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DESCRIPTION

Field of the Invention

[0001] The invention relates to an oncolytic immunotherapeutic agent and to the use of the oncolytic immunotherapeutic agent in treating cancer.

Background to the Invention

[0002] Viruses have a unique ability to enter cells at high efficiency. After entry into cells, viral genes are expressed and the virus replicates. This usually results in the death of the infected cell and the release of the antigenic components of the cell as the cell ruptures as it dies. As a result, virus mediated cell death tends to result in an immune response to these cellular components, including both those derived from the host cell and those encoded by or incorporated into the virus itself.

[0003] Viruses also engage with various mediators of the innate immune response as part of the host response to the recognition of a viral infection through e.g. toll-like receptors and cGAS/STING signalling resulting in the activation of interferon responses and inflammation which are also immunogenic signals to the host. These immune responses may result in the immunogenic benefit to cancer patients such that immune responses to tumor antigens provide a systemic overall benefit resulting in the treatment of tumors which have not been infected with the virus, including micro-metastatic disease, and providing vaccination against relapse.

[0004] The combined direct ('oncolytic') effects of the virus, and immune responses against tumor antigens (including non-self `neo-antigens', i.e. derived from the particular mutated genes in individual tumors) is termed `oncolytic immunotherapy'.

[0005] Viruses may also be used as delivery vehicles ('vectors') to express heterologous genes inserted into the viral genome in infected cells. These properties make viruses useful for a variety of biotechnology and medical applications. For example, viruses expressing heterologous therapeutic genes may be used for gene therapy. In the context of oncolytic immunotherapy, delivered genes may include those encoding specific tumor antigens, genes intended to increase the immunogenicity of antigens released following virus replication and cell death, to increase the general immune activation status of the tumor, or to increase the direct oncolytic properties (i.e. cytotoxic effects) of the virus.

[0006] It has been demonstrated that a number of viruses including herpes simplex virus (HSV) have utility in the oncolytic treatment of cancer. HSV for use in the oncolytic treatment of cancer must be disabled such that it is no longer pathogenic, but can still enter into and kill

tumor cells. A number of disabling mutations to HSV, including disruption of the genes encoding ICP34.5, ICP6, and/or thymidine kinase, have been identified which do not prevent the virus from replicating in culture or in tumor tissue *in vivo,* but which prevent significant replication in normal tissue. HSVs in which only the ICP34.5 genes have been disrupted replicate in many tumor cell types *in vitro,* and replicate selectively in tumor tissue, but not in surrounding tissue, in mouse tumor models. Clinical trials of ICP34.5 deleted, or ICP34.5 and ICP6 deleted, HSV have also shown safety and selective replication in tumor tissue in man.

[0007] As discussed above, an oncolytic virus, including HSV, may also be used to deliver a therapeutic gene in the treatment of cancer. An ICP34.5 deleted virus of this type additionally deleted for ICP47 and encoding a heterologous gene for GM-CSF has also been tested in clinical trials, including a phase 3 trial in melanoma in which safety and efficacy in man was shown. The trial data demonstrated that tumor responses could be seen in injected tumors, and to a lesser extent in uninjected tumors. Responses tended to be highly durable (months-years), and a survival benefit appeared to be achieved in responding patients. Each of these indicated engagement of the immune system in the treatment of cancer in addition to the direct oncolytic effect. However, this and other data with oncolytic viruses generally showed that not all tumors respond to treatment and not all patients achieve a survival advantage.

[0008] Liu et al. (2003, Gene Therapy, 10 (4): 292-303) describes ICP34.5 deleted herpes simplex viruses with enhanced oncolytic, immune stimulating and anti-tumour properties.

[0009] Senzer et al. (2009, Journal of Clinical Oncology, 27(34): 5763-5771) describes a phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second generation oncolytic herpes virus in patients with unresectable metastatic melanoma.

[0010] Simpson et al. (2006, Cancer Research, 66(9): 4853-4842) discloses the combination of a fusogenic glycoprotein, prodrug activation, and oncolytic herpes simplex virus for enhanced local tumour control.

[0011] Piasecki et al. discloses that Talilmogene laherparepvec increases the anti-tumour efficacy of the anti-PD-1 immune checkpoint blockade (2015, URL: http://www.abstractsonline.com/Plan/View Abstract.aspx?mID=3682&sKey=ebc7290a-af22-4fa6-9436-c276011fb181&cKey=b61c1150-5517-456b-acd3-2f8048807587&mKey=19573a54-ae8f-4e00-9c23-bd6d62268424).

