1 ²⁵ -P2A ¹² -PA ⁴ (SEQ ID 73)
NG-1126 (SEQ ID NO: 99)	SSA ¹ -anti-HER2ScFv-GS_linker-CD19 ²⁶ -P2A ¹² -PA ⁴ (SEQ ID 74)

¹SEQ ID NO. 1; ²SEQ ID NO. 28; ³SEQ ID NO. 12; ⁴SEQ ID NO. 4; ⁵SEQ ID NO. 29 ⁶SEQ ID NO. 7; ⁷SEQ ID NO. 13; ⁸SEQ ID NO. 5; ⁹SEQ ID NO. 17; ¹⁰SEQ ID NO. 6; ¹¹SEQ ID NO. 19; ¹²SEQ ID NO. 8; ¹³SEQ ID NO. 21; ¹⁴SEQ ID NO. 30; ¹⁵SEQ ID NO. 32; ¹⁶SEQ ID NO. 33; ¹⁷SEQ ID NO. 34; ¹⁸SEQ ID NO. 39; ¹⁹SEQ ID NO. 40; ²⁰SEQ ID NO. 41; ²¹SEQ ID NO. 42; ²²SEQ ID NO. 43; ²³SEQ ID NO. 44; ²⁴SEQ ID NO. 46; ²⁵SEQ ID NO. 47; ²⁶SEQ ID NO. 48;

Virus Production

The plasmid pColoAd2.4 was used to generate the different viral vectors by direct insertion of transgene cassette sequences taken from pU57 plasmids by restriction enzyme digestion. The pColoAd2.4 plasmid was digested using AsiSI and SbfI restriction enzymes and each excised transgene cassette was directly ligated into the digested pColoAd2.4 plasmid. Construction of plasmid DNA for each viral vector was confirmed by restriction analysis and Sanger sequencing.

To generate viruses, the plasmids were linearised by restriction digest with the enzyme AscI to produce the virus genomes. The viruses were amplified and purified according to methods given below.

Digested DNA was purified by phenol/chloroform extraction and precipitated for 16±2hrs, -20°C in 600µl >95% molecular biology grade ethanol and 15µl 3M Sodium Acetate. The precipitated DNA was pelleted by centrifuging at 13000rpm, 5 mins and was washed twice in 500µl 70% ethanol. 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 HEK-293 cells grown to 70% confluency. After incubation of the cells with the

transfection mix for approximately 2hrs at 37°C, 5% CO₂ 4mls of cell media (DMEM high glucose with glutamine supplemented with 2% FBS) was added to the cells and the flasks was incubated 37°C, 5% CO₂.

The transfected HEK-293 cells were monitored every 24hrs and were supplemented with additional media as required. 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 the HEK-293 cells by three freeze-thaw cycles. The harvested viruses were used to re-infect HEK-293 cells 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 HEK-293 cells by three freeze-thaw cycles. The amplified stocks of viruses were used for further amplification before the viruses were purified by density gradient centrifugation to produce purified virus stocks.

EXAMPLE 2: Screening of bispecific protein designs for ability to bind to tumour cells *in vitro*.

With the purpose of verifying correct folding of bispecific proteins prior to producing viral vectors encoding them, transgene cassette designs NG-1100 to NG-1126 were created as pUC57 plasmid DNA vectors. These plasmids were transfected into A549 human lung tumour cells and cells cultured for 72 hours. Cell culture supernatants (SN) containing secreted bispecific proteins were collected and added to fresh untransfected A549 cells in suspension and incubated for 1.5 hours at room temperature. During this time, the bispecific proteins present in the SN can bind to the A549 cells through their anti-EpCAM ScFv or anti-HER2 ScFv portion. Binding to A549 was detected by flow cytometry through a) reduction of the mean fluorescence intensity (MFI) of EpCAM staining (due to competitive binding between the anti-EpCAM ScFv and the anti-EpCAM antibody clone 9C4); b) detection of the 2A peptide tag using an anti-P2A antibody for designs in which such a tag was present. For designs having CD19 as CAR-T target, the CD19 extracellular domain portion of the protein was detected by flow cytometry using an anti-CD19 antibody. For all these readouts, background level for signal intensity was established using SN from cells transfected with a control plasmid encoding an irrelevant protein.

