Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3123973 A1 2020/04/16

(21) 3 123 973

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/10/10
(87) Date publication PCT/PCT Publication Date: 2020/04/16
(85) Entrée phase nationale/National Entry: 2021/04/06
(86) N° demande PCT/PCT Application No.: GB 2019/052877
(87) N° publication PCT/PCT Publication No.: 2020/074902
(30) Priorité/Priority: 2018/10/10 (GB1816547.2)

(51) Cl.Int./Int.Cl. *A61K 39/285* (2006.01), *A61K 38/20* (2006.01), *A61K 39/00* (2006.01)

(71) Demandeur/Applicant:
QUEEN MARY UNIVERSITY OF LONDON, GB

(72) Inventeurs/Inventors:
WANG, YAOHE, GB;
YUAN, MING, GB

(74) Agent: LEUNG, JASON C.

(54) Titre : VIRUS DE LA VACCINE ONCOLYTIQUE AVEC GENE B5R MODIFIE POUR LE TRAITEMENT DU CANCER
(54) Title: ONCOLYTIC VACCINIA VIRUS WITH MODIFIED B5R GENE FOR THE TREATMENT OF CANCER

(57) Abrégé/Abstract:
The present invention relates to a vaccinia virus vector comprising a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$SCR3$^-$ SCR4$^-$) inserted into the TK gene of the vaccinia virus. The invention also relates to compositions comprising the vaccinia virus vector, methods of treatment using the compositions, medical uses of the compositions and kits comprising the vaccinia virus vector. The invention also relates to a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) of vaccinia virus.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 April 2020 (16.04.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/074902 A1

(51) International Patent Classification:
A61K 39/285 (2006.01) *A61K 39/00* (2006.01)
A61K 38/20 (2006.01)

(21) International Application Number:
PCT/GB2019/052877

(22) International Filing Date:
10 October 2019 (10.10.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1816547.2 10 October 2018 (10.10.2018) GB

(71) Applicant: **QUEEN MARY UNIVERSITY OF LONDON** [GB/GB]; Mile End Road, London E1 4NS (GB).

(72) Inventors: **WANG, Yaohe**; Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, Ground Floor, John Vane Science Centre, London Greater London EC1M 6BQ (GB). **YUAN, Ming**; Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, Ground Floor, John Vane Science Centre, London Greater London EC1M 6BQ (GB).

(74) Agent: **BASSIL, Nicholas Charles**; Kilburn & Strode LLP, Lacon London, 84 Theobalds Road, London Greater London WC1X 8NL (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: ONCOLYTIC VACCINIA VIRUS WITH MODIFIED B5R GENE FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to a vaccinia virus vector comprising a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$SCR3$^-$ SCR4$^-$) inserted into the TK gene of the vaccinia virus. The invention also relates to compositions comprising the vaccinia virus vector, methods of treatment using the compositions, medical uses of the compositions and kits comprising the vaccinia virus vector. The invention also relates to a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) of vaccinia virus.

WO 2020/074902 A1

ONCOLYTIC VACCINIA VIRUS WITH MODIFIED B5R GENE FOR THE TREATMENT OF CANCER

The present disclosure relates to modified oncolytic viruses for use in the treatment of cancer.

Despite advances in new therapeutics, the survival rates for patients with many solid tumour types remain as one of the biggest challenges. More effective therapeutics are in much need. Oncolytic viruses are attractive therapeutics for treatment of cancers that are resistant to conventional therapies (Wong et al., Viruses 2, 78-106 (2010)).

Oncolytic viruses are viruses that can specifically target and kill cancer cells. Additionally, oncolytic viruses provide the danger signals essential for induction and amplification of the host's anti-tumour immune response.

Vaccinia virus (VV) is a virus with double stranded DNA bearing many features that make it an attractive candidate for oncolytic therapy (Al Yaghchi C. et al., Immunotherapy 7(12):1249-58 (2015)). It can replicate rapidly in the cells, spread efficiently within tumours and is able to lyse infected cells. Additionally, VV has been studied extensively and has a well-defined molecular biology with a large cloning capacity and a variety of natural and synthetic promoters making it ideal as a vector for carrying heterologous nucleic acid sequences. VV has well established safety profile subsequent to its use for the eradication of small pox, and as such, treatments for uncontrolled infections are readily available. Furthermore, the hypoxic microenvironment commonly found in solid tumours is detrimental to the replication and efficacy of many types of oncolytic viruses, but VV replicates effectively in this environment (Hiley et al., Gene Therapy 17, 281-287 (2010)). Early clinical results using either vaccine strains or genetically modified strains have demonstrated antitumor effects (Haddad et al., Annals of Surgical Oncology 19 Suppl 3, S665-674 (2012); Park et al., Lancet Oncol 9:533-542, 2008; Breitbach et al., Nature 477:99-102, 2011)).

Various deletion mutants of vaccinia virus have been reported. Western Reserve strain mutants with the deletion of the thymidine kinase (TK) gene and the viral growth factor (VGF) gene are capable of efficiently priming the immune system against tumour antigens (McCart et al., Cancer Res 61, 8751-8757 (2001)). In addition, arming the virus with heterologous genes for example cytokine encoding genes can further activate the anti-tumour immune response.

Release of VV from infected cells via cell lysis enables the virus to infect more cells locally and distantly via circulating blood. There are two major forms of infectious VV, intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Appleyard et al., J. Gen. Virology 13, 9-17 (1971)), IMV is the first infectious form of virus and represents the majority of infectious progeny. EEV is coated with host cellular proteins and is relatively quiet antigenically, with the ability to antagonise innate (complement) and adaptive (neutralising antibodies) systemic host defences that permits widespread and long-distance dissemination within the host (Smith, G.L. & Vanderplasschen, A. &

Law, M., J. Gen. Virol. 83, 2915-2931 (2002); Payne, L.G. & Kristensson, K, J. Gen. Virol. 66 (3), 643-646 (1985)). However, EEV is produced only in low numbers by most strains of VV (<1% of all infectious progeny).

Six genes are known to encode EEV-specific proteins. These are A56R, F13L, B5R, A34R, A36R and A33R. B5R, encodes a 42-kDa glycoprotein which contains four copies of a 50- to 70-amino-acid repeat called 'short consensus repeat' (SCR). Deletion of B5R leads to a small plaque size and a huge decrease (≤10-fold) in EEV formation (Blasco, R. & Moss, B., J. Virol. 65, 5910-5920 (1991); Engelstad, M. & Smith, G.L., Virology 194, 627-637 (1993)). Sequences within the transmembrane and cytoplasmic tail of B5R are important for targeting the protein to the wrapping membrane (Katz et al., J. Virol. 71, 3178-3187 (1997)). Mutant VV with deletion of SCR4, SCR3,4 or SCR 2,3,4 generate small plaques, but produce approximately 50-fold more infectious EEV than wild-type virus and formed comet-shaped plaques (Sanderson et al., J. Gen. Virol. 79 (6), 1415-1425 (1998); Mathew et al., J. Virol. 72, 2429-2438 (1998)).

Despite the progress that has been made in the field of oncolytic viruses, no therapeutic product based on vaccinia has yet reached the market. There is therefore an unmet need for more effective forms of oncolytic vaccinia virus for use in the treatment of cancer.

According to a first aspect of the invention there is provided a vaccinia virus vector comprising a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) inserted into the TK gene of the vaccinia virus.

The nucleic acid sequence may be natural, synthetic or recombinant. It may, for example, be cDNA, PCR product or a genomic sequence. It may be isolated, or as part of a plasmid, vector or host cell. A plasmid is a circular extrachromosomal DNA molecule with the ability to replicate independently of chromosomal DNA.

The term "nucleic acid," in its broadest sense, refers to any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. In some embodiments, "nucleic acid" refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising individual nucleic acid residues. As used herein, the terms "oligonucleotide" and "polynucleotide" can be used interchangeably. In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA and/or cDNA. Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, i.e., analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

The term “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and/or encode the same amino acid sequence. Nucleotide sequences that encode proteins and/or RNA may include introns. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc. Where appropriate, e.g., in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, etc. A nucleic acid sequence is presented in the 5′ to 3′ direction unless otherwise indicated. The term “nucleic acid segment” is used herein to refer to a nucleic acid sequence that is a portion of a longer nucleic acid sequence. In many embodiments, a nucleic acid segment comprises at least 3, 4, 5, 6, 7, 8, 9, 10, or more residues. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2′-fluororibose, ribose, 2′-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5′-N-phosphoramidite linkages). In some embodiments, the present invention is specifically directed to “unmodified nucleic acids,” meaning nucleic acids (e.g., polynucleotides and residues, including nucleotides and/or nucleosides) that have not been chemically modified in order to facilitate or achieve delivery.

The B5R gene of vaccinia virus has an open reading frame (ORF) which encodes a membrane protein that is essential for EEV formation. Deletion of the B5R ORF results in a dramatic reduction of EEV, and as a consequence, the virus produces small plaques in vitro and is highly attenuated in vivo. The extracellular portion of B5R is composed mainly of four domains that are similar to the short consensus repeats (SCRs) present in complement regulatory proteins. The partial B5R gene of the invention suitably comprises nucleic acid regions encoding the signal peptide (SP), the stalk region (STALK), the transmembrane region (TM) and the cytoplasmic tail (CT) of the native B5R protein of vaccinia virus.

The partial B5R gene may be present in the form of an expression cassette with an upstream promoter to drive expression. The nucleic acid sequence of the first aspect may therefore be part of an expression cassette. An expression cassette may be part of a vector. It comprises a promoter, an open reading frame and a 3’ untranslated region. A promoter is a region of DNA with a specific sequence that initiates the transcription of a particular gene or genes. Promoters used for the expression of heterologous genes in vaccinia include promoters controlling early and late transcriptional activity, for example mH5, H5, P7.5 and PE/L. A heterologous gene, as used herein,

is a gene that is not normally found in the virus. The modified H5 promoter, mH5, has a predominantly early activity and shows greater stability than the naturally occurring H5.

Suitably, the promoter may be an H5 promoter or p7.5 Early/late synthetic or pH5R promoter. The expression cassette may further comprise a reporter protein, such Red Fluorescent Protein (RFP). A second promoter may be provided upstream of the reporter protein and used to drive expression of the reporter protein, such as an H5 promoter or p7.5 Early/late synthetic or pH5R promoter.

In an embodiment of the first aspect, the nucleic acid sequence may be present in a vector. A vector as used herein refers to a construct for introducing a nucleic acid sequence into a cell or a virus for expression or replication. It refers to a recombinant construct for example a plasmid, a virus or any other construct capable of expression or replication of the nucleic acid sequence upon introduction into a cell or virus.

A plasmid may therefore be used to introduce an expression cassette into a host cell. Plasmids may also be used to express a polypeptide in a host cell. For example, a host cell may be transfected with a plasmid capable of encoding a particular polypeptide, in order to express that polypeptide.

The expression cassette may be inserted into the Thymidine Kinase (TK) gene of a vaccinia virus. Any convenient site-specific recombination or gene insertion technology may be used. Suitably, the insertion is made using homologous recombination. Any suitable homologous recombination system may be used, such as for example Cre-Lox, or Flp/FRT systems. For example, the integration sites may be added to the expression cassette around a nucleic acid sequence encoding a reporter protein and upstream of the promoter region driving expression of the partial B5R gene.

In a preferred embodiment of the invention the vaccinia virus vector comprises a nucleotide sequence that is substantially homologous to the sequence set forth in any one of Figure 31. Nucleic acid sequences with greater than 20% identity (for example 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99%) are considered to be homologous sequences. As used herein, substantially homologous refers to sequences exhibiting at least 60% or 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95%, 96%, 97%, 98%, 99% or greater identity. In an embodiment of the invention the sequence has at least 80% or more (e.g., 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) homology with the sequence set forth in any one of Figure 31.

As used herein, the terms homology and identity are interchangeable.

Sequence comparisons to determine homology can be carried out using readily available sequence comparison software. Examples include but are not limited to BLAST (see Ausubel et al., 1999 Short Protocols in Molecular Biology, 4th Ed - Chapter 18) and FASTA (Altschul et al., 1990 J. Mol. Biol. 403-410). Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, Short Protocols in Molecular Biology, pages 7-58 to 7-60).

In one embodiment of the invention the vaccinia virus vector comprises a nucleotide sequence that has at least 80% or more (e.g., 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) homology with the sequence set forth in any one of Figure 31.

As used herein, the term “identity” refers to the overall relatedness between polymeric molecules, e.g., between nucleic acid molecules (e.g., DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two nucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes).

In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or substantially 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4: 11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM 120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix.

In one embodiment of the invention the vaccinia virus vector comprises a nucleotide sequence that has at least 80% or more (e.g., 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) identity with the sequence set forth in any one of Figure 31.

As used herein, the term “isolated” refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or

manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. As used herein, calculation of percent purity of isolated substances and/or entities should not include excipients (e.g., buffer, solvent, water, etc.).

As used herein, a "polypeptide", generally speaking, is a string of at least two amino acids attached to one another by a peptide bond. In some embodiments, a polypeptide may include at least 3-5 amino acids, each of which is attached to others by way of at least one peptide bond. Those of ordinary skill in the art will appreciate that polypeptides sometimes include "non-natural" amino acids or other entities that nonetheless are capable of integrating into a polypeptide chain, optionally.

As used herein, the term "protein" refers to a polypeptide (i.e., a string of at least two amino acids linked to one another by peptide bonds). Proteins may include moieties other than amino acids (e.g., may be glycoproteins, proteoglycans, etc.) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete polypeptide chain as produced by a cell (with or without a signal sequence), or it can be a characteristic portion thereof. Those of ordinary skill will appreciate that a protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. Polypeptides may contain 1-amino acids, d-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, e.g., terminal acetylation, amidation, methylation, etc. In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. The term "peptide" is generally used to refer to a polypeptide having a length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or less than 10 amino acids. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

There are multiple strains of vaccinia with varying levels of virulence for humans and animals. A number of strains of the virus were used around the world as part of the smallpox eradication programme in the 1950s. Different strains were used in different areas of the world, for example, the New York City Board of Health (NYCBOH) strain and its derivative, Wyeth, were popular in the United States, whereas Copenhagen (CPN) and Lister strains were predominant in Europe. In a preferred embodiment the vaccinia strain is the Lister strain.

The vaccinia virus vector is a thymidine-kinase deficient (TK-deficient) vaccinia virus. A TK-deficient vaccinia virus as used herein refers to a vaccinia virus that shows a phenotype consistent with a lack of endogenous thymidine kinase (TK). A TK-deficient vaccinia virus is dependent on thymidine kinase produced by the host cell. Thymidine kinase is constitutively produced in tumour cells but not in normal cells. A TK-deficient vaccinia virus can therefore survive selectively in tumour cells, especially with activation of EGFR/Ras/ERK pathways.

In order to target the TK gene (L090) of vaccinia virus for homologous recombination, the expression cassette used to create the vector of the invention may be provided with additional sequences complementary to the TK gene and/or the gene adjacent to the TK gene. For example, the additional sequences may be provided on the upstream and downstream ends of the expression cassette respectively. In other words, a TK left arm (L-arm) may be provided to target the left side (L089) of the TK gene and a TK right arm (R-arm) to target the right side (L091) of the TK gene. The actual expression cassette comprising the partial B5R gene and gene insertion elements may then be located between TK L-arm and TK-R arm ready for insertion into the TK region of the vaccinia virus. The expression cassette may be suitably inserted into a shuttle vector prior to transformation of the vaccinia virus vector.

The expression cassette may therefore be composed of the following nucleic acid sequence elements:

INT-PRO-REP-INT-PRO-SP-STC

where INT is a gene insertion element, PRO is an optional promoter, REP is an optional nucleic acid sequence encoding a reporter protein, SP is a nucleic acid sequence encoding a signal peptide and STC is a nucleic acid encoding a partial B5R gene of the invention as defined above. The optional promoter is only present when the sequence encoding optional reporter protein is present. The signal peptide (SP) domain will not appear in the protein expressed by the recombinant virus since the SP (signal peptide) directs the modification of the partial B5R gene (STC) after protein synthesis in a cell infected by the virus. The signal protein (SP) will be cleaved from the STC after the partial B5R protein is expressed and subject to post-translational modification.

In one embodiment, the expression cassette may be composed of the following elements:

Loxp-H5-RFP-Loxp-H5-SP+STC

where the gene insertion element is a Loxp site, the promoter is an H5 promoter, and the optional reporter protein is RFP.

The nucleic acid sequence encoding the SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) is therefore an exogenous sequence which is inserted into the TK gene of the vaccinia virus.

Inactivation as described herein refers to silencing of the gene at the transcriptional or post transcriptional level, deletion of the gene, mutation in the gene, disruption of the gene by insertion of a nucleic acid sequence or any other method that renders the virus unable to create a fully functional gene product. Inactivation of a gene can be partial or complete. As described herein, the inactivation of the TK gene in the vaccinia virus of the invention is caused by insertion of a nucleic acid sequence encoding the partial B5R gene. The insertion can be facilitated by homologous recombination as described herein.

The vaccinia virus vector of the invention can be prepared as described above by means of insertion of the nucleic acid sequence encoding the partial B5R gene into the TK gene of the vaccinia virus. The insertion of the partial B5R nucleic acid sequence may be achieved by any suitable method of site-specific recombination. However, homologous recombination may be a generally applicable method for this purpose.

In one embodiment, the native B5R gene of the vaccinia virus vector may remain intact.

The vaccinia virus vector of the invention may further comprise additional nucleic acid sequences encoding further proteins. Suitably the protein may be a biologically active protein, i.e. it has a therapeutic effect, and/or it may be a reporter protein. It may be convenient for the nucleic acid sequence encoding the protein(s) to inserted into the vaccinia virus vector by site-specific recombination.

Any convenient site-specific recombination or gene insertion technology may be used. Suitably, the insertion is made using homologous recombination. Insertion of a nucleic acid sequence into a target sequence can be facilitated by methods well known to the person skilled in the art. For example, methods described in Molecular Cloning, A Laboratory Manual, Second Edition, by J. Sambrook, E. F. Fritsch and T. Maniatis (2003), Cold Spring Harbor Laboratory Press, Virology Methods Manual, edited by Brian W J Mahy and Hillar O Kangro (1996) Academic Press and Expression of genes by Vaccinia virus vectors. Current Protocols in Molecular Biology, published by John Wiley and Son (1998), Chapter 16.

Any suitable homologous recombination system may be used, such as for example Cre-Lox, or Flp/FRT systems for removing reporter genes. The gene insertion elements may be added to the expression cassette around the nucleic acid sequence encoding the protein(s) and upstream of the promoter region driving expression of the protein(s).

It may be suitable for further heterologous protein(s) to be inserted into the N1L gene of the vaccinia virus vector. In order to target the N1L gene (L025) of vaccinia virus for homologous recombination, the expression cassette may be provided with additional sequences complementary to the N1L gene and/or the gene adjacent to the N1L gene. For example, the additional sequences may be provided on the upstream and downstream ends of the expression cassette respectively. In other words, a N1L left arm (L-arm) may be provided to target the left side of the N1L gene and/or the next gene (L024) and a N1L right arm (R-arm) to target the right side of the N1L gene and/or the next gene (L026). The actual expression cassette comprising the nucleic acid sequence(s) encoding the protein(s) and gene insertion elements may then be located between N1L L-arm and N1L R-arm ready for insertion into the N1L gene of the vaccinia virus. The expression cassette may be suitably inserted into a shuttle vector prior to transformation of the vaccinia virus vector.

The expression cassette may therefore be composed of the following nucleic acid sequence elements:

INT-PRO-REP-INT-PRO-X

where INT is a gene insertion element, PRO is an optional promoter, REP is an optional nucleic acid sequence encoding a reporter protein, and X is nucleic acid sequence encoding a heterologous protein, for example a biologically active protein. The optional promoter is only present when the sequence encoding optional reporter protein is present. The heterologous protein may be a therapeutic protein with an anti-cancer activity, e.g. a cytostatic, cytotoxic, or immunogenic activity.

A polypeptide as used herein refers to a plurality of amino acid residues joined together by peptide bonds. It is used interchangeably with protein, peptide, oligopeptide and includes glycoproteins and derivatives thereof. The term “polypeptide” is also intended to cover analogues and derivatives of polypeptides which retain the same biological function or activity as the original polypeptide. A heterologous polypeptide as used herein refers to any polypeptide that is not normally expressed by the virus in nature. The heterologous polypeptide can be biologically active. A biologically active polypeptide as used herein refers to a polypeptide that has a biological function or activity.