[0012] Sokolowski et al. (2015, Oncolytic virotherapy, 4: 207-219) reviews how far have we come in oncolytic virotherapy using herpes simplex virus.

[0013] WO 2006/002394 discloses avirulent oncolytic herpes virus strains engineered to counter the innate host response.

[0014] Thus, improvements to the art of oncolytic therapy and oncolytic immunotherapy are clearly needed. These may serve to increase the direct oncolytic effects of therapy, the anti-

tumor immune stimulating effects of the therapy, or both of these effects together.

[0015] Recently it has been shown that oncolytic immunotherapy can result in additive or synergistic therapeutic effects in conjunction with immune checkpoint blockade (i.e. inhibition or 'antagonism' of immune checkpoint pathways), also referred to as immune co-inhibitory pathway blockade. Checkpoint (immune co-inhibitory pathway) blockade is intended to block host immune inhibitory mechanisms which usually serve to prevent the occurrence of auto-immunity. However, in cancer patients these mechanisms can also serve to inhibit or block the potentially beneficial effects of any immune responses induced to tumors. Alternatively, immune responses may not be fully potentiated due to a lack of activation or lack of full activation of immune potentiating pathways. Therefore, drugs which alleviate these blocks or stimulate immune potentiating pathways (i.e. which activate, or are 'agonists' of these immune potentiating pathways) are attractive for testing and developing cancer treatments. Targets for such approved or experimental drugs include CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, VISTA, CSF1R, IDO, CEACAM1, GITR, 4-1-BB, KIR, SLAMF7, OX40, CD40, ICOS or CD47.

[0016] For these approaches to be successful, pre-existing immune responses to tumors are needed, i.e. so that a pre-existing immune response can be potentiated or a block to an anti-tumor immune response can be relieved. The presence of an inflamed tumor microenvironment, which is indicative of such an ongoing response, is also needed. Pre-existing immune responses to tumor neo-antigens appear to be particularly important for the activity of immune co-inhibitory pathway blockade and related drugs. Only some patients may have an ongoing immune response to tumor antigens including neoantigens and/or an inflamed tumor microenvironment, both of which are required for the activity of these drugs. Therefore, oncolytic agents which can induce immune responses to tumor antigens, including neoantigens, and/or which can induce an inflamed tumor microenvironment are attractive for use in combination with immune co-inhibitory pathway blockade and immune potentiating drugs. This likely also explains the promising combined anti-tumor effects of oncolytic agents and immune co-inhibitory pathway blockade in mice and humans that have so far been observed.

[0017] The indoleamine 2,3-dioxygenase (IDO) pathway contributes to tumor-induced tolerance by creating a tolerogenic environment in the tumor and the tumor-draining lymph nodes, both by direct suppression of T cells and enhancement of local regulatory T cell (Treg)-mediated immunosuppression. IDO catalyses the rate-limiting step of tryptophan degradation along the kynurenine pathway, and both the reduction in local tryptophan concentration and the production of immunomodulatory tryptophan metabolites contribute to the immunosuppressive effects of IDO. IDO is chronically activated in many cancer patients with IDO activation correlating with more extensive disease. It can also function as an antagonist to other activators of antitumor immunity. Therefore, inhibitors of the IDO pathway are being developed as anticancer agents, particularly in combination with checkpoint blockade agents such as those which target CTLA-4, PD-1 or PDL-1. IDO inhibitors may also be synergistic with oncolytic immunotherapy, including together with drugs targeting other immune checkpoint or immune co-stimulatory pathways.

Summary of the Invention

[0018] The invention provides improved oncolytic viruses. The improved oncolytic viruses have improved direct oncolytic effects. The improved direct oncolytic effects provided by the viruses of the invention will also lead to improved systemic anti-tumor immune effects. The improved direct oncolytic effects provided by the viruses of the invention will also lead to improved therapeutic effects in patients. Enhanced replication in and killing of tumor cells will result in enhanced tumor antigen release and enhanced systemic immune responses to the released antigens. The expression levels of any genes inserted to augment the direct oncolytic effects and/or immune stimulation will also be increased.