We first tested a design encoding a transmembrane CD19 target protein, NG-1108. To verify transmembrane CD19 expression, A549 cells were transfected with a pUC57 plasmid encoding the NG-1108 transgene cassette (**SEQ ID NO: 56**) or a negative control plasmid and the transfected cells were stained with the anti-CD19 antibody by flow cytometry. High level transmembrane CD19 expression was detected on the pUC-1108-transfected A549 compared to controls (**Figure 3A**)

Data from a series of experiments with different sets of plasmids (**Figure 3B-F**) show that different secreted bispecific protein designs can bind to tumour cell surfaces. The data indicated that bispecific protein designs with anti-EpCAM ScFv as the tumour cell binding

component were only functional when the ScFv antibody portion was placed N-terminal to the antigen (RAET1G3 or CD19) but not when placed C-terminally, whereas the anti-Her2 ScFv antibody could function in either the N-terminal or C-terminal position.

EXAMPLE 3: Spreading of a virus-encoded bispecific protein from infected to uninfected tumour cells.

We hypothesized that when a secreted bispecific protein is encoded by a viral vector, the secreted protein will be able to bind both the infected tumour cells and nearby uninfected tumour cells, thus spreading the target antigen in the microenvironment. To demonstrate this in vitro, two different cell culture systems were employed. In the first model, A549 cells were infected with EnAd or NG-1124 (**SEQ ID NO: 97**) viral vector at 0.1 or 1 ppc for 7 days or at 10 or 50 ppc for 3 days and infection supernatants were collected and incubated with uninfected A549 or SKOV3 cells for 1.5 hours before cell were analysed for CD19 expression by flow cytometry. Presence of anti-HER2 ScFc-CD19m1 bispecific protein (**SEQ ID NO: 47**) in the SN was demonstrated by presence of positive CD19 staining on both SKOV3 and A549 in all the conditions tested (**Figure 4A&B**). By contrast, when CD19 is encoded in a virus as transmembrane protein rather than as part of a bispecific soluble molecule (as for NG-1108), CD19 expression is expected to be detected only in infected cells but not to be transferred to non-infected cells. Consistently, we found that CD19 was expressed on the membrane of A549 cells infected with NG-1108 (**SEQ ID NO: 81**) after 3 days from infection at 1 ppc. However, when infection supernatants from these cells were transferred and incubated with uninfected A549 cells, no CD19 was detected on the latter (Figure 4C).

In the second model, a cell type which easily allows virus replication and transgene expression during a short culture period, specifically A549 lung tumour cells, and a cell line poorly permissive to virus replication and transgene expression over this time frame, specifically SKOV3 ovarian cancer cells, were cultured and infected either separately or in a co-culture containing both cell types. Both cell lines were shown to express EpCAM and HER2 cell surface target antigens before use in the assay. The SKOV3 cells were labelled with Cell Trace Violet dye to allow them to be distinguished from the A549 cells using flow cytometry. Cells were infected with either EnAd, NG-1100 (**SEQ ID NO: 75**) or NG-1124 (**SEQ ID NO: 97**) viral vectors at 10 ppc, and after 3 days cells were harvested and analysed by flow cytometry to quantify bispecific protein binding to each cell type based on either the P2A tag or CD19 detection. Following NG-1100 infection, SKOV3 did not show any P2A signal above background (EnAd infection) when cultured and infected alone, due to the inability of the virus to produce enough transgene protein in this cell type in the time of the assay (**Figure 5A**). However, when cultured together with A549, about 13% of SKOV3 cells were positive for 2A staining, indicating transfer and binding of bispecific proteins produced by the A549 cells present in the culture (**Figure 5A**). As a control, A549 could produce bispecific proteins and show positivity for P2A staining even when cultured without the SKOV3, due to the high capability of A549 for viral