The heterologous protein may be a cytokine or a protein which acts to neutralise suppressive cytokines in order to promote anti-tumour immunity, for example an antibody or antibody fragment such as fragment antigen-binding (Fab) or single-chain variable fragment (scFv), a cytokine receptor or cytokine receptor fragment.

The terms "antibody" and "immunoglobulin" are used herein interchangeably. An antibody molecule is made up of two identical heavy (H) and two identical light (L) chains held together by disulphide bonds. Each heavy chain comprises an Fc polypeptide. The two Fc polypeptides from the two

heavy chains dimerise to form the Fc region of the antibody molecule. The term "Fc region" refers to the constant region of an antibody excluding the first constant region immunoglobulin domain of the heavy chain (CH1) that interacts with the constant portion of the light chain (CL) forming a CH1 -CL domain pair. Thus, Fc region comprises the last two constant region immunoglobulin domains (CH2 and CH3) of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM (CH2, CH3 and CH4), Any polypeptide of the various immunoglobulin constant domains may therefore be used in accordance with the present invention as a dimerisation domain.

Several antibody effector functions are mediated through the binding of the Fc region to Fc receptors (FcR) found on the surface of many cells for example lymphocytes, macrophages, natural killer cells, etc. FcRs are defined by their specificity for antibody isotypes. For example, Fc receptors for IgG antibodies are referred to as FcγR.

An "antibody fragment" as referred to herein means any portion of a full length antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, scFv, Fv, dsFv diabody and Fd fragments.

The term "single chain variable fragment" or "scFv" refers to an Fv fragment in which the heavy chain domain and the light chain domain are linked. One or more scFv fragments may be linked to other antibody fragments (such as the constant domain of a heavy chain or a light chain) to form antibody constructs having one or more antigen recognition sites.

The heterologous protein may be a cytokine selected from the group consisting of IL-21, GM-CSF, IL-2, IL-7, IL-12, IL-15, IL-18 and IFN-α, or any combinations thereof. Other suitable heterologous proteins include antigen presentation enhancing molecule (HSP96), or immune checkpoint blocking inhibitors such as PD-L1 or anti-PD-1 inhibitors (for example anti-PD-L1 or anti-PD-1 antibodies). Example anti-PD-L1 antibodies include atezolizumab, avelumab and durvalumab. Example anti-PD-1 antibodies include pembrolizumab, nivolumab, pidilizumab and cemiplimab. Other suitable PD-L1 or anti-PD-1 inhibitors include fusion proteins of fragments of antibodies, e.g. the Fc domain of an antibody fused to the extracellular domain of the PD-1 ligand programmed cell death ligand 2 (PD-L2), such as AMP-224. Other immune checkpoint blocking inhibitors include PD-1, PD-L1, TIM-3 or CTLA-4, or any combinations thereof. The immune checkpoint blocking inhibitor molecules may be soluble. Immune checkpoint blocking inhibitors can also be referred to as immune checkpoint blocking molecules.

The terms "antibody" and "immunoglobulin" are used herein interchangeably. An antibody molecule is made up of two identical heavy (H) and two identical light (L) chains held together by disulphide bonds. Each heavy chain comprises an Fc polypeptide. The two Fc polypeptides from the two heavy chains dimerise to form the Fc region of the antibody molecule. The term "Fc region" refers to the constant region of an antibody excluding the first constant region immunoglobulin domain of

the heavy chain (CH1) that interacts with the constant portion of the light chain (CL) forming a CH1 -CL domain pair. Thus, Fc region comprises the last two constant region immunoglobulin domains (CH2 and CH3) of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM (CH2, CH3 and CH4), Any polypeptide of the various immunoglobulin constant domains may therefore be used in accordance with the present invention as a dimerisation domain.

Several antibody effector functions are mediated through the binding of the Fc region to Fc receptors (FcR) found on the surface of many cells for example lymphocytes, macrophages, natural killer cells, etc. FcRs are defined by their specificity for antibody isotypes. For example, Fc receptors for IgG antibodies are referred to as FcyR.

An "antibody fragment" as referred to herein means any portion of a full length antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, scFv, Fv, dsFv diabody and Fd fragments.

The term "single chain variable fragment" or "scFv" refers to an Fv fragment in which the heavy chain domain and the light chain domain are linked. One or more scFv fragments may be linked to other antibody fragments (such as the constant domain of a heavy chain or a light chain) to form antibody constructs having one or more antigen recognition sites.

In one embodiment, the expression cassette encoding the heterologous protein may be composed of the following elements:

FRT-H5-RFP-FRT-H5-X

where the gene insertion element is a FRT site, the promoter is an H5 promoter, and the optional reporter protein is RFP. In some further embodiments, the heterologous protein (X) may be a cytokine, for example an interleukin, such as IL-21, IL-15, IL-12 or GM-CSF.

In a further embodiment, the cytokine may be an IL-15/Receptor fused gene. The IL-15/Receptor fused gene may encode an IL-15 / IL-15 Receptor fusion protein. For example, the fusion protein may be a soluble IL-15/IL-15Ralpha complex. Without being bound by theory, IL-15 has substantial potential as an immunotherapeutic agent for augmenting immune responses. However, the activity of IL-15 is mediated by a unique mechanism in which the cytokine is transpresented by cell-bound high-affinity IL-15Ralpha to target cells expressing the IL-15Rbeta and the common gamma-chain. Thus, the efficacy of administered IL-15 alone may be limited by the availability of free IL-15Ralpha. Soluble IL-15/IL-15Ralpha complexes may greatly enhance IL-15 half-life and bioavailability in vivo, thus maximizing IL-15 activity.

References to IL-12 herein include IL-12A (for example GenBank Accession no. AF404773.1 GI:15128214) and/or IL-12B (for example GenBank Accession no. AY008847.1 GI:11192034). The mature IL-12 protein includes both subunits. References to IL-21 herein include isoform 1 (for example GenBank Accession no. NP_068575.1 / GI:11141875) and/or isoform 2 (GenBank Accession no. NP_001193935.1 / GI:333033767). References to GM-CSF herein include GenBank Accession no. AF373868.2 / GI:14278709. References to IL-15 herein include GenBank Accession no. U14407.1. Generally, the sequences are human sequences.

Reporter polypeptide as used herein refers to a polypeptide whose expression is indicative of the presence of the nucleic acid sequence, expression cassette or vector in a host cell or virus. Examples of reporter polypeptides include but are not limited to fluorescent polypeptides, chemiluminescent polypeptides, bioluminescent polypeptides, phosphorescent polypeptides as well as enzymes.

In an embodiment of the invention the reporter polypeptide is a fluorescent polypeptide. Fluorescent polypeptides include but are not limited to green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein and their derivatives.

However, in clinical use for the treatment of cancer in patient, it is preferred that the expression cassette does not include a reporter protein.

Restriction sites are specific nucleotide sequences that are recognised and cleaved by restriction enzymes. Examples of restriction enzymes are SalI, BglII, HindIII, SmaI, BamHI and MluI. A BamHI restriction site is a restriction site recognised by BamHI. The restriction sites for other enzymes are similarly named.

In an embodiment of the invention, the nucleic acid sequence or vector comprises one or more restriction sites. A preferred embodiment of the invention is a nucleic acid sequence or vector comprising SalI, BglII, HindIII, SmaI, BamHI and MluI restriction sites.

In an embodiment of the invention the nucleic acid sequence or vector is comprised within a vaccinia virus. In a specific embodiment of the invention the nucleic acid sequence has the formula shown in any one of Figure 31.

According to a second aspect of the invention there is provided a composition comprising a vaccinia virus vector of the first aspect of the invention. In an embodiment of the invention according to this aspect the composition optionally comprises a pharmaceutically acceptable carrier, diluent or excipient. The invention therefore includes a pharmaceutical composition as described herein.

The composition may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), topical (including buccal, sublingual or transdermal), or parenteral (including subcutaneous, intramuscular, intravenous, intra-arterial, intra-thecal, intra-pleural, intra-ophthalmological, intra-cardiac, intraperitoneal or intradermal) route.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents.

Excipients which may be used for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The compositions may be presented in unit-dose or multidose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carried, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

According to a third aspect of the invention there is provided a method of treatment comprising administering a composition of the second aspect of the invention to a subject in need thereof for the treatment of cancer. The composition may be optionally formulated with a pharmaceutically acceptable adjuvant, diluent and/or buffer. The composition may therefore be formulated as a pharmaceutical composition.

Embodiments of this aspect of the invention extend to a composition comprising a vaccinia virus vector as defined herein for use in the treatment of cancer. Such embodiments include a nucleic acid sequence or a vaccinia virus vector as defined herein in the manufacture of a medicament for use in the treatment of cancer.

As used herein, a subject refers to an animal, including a human being. An animal can include mice, rats, fowls such as chicken, ruminants such as cows, goat, deer, sheep and other animals such as pigs, cats, dogs and primates such as humans, chimpanzees, gorillas and monkeys.

A therapeutically effective amount is the dose sufficient to induce oncolysis. Doses for delivery and administration can be based upon current existing protocols, empirically determined, using animal disease models or optionally in human clinical trials. Initial study doses can be based upon animal studies set forth herein, for a mouse, for example. Doses can vary and depend upon whether the treatment is prophylactic or therapeutic, the type, onset, progression, severity, frequency, duration, or probability of the disease to which treatment is directed, the clinical endpoint desired, previous or simultaneous treatments, the general health, age, gender, race or immunological competency of the subject and other factors that will be appreciated by the skilled artisan. The dose amount, number, frequency or duration may be proportionally increased or reduced, as indicated by any adverse side effects, complications or other risk factors of the treatment or therapy and the status of the subject. The skilled artisan will appreciate the factors that may influence the dosage and timing required to provide an amount sufficient for providing a therapeutic or prophylactic benefit.
In an embodiment of this aspect of the invention, the method further comprises administering to the subject an additional cancer therapy. Cancer therapy as used herein refers to refers to treatment of cancer by any medical or physical means. The additional cancer therapy can be chemotherapy, biological therapy, radiotherapy, immunotherapy, hormone therapy, anti-vascular therapy, cryotherapy, toxin therapy and/or surgery, including combinations thereof.

Methods and uses of the invention as disclosed herein can be practiced immediately or days, months or years after a subject has been identified as having the disease targeted for treatment.

The methods include administering the virus at different schedules. A single dose of the virus may be administered to a subject or a tumour over a 1, 2, 5, 10, 15, 20, or 24-hour period. The virus may be administered over 1, 2, 3, 4, 5, 6, 7 or more days or weeks. The interval between injections can be 1, 2, 3, 4, 5, 6, 7 days or weeks. Typically, multiple doses are administered to the same general target region, such as in the proximity of a tumour or in the case of intravenous administration a particular entry point in the blood stream or lymphatic system of a subject. The vaccinia virus vector may be administered 2, 3, 4, 5, or more times. The vaccinia virus vector could be given before resection of tumours at different schedule and doses.

The methods and uses may also include administering one, two, three or four different embodiments of the virus separately, subsequently or simultaneously. A first virus comprising a nucleic acid sequence encoding a first heterologous protein and a second virus comprising a nucleic acid sequence encoding a second heterologous protein may be administered separately, subsequently or simultaneously. The interval between subsequent administration of the different embodiments of the virus may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or 24 hours.

For example, a first virus comprising a nucleic acid sequence encoding an immune checkpoint inhibitor may be administered subsequent to a second virus comprising a nucleic acid sequence encoding a cytokine.

The immune checkpoint inhibitor protein may be PD1, PD-L1, TIM-3 or CTLA-4, or any combinations thereof. The immune checkpoint inhibitor protein may be soluble.

The cytokine may be IL-21, GM-CSF, IL-2, IL-7, IL-12, IL-15, IL-18 and IFN-α, or any combinations thereof.

In one embodiment, a vaccinia virus vector in which the biologically active protein is soluble PD1 is administered subsequently to a vaccinia virus vector in which the biologically active protein is IL-12.

The methods and uses may also include administering a virus and a heterologous protein separately, subsequently or simultaneously. The interval between subsequent administration may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or 24 hours. For example, an antibody may be administered subsequent to a virus comprising a nucleic acid sequence encoding a cytokine.

The separate, subsequent or simultaneous administration of the first or second aspect of the invention or the heterologous protein may be achieved by any convenient route whereby the administration is intravenously, intraperitoneally, intramuscularly, orally, intranasally or subcutaneously. The first or second aspect of the invention and the heterologous protein may be prepared as a combined preparation or may be prepared as separate components.

The methods include administering the virus at different viral concentrations. In certain aspects, the subject is administered at least $5x10^7$, $1x10^8$, 2×10^8, 5×10^8, 1×10^9,2×10^9, 5×10^9, 1×10^{10}, 5×10^{10}, 1×10^{11}, 5×10^{11}, 1×10^{12} or more viral particles or plaque forming units (pfu), including the various values and ranges there between. The viral dose can be administered in 0.1mL, 1mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL or more, including all values and ranges there between. The dose may be spread over time or by separate injection.

In certain embodiments the subject is a human with cancer and/or a tumour. The cancer may be a gastrointestinal cancer, a respiratory tract cancer, a genitourinary tract cancer, a hematopoietic cancer, a sarcoma, an adenocarcinoma, a squamous cell carcinoma or a non-malignant tumor/hyperplasia. The tumour may be non-resectable prior to treatment and resectable after treatment. The tumour can be a recurrent, primary, metastatic, and/or multi-drug resistant tumour. In certain aspects the tumor is located on or in the pancreas. In other aspects, the tumour can be a neuroendocrine tumour, an endocrine tumour, a peripheral central nervous system tumour, a brain cancer tumor, a head and neck cancer tumor, an esophageal cancer tumour, a skin cancer tumor, a lung cancer tumor, a liver tumour, a thymic tumor, a stomach cancer tumor, a colon cancer tumour, an ovarian cancer tumor, a uterine cancer tumor, a bladder cancer tumor, a testicular cancer tumour, a bladder tumour, a rectal cancer tumour, melanoma or a breast cancer tumour.

The compositions and methods disclosed in the present invention may be used in different types of gene therapy for example tumor suppressor gene therapy, suicide gene therapy, viral vector

immunisation strategies, anti-angiogenic therapy, pro-apoptosis gene therapy and gene replacement therapy. “Oncolytic Viruses for Cancer Therapy: Overcoming the Obstacles” (Wong et al. Viruses 2010, 2, 78-106) is incorporated herein by reference in its entirety.

The compositions and methods disclosed in the present invention may be used in combination with additional therapeutic means or methods in the treatment of cancer, for example surgery, chemotherapy, radiation therapy, molecular cancer therapy or a further gene therapy, which may be used for administering genes that are different from the herein described nucleic acids of the invention.

According to a fourth aspect of the invention there is provided a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1^{-} SCR2^{-} SCR3^{-} SCR4^{-}) of vaccinia virus.

According to a fifth aspect of the invention there is provided a kit comprising a vaccinia virus vector as defined herein and a pharmaceutically acceptable adjuvant, diluent and/or buffer. The kit may therefore be useful in the treatment of cancer. The kit may further comprise a further therapeutic agent, such as a chemotherapeutic agent, and/or instructions for use.

The present disclosure describes a novel recombinant vaccinia virus expression vector and its application in cancer treatment and vaccination. The inventive vector (named VVL15TK STC) is derived from a vaccine strain Lister vaccinia virus that has been safely used in hundreds of millions of people for prevention of small pox, more particularly comprising deletion of one viral gene (Thymidine kinase gene). This novel mutant VV results in 10-30-fold more infectious EEV produced than its parental virus while the total replication of the mutant VV is not attenuated.

In one embodiment of this disclosure, a replication-competent Lister strain vaccinia virus (VV15 TK-STC) is described comprising a part of B5R gene (STC) in thymidine kinase (TK) region and intact B5R gene, which shows a better comet tail formation and EEV production with normal size of plaques. The partial B5R gene (STC) can be inserted into any non-vital region of vaccinia virus genome with B5R gene intact, such a modification would render the modified virus the same features as VV15 TK-STC. VV15 TK-STC virus has an enhanced anti-tumour efficacy in vivo. VV15 TK-STC virus armed with therapeutic gene IL-21 improves anti-tumour immunity. VV15 TK-STC virus can carry any other therapeutic gene, such as IL-12. The VV15 TK-STC virus can be administered locally (intra-tumoural injection) and systemically (intravenous injection).

In one embodiment of this disclosure, a novel VV vector is described which is derived from a Lister strain VV, with particular manipulations of viral genes, insertion of a partial B5R gene (STC) in the thymidine kinase region and its use for cancer treatment.

Introducing a mutant B5R gene (STC) into the TK region with retention of the original B5R gene at its original position creates a virus that produces normal plaque sizes in vitro, which is important for better virus spreading. This modification results in the production of more EEV, which benefits the ability of the virus for long range dissemination. Thus, this modification enhances the virus's anti-tumour potency and creates a virus more suitable for intravenous delivery, as shown in the present disclosure.

Intravenous delivery of oncolytic viruses is desirable for improved anti-tumour efficacy in vivo as i.v. delivered virus can be expected to more readily target metastatic and circulating tumour cells, in addition to the primary tumour within the patient. A key advantage of the present disclosure is that the oncolytic virus can be delivered more effectively intravenously. The mutant virus described herein has an enhanced ability to form EEV which is further advantageous since EEV forms of Vaccinia virus are inherently more resistant to immune-mediated virus clearance. The present disclosure shows that a tumour tropic (by virtue of TK and N1L deletions) VV can be modified to enhance EEV formation by incorporation of a second copy of the STC region of the viral B5R gene within the modified VV, while maintaining expression of the original, full length B5R region. This disclosure represents a step forward for i.v.-deliverable oncolytic virotherapies, which have yet to demonstrate efficacy when delivered i.v. clinically. The modified virus can also incorporate further transgenes to enhance immune responses against the tumor at both the primary and metastatic sites.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

The present invention will now be described by way of reference to the accompanying drawings with are included for the purposes of illustration only and are not to be construed as being limitations to the invention.

FIGURE 1 shows the expression cassette in the pGEMT-B5R-STC and pGEMT-B5R S-STC shuttle vector. The top panel shows the structure of B5R protein and the expression cassette in the pGEMT-B5R STC and S-STC shuttle vector. The expression STALK-TM-CT(STC) containing signal peptide (SP) and SCR1-STALK-TM-CT(S-STC) containing signal peptide (SP). The bottom panel is the expression cassette in the pGEMT-B5R STC shuttle vector, both the marker gene RFP and the STALK-TM-TC (STC) are driven by H5 promotor. The expression STALK-TM-CT(STC) containing signal peptide (SP) is driven by the H5 promoter and SCR1-STALK-TM-CT(S-STC) containing signal peptide (SP) is driven by the native B5R promoter, the marker gene RFP is driven by H5 promotor. SP=the signal peptide of B5R gene. Homologous recombination section of pGEMT-B5R-STC and pGEMT-B5R S-STC shuttle vector. TK L arm is to target the left side (L089) of the TK

gene, TK R arm is to target the right side (L091) of the TK gene. Expression cassette is located between TK L arm and TK R arm.

FIGURE 2 shows a comparison of the plaque and comet tail formation of vaccinia viruses in vitro. CV-1 cells in 6-well plates were infected by diluted control virus VVL15, recombinant VVL15 B5R-STC, recombinant VVL15 B5R S-STC. Three days post infection, the infected cells were stained using crystal violet as described previously. The plates were photo scanned.

FIGURE 3 shows expression cassette and TK-STC shuttle vector and homologous recombination. Top panel: The first H5 promoter (from left) drives the RFP expression, the second H5 promoter drives the STC expression. SP: signal peptide (from B5R); STC: Stalk (S), TM (T), CT (C). TM is the transmembrane domain of B5R protein, CT is the cytoplasmic tail of B5R protein. Bottom panel: Homologous recombination section of TK-STC shuttle vector. TK left arm (L-arm) is to target the left side (L089) of the TK gene, TK right arm (R-arm) is to target the right side (L091) of the TK gene. Expression cassette (Loxp-H5-RFP-Loxp-H5-SP+STC) is located between TK L-arm and TK-R arm in the TK region.