[0019] Virus species naturally exist in a range of variants (strains) within the natural population which may differ by a small or larger number of nucleotides while still retaining the antigenic characteristics and sufficient sequence identity to still be recognized as the same virus species. These strains, due to their differing sequences, may exhibit a range of differing properties, including properties which have been selected for by natural selection in their natural host or hosts (for example the ability to infect or replicate in the target cell types of the virus in question, spread between these cells, or to evade the host innate or adaptive immune system, or to spread between infected individuals of the host species) and properties which have not been specifically selected for (e.g. the ability to replicate in and kill or spread between cell types which are not the natural targets of the virus in question, including tumor or other non-target cell types or tissues). The inventors have recognised that sampling a range of viral strains of a particular viral species which are present in the natural host population (in the case of viruses infecting humans, here termed `clinical isolates') and comparing these to each other to select for the strain with the best properties for the intended purpose for which it is to be used (e.g. infection and killing of tumor cells) can be used to identify a virus (i.e. a virus strain) with optimal properties for that purpose. The optimal properties may be properties that offer the best starting point for development to produce a virus that can be used as a therapeutic. A virus identified by this approach is likely to have more optimal properties for the intended purpose than a 'prototype' or `laboratory' virus strain or a clinical strain which has not been selected for the required property or properties from a broad group of viral strains. This is because the full biological complexity in the natural population, particularly with respect to the particular desirable property or properties, is unlikely to have been sampled through taking a narrow approach to screening for the desired property or properties, bearing in mind the degree of sequence variation present in natural virus populations. In particular, these may vary in sequence within an infected host (as is often the case with RNA or retroviral populations where so-called quasi-species are often present), between individual infected hosts, or between different geographically separated viral populations.

[0020] Viruses of the invention have therefore been selected by sampling a range of viral strains present in the natural population of a particular viral species and testing these against each other for the desired property or properties (e.g. the ability to infect and kill tumor cells).

The virus strain or strains with the best properties for the intended purpose are used for further development.

[0021] Where the intended use is oncolytic viral therapy, taking such an approach provides an improved starting point for development of an oncolytic agent, which may require further manipulation of the advantageous virus strains. Such manipulation includes the deletion of viral genes to provide, for example, tumor selectivity, and/or the insertion of exogenous genes to improve oncolytic or immune potentiating properties further.

[0022] The viruses of the invention therefore include novel clinical isolates of a viral species that have better anti-tumor effects than the other clinical isolates to which they were compared and through which comparison they were identified. In particular, the viruses of the invention kill tumor cell lines *in vitro* more quickly and/or at a lower dose than these reference clinical isolates of the same virus type. The viruses of the invention have been identified through comparison of >20 clinical isolates of the viral species.

[0023] Typically, a virus of the invention can kill two or more tumor cell lines *in vitro* within 24 to 48 hours after infection at a multiplicity of infection (MOI) of 0.01 to 0.001 or less.

[0024] The viruses of the invention may be modified to further enhance their anti-tumor effects. The genome of a virus of the invention may be modified to delete or alter expression of one or more viral genes, and/or the genome of the clinical isolate may be modified to express one or more heterologous genes, such as genes encoding a fusogenic protein and/or an immune stimulatory molecule or molecules.

[0025] Oncolytic viruses of the invention provide improved treatment of cancer through improved direct oncolytic effects, viral replication and spread through tumors, which (i) increases the amount of tumor antigens, including neoantigens, which are released for the induction of an anti-tumor immune response; and (ii) enhances the expression of the virus-encoded immune stimulatory molecule(s). Expression of immune stimulatory molecule(s) by the virus can further enhance and potentiate the anti-tumor immune effect. Expression of fusogenic protein(s) by the virus can further enhance viral spread through tumors. Expression of fusogenic protein(s) by the virus can further enhance tumor cell killing.

[0026] Anti-tumor efficacy of an oncolytic virus of the invention is achieved when the virus is used as a single agent and also when the virus is used in combination with other anti-cancer modalities, including chemotherapy, treatment with targeted agents, radiation, immune checkpoint blockade (i.e. administration of one or more antagonist of an immune co-inhibitory pathway) and/or immune potentiating drugs (e.g.one or more agonists of an immune co-stimulatory pathway). The improved direct oncolytic effects (i.e. virus replication in, spread between, and direct killing of tumor cells) and improved systemic anti-tumor immune effects of the viruses of the invention improve on the combined benefits of oncolytic therapy and immune co-inhibitory pathway blockade and/or immune co-stimulatory pathway activation.