replication (**Figure 5B**). In a similar experiment, SKOV3 cells were shown not to produce or secrete any bispecific protein following NG-1124 (**SEQ ID NO: 97**) infection, however, when cultured together with A549 cells, SKOV3 cells could bind the bispecific construct (**SEQ ID NO: 47**) secreted by A549 cells, resulting in approximately 100% CD19 positivity of SKOV3 cells (**Figure 5C**). As a control, A549 could produce and bind bispecific protein and display positive CD19 signal both when cultured alone and in a co-culture with SKOV3 cells (**Figure 5D**).

EXAMPLE 4: Anti-CD19 CAR-T mediated cytotoxicity against SKOV3 and A549 tumour cells in presence of cell culture supernatants from NG-1124 infected cells.

The ability of anti-HER2 ScFv-CD19m1 fusion protein (**SEQ ID NO: 47**) encoded by NG-1124 (**SEQ ID NO: 97**) to engage anti-CD19 CAR-T cells and direct their cytotoxic activity against HER2-expressing tumour cells was assessed through Real Time Cytotoxicity Analysis (RTCA) using either SKOV3 or A549 cell lines as target cells. In assays using the SKOV3 cell line, cells were incubated in presence of cell culture supernatants (diluted 1:10 in culture medium) from A549 cells infected with either NG-1124 or EnAd at 10 ppc for 3 days. After 1.5 hrs incubation, anti-CD19 or control (Ctrl) T cells were added to SKOV3 tumour cells at a 3:1 T cell: tumour cell ratio and tumour cell death was monitored by RTCA using an xCELLigence instrument. Complete tumour cell death was set using control conditions with media containing 4% Tween20 (100% lysis control). Addition of anti-CD19 CAR-T cells to SKOV3 target cells in presence of SN from NG-1124 infection, but not from EnAd infection, resulted in complete killing of target cells in less than 24 hrs from CAR-T addition (**Figure 6A&B**). As a control, Ctrl T cells non-specifically pre-activated with anti-CD3/anti-CD28 antibodies did not show any detectable cytotoxicity in presence of NG-1124 infection SN (**Figure 6A&B**).

Since A549 tumour cells are highly susceptible to adenoviral replication and adenovirus-induced oncolysis, to assess CAR-T specific cytotoxicity against these cells, without any confounding virus-related cell death, cell culture supernatants with reduced viral content (VR SN) were generated for testing in CAR-T cell-A549 RTCA assays. To generate VR SN, we infected A549 cells with either NG-1124 or EnAd at 0.1 ppc for 7 days, collected the cell culture SN and filtered them through 300 KDa size exclusion columns to separate the viral particles from the remaining SN containing the bispecific protein. The filtration process had minimal effect on anti-HER2-CD19 protein content in the SN, as assessed by incubation of SN pre and post viral removal with A549 cells and detection of CD19 expression (**Figure 7A**). To assess the ability of the secreted anti-HER2-CD19 bispecific protein to engage anti-CD19 CAR-T cells against tumour cells, A549 cells were incubated for 1.5 hrs with VR SN, CAR-T cells or Ctrl T cells were then added at a 3:1 T cell:tumour cell ratio and tumour cell cytotoxicity was monitored through RTCA. Complete tumour cell death was defined as 100% lysis control in presence of 4% Tween20. Complete A549 cell death was observed less than 24 ours post CAR-T addition when cells were incubated in presence of NG-1124 VR SN containing anit-Her2 ScFv-CD19 protein, but not in

presence of EnAd VR SN or normal cell culture media (**Figure 7B-D**). Control activated T cells (generated as described for figure 5) showed low level non-specific killing activity independent from the presence or type of viral SN added.