FIGURE 4 shows confirmation of VVL15 TK-STC virus. A: VVL15 RFP virus. B: VVL15 TK-STC virus. Final round of VVL15 TK-STC plaque purification confirmed that all colonies within a well of CV1 cells were expressed RFP. The specific primer pairs to amplify the STC gene and part of the downstream TK gene by PCR from viral DNA extracted from CV1 lysates were used. The PCR product was absent in the VVL15 RFP DNA but present in the VVL15 TK-STC DNA, which indicates a TK gene deletion in the VVL15 TK-STC and the presence of STC in the TK region. Forward primer on SP of B5R gene and reverse primer on B5R cytoplasmic tail were used in the PCR to amplify the whole B5R gene and mutant B5R STC. Full length B5R was present in VVL15 RFP and VVL15 TK-STC, while mutant SP-STC only was present in VVL15 TK-STC as expected.

FIGURE 5 shows a comparison of the plaques of vaccinia viruses. CV-1 cells in 6-well plates were infected by diluted control virus VVL15 RFP, recombinant VVL15 TK-STC and overlayed with agarose gel. Three days later, the agarose gel was removed and the cells stained using crystal violet. Plates were photo scanned shown on the left. The size of plaques was measured using Image J shown on the right.

FIGURE 6 shows a comparison of the plaque and comet tail formation of vaccinia viruses. CV-1 cells in 6-well plates were infected by control virus VVL15 RFP and recombinant VVL15TK STC. Three days post infection, the cells were stained using crystal violet and the plates photo scanned.

FIGURE 7 shows EEV production by VVL15 RFP and VVL15TK STC viruses in CV1 cell line. 0.01 PFU/cell VV was used to infect CV-1 cells in 6-well plates. Cell culture medium and the infected cells were collected into separate tubes 48 and 72 hours post infection and viruses were titrated respectively. The ratio of EEV produced was calculated by comparing the total amount of virus recovered from the cell culture medium with the total amount of virus recovered from infected cells.

FIGURE 8 shows EEV production by VVL-DD and VVL-DD STC viruses in pancreatic cancer cell lines. 0.01 PFU/cell VV was used to infect cells in 6-well plates. Cell culture medium and the infected cells were collected into separate tubes 18, 24 and 48 hours post infection and viruses were titrated respectively. The ratio of EEV produced was calculated by comparing the total amount of virus recovered from the cell culture medium with the total amount of virus recovered from infected cells. TB11831: mouse pancreatic cancer cell line, STUIT-2 and MIA PaCa-2 are human pancreatic cancer cell lines.

FIGURE 9 shows EEV production by VVL-DD and VVL-DD STC viruses in lung cancer cell lines. 0.01 PFU/cell VV was used to infect cells in 6-well plates. Cell culture medium and the infected cells were collected into separate tubes 18, 24 and 48 hours post infection and viruses were titrated respectively. The ratio of EEV produced was calculated by comparing the total amount of virus recovered from the cell culture medium with the total amount of virus recovered from infected cells. LLC: mouse lung cancer cell line. H460, H1299MIA and A549 are human lung cancer cell lines.

FIGURE 10 shows replication VVL15 RFP and VVL15TK STC viruses in CV1 cell line. Viral titres in PFU/cell were determined by performing TCID50 assays on viral lysates collected at 24, 48 and 72 hours after infection with 1 PFU/ tumour cell. Each assay was performed in triplicate.

FIGURE 11 shows a comparison of lytic potency between VVL15 RFP and VVL15TK STC viruses. MTS assays were performed to measure the cytotoxic potency of VVL15 RFP and VVL15TK STC viruses on cell lines CV1, CT26 and DT6606. The graph is a plot of EC50 values (virus doses used to kill 50% of the cells) taken from corresponding viral dose-response (percentage cell death) curves (not shown).

FIGURE 12 shows VVL15TK STC was more efficacious than VVL15 RFP in a colon cancer model. Syngeneic CT26 subcutaneous flank models were established in Balb/C immunocompetent mice. When tumour volumes reached an average of 100 mm^3, daily doses (5 in total) of $1x10^8$ PFUs of virus or the equivalent volume of vehicle buffer (50 µl of

PBS) were injected IT (n=5-7 per group). Tumour growth was followed up via twice weekly calliper measurement.

FIGURE 13 shows VVL15TK STC was more efficacious than VVL15 RFP in a pancreatic cancer model. Syngeneic DT6606 subcutaneous flank models were established in immunocompetent C57/Bl6 mice. When tumour volumes reached an average of 100 mm^3, daily doses (5 in total) of $1x10^8$ PFUs of virus or the equivalent volume of vehicle buffer (50 µl of PBS) were injected IT (n=5-7 per group). Tumour growth was followed up via twice weekly calliper measurement.

FIGURE 14 shows expression cassette and N1L shuttle vector expressing mIL-21 or hIL-21 and homologous recombination. Modified VV TK-STC with RFP excised by Cre recombinase was used for making a virus expressing a cytokine transgene in the N1L region. Expression of mIL-21 or hIL-21 and RFP in the N1L shuttle vector was driven by the H5 promoter. RFP and its promoter H5 was flanked by FRT, thus RFP and its promoter H5 can be excised by Flipase acting on FRT in the final modified virus to create a modified, marker-free virus.

FIGURE 15 shows confirmation of hIL-21 expression in the VTK-STC hIL-21 virus. The expression of hIL-21 in CV-1 cells was measured by ELISA three days post infection of wild-type VV (VV WT), TK-STC VV (CTRL), VVL12 N1L hIL-21(hHIL21) and TK STC hIL-21.

FIGURE 16 shows confirmation of mIL-21 expression in the TK-STC mIL-21 virus. The expression of mIL-21 in DT6606 cells at indicated post infection time was measured by ELISA All the viruses used are TK and N1L gene deleted. mIL21 is expressed in the cells after virus infection. d=deletion.

FIGURE 17 shows VV presence within DT6606 tumours established subcutaneously in immunocompetent C57/Bl6 mice. Once palpable, mice were treated with CAL101 (10mg/Kg) by oral gavage followed 3 hours later by intravenous injection using $1x10^8$ PFU/injection VVLΔTKΔN1L that doesn't contain a modified second copy of B5R, or VVLΔTK-STCΔN1L. Treatments were given on days 1,3 and 5. 5 days following the last treatment, tumours were excised and viral load analysed using qPCR (n=3/group). A Students unpaired T test was used to compare viral load in the two groups (*p>0.05).

FIGURE 18 shows SHPC6 tumours established intra-peritoneally in immunocompetent Syrian hamsters. Hamsters were treated with $1x10^7$ PFU/injection VVLΔTKΔN1L or VVLΔTK-STCΔN1L on days 4,6 and 8 post tumour implantation. Kaplan-Meier survival analysis with Log rank (Mantel-cox) tests were used to assess survival (n=10/group).

FIGURE 19 shows SHPC6 tumours established intra-peritoneally in immunocompetent Syrian hamsters. Hamsters were treated with 1x10^7 PFU/injection VVLΔTK-STCΔN1L and VVLΔTK-STC-ΔN1L-hIL21 on days 4,6 and 8 post tumour implantation. Kaplan-Meier survival analysis with Log rank (Mantel-cox) tests were used to assess survival (n=10/group).

FIGURE 20 shows treatment of cancer by combination of Cal101 (a transient inhibitor of macrophage) and VV. Cal 101 was administered by oral gavage 3 hours prior to i.v. injection of vaccinia virus, PBS, TK STC control virus (STC ctrl) and TK STC mIL-21 (STC VVl21). Tumour size was measured twice a week.

FIGURE 21 shows treatment of cancer by combination of Cal101, anti-PD1 antibody and VV. Cal 101 was administered by oral gavage 3 hours prior to i.v. injection of PBS, TK STC control virus (STC ctrl) and TK STC mIL-21 (STC VVl21). Tumour size was measured twice a week. VVL-DD is also named as VVDTK-DN1L, VVL-DD STC is also named as VVDTK-STC-DN1L.

FIGURE 22 shows confirmation of mIL-12 expression in the TK-STC mIL-12 virus. The expression of mIL-12 in Suit-2 cells was measured by ELISA three days post infection of TK-STC vaccinia virus (dTK-STC-dN1L), TK-STC mIL-12(dTK-STC-dN1L-mIL-12). VVL-DD is also named as VVDTK-DN1L, VVL-DD STC is also named as VVDTK-STC-DN1L.

FIGURE 23 shows confirmation of hIL-12 expression in the TK-STC hIL-12 virus. The expression of mIL-12 in CF Pac1 cells was measured by ELISA three days post infection of TK-STC vaccinia virus (dTK-STC-dN1L), TK-STC hIL-12(dTK-STC-dN1L-hIL-12). VVL-DD is also named as VVDTK-DN1L, VVL-DD STC is also named as VVDTK-STC-DN1L.

FIGURE 24 show LLC lung cancer tumours established subcutaneously in immunocompetent C57/Bl6 mice. Once palpable, mice were treated with PBS intratumorally on days 1,3,5,7,9,11 or αPD-1 antibody administered intraperitoneally at 200μg/injection on days 1,4,7 or intertumoral injections of 1x108PFU/injection VVLΔTK-STCΔN1L –mIL12 on days 1,3,5 (followed by intraperitoneal injections of PBS on days 7,9,11) or VVLΔTK-STCΔN1L-mIL12 on days 1,3,5 followed by intraperitoneal injections of αPD-1 antibody on days 7,9,11). Tumour growth was monitored and is shown until the death of the first animal in each group. A two-way ANOVA with Bonferroni post-test was used to compare the significance at each timepoint.

FIGURE 25 show LLC lung cancer tumours established subcutaneously in immunocompetent C57/Bl6 mice. Once palpable, mice were treated with PBS

intratumorally on days 1,3,5,7,9,11 or αPD-1 antibody administered intraperitoneally at 200µg/injection on days 1,4,7 or intertumoral injections of 1x108PFU/injection VVLΔTK-STCΔN1L –mIL12 on days 1,3,5 (followed by intraperitoneal injections of PBS on days 7,9,11) or VVLΔTK-STCΔN1L-mIL12 on days 1,3,5 followed by intraperitoneal injections of αPD-1 antibody on days 7,9,11). Kaplan-Meier survival analysis with Log rank (Mantel-cox) tests were used to assess survival (n=10/group).

FIGURE 26 shows LLC lung cancer tumours established subcutaneously in immunocompetent C57/Bl6 mice. Once palpable, mice were treated with PBS intratumorally on days 1,3,5,7,9,11 or αPD-1 antibody administered intraperitoneally at 200µg/injection on days 1,4,7 or intertumoral injections of 1x108PFU/injection VVLΔTK-STCΔN1L –mIL12 on days 1,3,5 (followed by intraperitoneal injections of PBS on days 7,9,11) or VVLΔTK-STCΔN1L-mIL12 on days 1,3,5 followed by intraperitoneal injections of αPD-1 antibody on days 7,9,11) or VVLΔTK-STCΔN1L –mIL12 on days 1,3,5 followed by VVLΔTKΔN1L-sPD1 (that expressed soluble PD1) on days 7,9,11. Tumour growth was monitored and is shown until the death of the first animal in each group.

FIGURE 27 shows CMT64 lung cancer tumours established subcutaneously in immunocompetent C57/Bl6 mice. Once palpable, mice were treated with PBS intratumorally on days 1,3,5,7,9,11 or with 1x108PFU/injection VVLΔTK-STCΔN1L –mIL12 on days 1,3,5 followed with VVLΔTKΔN1L-sPD1 on days 7,9,11 or vice versa. Tumour growth was monitored and is shown until the death of the first animal in each group.

FIGURE 28 shows the nucleic acid sequence for the construct B5R-S-STC from Figure 1 including the corresponding amino acid sequence encoded by the nucleic acid sequence. The construct B5R-S-STC comprises the domains SP-SCR1-STALK-TM-CT-H5-RFP as shown. (a) The signal peptide domain SP consists of amino acid residues 1-19. (b) The domain SCR1 consists of amino acid residues 20-72. (c) The domain STALK consists of amino acid residues 237-275. (d) The transmembrane domain TM consists of amino acid residues 276-303. (e) The C-terminal domain CT consists of amino acid residues 304-317. The numbering of the amino acid residues in the construct B5R-S-STC is given with respect to the amino acid sequence of the native B5R protein encoded by the unmodified B5R gene. (f) The H5 promoter has the nucleic acid sequence as indicated. (g) The Red Fluorescent Protein (RFP) expressed by the construct when present consists of amino acid residues 1-225 encoded by the nucleic acid sequence as indicated. The domain H5-RFP may be replaced by a single H5 promoter. (h) SP(aa 1-19)-SCR1(aa 20-72)-STALK(aa 237-275)-TM(aa 276-303)- CT(aa 304-317).

FIGURE 29 shows the nucleic acid sequence for the construct B5R-STC from Figure 1 including the corresponding amino acid sequence encoded by the nucleic acid sequence.

The construct B5R-STC comprises the domains (H5-RFP-H5)-SP-STALK-TM-CT as shown. The optional domain H5-RFP-H5 consists of 2 copies of the nucleic acid sequence of the H5 promoter (a) and (c) as shown either side of a nucleic acid sequence encoding the Red Fluorescent Protein (RFP) (b) expressed by the construct when present consists of amino acid residues 1-225 encoded by the nucleic acid sequence as indicated. The domain H5-RFP-H5 may be replaced by a single H5 promoter. (d) The signal peptide domain SP consists of amino acid residues 1-19. (e) The domain STALK consists of amino acid residues 237-275. (f) The transmembrane domain TM consists of amino acid residues 276-303. (g) The C-terminal domain CT consists of amino acid residues 304-317. The numbering of the amino acid residues in the construct B5R-STC is given with respect to the amino acid sequence of the native B5R protein encoded by the unmodified B5R gene.

FIGURE 30 shows the nucleic acid sequence for the construct TK-STC from Figures 2 and 3 including the corresponding amino acid sequence encoded by the nucleic acid sequence. The construct comprises the domains (Loxp-H5-RFP-Lox-H5)-SP-STALK-TM-CT. The domain (Loxp-H5-RFP-Lox-H5) consists of two Loxp elements (a) and (d), an H5 promoter (b) and (e) and an optional nucleic acid sequence encoding Red Fluorescent Protein (RFP) (c) expressed by the construct when present consisting of amino acid residues 1-225. The Loxp elements and H5 promoter have the nucleic acid sequences shown. (f) The signal peptide domain SP consists of amino acid residues 1-19. (g) The domain STALK consists of amino acid residues 237-275. (h) The transmembrane domain TM consists of amino acid residues 276-303. (i) The C-terminal domain CT consists of amino acid residues 304-317. The numbering of the amino acid residues in the construct TK-STC is given with respect to the amino acid sequence of the native B5R protein encoded by the unmodified B5R gene.

FIGURE 31 shows (a) the nucleic acid sequence for the construct STC and (b) the corresponding amino acid sequence encoded.

Materials and Methods:

Cell Lines: All tumour cell lines used were stored in our lab, either from ATCC or Cancer Research UK Cell line Service Unit or kindly provided by or collaborators. All human cancer cell lines were genotyped by STR assay. The murine tumour cell lines used in this study include: The colorectal cancer cell line CT26 was derived from the BALB/c strain. DT6606 (pancreatic carcinoma) originated from a C57BL/6 strain transgenic mouse with mutations in the K-Ras and p53 genes conditional to the pancreas. This was a kind gift of Professor David Tuveson (CRUK, Cambridge Research Institute, Cambridge, UK). CV1 is an African Green Monkey “normal” kidney cell line obtained from the ATCC, VA, USA and was used as a stock cell line to facilitate the mass production of viruses as well as in all viral titration assays.

Viruses: VVL15 was constructed by the insertion of the lacZ reporter and the firefly luciferase genes into the TK region of the Lister vaccine strain of vaccinia virus (VV Lister) under the control of the synthetic early/late and p7.5 promoters respectively (Hung, C.F. et al., Gene Ther 14, 20-29 (2007)) - using an in vitro intracellular recombination technique previously described. VVL15 TK-RFP was constructed previously (data not published) with RFP replacing TK gene.

Construction of VVL15 B5R-STC shuttle vector:

RFP was amplified by PCR from DsRed plasmid (Clontech) with H5-RFP forward primer (5'-AGATCTAAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAA ATAATCATAAATAGCTACCGGACTCAGATCCA-3') (BglII is underlined) and H5 RFP reverse primer (5'-ACGCGTCCCGGGAAGCTTTATTTATGATTATTTCTCGCTTTCAATTTAACACAACC CTCAAGAACCTTTGTATTTATTTTCAATTTTTCGCCTTAAGATACATTGATGAG-3') (M1UI, SmaI and HindIII sites are underlined). SP+STC+B5R right arm was amplified by PCR from WR-STC genome (from Rafeal Blasco, Spain) with left arm forward primer (5'-AAGCTTAAATAAAA ATGAAAACGATTTCC-3') (HindIII site is underlined) and right arm reverse primer (5'-CCCGGGGAATTCAGATCTTTTTATTTATGAGCGTTAAAAATAGTATA-3') (SmaI and BglII sites are underlined). B5R left arm was amplified from VV lister genome with forward primer (5'-TATACTGCGTGTATGACCG-3') and reverse primer (5'-CCCGGGGAATTCAGATCTTTTTA TTTATGAGCGTTAAAAATAGTATA-3') (SmaI and BglII sites are underlined). All PCR products were cloned into pGEMT-easy vector (Promega) following the manufacturer's instructions. Correct sequences were verified by sequencing. pGEMT-easy-B5R left arm was linearized with BglII and SmaI restriction enzymes. H5-RFP-H5 was released from pGEMT-easy-H5-RFPH5 with BglII and HindIII restriction enzymes. B5R right arm was released from pGEMT-easy-B5R right arm with HindIII and SmaI restriction enzymes. pGEMT-easy-left arm was linearized with BglII and SmaI restriction enzymes. H5-RFP-H5 (BglII+HindIII), STC+B5R right arm (HindIII+SmaI) were ligated into linearized pGEMT-easy B5R left arm (BglII+SmaI). The resulted shuttle vector pGEMT-easy-B5R left arm+H5-RFP-H5+STC+B5R right arm was verified by sequencing. The shuttle vector is designated as VVL15 B5R STC shuttle vector hereafter. The illustration of expression cassette of VVL15 B5R STC shuttle vector is shown in Fig1.

Construction of VVL15 B5R S-STC shuttle vector:

B5R S-STC (signal peptide/SP+SCR1+stalk+transmembrane domain+cytoplasmic tail/STC: SP-SCR1-STC). The left arm and SP+SCR1 were amplified by PCR from the VV lister genome with primers B5R left arm forward (5'-TATACTGCGTGTATGACCG-3') and B5R SCR1 reverse (5'-CTCGAGGAATTCAAGCTTGCATGGATTTTCGTATTTC-3') (XhoI and HindIII sites are underlined). STC was amplified by PCR with B5R stalk primer forward (5'-AAGCTTTGTGTAC GAACTAACGAAAAA-3') (HindIII site is underlined) and cytoplasmic tail reverse primer (5'-AGATCTTCACGGTAGCAATTTATGG-3') (BglII site is underlined). H5-RFP was amplified by PCR with forward primer (5'-AGATCTAAAAATTGAAAATAAATACAAAGGTTCTTGAGGGT

TGTGTTAAATTGAAAGCGAGAAATAATCATAAATAGC-3') (BgLII site is underlined) and reverse primer (5'-ACGCGTCGCCTTAAGATACATTGATGAG-3') (M1UI site is underlined). B5R right arm was amplified by PCR with B5R right arm forward primer (5'-ACGCGTCTACCGTGAA TATAAATCCGT-3') (M1UI site is underlined) and B5R right arm reverse primer (5'-CTCGAGGGATGTATATACCATCGTCGT-3') (XhoI site is underlined). All PCR products were cloned into pGEMT-easy vector (promega) following the manufacturer's instructions. The correct sequences were verified by sequencing. pGEMT-easy-B5R left arm+SP+SCR1 was linearized with XhoI and HindIII restriction enzymes. STC was released from pGEMT-easy-STC with HindIII and BgLII restriction enzymes. H5-RFP was released from pGEMT-easy-H5-RFP with BgLII and M1UI restriction enzymes. B5R right arm was released from pGEMT-easy-B5R right arm with M1UI and XhoI restriction enzymes. Digested STC (HindIII+BgLII), H5-RFP (BgLII+M1UI) and B5R right arm (M1UI+XhoI) were ligated into linearized pGEMT-easy-B5R left arm+SP+SCR1 (HindIII+XhoI). The resulted shuttle vector pGEMT-easy-B5R left arm+SP+SCR1+STC+H5-RFP+B5R right arm was verified by sequencing. The shuttle vector is designated as VVL15 B5R S-STC shuttle vector hereafter. The illustration of expression cassette of VVL15 B5R S-STC shuttle vector is shown in Fig1.