These RTCA-based assays demonstrated that the anti-Her2 ScFv-CD19m1 bispecific construct (**SEQ ID NO: 47**) encoded by NG-1124 adenovirus (**SEQ ID NO: 97**) is functional and able to re-direct the cytotoxic activity of anti-CD19 CAR-T cells against HER2+ tumour cells that do not endogenously express CD19.

EXAMPLE 5: Impact of encoding multiple transgenes on bi-specific protein activity.

With the intent of maximizing the ability of adenoviruses to synergistically enhance cell therapy efficacy, a series of viruses encoding CAR-T-cell-targeting bispecific proteins together with further transgenes (e.g. chemokines, cytokines and other immunomodulators), that can provide additional signals to promote activity of cell therapies, such as CAR- or TCR T-cell therapies, were generated. To verify that these more complex designs were still able to effectively express bispecific proteins, A549 cells were infected with various transgene-encoding NG viruses or with EnAd at 0.1 ppc for 7 days. SN from cell cultures infected with NG-1100 (**SEQ ID NO: 75**), NG-1101 (**SEQ ID NO: 76**), NG-1124 (**SEQ ID NO: 97**), NG-1125 (**SEQ ID NO: 98**), NG-611 (**SEQ ID NO: 77** from patent filing WO2019/043020), NG-1104 (**SEQ ID NO: 78**) or EnAd were collected and incubated with uninfected A549 cells for 1.5 hrs, after which presence of bispecific protein binding to the cells was determined by flow cytometry detection of either P2A tag, CD19 antigen or OKT3 ScFv, depending on the viral design. For each staining, either an anti-2A peptide antibody, an anti-CD19 antibody or an anti- OKT3 ScFV antibody was employed, and fluorescence signal was considered positive when above background fluorescence measured in cells incubated with SN from EnAd infection. Surprisingly, we found that addition of two or more transgenes to the same transgene cassette encoding a bispecific protein resulted in a clearly increased bispecific protein expression as compared to the corresponding virus design encoding for the bispecific protein alone. Specifically: I) ~45% of A549 cells incubated with SN from NG-1101 (**SEQ ID NO: 76**) infection were positive for the 2A tag peptide (indicating binding of anti-EpCAM ScFv-RAET1G3 protein) compared to ~15% of cells incubated with NG-1100 SN ; II) ~12% of A549 cells incubated with SN from NG-1104 (**SEQ ID NO: 78**) infection showed a positive signal for anti-OKT3 ScFv (representative of anti-EpCAM-OKT3 ScFv protein binding) versus ~7.5% of cells incubated with NG-611 SN; III) ~55% of A549 cells incubated with SN from NG-1125 (**SEQ ID NO: 98**) infection were positive for CD19 staining (indicating binding of anti-HER2-CD19m1 protein) versus ~20% of cells incubated with NG-1124 SN (**Figure 8A**). Production of the other encoded transgenes (CXCL9, CCL21, IFN α & IL-15) following treatment with NG-1101, NG-1104 or NG-1125 was demonstrated by specific ELISA assays (**Figure 8B**).

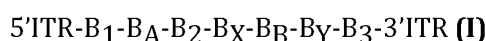
EXAMPLE 6: *In vivo* binding of NG-1125-encoded anti-HER2 ScFv-CD19 bispecific protein in A549 tumours. To investigate the efficiency of anti-HER2 ScFv-CD19m1 bispecific protein production and tumour cell-binding *in vivo*, NSG mice were inoculated subcutaneously with 5×10^9 A549 lung tumour cells to generate xenografts and, when tumours reached approximately 100-200mm³, the mice were dosed intravenously with 5×10^9 viral particles (VP) of either EnAd or NG-1125 (**SEQ ID NO: 98**) (administered at day 0 and 3). Mice were euthanized 9 days after the first virus dose and tumours processed into single cell suspension and analysed by flow cytometry. Tumour cells were gated as live (i.e. negative for LIVE/DEAD™ Fixable Aqua Dead Cell viability dye staining), CD45⁻ EpCAM⁺ cells. Based on intranuclear staining for adenoviral capsid protein, uninfected tumour cells (no positive adenovirus staining above negative isotype antibody control) could be distinguished from infected tumour cells with low VP load (dim fluorescence intensity for adenovirus staining) and infected tumour cells with high VP load (bright fluorescence intensity for adenovirus staining) (**Figure 9A**). Infected tumour cells with low and high VP load represented on average 20% and 5% of total tumour cells, respectively (**Figure 9B**). Within each tumour cell subset, we used surface CD19 staining to quantify the percentage of tumour cells that had bound NG-1125-encoded anti-HER2-ScFv-CD19m1 protein (**SEQ ID NO: 47**). Within both uninfected and infected tumour cell populations, we found a subset of tumour cells that were CD19⁺ (**Figure 9C**). These data indicated that anti-HER2 ScFv-CD19 bi-specific protein was effectively secreted *in vivo* by tumour cells infected by NG-1125 virus and could spread in the TME and bind to both infected and uninfected tumour cells, potentially allowing recognition and disruption of these cells by anti-CD19 CAR-T cells.

In a further *in vivo* study, NSG mice were inoculated subcutaneously with 5×10^6 A549 lung tumour cells to generate xenografts and, when tumours reached approximately 100-200mm³, the mice were dosed intravenously with 5×10^6 viral particles (VP) of either EnAd or NG-1125 (**SEQ ID NO: 98**) (administered at day 0 and 3). On day 6, the mice were dosed intravenously with 1.7×10^7 CD19-specific human CAR-T cells (generated and provided by ProMab Biotechnologies Inc, Richmond, CA, USA). The mice were then re-dosed with the two further intravenous injections of the same viruses at day 30 and day 34 before assessing the accumulation of the adoptively transferred human T-cells in tumours on day 50. Tumours were removed and gently disrupted to form cell suspensions and flow cytometry used to determine the numbers of total human CD45⁺ cells and of activated human CD8⁺ and CD4⁺ T cells expressing the CD107a degranulation marker, a biomarker for activation of functional cytotoxicity. **Figure 10** shows that NG-1125 (**SEQ ID NO: 98**), expressing the anti-HER2 ScFv-CD19m1 bispecific protein (**SEQ ID NO: 47**) together with human CXCL9 and human IFN α , led to a higher density of total human T-cells (A) and cytotoxically activated CD8⁺ (B) and CD4⁺ (C) T cells in the human tumour xenografts, compared to NG-1124 (**SEQ ID NO: 97**) which expresses just the CD19 bispecific protein (**SEQ ID NO: 47**) alone or the empty vector (EnAd).