Construction of TK-STC shuttle vectors:

TK-directed shuttle vector containing RFP flanked by LoxP sites was constructed previously (Yuan, M. et al., Mol Ther-Meth Clin D 2 (2015)). Signal peptide of B5R gene (SP) was amplified by PCR using forward primer (5'-TTAATTAAAAATAAAAATGAAAACGATTTCCG-3') (PacI is underlined) and reverse primer (5'-GCTAGCGAATTCAAGCTTTGAATAAACAACAGC-3') (NheI, EcoRI and HindIII are underlined). B5R STC fragment (STALK+TM+TC) was amplified by PCR using forward primer (5'-AAGCTTTGTGTACGAACTAACGAAAAA-3') (HindIII is underlined) and reverse primer (5'-GCTAGCTCACGGTAGCAATTTATGGAACT-3') (NheI is underlined). The SP fragment was cloned into the pGEMT easy vector, designated as pEGMT easy-SP. STC fragment was cloned into HindIII and NheI sites of pGEMT easy-SP to obtain pEGMT easy-SP+STC. SP+STC was released from pEGMT easy-SP+STC using PacI and NheI restriction enzymes and cloned into PacI and NheI sites of the TK-directed shuttle vector containing RFP flanked by LoxP sites. The resulted shuttle vector is named as TK STC shuttle vector. The illustration of expression cassette of TK STC shuttle vector is shown in Fig 3.

Construction of cytokine N1L-mIL-12 and N1L-hIL12 shuttle vectors:

The N1L-directed shuttle vector containing RFP flanked by FRT sites for homologous recombination was generated previously (Yuan et al., Mol Ther-Meth Clin D 2 (2015)), this shuttle vector is designated as N1L shuttle vector hereafter. mIL-12 and hIL-12 were cloned into PmeI and NheI sites of the N1L shuttle vector to obtain N1L-mIL-12 and N1L-hIL12 shuttle vectors (Fig 22).

Construction of N1L-mIL-21 and N1L-hIL21 shuttle vectors:

mIL-21 and hIL-21 were cloned into SalI and BglII sites of the N1L shuttle vector to obtain N1L-mIL-21 and N1L-hIL21 shuttle vectors (Fig 14).

Cas9-mediated homologous recombination as stated previously:

Briefly, 3×10^5 CV-1 cells were seeded into one well of a six-well plate the day before transfection. gRNA vector (N1LgRNA for targeting N1L region, TK gRNA for targeting TK region) was co-transfected with Cas9 into CV-1 cells in the six-well plate. The next day, the transfected well was infected with 0.01 PFU/cell of backbone virus. The shuttle vector for homologous recombination was transfected into infected wells 2 hours after virus infection. Cells were harvested 24 hours later, and frozen at −80 °C for plaque purification.

Purification of desired virus:

Thaw the cell lysates collected from Cas9-mediated homologous recombination, and 1 µl of this lysates was used to infect all 6 wells of a six well plate containing CV1 cells grown to 80-90% confluence. This low viral load would ensure the emergence of well separated PFUs. A further 48 hr later, each well was carefully scrutinized under green light searching for those viral PFUs that fluoresced red. Upon identification of positive colonies, their location was marked on the under surface of the plate with a fine tipped permanent marker. The colony was carefully picked with a 20 µl tip after aspirating the media from the well. The tip was then submerged into a cryotube containing 250 µl of 5% FCS CM. Following further freeze-thaw cycles, 5-20 µl of this virus solution was added to each well of a new 6-WP containing CV1 cells as before. This process was repeated until every PFU fluoresced red i.e. all viral colonies were due to recombinant virus. In general it took between 6-10 rounds of plaque purification to obtain a pure batch of recombinant virus. At this point, the viral lysate was scrape-harvested and viral DNA was extracted via a column based system (i.e. the Blood Mini Kit from Qiagen). The purity of virus was confirmed by PCR amplification of the target gene from extracted viral DNA. Its presence would indicate contamination with the parental virus, VVL (Wang et al., J Clin Invest 119, 1604-1615 (2009)).

Once preliminary investigations had confirmed the likely creation of a pure recombinant virus that expressed the STC, 50µl of viral lysate was added to a T175 flask containing CV1 cells, again grown to 80-90% confluence in approximately 30 ml of 5% FCS CM. Cells and media were scrape-harvested 48 hr later and kept as a “primary viral expansion”.

Verification of TK STC VACV:

VACV DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s protocol. To verify the insertion of STC in the TK region, forward primer (5’-AAATAAAAATGAAAACGATTTCCG-3’) targeting SP part of SP-STC and reverse primer (5’-GGATGTATATACCATCGTCGT-3’) targeting right arm side of TK gene. A control DNA fragment spanning the A46R and A47L genes was amplified by PCR using forward primer (5’-TTGGCTATTAAACAGTATGGA-3’) and reverse primer (5’-GGATCCCGATAACAAATG-3’).

Extensor Long PCR ReddyMix Master Mix was used for all PCR reactions. The PCR products were analysed by 1% agarose gel electrophoresis.

Verification of mutant N1L VACV:

CV-1 Cells were infected with purified plaques. Infected cells were harvested after 2 days of infection. VACV DNA was extracted using DNeasy Blood & Tissue Kit according to the manufacturer's protocol. To verify the deletion of the N1L gene, a DNA fragment spanning the N1L gene and the L026 gene was amplified by PCR using forward primer (5'-TATCTAGCA ATGGACCGT-3') (within the N1L gene) and reverse primer (5'-CCGAAGGTAGTAGCATGGA -3') (within the L026 gene). A control DNA fragment spanning the A46R and A47L genes was amplified by PCR using forward primer (5'-TTGGCTATTAAACAGTATGGA-3') and reverse primer (5'-GGATCCCGATAACAAATG-3'). Extensor Long PCR ReddyMix Master Mix was used for all PCR reactions. The PCR products were analysed by 1% agarose gel electrophoresis.

Excision of RFP using Cre recombinase:

pCAG-Cre (from Addgene) was transfected into CV-1 cells in one well of six-well plate. Twenty-four hours after transfection with pCAG-Cre, CV-1 cells were infected with 100–200 PFU of Cre-RFP VACV. Two days later, RFP-negative plaques were picked, and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. Then RFP-negative plaques were picked and CV-1 cells were infected until no RFP-positive plaques were seen under fluorescence microscopy every 2 days. The excision of RFP from the virus by Cre recombinase was tested by PCR of RFP gene.

Excision of RFP using Flp recombinase:

pCAG-Flpe (from Addgene) was transfected in CV-1 cells in one well of six-well plate. Twenty-four hours after transfection with pCAG-Flpe, CV-1 cells were infected with 100–200 PFU of Flp-RFP VACV. Two days later, RFP-negative plaques were picked, and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. Then RFP-negative plaques were picked and CV-1 cells were infected until no RFP-positive plaques were seen under fluorescence microscopy every 2 days.

Generation of VVL-DD and VVL-DD STC viruses:

The generation of VVL-DD virus without RFP was described previously (Yuan, M. et al., Mol Ther-Meth Clin D 2 (2015)). The VVL-DD STC was created in the same way as with the VVL-DD virus, but using TK-STC shuttle vector (Fig 3) instead of TK shuttle (Yuan, M. et al., Mol Ther-Meth Clin D 2 (2015)). VVL-DD STC virus is a FRP-negative virus with N1L deletion and STC inserted into TK region.

Enzyme-linked immunosorbent assay:

The expression of mIL-12, hIL-12, mIL-21 and hIL-21 was detected by enzyme-linked immunosorbent assay (eBioscience, UK) following the manufacturer's instructions.

Mass viral production:

The primary viral expansion from above was rapidly freeze-thawed twice and diluted into the necessary volume of 5% FCS CM required to infect between 36-40 T175 flasks containing CV1 cells (at 80-90% confluence). 48 hr later, infected CV1 cells were scrape harvested and through repeated rounds of centrifugation at a speed of 2,000 rpm (at 4°C), collected into a single pellet. The pellet was washed in PBS, re-suspended in 12 mls of 10 mM Tris-HCl (pH 9) buffer and stored at -80°C for purification at a later date.

Viral purification:

The concentrated viral lysate suspension from above was freeze-thawed twice and transferred to a dounce homogeniser (Thermofisher) and homogenised via 60 strokes. It was then ultrasonicated for 30 seconds. Following centrifugation at 2,000 rpm at 4°C for 5 minutes, the supernatant (containing released virion particles) was collected and diluted to a total volume of 30 mls with 10 mM Tris-HCl buffer. The solution was divided into four; each layered gently onto 17 ml of a 36% glucose solution in a 36ml Beckman ultracentrifuge tube and centrifuged at 13,500 rpm for 80 minutes at 4°C. The resulting pellets were re-suspended to a total of 16 mls in 10 mM Tris-HCl, divided into 4 again and carefully layered onto another four glucose gradients, this time graded from 25% w/m near the surface to 40% at the base of each tube. A second round of ultracentrifugation was performed. This was necessary to remove further particulate cellular debris, which could be toxic when administered intravenously into mice. The final pellets were re-suspended in 1-4 mls of viral re-suspension buffer (PBS; 10% glycerol; 138 mM NaCl; pH 7.4). A sample of purified virus was titrated via a TCID50 assay as described below.

Viral replication

Cells were seeded at 2 to 4 $\times 10^5$ cells per well, depending on growth rates, in three wells of 6-well plates in media with 10% FCS, and infected with 1 PFU/cell of vaccinia viruses 16-18 hours later. Samples were harvested in triplicate at 24-hour intervals up to 144 hours. Viral replication was detected by TCID50 (50% tissue culture infective dose) as previously described (Wang, Y. et al., J. Clin. Invest. 119, 1604-1615 (2009)).

Evaluation of viral cytotoxicity in vitro

Cells were seeded at $1x10^3$ and $1x10^4$ cells per well, depending on growth rates, in 96-well plates, and infected with viruses 16-18 hours later. Cell survival on day 6 after viral infection was determined by MTS assay and EC50 value (viral dose killing 50% of tumour cells) was calculated as previously described (Wang, Y. et al., J. Clin. Invest. 119, 1604-1615 (2009)). All assays were performed at least three times.

In vivo efficacy experiments for comparison of different strain VVs:

Flank tumours were established in 10 mice per treatment group through subcutaneous injection of 1-5x10^6 cancer cells and allowed to reach 0.4-0.5cm in diameter, then the mice were regrouped by tumour size and received three 50µl IT injections of 1.0×10^7 PFU (nude mice) or 1 x 10^8 PFU (immunocompetent mice) or PBS on days 1, 3 and 5 or days 1, 2, 3, 4, 5. Tumour volumes were estimated (Volume = (length × width^2 × π)/6) twice weekly until mice were sacrificed when tumour volume reached 1.00 cm^3 or had been present for three months. 4-5 week male mice strains BALB/c and C57BL/6 were obtained from Harlan UK Ltd.

Efficacy of IT injected VVL recombinant against pancreatic and colorectal flank tumour models:

Either 2x10^6 CT26 cells or 3x10^6 DT6606 cells were subcutaneously implanted into the shaved right flanks of BALB/c or C57BL/6 male mice. Once tumour volumes had reached approximately 100mm^3, they were randomised into three groups and a dose of 1x10^8 PFU of virus in 50 µl PBS or 50 µl PBS vehicle buffer control was injected as per the treatment schedules outlined in Table 3 (schedule 1 and 2). Tumour volumes were monitored via twice weekly calliper measurement and mice were weighed weekly.

Efficacy of IV injected VVL recombinant against pancreatic tumour models:

3x10^6 DT6606 were subcutaneously implanted into the shaved right flanks of C57BL/6 male mice. DT6606 cells (3x10^6 cells/mouse) were implanted subcutaneously into the right flanks of eight week-old male C57/Bl6 mice. When the tumours were palpable mice were stratified into treatment groups. Mice received 10mg/kg CAL101 or vehicle buffer via oral gavage three hours prior to virus (or PBS) injection at 1x10^8 PFU/injection on days 1, 3 and 5 and tumour growth measured twice a week. Viruses were re-suspended in PBS injected intravenously via a tail vein. αPD-1 antibody was re-suspended in PBS at final concentration of 200µg/mouse and injected at day 3, 6 and 8. Tumour volumes were monitored via twice weekly calliper measurement and mice were weighed weekly.

Efficacy of intraperitoneally (IP) injected VVL recombinant against disseminated pancreatic tumour models in Syrian hamsters:

1x10^7 SHPC6 cells were seeded into the lower right peritoneal cavity of Syrian hamsters. Four days later, 10 hamsters per group were injected i.p with 500 µl PBS or 2x10^7 PFU virus on days 0, 2, 4. The survival of hamsters was monitored.

Quantitative polymerase chain reaction to assess viral load in tumours:

Viral DNA extraction was performed using the Qiagen DNeasy Blood & Tissue Kit. Quantification of viral genome copy number was achieved using the TaqMan® PCR system provided by Applied Biosystems. For VV quantification, the primers and probe were designed for the Vaccinia virus late transcription factor 1 (VLTF-1) gene: Forward; 5'-AACCATAGAAGCCAACGAATCC, reverse; 5'-TGAGACATACAAGGGTGGTGAAGT, probe; sequence ATTTTAGAACAGAAATACCC. The

primers were supplied by Sigma-Aldrich. The standard was WT VV DNA, and 40ng of DNA was used per sample as the template. Viral genome copy number was normalised by total DNA loaded.

Statistical Analysis:

Unless otherwise mentioned, Graphpad Prism 5 was used for comparative statistical analysis. Dual condition comparisons were made using the unpaired student t test. For more than one condition or for an additional variable such as time, a 1 or 2-way ANOVA respectively was performed. Post hoc tests (Knewman-Keuls for 1-way ANOVA and Bonferroni for 2-way ANOVA) compared specific pairs of conditions within the experiment. Survival data was represented as a Kaplan-Meier plot with log rank analysis to delineate whether any differences between groups were statistically significant.

Example 1: Comparison of the plaque and comet tail formation of VVL15, VVL15-B5R-STC and VVL15 B5R S-STC VV in vitro

To generate a virus which produces more EEV than the VVL15 virus, VVL15-B5R-STC and VVL15 B5R S-STC VV were created. B5R-STC in VVL15-B5R-STC virus or B5R S-STC in VVL15 B5R S-STC replaces the full length of B5R gene respectively (Fig 1). CV-1 cells in 6-well plates were infected by equal amount of control virus VVL15, VVL15-B5R-STC and VVL15 B5R S-STC VV. Three days post infection, the infected cells were stained using crystal violet as described previously 16. VVL15-B5R-STC and VVL15 B5R S-STC VV formed smaller size plaques and more comet tails indicating that they produced more EEV compared to VVL15 (Fig 2).

Example 2: Generation of VVL15 TK-STC virus

The modified VVL15-B5R-STC and VVL15 B5R S-STC VV (Fig 1) formed smaller sized plaques and more comet tails (Fig 2) than the control virus VVL15. To make a modified VV which produces normal plaque size and more EEV compared to the backbone virus VVL15, the VVL TK-STC vaccinia virus was generated as illustrated in Fig 3, the STC replacing the TK gene (Stalk, TM and CT), and this virus retains an intact copy of B5R. For purification of the VVL TK-STC vaccinia virus, visual inspection (under red fluorescent light) of the final round of plaque purification confirmed that all plaques within a well of infected CV1 cells expressed RFP from the STC-TK shuttle vector (Fig 4). The specific primer pairs to amplify the STC gene and partial of TK downstream gene by PCR from viral DNA extracted from CV1 lysates were used for validation of the virus. The PCR product was absent in the VVL15 RFP DNA but present in the VVL15 TK-STC DNA, which indicated the TK gene was deleted in the VVL15 TK-STC and the STC was in the TK region. Forward primer designed against SP of B5R gene and reverse primer recognising the B5R cytoplasmic tail were used in the PCR to amplify the whole B5R gene and STC. Full length B5R was present in VVL15 RFP and VVL15 TK-STC, while STC was only present in VVL15 TK-STC as expected (Fig 4).

Example 3: Comparison of the plaque and comet tail formation of VVL15 TK-RFP and VVL TK - STC VVin vitro

The VVL15 TK-STC virus retains its copy of the B5R gene intact and has an additional STC insertion into the TK gene region, inactivating viral TK for tumour selectivity. To evaluate the plaque and comet tail formation of the VVL15 TK-STC virus, CV-1 cells in 6-well plates were infected by equal amount of control virus VVL15 TK-RFP and VVL15 TK-STC. Three days post infection, the infected cells were stained using crystal violet as described previously 16, and the plates were photo scanned (Fig 5 and 6). VVL15 TK-STC generated normal sized plaques (Fig 5) and produced more comet tails (Fig 6) compared to VVL15 TK-RFP.

Example 4: Assessment of EEV production in recombinant VVL15 TK-STC

To quantify the EEV produced in the VVL15 TK-STC virus and its control virus, 0.01 pfu/cell of vaccinia viruses were used to infect CV-1 cells in a 6-well plate. Cell culture medium and the infected cells were collected into separate tubes 48 and 72 hours post infection and viruses were titrated respectively. The amount of EEV produced was calculated by comparing the total amount of VV in the cell culture medium with the total amount of VV produced by the infected cells. VVL15 TK-STC produces 10 times more EEV at 48-hour post-infection and 30 times more EEV at the 72 hour time-point compared to its parental virus VVL15 RFP (Fig 7).

Example 5: Assessment of EEV production in recombinant VVL-DD STC

Recombinant VVL-DD and VVL-DD STC viruses were created. VVL-DD is the virus with TK and N1L regions deleted (also named as VVΔTK-ΔN1L) . VVL-DD STC is the virus with TK and N1L regions deleted and STC inserted into TK region (also named as VVΔTK-STC-ΔN1L). To quantify the EEV produced in the VVL-DD STC virus and its control virus (VVL-DD), 0.01 pfu/cell of vaccinia viruses were used to cells in a 6-well plate. Cell culture medium and the infected cells were collected into separate tubes 48 and 72 hours post infection and viruses were titrated respectively. The amount of EEV produced was calculated by comparing the total amount of VV in the cell culture medium with the total amount of VV produced by the infected cells. VVL-DD STC virus produces more EEV at the indicated time-points post-infection compared to its parental virus VVL-DD in pancreatic cancer cells (Fig 8) and in lung cancer cells (Fig 9).

Example 6: Comparison of the replication and cytotoxicity of VV in vitro

The replication of VVL15 TK-STC and its parental VVL15 RFP virus was compared (Fig 10). VVL15 TK-STC replicates more effectively than VVL15 RFP in CT26 (mouse colon cancer cell line) and DT6606 (mouse pancreatic cancer cell line) cancer cell lines.

The cytotoxicity of VVL15 TK-STC and VVL15 RFP were measured in CV1, CT26 and DT6606 cell lines (Fig 10). There was no significant difference in cytotoxicity between the two viruses in DT6606 cells. VVL15 TK-STC was significantly more potent than the parental VVL15 RFP at killing CV-1 and CT26 cells (Fig 11).

Example 7: Comparison of the anti-tumour potency of VV in vivo

To test the anti-tumour potency of VVL15 TK-STC virus, subcutaneous models of CT26 colon cancer CT26 (Fig 12) and DT6606 pancreatic cancer models (Fig 13) were used. VVL15 RFP and VVL15 TK-STC viruses were injected intra-tumourally (Fig 12 and 13). For the CT26 tumour model, three doses of viruses at 2x107 PFU/injection were used at day 1, day 3 and day 5. For the DT6606 tumour model, five doses of viruses at 2x108 PFU/injection were used at the indicated time points (Fig 13). VVL15 TK-STC consistently demonstrates improved anti-tumour efficacy compared to the control virus VVL15 RFP.

Example 8: Creation of human IL-21 (hIL-21) and mouse IL-21 (mIL-21) expressing VVL15 TK-STC virus

To improve anti-tumour immunity, VVL15 TK-STC virus was armed with interleukin-21 (IL-21), the NK cell and T cell stimulator cytokine. Human IL-21 (hIL-21) and mouse IL-21 (mIL-21) expressing viruses were created using the VVL15 TK-STC virus with RFP deletion as shown in Fig 14, hIL-21 and mIL-21 were cloned into the N1L region (Fig 14). The expression of hIL-21 and mIL-21 by the virus in infected cells was confirmed (Fig 15 and 16).

Example 9: Intravenous injection of TK STC mIL-21 virus shows superior anti-tumour ability compared the control virus without mIL-21

To prolong the persistence of VV after intravenous injection, a transient inhibitor of macrophage function, Cal101, was delivered three hours before VV was injected intravenously (i.v). The anti-tumour potency of TK STC mIL-21 was tested in the subcutaneous DT6606 tumour model. I.V delivery of TK STC mIL-21 shows improved anti-tumour potency compared to the unarmed TK STC control virus (no mIL-21 expression) (Fig 16). Accumulation of Vaccinia virus DNA in tumours after injection was determined using qPCR 5 days after the last of 3 injections given on days 1,3 and 5 ($1x10^8$PFU/injection) (Fig. 17). The STC virus accumulated to higher levels compared to the control virus. The anti-tumour potency of TK STC mIL-21 was tested in the peritoneally disseminated SHPC6 pancreatic cancer model in Syrian hamsters. Intraperitoneal delivery of TK STC N1L deleted virus shows improved efficacy compared to the control virus in which no STC was present in the TK domain (Fig. 18). Furthermore, arming the TK-STC-N1L with IL-21 could cure the peritoneally disseminated pancreatic cancer in Syrian hamsters (Fig 19).

Example 10: Check point inhibitor anti-PD-1 antibody improves the anti-tumour effect of TK STC mIL-21 virus

Anti-PD-1 antibody is being widely used to enhance anti-tumour immunity in a range of tumour types and clinical evidence demonstrates a significant improval in survival of some cancer patients. To investigate whether anti-PD1 antibody could enhance the anti-tumour potency of TK STC mIL-21, anti-PD1 antibody was used in combination with Cal101 and i.v. injection of VV. The anti-PD1 antibody dramatically improves the anti-tumour potency of TK STC mIL-21 virus (Fig 21). When the Cal101 and anti-PD1 antibody combination was used to treat tumours, inclusion of TK STC mIL-21

(STC VVI21) in the treatment regime significantly enhanced the anti-tumour effect of theCal101 and anti-PD1 antibody combination (Fig 21).

Example 11: Creation of human IL-12 (hIL-12) and mouse IL-12 (mIL-12) expressing TK STC virus

To improve anti-tumour immunity, VVL15 TK-STC virus was armed with interleukin-12 (IL-12), which stimulates most cells of the adaptive and innate immune system. Human IL-12 (hIL-12) and mouse IL-12 (mIL-12) expressing viruses were created using the VVL15 TK-STC virus with RFP deletion as the same strategy of making VVL15 TK-STC expressing IL-21 shown in Fig 14, hIL-12 and mIL-12 were cloned into the N1L region (Fig 14). The expression of mIL-12 and hIL-12 in infected cells by the virus was confirmed (Fig 22 and 23).

Example 12: IL-12 expressing TK STC virus is effective in lung cancer models after intratumoral administration and enhanced the antitumour efficacy of checkpoint inhibitor anti-PD1 antibody.

A subcutaneous lung cancer model was established in immunocompetent mice using Lewis Lung Carcinoma cells (LLC). Mice were treated intratumorally three times with STC-mIL12 virus ($1x10^8$PFU/injection) on days 1,3,5. Where appropriate, α-PD1 antibody was administered on days 7,9,11 (200μg/mouse). STC-mIL12 virus was more effective than PBS or α-PD1 antibody therapy alone at reducing tumor growth rate. Addition of α-PD1 antibody to the virus treatment further enhanced long term efficacy (Fig 24 and 25).

Example 13 The sequential use of viruses, first expressing IL12 and second expressing soluble PD1 is as effective as using virus expressing IL12 followed by α-PD1 antibody. The use of a virus expressing soluble PD1 was as effective as the use of an α-PD1 antibody (Fig 26).

Example 14 Administration of the viruses expressing different immune-modulator molecules in certain orders is important (Fig 27), i.e virus expressing mIL-12 must be delivered before the virus expressing soluble PD1 (sPD1) as if they are given in the other way round the superior anti-tumour effect is lost.

CLAIMS

1. A vaccinia virus vector comprising a nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) inserted into the TK gene of the vaccinia virus.

2. A vaccinia virus vector according to claim 1, wherein the native B5R gene remains intact.

3. A vaccinia virus vector according to claim 1 which further comprises a nucleic acid sequence encoding a biologically active protein inserted into the N1L gene of the vaccinia virus.

4. A vaccinia virus vector according to claim 3, in which the biologically active protein is selected from the group consisting of cytokines, antibodies, antibody fragments, cytokine receptors and cytokine receptor fragments.

5. A vaccinia virus vector according to claim 4, in which the biologically active protein is a cytokine.

6. A vaccinia virus vector according to claim 5, in which the cytokine is selected from the group consisting of IL-21, GM-CSF, IL-2, IL-7, IL-12, IL-15, IL-18 and IFN-α, or any combinations thereof.

7. A vaccinia virus vector according to claim 1 and any one of claims 3 to 5, in which the nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) is defined in Figure 31.

8. A vaccinia virus vector according to claim 4, in which the biologically active protein is an immune checkpoint inhibitor molecule.

9, A vaccinia virus vector according to claim 8, in which the immune checkpoint inhibitor molecule is selected from the group consisting of soluble PD1, soluble PD-L1, soluble TIM-3, soluble CTLA-4, or any combinations thereof.

10. A vaccinia virus vector according to any one of claim 8 or claim 9, in which the nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) is defined in Figure 31.

11. A composition comprising a vaccinia virus vector according to any one of claims 1 to 7.

12. A composition comprising a vaccinia virus vector according to any one of claims 8 to 10.

13. A method of treatment comprising administering a composition according to claim 11 to a subject in need thereof for the treatment of cancer.

14. A method of treatment comprising administering the composition according to claim 12 to a subject in need thereof for the treatment of cancer.

15. A method of treatment comprising administering the composition according to claim 12 and the composition according to claim 11 separately, subsequently or simultaneously.

16. A composition comprising a vaccinia virus vector according to claim 11 for use in the treatment of cancer.

17. A composition comprising a vaccinia virus vector according to claim 12 for use in the treatment of cancer.

18. A composition comprising a vaccinia virus vector according to claim 12 and a composition comprising a vaccinia virus vector according to claim 11 for separate, subsequent or simultaneous use in the treatment of cancer.

19. A nucleic acid sequence encoding a SCR1-, SCR2-, SCR3-, and SCR4- domain deleted B5R gene (B5R SCR1$^-$ SCR2$^-$ SCR3$^-$ SCR4$^-$) of vaccinia virus.

20. A kit comprising a vaccinia virus vector according to any one of claims 1 to 6 and a pharmaceutically acceptable adjuvant, diluent and/or buffer.

21. A kit comprising a vaccinia virus vector according to any one of claims 8 to 9 and a pharmaceutically acceptable adjuvant, diluent and/or buffer.

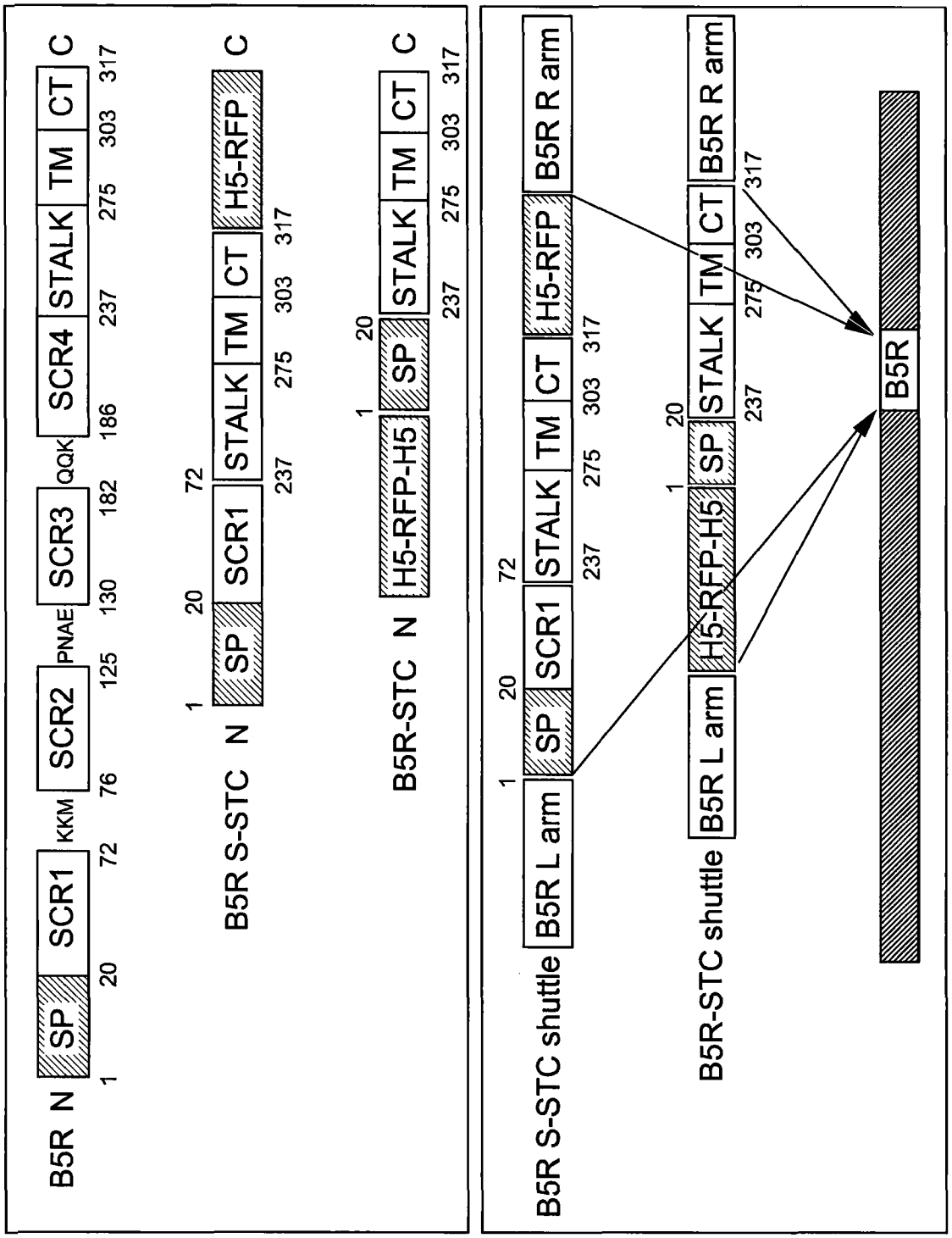

B5R N
SP
SCR1
KKM
SCR2
PNAE
SCR3
QQK
SCR4
STALK
TM
CT
C
1
20
72
76
125
130
182
186
237
275
303
317
B5R S-STC N
SP
SCR1
STALK
TM
CT
H5-RFP
B5R-STC N
H5-RFP-H5
B5R S-STC shuttle
B5R L arm
B5R R arm
B5R-STC shuttle
B5R

Fig.1

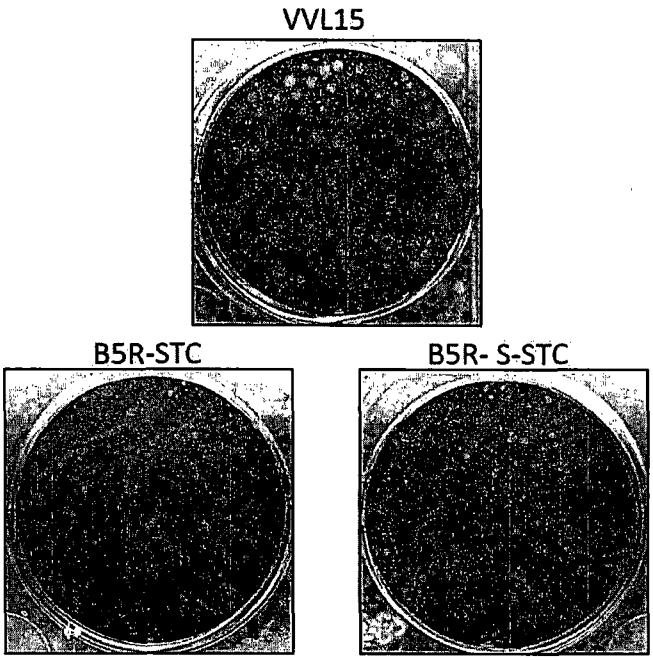

VVL15
B5R-STC
B5R- S-STC

Fig.2

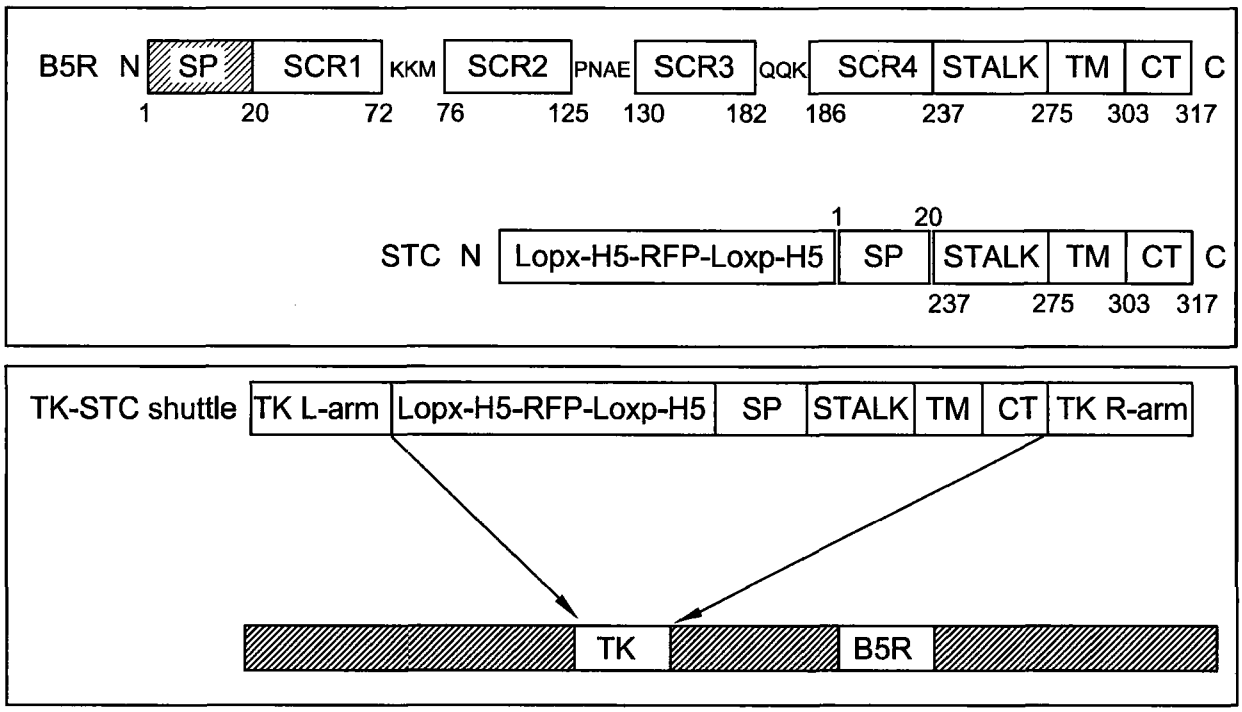

B5R N
SP
SCR1
KKM
SCR2
PNAE
SCR3
QQK
SCR4
STALK
TM
CT
C
1
20
72
76
125
130
182
186
237
275
303
317
STC N
Lopx-H5-RFP-Loxp-H5
SP
STALK
TM
CT
C
1
20
237
275
303
317
TK-STC shuttle
TK L-arm
Lopx-H5-RFP-Loxp-H5
SP
STALK
TM
CT
TK R-arm
TK
B5R

Fig.3

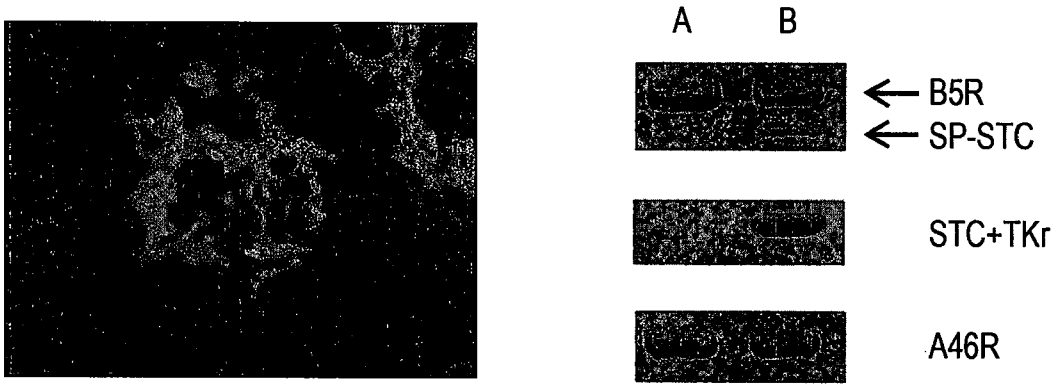

A
B
B5R
SP-STC
STC+TKr
A46R

Fig.4

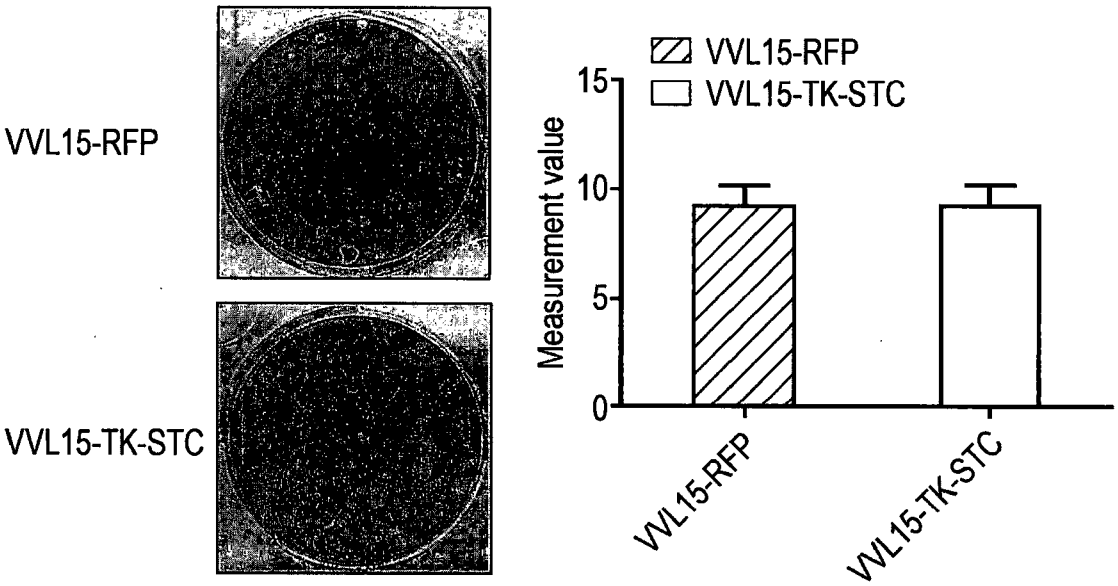

VVL15-RFP
VVL15-TK-STC
VVL15-RFP
VVL15-TK-STC
Measurement value
15
10
5
0
VVL15-RFP
VVL15-TK-STC