Example 7: *In vivo* enhancement of recruitment of T-cells into tumors by NG-641 and NG-1125. In a further *in vivo* study, NSG immunodeficient mice were inoculated subcutaneously with A549 tumour cells as described in Example 6 and, when tumours reached approximately 100-200mm³, the mice were dosed intravenously with 5x10⁹ viral particles (VP) of either NG-641 (SEQ ID NO: 84 in WO2019/043020, NG-1125 (**SEQ ID NO: 98**) or the empty vector (EnAd), all administered on days 0, 3 and 5, or received no virus treatment. NG-641 expresses CXCL9 and CXCL10 chemokines, IFN α , and FAP-TAC, a bispecific molecule composed of an antibody fragment targeting human FAP fibroblast activation protein (FAP), which does not bind to mouse FAP (and human FAP is not expressed in the tumor microenvironment of A549 xenografts) linked to an anti-CD3 agonistic antibody fragment. In this model, the anti-FAP-anti-CD3 construct is expected to be inactive and therefore the NG-641 virus was used as “no antigen” control to account for the impact of CXCL9 and IFN α on CAR-T cell recruitment and activation independently from the presence of the CD19 antigen. On day 12, the mice were dosed intravenously with 2.5x10⁷ CD19-specific human CAR-T cells (generated and provided by ProMab Biotechnologies Inc, Richmond, CA, USA). Ten days after CAR-T cell injection, tumour-derived single cell suspensions and blood samples were analysed by flow cytometry to determine the frequency of human CD45⁺ cells (corresponding to all transferred T cells) and expression of the CD25 activation marker and CD107a degranulation marker, the latter used to identify actively cytotoxic T cells. **Figure 12** shows that both NG-641 and NG-1125 (**SEQ ID NO: 98**) dosing resulted in an increased intra tumoral density of total human CD45⁺ cells (**Figure 12A**) and activated human CD45⁺CD25⁺ (**Figure 12B**) and CD45⁺CD107⁺ (**Figure 12C**) cells compared to the EnAd and no virus dosing conditions, indicating an effect of CXCL9 and IFN α (expressed by both of these viruses) on CAR-T cell recruitment and activation in the tumor. NG-1125, which, alongside CXCL9 and IFN α , also expresses the anti-HER2 ScFv-CD19m1 bispecific protein (**SEQ ID NO: 47**) induced an overall higher frequency of total and activated human CD45⁺ cells in the tumors, indicating an additive effect of tumor-specific CD19 antigen expression over CXCL9 and IFN α -mediated CAR-T cell boosting, most likely as a consequence of antigen-specific engagement of the anti-CD19 CAR-T receptor (**Figure 12 A,B and C**). The frequency of human CD45⁺ T cells in the blood was higher in mice that received no viral vector as compared with NG-641, NG-1125 and EnAd-dosed mice (**Figure 12 D**), suggesting that virus infection alone can promote recruitment of circulating CAR-T cells into solid tumors, and (as shown in **Figure 12A-C**) that this can then be further enhanced by selected transgene expression.

CLAIMS

1. An oncolytic group B adenovirus suitable for treating a solid tumour (for example sarcoma, carcinoma and/or lymphoma) comprising a sequence of formula (I):



wherein:

B_1 is bond or comprises: E1A, E1B or E1A-E1B;

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

B_2 is a bond or comprises: E3;

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 is a DNA sequence encoding at least two transgenes namely a first transgene and a second transgene, for example under the control of the major late promoter; and

B_3 is a bond or comprises: E4

wherein:

- a first transgene encodes a polypeptide comprising a **target-sequence** specific for a binding domain on the cells of a cell-based immunotherapy, for example bearing a (exogenous) recombinant surface expressed protein, such as a chimeric antigen receptor or an NKG2D receptor, in particular wherein the target-sequence specifically binds to said surface expressed protein (more especially the chimeric antigen receptor) on the immunotherapy cell, and
- a second transgene encodes a polypeptide comprising a molecule that promotes trafficking of the cell-based immunotherapy into and within the tumour.

2. An oncolytic group B adenovirus according to claim 1, wherein expression of said first transgene increases the local concentration of the **target-sequence** within the tumour.
3. An oncolytic group B adenovirus according to claim 1 or 2, wherein the **target-sequence** is specific to a recombinant receptor on the immunotherapy cell
4. An oncolytic group B adenovirus according to any one of claims 1 to 3, wherein the **target-sequence** is a tumour antigen, for example CD20, CD19, CD22, CD33, CD34, CD37, CD38, CD47, CD52, CD56, CD70, CD74, CD133, CD138, CD147, CD152, CD221, CD254, CD261, CD262, CD309, CD340, BCMA, C-MYC, CAIX, Claudins [such as claudin 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and/or 24], EGFRvIII, EPHA3, Folate Receptor alpha [FR α] GPC3, WT1, CEA, MUC-1, EpCAM, MAGE, Mesothelin, PRAME, NYESO AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1 [EGFR], HER2, HER3, HER4, MCAM, PEM, A33, G250,

carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3, episialin, FOLR-1, 5T4, GPNMB, integrin α V β 3, integrin α 5 β 1, Lewis-Y antigen, MET [HGFR], mucin, PMSA, TAG-72, VEGFR, PDL1 [or an antigenic fragment of any one of the same] for example a tumour antigen, such as CD19, BCMA, CEA, Claudin-6, Claudin-18.2, EGFRvIII, FR α , GPC3, MCAM, Mesothelin, MUC-1, EpCAM, MAGE, PRAME, AFP, CA-125, ETA, tyrosinase, RAS, p53, HER receptors HER1, HER2, HER3, HER4, PEM, A33, G250, carbohydrate antigens Le^y, Le^x, Le^b, PSMA, TAG-72, STEAP1, CD166, CD24, CD44, E-cadherin, SPARC, ErbB2 and ErbB3 or an antigenic fragment of any one of the same.

5. An oncolytic group B adenovirus according to any one of claims 1 to 4, wherein the **target-sequence** is CD19 or an antigen fragment thereof.
6. An oncolytic virus according to any one of claims 1 to 5, wherein the **target-sequence** is a ligand (including an antibody or an antigen binding fragment thereof, such as an idiotypic antibody), for example a ligand (including an antibody binding domain) which activates signalling through a “native” receptor on the immunotherapy cell, such as NKG2D ligands (the latter interacting with native NKG2D on NK and CD8 T cells stimulates cytotoxic activity) or CD40L, OX40L, CD80, CD86, 4-1BBL (TNFSF9), CD70, LIGHT (TNFSF14), GITRL (TNFSF18), CD258 (HVEM, TNFSF14), ICOSL (B7-H2), interacting with their “native” receptors on immune cells, or wherein the target-sequence is a ligand (including an antibody binding domain) which inhibits signalling through a “native” receptor, such as PD1, TIM3, LAG3, VISTA, TIGIT, B7-H3, B7-H4, HVEM, ILT-2, ILT-3, ILT-4, BTLA, CD160 on the immunotherapy cell, for example PD1, TIM3, LAG3, VISTA, TIGIT on T-cells.
7. An oncolytic group B adenovirus according to any one of claims 1 to 6, wherein the first transgene encodes a membrane anchored form of the **target-sequence** suitable for expression on the surface of an infected cancer cell, in particular allowing the cell-based therapy to bind directly to the cancer cell, for example wherein the membrane anchored form comprises a transmembrane domain or GPI anchor.
8. An oncolytic group B adenovirus according to any one of claims 1 to 7, wherein the **target-sequence** is a non-human, for example a mouse or a yeast antigen, such as GCN4 or a fully synthetic molecule (in particular non-human origin) comprising and amino acid sequence selected for recognition by specific receptors embodied by the cell-based immunotherapy.
9. An oncolytic group B adenovirus according to any one of claims 1 to 8, wherein the **target-sequence** is a label, for example a HA-Tag (amino acids 98 to 106 of human influenza hemagglutinin), His-tag (for example comprising at least 6 histidine residues), FLAG-tag or a 2A peptide tag (such as P2A, T2A, E2A and/or F2A).