Fig.5

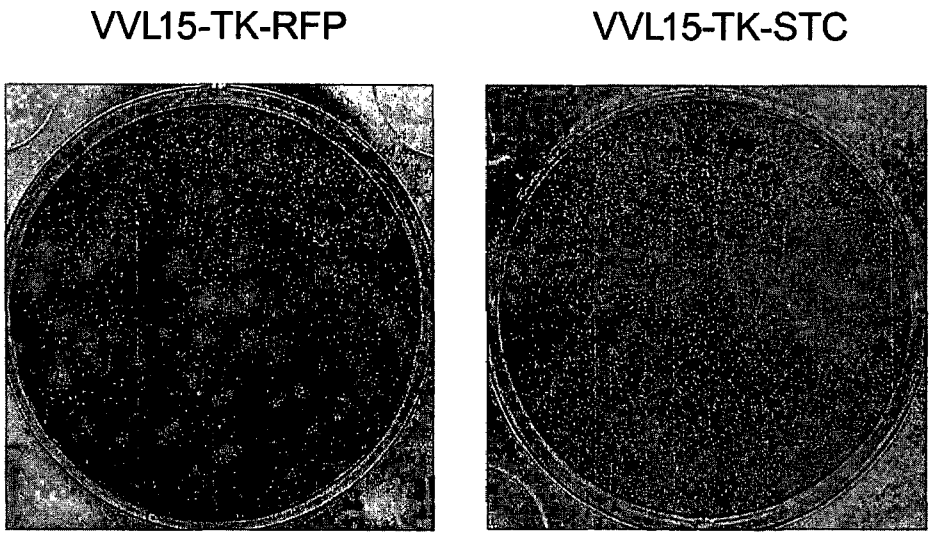

VVL15-TK-RFP
VVL15-TK-STC

Fig.6

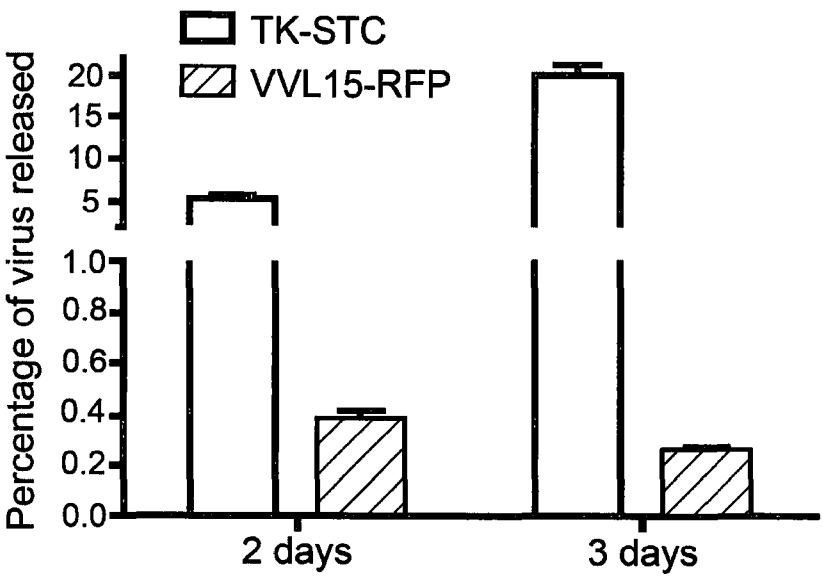

TK-STC
VVL15-RFP
Percentage of virus released
20
15
10
5
1.0
0.8
0.6
0.4
0.2
0.0
2 days
3 days

Fig.7

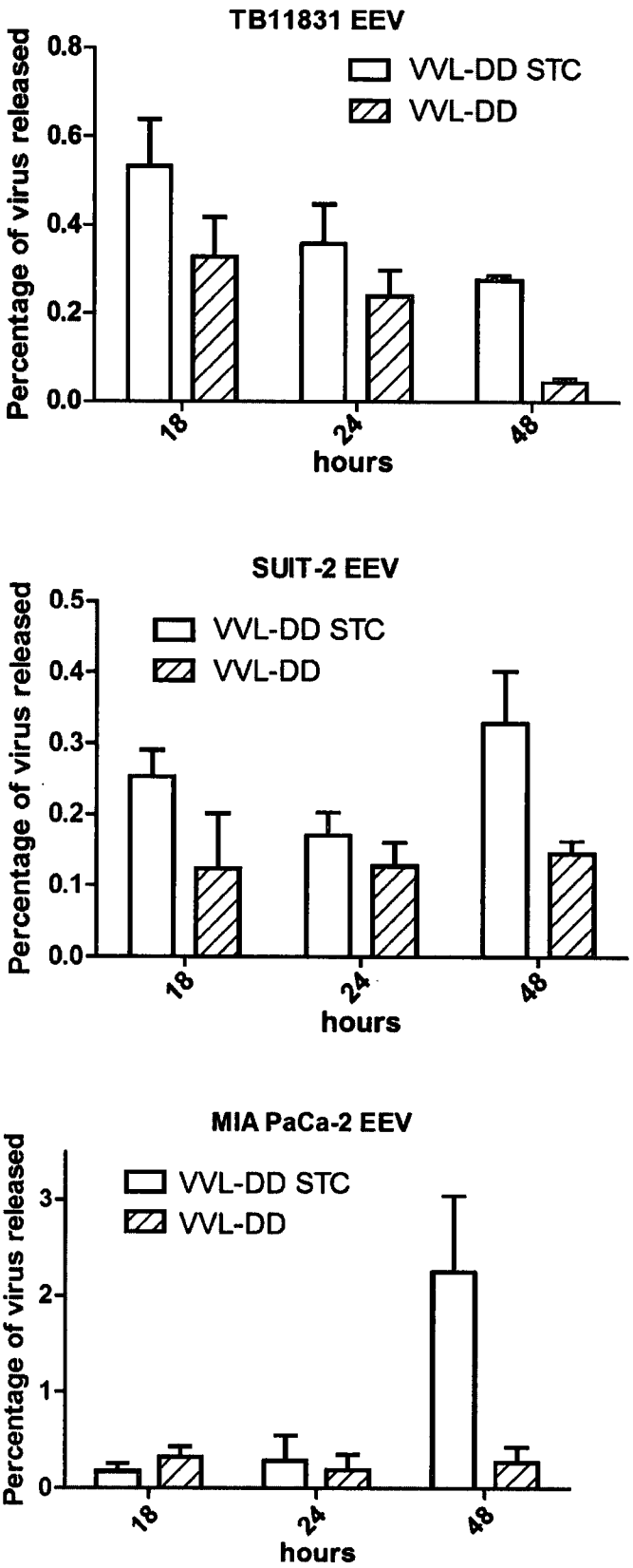
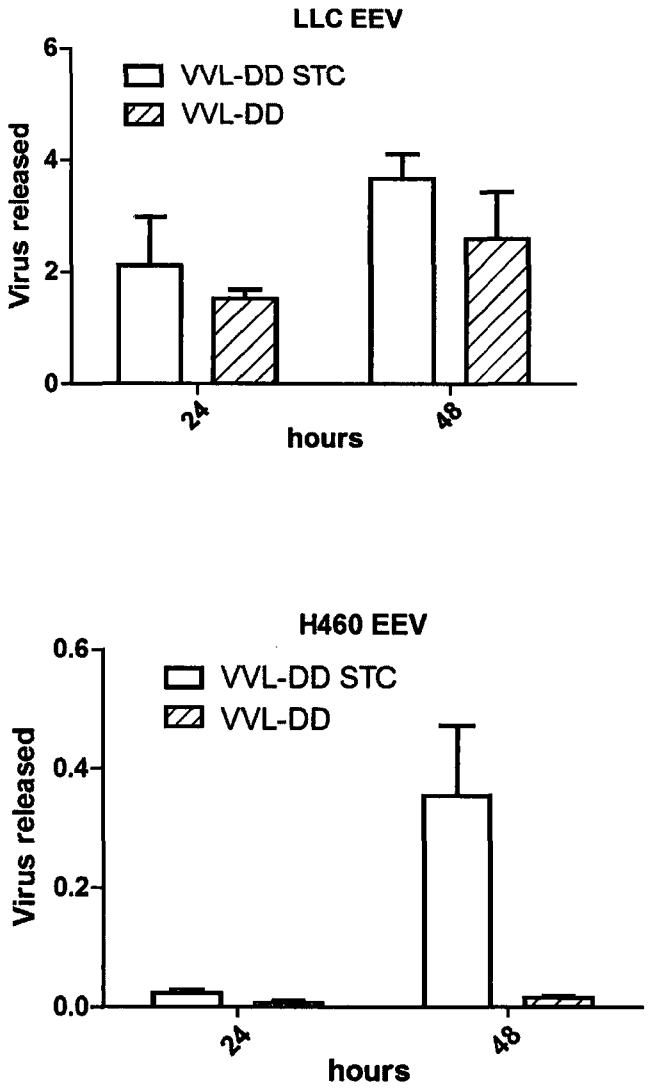

TB11831 EEV
VVL-DD STC
VVL-DD
Percentage of virus released
0.8
0.6
0.4
0.2
0.0
18
24
48
hours
SUIT-2 EEV
VVL-DD STC
VVL-DD
Percentage of virus released
0.5
0.4
0.3
0.2
0.1
0.0
18
24
48
hours
MIA PaCa-2 EEV
VVL-DD STC
VVL-DD
Percentage of virus released
3
2
1
0
18
24
48
hours

Fig.8

Fig.8 (continued)



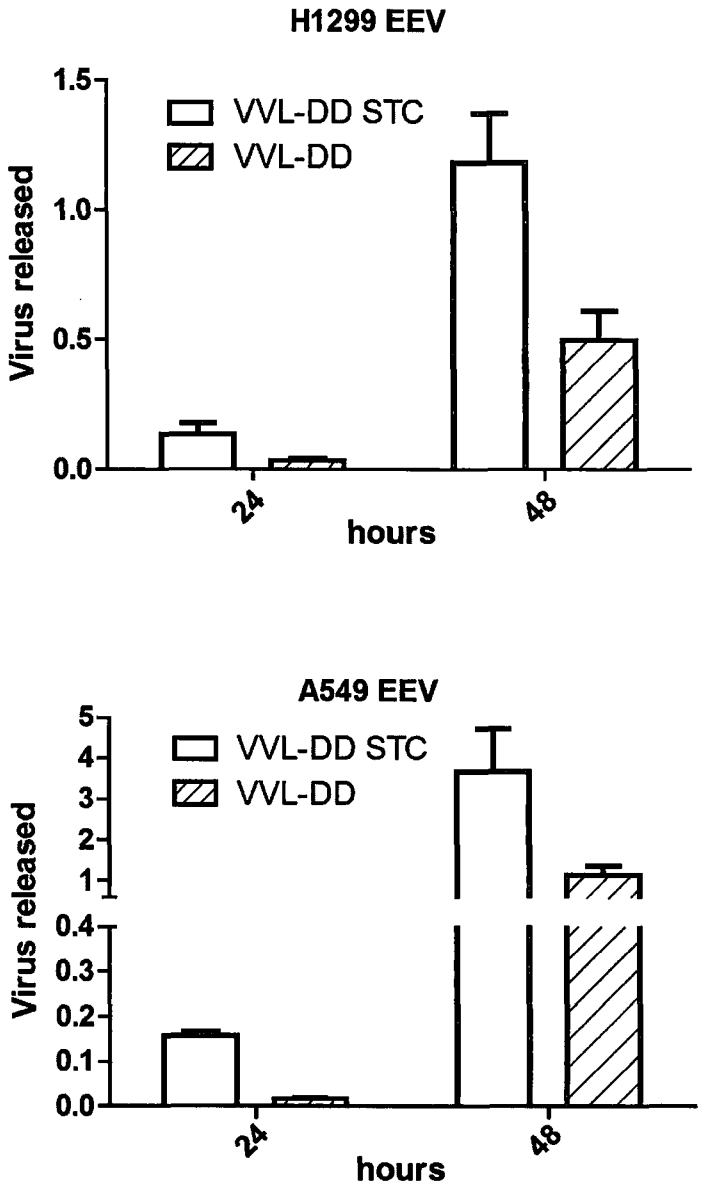

H1299 EEV
VVL-DD STC
VVL-DD
Virus released
1.5
1.0
0.5
0.0
24
48
hours
A549 EEV
VVL-DD STC
VVL-DD
Virus released
5
4
3
2
1
0.4
0.3
0.2
0.1
0.0
24
48
hours

Fig.9

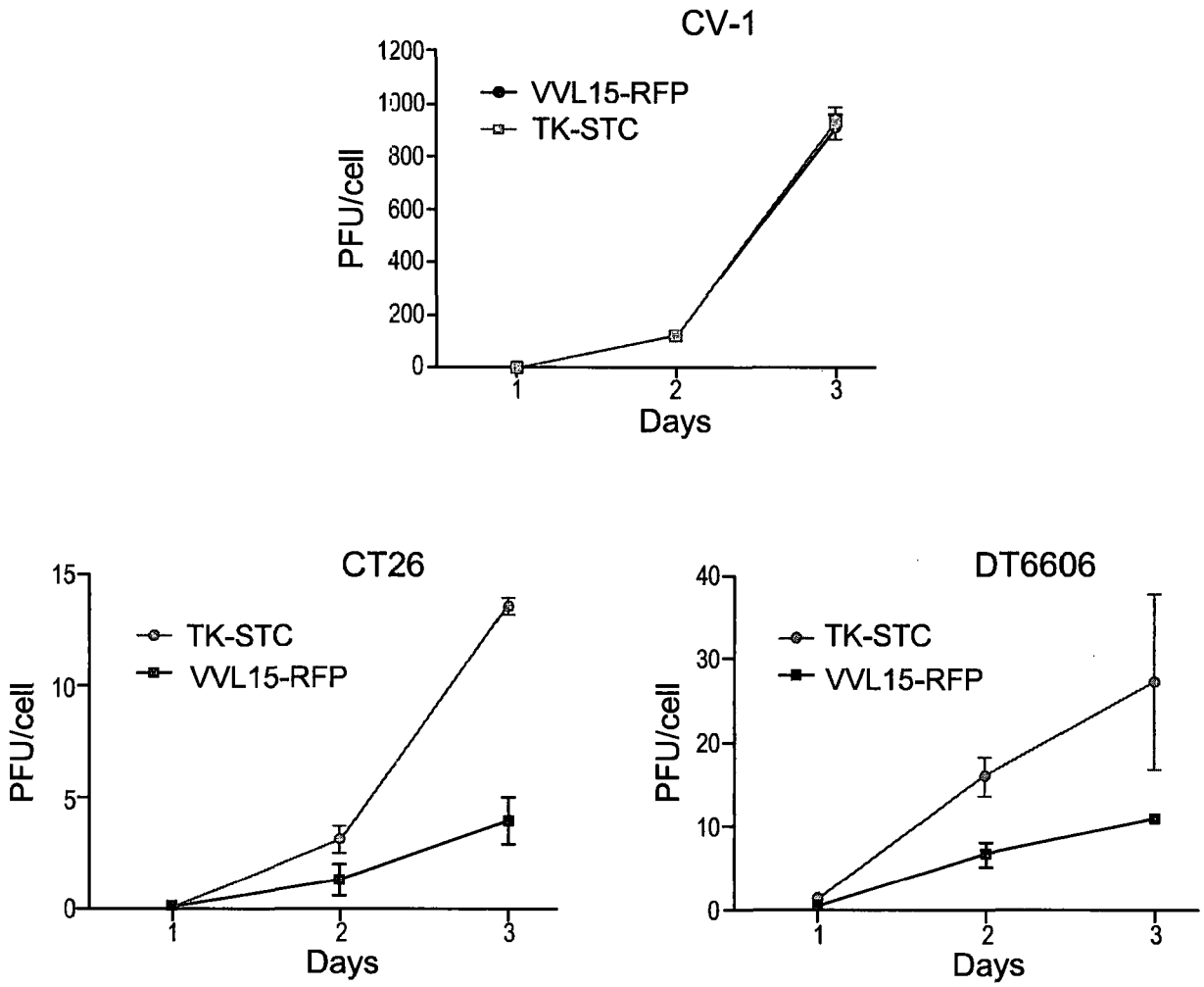

CV-1
1200
1000
800
600
400
200
0
PFU/cell
VVL15-RFP
TK-STC
1
2
3
Days
CT26
15
10
5
0
PFU/cell
TK-STC
VVL15-RFP
1
2
3
Days
DT6606
40
30
20
10
0
PFU/cell
TK-STC
VVL15-RFP
1
2
3
Days

Fig.10

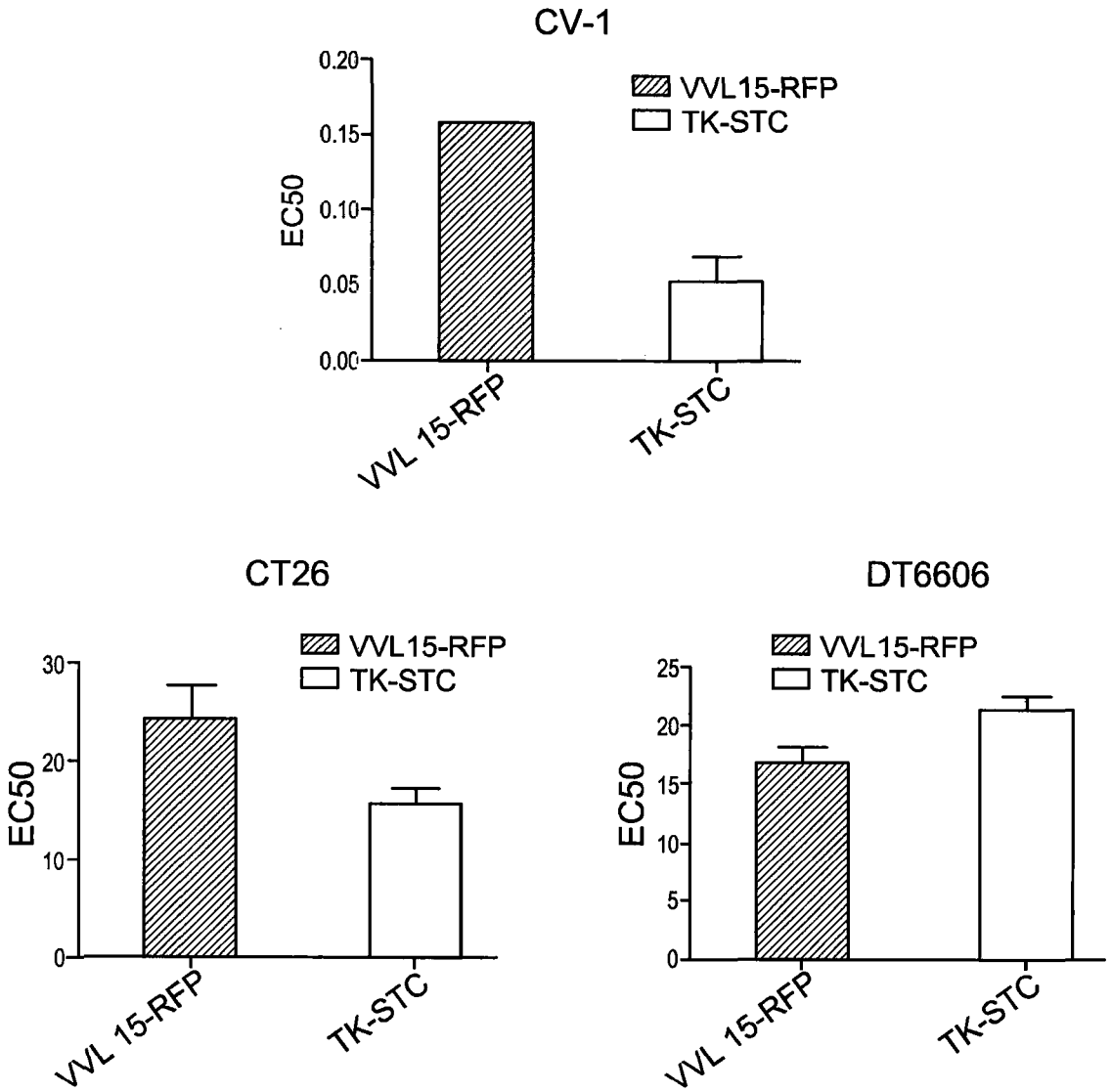

CV-1
EC50
0.20
0.15
0.10
0.05
0.00
VVL15-RFP
TK-STC
VVL 15-RFP
TK-STC
CT26
EC50
30
20
10
0
VVL15-RFP
TK-STC
VVL 15-RFP
TK-STC
DT6606
EC50
25
20
15
10
5
0
VVL15-RFP
TK-STC
VVL 15-RFP
TK-STC