10. An oncolytic group B adenovirus according to any one of claims 1 to 9, wherein the first transgene encodes a fusion protein comprising:
 - a. the **target-sequence** which binds and activates the cell-based immunotherapy, and
 - b. a first binding protein specific to a protein expressed on a cancer cell, on a stromal cell, or in stromal tissue, in particular allowing the cell-based immunotherapy to indirectly bind the cancer cell and/ or stromal cells or stromal tissue through said fusion protein
11. An oncolytic group B adenovirus according to claim 10, wherein the virus encodes at least two fusion proteins, wherein the first binding protein in part b) is different in each fusion protein, for example where one fusion protein is encoded by the first transgene and the second fusion protein is encoded by the second transgene.
12. An oncolytic group B adenovirus according to claim 11, wherein the **target-sequence** of part a) is the same for said at least two fusion proteins i.e. both fusion proteins bind the same entity on the immunotherapy cells.
13. An oncolytic group B adenovirus according to any one of claims 11 or 12, wherein the **target-sequence** of part a) is different in said two fusion proteins (i.e. said fusion proteins bind different entities on the same or different immune cells).
14. An oncolytic group B adenovirus according to any one of claims 10 to 13, wherein the binding protein of part b) is a ligand, for example to a protein or receptor found on the cancer and/or stromal cells.
15. An oncolytic group B adenovirus according to any one of claims 1 to 14, wherein encoding the second transgene increases the effectiveness of the cell-based immunotherapy.
16. An oncolytic group B adenovirus according to claim 15, wherein a further transgene (for example the second transgene or a third transgene) increases the effectiveness of the of the cell-based immunotherapy by modulating the tumour microenvironment (for example blocking inhibitory features of the tumour microenvironment), for example wherein the microenvironment is modulated to render it more pervasive to the cell-based therapy and/or to render the microenvironment more inflammatory.
17. An oncolytic group B adenovirus according to any one of claims 1 to 36, wherein the second transgene or a further transgene encodes a chemokine to aid recruitment of immunotherapy cells into the tumour.
18. An oncolytic according to any one of claims 1 to 17, wherein a further transgene encodes a synthetic protein designed to engage with additional recombinant receptors expressed by the cells of the therapy to enhance their ability to enter and act within the tumour and/or their survival within the patient, for example Ortho IL-2 as synthetic ligand for ortho IL-2R β (synthetic receptor – e.g. Zhang et al 2021 Sci. Transl. Med. 13 (625) eabg6986 incorporated herein by reference; TIM3/CD28 switch receptors in CAR-T interacting with secreted TIM3 ligands e.g.

galectin 9, HMGB1) which may be engineered as fusions with antibody fragments so they bind tumour cells (Zhao et al 2021 J. Immunotherapy Cancer 9, e003176 incorporated herein by reference); synNotch fusion receptors on CAR-T (e.g. the CAR is an anti-CD19 scFv coupled to IC domain of Notch to signal responses in engineered CAR when they recognize CD19 (including wherein the CD19 is in a fusion protein, such as anti-HER2 ScFv-CD19 fusion protein) – Roybal et al 2016 Cell 167(2), 419-432 incorporated herein by reference.

19. An oncolytic group B adenovirus according to any one of claims 1 to 18, where the cells expressing the (exogenous) recombinant antigen receptor are selected from the group comprising or consisting of: T-cells (T), macrophages (Mac), natural killer cells (NK), natural killer T-cells (NKT) or innate lymphoid cells (ILC).
20. An oncolytic group B adenovirus according to any one of claims 1 to 19, where the exogenous recombinant antigen receptor is a chimeric antigen receptor (CAR) or a T-cell receptor (TCR).
21. An oncolytic group B adenovirus according to any one of claims 1 to 42, for use in treatment, in particular for use the treatment of cancer, such as solid tumours, for example where cancer is transformed epithelial cancer cells.
22. An oncolytic group B adenovirus according to any one of claims 1 to 20, for use in the manufacture of a medicament for the treatment of cancer, such as solid tumours, in particular transformed epithelial cancer cells.
23. A method of treating a patient comprising administering a therapeutically effective amount of an oncolytic group B adenovirus as defined in any one of claim 1 to 20, in particular for the treatment of cancer, more specifically a solid tumour, such as transformed epithelial cancer cells.
24. A combination therapy comprising an oncolytic group B adenovirus according to any one of claims 1 to 20 and an engineered immunotherapy cell, for example for the treatment of cancer.
25. A method of producing a virus according to any one of claims 1 to 20, wherein the virus is replicated in a host cell, for example a mammalian cell, such as a HEK cell.
26. A virus obtainable from claim 25.