Fig.11

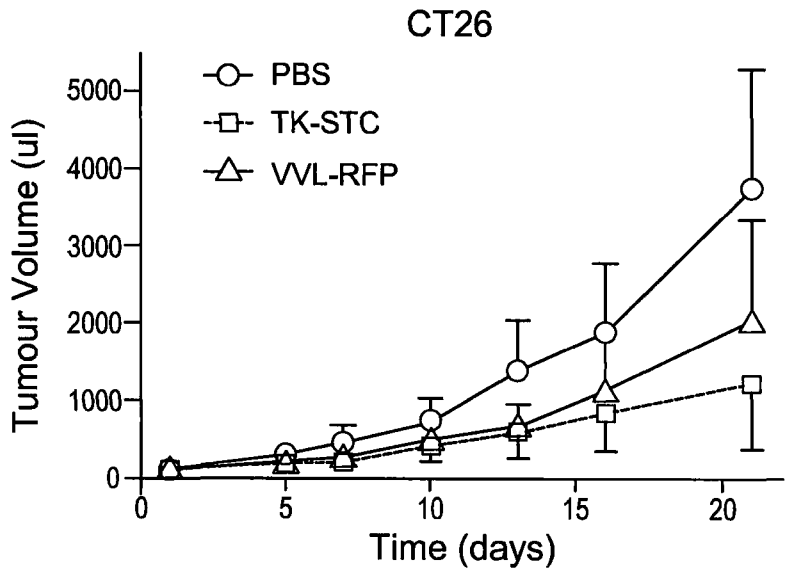

CT26
PBS
TK-STC
VVL-RFP
Tumour Volume (ul)
5000
4000
3000
2000
1000
0
0
5
10
15
20
Time (days)

Fig.12

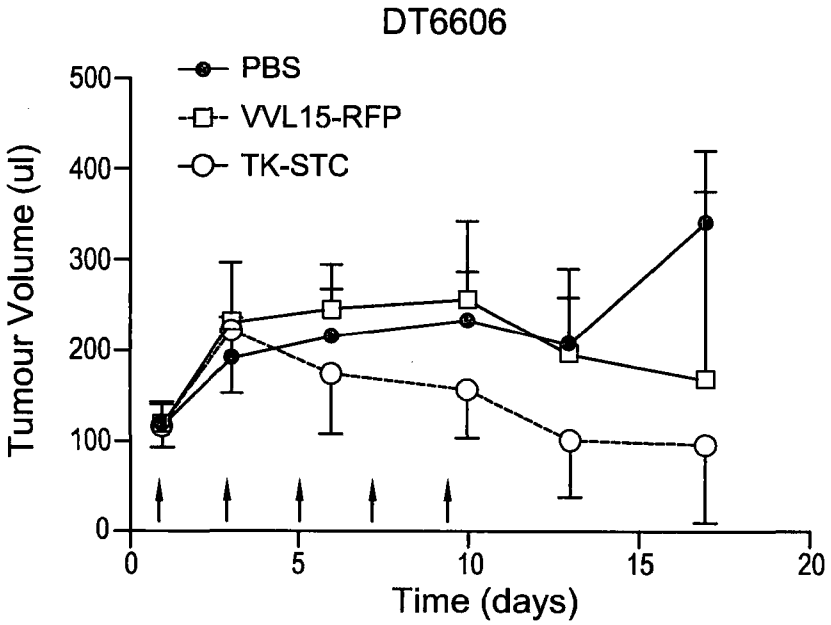

DT6606
PBS
VVL15-RFP
TK-STC
Tumour Volume (ul)
500
400
300
200
100
0
0
5
10
15
20
Time (days)

Fig.13

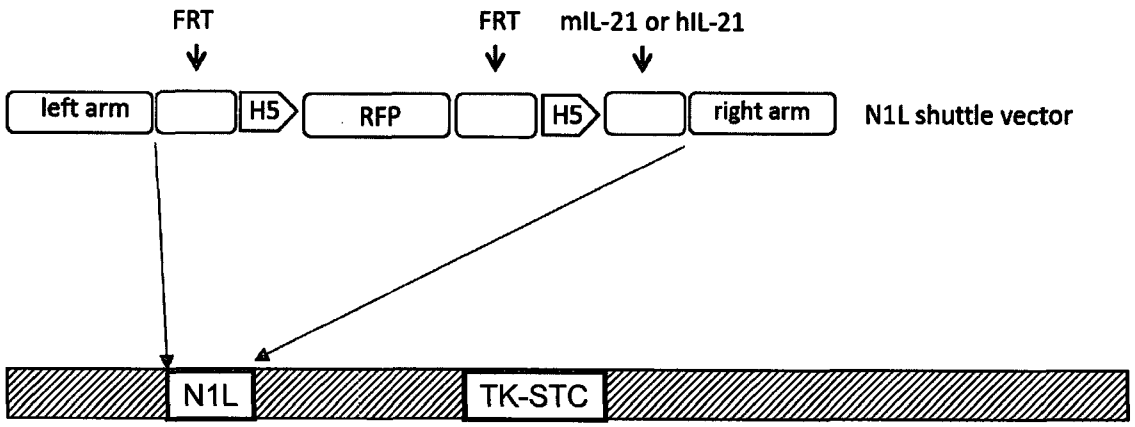

FRT
FRT
mIL-21 or hIL-21
left arm
H5
RFP
H5
right arm
N1L shuttle vector
N1L
TK-STC

Fig.14

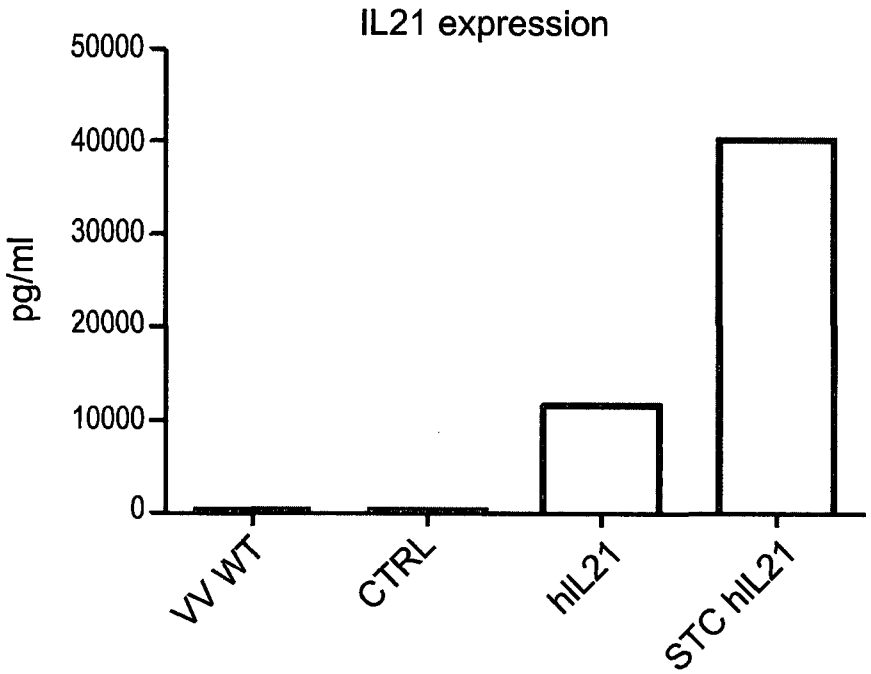

IL21 expression
pg/ml
50000
40000
30000
20000
10000
0
VV WT
CTRL
hIL21
STC hIL21

Fig.15

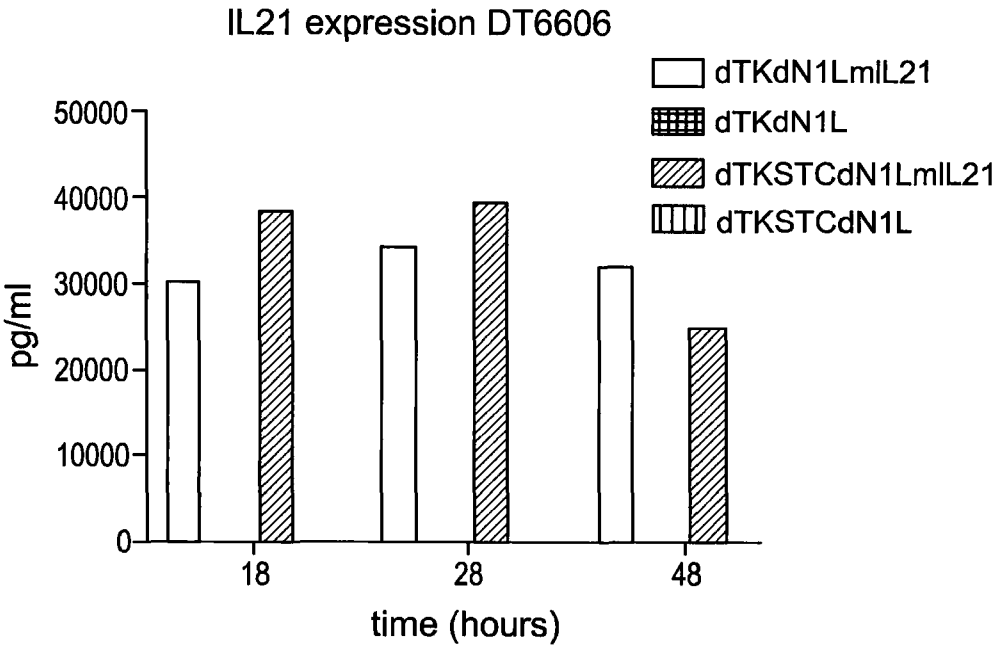

IL21 expression DT6606
dTKdN1LmIL21
dTKdN1L
dTKSTCdN1LmIL21
dTKSTCdN1L
50000
40000
30000
20000
10000
0
pg/ml
18
28
48
time (hours)

Fig.16

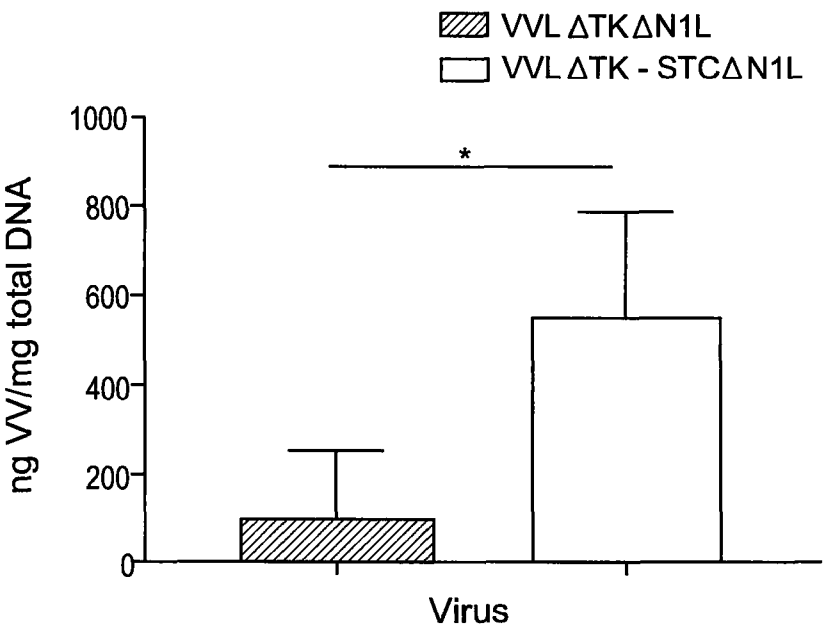

VVL ΔTKΔN1L
VVL ΔTK - STCΔN1L
1000
800
600
400
200
0
ng VV/mg total DNA
*
Virus

Fig.17

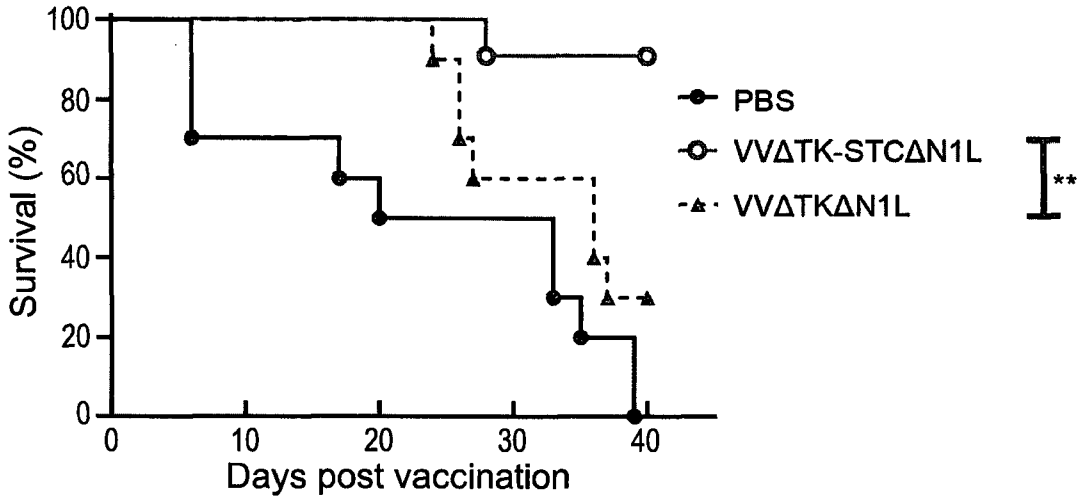

100
80
60
40
20
0
Survival (%)
0
10
20
30
40
Days post vaccination
PBS
VVΔTK-STCΔN1L
VVΔTKΔN1L
**

Fig.18

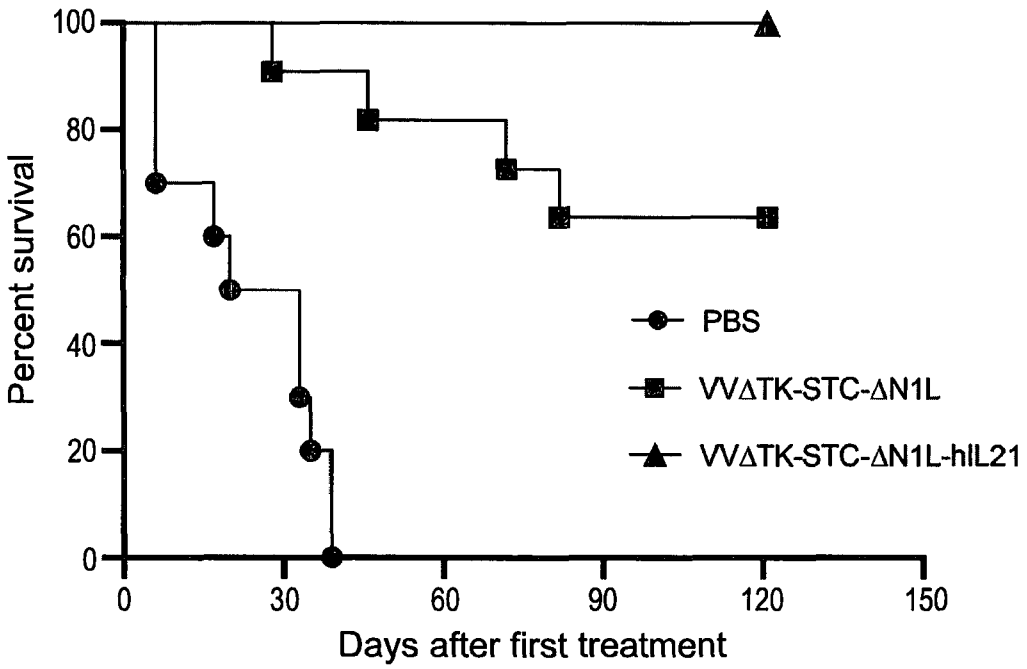

100
80
60
40
20
0
Percent survival
0
30
60
90
120
150
Days after first treatment
PBS
VVΔTK-STC-ΔN1L
VVΔTK-STC-ΔN1L-hIL21

Fig.19

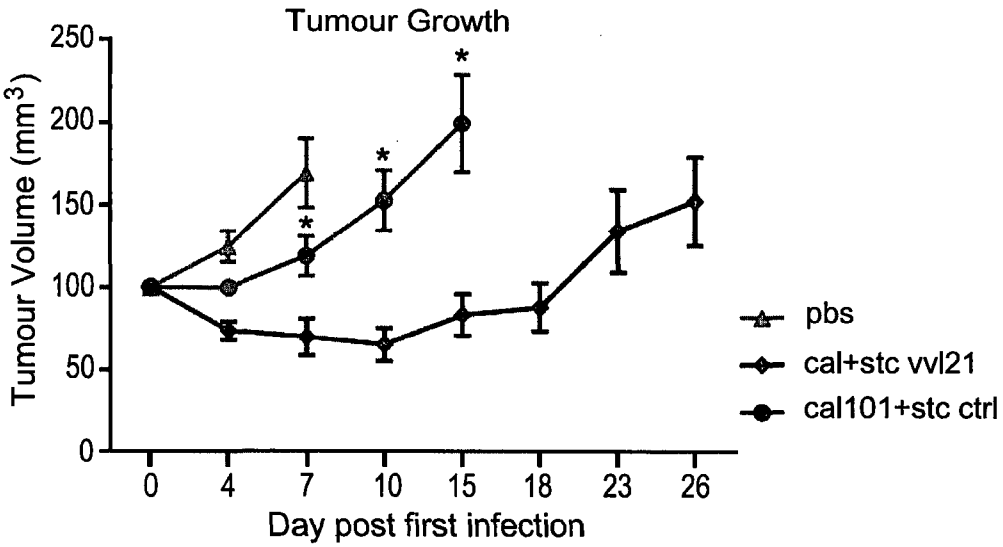

Tumour Growth
Tumour Volume (mm3)
250
200
150
100
50
0
0 4 7 10 15 18 23 26
Day post first infection
*
*
*
pbs
cal+stc vvl21
cal101+stc ctrl

Fig.20

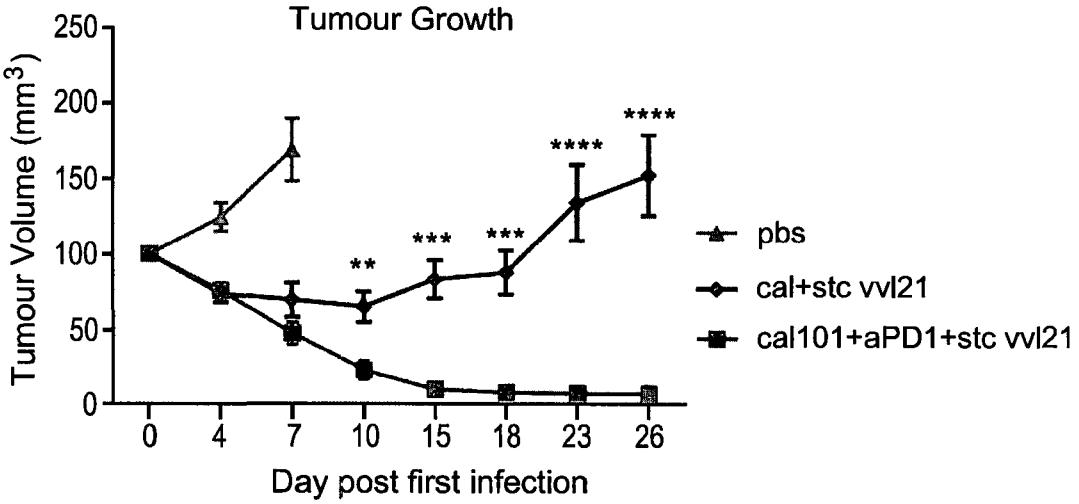

Tumour Growth
Tumour Volume (mm3)
250
200
150
100
50
0
0 4 7 10 15 18 23 26
Day post first infection
**
***
***
****
****
pbs
cal+stc vvl21
cal101+aPD1+stc vvl21

Fig.21

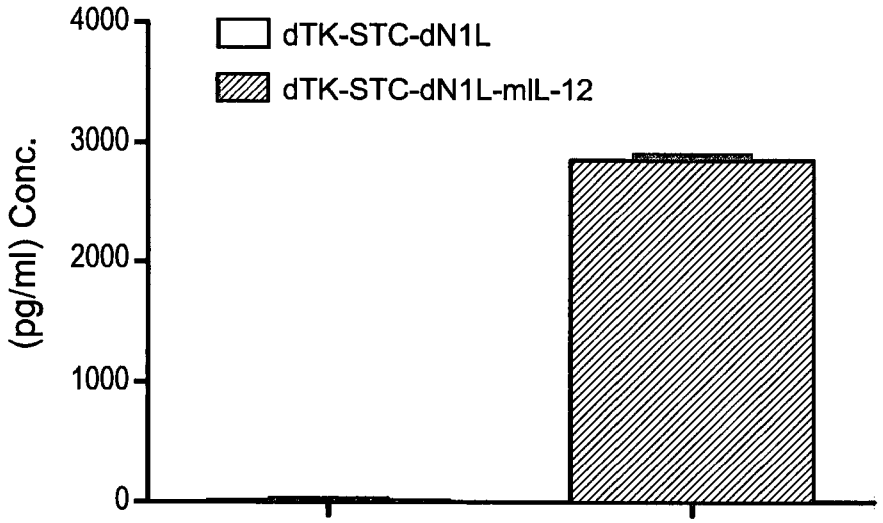

4000
3000
2000
1000
0
(pg/ml) Conc.
dTK-STC-dN1L
dTK-STC-dN1L-mIL-12

Fig.22

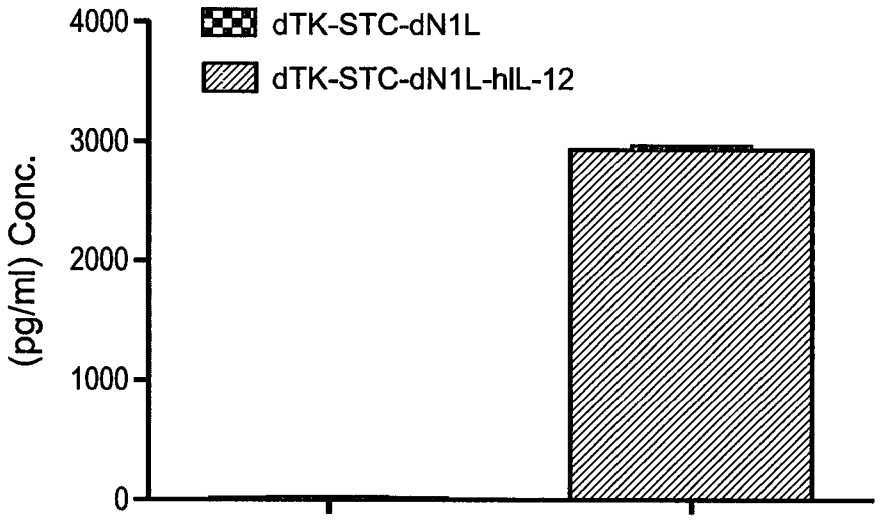

4000
3000
2000
1000
0
(pg/ml) Conc.
dTK-STC-dN1L
dTK-STC-dN1L-hIL-12

Fig.23

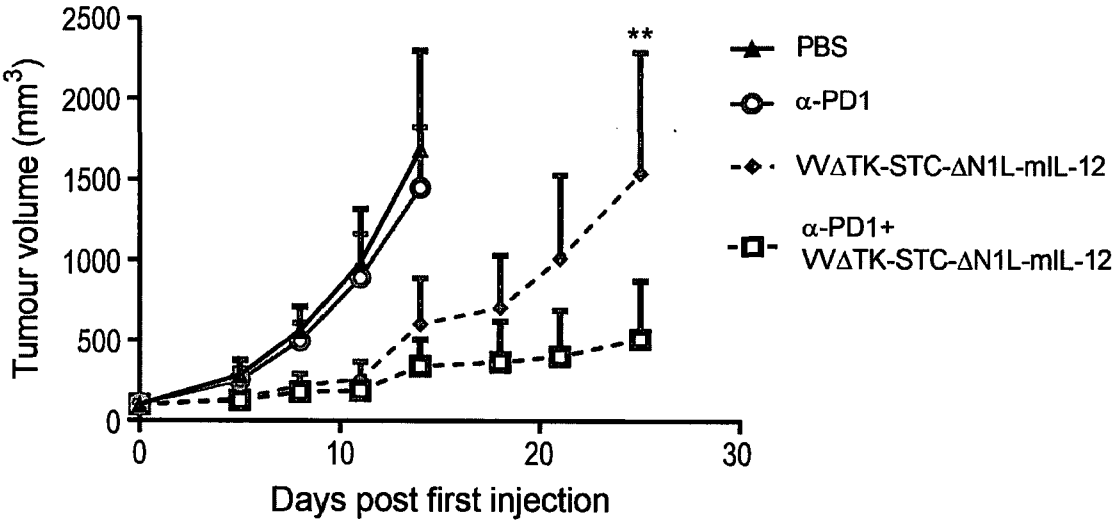

Tumour volume (mm3)
2500
2000
1500
1000
500
0
0
10
20
30
Days post first injection
**
PBS
α-PD1
VVΔTK-STC-ΔN1L-mIL-12
α-PD1+
VVΔTK-STC-ΔN1L-mIL-12

Fig.24

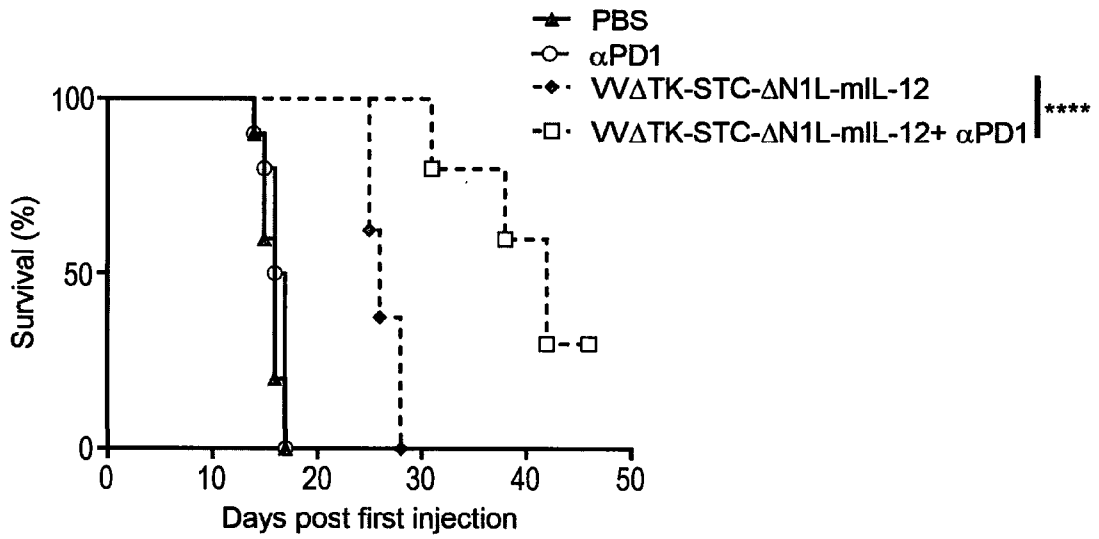

Survival (%)
100
50
0
0
10
20
30
40
50
Days post first injection
PBS
αPD1
VVΔTK-STC-ΔN1L-mIL-12
VVΔTK-STC-ΔN1L-mIL-12+ αPD1
****

Fig.25

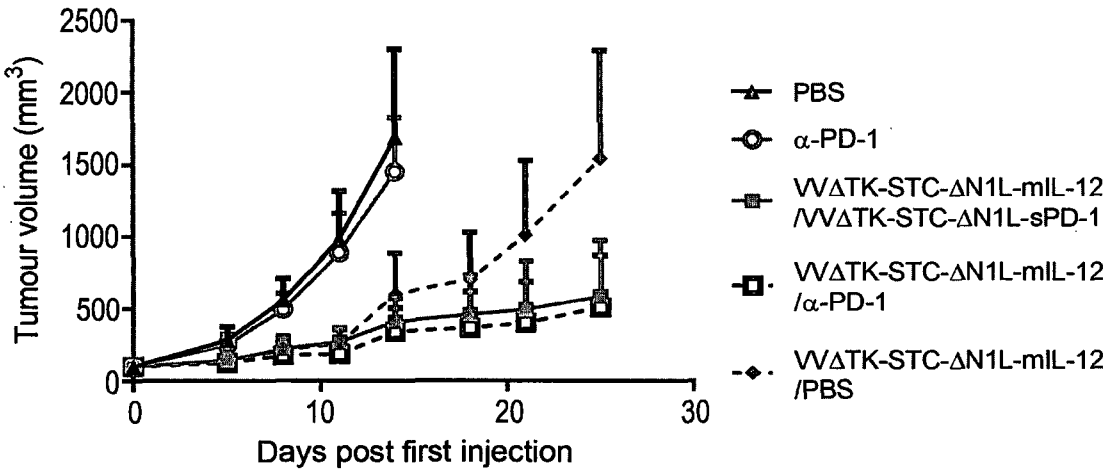

Tumour volume (mm3)
2500
2000
1500
1000
500
0
0
10
20
30
Days post first injection
PBS
α-PD-1
VVΔTK-STC-ΔN1L-mIL-12 /VVΔTK-STC-ΔN1L-sPD-1
VVΔTK-STC-ΔN1L-mIL-12 /α-PD-1
VVΔTK-STC-ΔN1L-mIL-12 /PBS

Fig.26

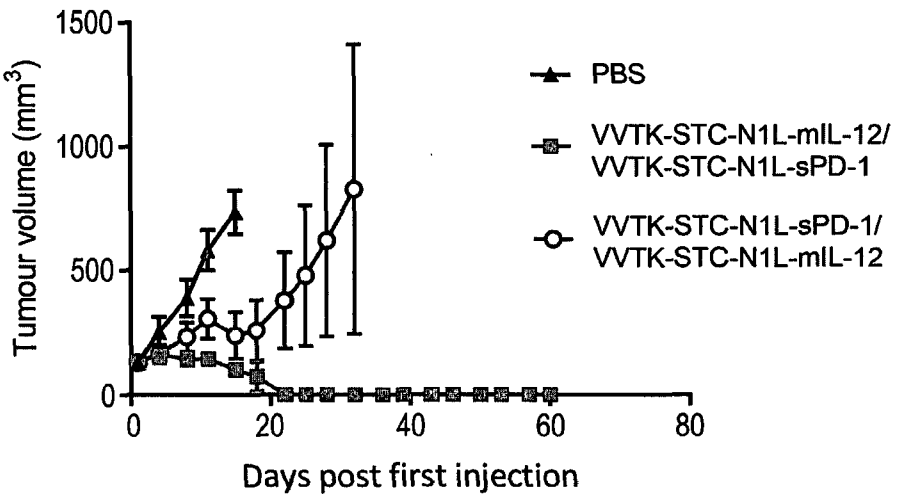

Tumour volume (mm3)
1500
1000
500
0
0
20
40
60
80
Days post first injection
PBS
VVTK-STC-N1L-mIL-12/ VVTK-STC-N1L-sPD-1
VVTK-STC-N1L-sPD-1/ VVTK-STC-N1L-mIL-12

Fig.27

Fig.28

Fig. 28(a) SP(aa 1-19) :

M K T I S V V T L L C V L P A V V Y S T

atgaaaacgatttccgttgttacgttgttatgcgtactacctgctgttgtttattca

Fig. 28(b) SCR1(aa 20-72):

T C T V P T M N N A K L T S T E T S F N D K Q K V T F T C D Q G Y H S S D P N A V C E T D K W K Y E N P

acaactgtacccactatgaataacgctaaattaacgtctaccgaaacatcgtttaatgataaacagaaagttacatttacatgtgatcagggatatcattc ttcggatccaaatgctgtctgcgaaacagataaatggaaatacgaaaatcca

Fig. 28(c) STALK(aa 237-275)

C V R T N E K F D P V D D G P D D E T D L S K L S K D V V Q Y E Q E I E S L E

tgtgtacgaactaacgaaaaatttgatccagtggatgatggtcccgacgatgagacagatttgagcaaactctcgaaagacgttgtacaatatgaaca agaaatagaatcgttagaa

Fig. 28(d) TM(aa 276-303)

A T Y H I I I V A L T I M G V I F L I

gcaacttatcatataatcatagtggcgttgacaattatgggcgtcatatttttaatc

Fig. 28(e) CT(aa 304-317)

S V I V L V C S C D K N N D Q Y K F H K L L P

tccgttatagtattagtttgttcctgtgacaaaaataatgaccaatataagttccataaattgctaccgtga

Fig. 28(f) H5

aaaaattgaaaataaatacaaaggttcttgagggttgtgttaaattgaaagcgagaaataatcataaata

Fig. 28(g) RFP aa 1-225

M A S S E N V I T E F M R F K V R M E G T V N G H E F E I E G E G E G R P Y E G H N T V K
L K V T K G G P L P F A W D I L S P Q F Q Y G S K V Y V K H P A D I P D Y K K L S F P E G F
K W E R V M N F E D G G V A T V T Q D S S L Q D G C F I Y K V K F I G V N F P S D G P V M
Q K K T M G W E A S T E R L Y P R D G V L K G E T H K A L K L K D G G H Y L V E F K S I
Y M A K K P V Q L P G Y Y Y V D A K L D I T S H N E D Y T I V E Q Y E R T E G R H H L F L

atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcaccgtgaacggccacgagttcgagatcgagggcg
agggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtgaccaagggcggccccctgcccttcgcctgggacatcctgtccc
cccagttccagtacggctccaaggtgtacgtgaagcaccccgccgacatccccgactacaagaagctgtccttccccgagggcttcaagtgggag
cgcgtgatgaacttcgaggacggcggcgtggcgaccgtgacccaggactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcgg
cgtgaacttcccctccgacggccccgtgatgcagaagaagaccatgggctgggaggcctccaccgagcgcctgtacccccgcgacggcgtgct
gaagggcgagacccacaaggccctgaagctgaaggacggcggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgca
gctgcccggctactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcaccgagggc
cgccaccacctgttcctgtag

Fig. 28(h)

SP(aa 1-19)-SCR1(aa 20-72)-STALK(aa 237-275)-TM(aa 276-303)- CT(aa 304-317)

MKTISVVTLLCVLPAVVYSTTCTVPTMNNAKLTSTETSFNDKQKVT
FTCDQGYHSSDPNAVCETDKWKYENPCVRTNEKFDPVDDGPDDET
DLSKLSKDVVQYEQEIESLEATYHIIIVALTIMGVIFLISVIVLVCSC
DKNNDQYKFHKLLP

atgaaaacgatttccgttgttacgttgtttatgcgtactacctgctgttgtttattcaacaactgtacccactatgaataacgctaaattaacgtctaccgaaa
catcgtttaatgataaacagaaaagttacatttacatgtgatcagggatatatcattcttcggatccaaatgctgtctgcgaaacagataaatggaaatacga
aaatccatgtgtacgaactaacgaaaaaatttgatccagtggatgatgggtcccgacgatgagacagatttgagcaaactctcgaaagacgttgtacaat
atgaacaagaaatagaatcgttagaagcaacttatataatcatagtggcgttgacaattatgggcgtcatatttttaatctccgttatagtattagtttgt
tcctgtgacaaaaaataatgaccaatataaagttccataaattgctaccgtga

Fig.29

Fig 29.

Fig 29(a) H5:

aaaaattgaaaataaatacaaaggttcttgagggttgtgttaaattgaaagcgagaaataaatcataaata

Fig 29(b) RFP aa 1-225

MASSENVITEFMRFKVRMEGTVNGHEFEIEGEGRPYEGHNTVK
LKVTKGGPLPFAWDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGF
KWERVMNFEDGGVATVTQDSSLQDGCFIYKVKFIGVNFPSDGPVM
QKKTMGWEASTERLYPRDGVLKGETHKALKLKDGGHYLVEFKSI
YMAKKPVQLPGYYYVDAKLDITSHNEDYTIVEQYERTEGRHHLFL

atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcaccgtgaacggccacgagttcgagatcgagggcg
agggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtgaccaagggcggccccctgccctgggacatcctgtccc
cccagttccagtacggctccaaggtgtacgtgaagcaccccgccgacatcccgactacaagaagctgtccttccccgagggcttcaagtgggag
cgcgtgatgaacttcgaggacggcggcgtggccgtgaccgtgacccaggactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcgg
cgtgaacttcccctccgacggccccgtgatgcagaagaaagaccatgggctggagggcctccaccgagcgcctgtaccccgcgacggcgtgct
gaagggcgagacccacaaggccctgaagctgaaggacggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgca
gctgcccggctactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcaccgagggc
cgccaccacctgttcctgtag

Fig. 29(c) H5:

aaaaattgaaaataaatacaaaggttcttgagggttgttgttaaattgaaagcgagaaataatcataaata

Fig. 29(d) SP(aa 1-19):

M K T I S V V T L L C V L P A V V Y S T

atgaaaacgattccgttgttacgttgttatgcgtactacctgctgttgtttattca

Fig. 29(e) STALK(aa 237-275)

C V R T N E K F D P V D D G P D D E T D L S K L S K D V V Q Y E Q E I E S L E

tgtgtacgaactaacgaaaaatttgatccagtggatgatggtcccgacgatgagacagattgagcaaactctcgaaagacgttgtacaatatgaaca
agaaatagaatcgttagaa

Fig. 29(f) TM(aa 276-303)

A T Y H I I I V A L T I M G V I F L I

gcaacttatcatataatcatagtggcgttgacaattatgggcgtcatattttaatc

Fig. 29(g) CT(aa 304-317)

S V I V L V C S C D K N N D Q Y K F H K L L P

tccgttatagtattagtttgttcctgtgacaaaaataatgaccaatataagttccataaaattgctaccgtga

Fig.30

Fig. 30(a) Loxp

acttcgtatagcatacattatacgaagttat

Fig. 30(b) H5:

aaaaattgaaaataaatacaaaggttcttgagggttgtgttaaattgaaagcgagaaataatcataaata

Fig. 30(c) RFP aa 1-225:

M A S S E N V I T E F M R F K V R M E G T V N G H E F E I E G E G E G R P Y E G H N T V K
L K V T K G G P L P F A W D I L S P Q F Q Y G S K V Y V K H P A D I P D Y K K L S F P E G F
K W E R V M N F E D G G V A T V T Q D S S L Q D G C F I Y K V K F I G V N F P S D G P V M
Q K K T M G W E A S T E R L Y P R D G V L K G E T H K A L K L K D G G H Y L V E F K S I
Y M A K K P V Q L P G Y Y Y V D A K L D I T S H N E D Y T I V E Q Y E R T E G R H H L F L

atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcaccgtgaacggccacgagttcgagatcgagggcg
agggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtgaccaagggcggccccctgccctcgccttcgcctgggacatcctgtccc
cccagttccagtacggctccaaggtgtacgtgaagcacccccgccgacatcccccgactacaagaagctgtccttcccccgagggcttcaagtgggag
cgcgtgatgaacttcgaggacggcggcgtggtggcgaccgtgacccaggactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcgg
cgtgaacttcccctccgacggccccgtgatgcagaagaagaccatgggctgggaggcctccaccgagcgcctgtaccccccgcgacggcgtgct
gaagggcgagacccacaaggccctgaagctgaaggacggcggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgca
gctgcccggctactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcaccgagggc
cgccaccacctgttcctgtag

Fig. 30(d) Loxp:

acttcgtatagcatacattatacgaagttat

Fig. 30(e) H5:

aaaaattgaaaataaatacaaaggttcttgagggttgtgttaaattgaaagcgagaaataatcataaata

Fig. 30(f) SP(aa 1-19) :

M K T I S V V T L L C V L P A V V Y S T

atgaaaacgatttccgttgttacgttgttatgcgtactacctgctgttgtttattca

Fig. 30(g) STALK(aa 237-275):

C V R T N E K F D P V D D G P D D E T D L S K L S K D V V Q Y E Q E I E S L E

tgtgtacgaactaacgaaaaatttgatccagtggatgatggtcccgacgatgagacagatttgagcaaactctcgaaagacgttgtacaatatgaaca
agaaatagaatcgttagaa

Fig. 30(h) TM(aa 276-303):

A T Y H I I I V A L T I M G V I F L I

gcaacttatcatataatcatagtggcgttgacaattatgggcgtcatatttttaatc

Fig. 30(i) CT(aa 304-317):

S V I V L V C S C D K N N D Q Y K F H K L L P

tccgttatagtattagtttgttcctgtgacaaaaataatgaccaatataagttccataaattgctaccgtga

Fig.31

(a) STC nucleic acid

tgtgtacgaactaacgaaaaatttgatccagtggatgatggtcccgacgatgagacagatttgagcaaactctcgaaagacgttgtacaatatgaaca
agaaatagaatcgttagaagcaacttatcatataatcatagtggcgttgacaattatgggcgtcatatttttaatctccgttatagtattagtttgttcctgtg
acaaaaataatgaccaatataagttccataaattgctaccgtga

(b) STC protein sequence

C V R T N E K F D P V D D G P D D E T D L S K L S K D V V Q Y E Q E I E S L E A T Y H I I I V
A L T I M G V I F L I S V I V L V C S C D K N N D Q Y K F H K L L P