[0027] Accordingly, the present invention provides an oncolytic virus which is a herpes simplex virus (HSV) 1 strain RH018A having the accession number ECCAC 16121904; RH004A having the accession number ECCAC 16121902; RH031A having the accession number ECCAC 16121907; RH040B having the accession number ECCAC 16121908; RH015A having the accession number ECCAC 16121903; RH021A having the accession number ECCAC 16121905; RH023A having the accession number ECCAC 16121906; or RH047A having the accession number ECCAC 16121909.

[0028] The invention also provides an oncolytic virus which is a herpes simplex virus (HSV) 1 strain RH018A having the accession number ECCAC 16121904; RH004A having the accession number ECCAC 16121902; RH03 1A having the accession number ECCAC 16121907; RH040B having the accession number ECCAC 16121908; RH015A having the accession number ECCAC 16121903; RH021A having the accession number ECCAC 16121905; RH023A having the accession number ECCAC 16121906; or RH047A having the accession number ECCAC 16121909, comprising (a) a mutation that prevents the expression of functional ICP34.5, ICP6 and/or thymidine kinase; (b) one or more immune stimulatory molecules or one or more immune stimulatory molecule encoding genes; and/or (c) one or more fusogenic protein-encoding genes.

[0029] The HSV typically does not express functional ICP34.5 and/or functional ICP47 and/or expresses the US 11 gene as an immediate early gene.

[0030] The virus may comprise (i) a fusogenic protein-encoding gene; and/or (ii) an immune stimulatory molecule or an immune stimulatory molecule-encoding gene. The virus may encode more than one fusogenic protein and/or more than one immune stimulatory molecule. The fusogenic protein is preferably the glycoprotein from gibbon ape leukemia virus (GALV) and has the R transmembrane peptide mutated or removed (GALV-R-). The immune stimulatory molecule is preferably GM-CSF and/or an agonist of an immune co-stimulatory pathway including GITRL, 4-1-BBL, OX40L, ICOSL or CD40L, or a protein capable of blocking signaling through CTLA-4, for example an antibody or a fragment thereof which binds CTLA-4.

[0031] The invention also provides:

- a pharmaceutical composition comprising a virus of the invention and a pharmaceutically acceptable carrier or diluent;
- the virus of the invention for use in a method of treating the human or animal body by therapy; and
- the virus of the invention for use in a method of treating cancer, wherein the method optionally comprises administering a further anti-cancer agent.

[0032] The further anti-cancer agent may be an antagonist of an immune co-inhibitory pathway or an agonist of an immune co-stimulatory pathway

Brief Description of the Figures

[0033]

Figure 1 depicts the structure of an exemplary virus of the invention that comprises a gene encoding GALV-R- and a gene encoding GM-CSF inserted into the ICP34.5 gene locus, and in which the ICP47 gene is deleted such that the US11 gene is under the control of the ICP47 immediate early promoter (top panel). Figure 1 also shows similar exemplary viruses of the invention expressing only a GALV-R-encoding gene (second panel), or only a GM-CSF-encoding gene (third panel) Also shown is an exemplary virus in which the ICP34.5 gene and the ICP47 gene are deleted.

Figure 2 depicts the structure of an exemplary virus of the invention that comprises a gene encoding GALV-R-, a gene encoding GM-CSF and a gene encoding CD40L.

Figure 3 shows the differential abilities of the eight top ranking HSV1 clinical isolate strains as assessed by crystal violet staining 24 hours or 48 hours after infection with a MOI of 0.1, 0.01 or 0.001 as indicated in the Figure to kill Fadu, SK-mel-28, A549, HT1080, MIA-PA-CA-2, HT29 and MDA-MB-231 human tumor cell lines. The virus strains ranked first and second on each cell line are indicated. The virus RH018A was ranked first on each of the Fadu, HT1080, MIA-PA-CA-2 and HT29 cell lines and second on each of the SK-mel-28, A549 and MDA-MB-231 cell lines. RH004A was ranked joint first with RH018A and RH015A on the HT29 cell line, first on the SK-mel-28 and A549 cell lines and second on the Fadu cell line. RH023A was ranked first on the MDA-MB-231 cell line and second on the HT1080 cell line. RH031A was ranked second on each of the MIA-PA-CA-2 and HT29 cell lines. RH040A was ranked joint second on the HT29 cell line.

Figure 4 shows a comparison between strain RH018A, the strain ranked first of all the strains tested, with an 'average' strain from the screen (i.e. strain RH065A). Approximately 10 fold less of strain RH018A was needed to kill an equal proportion of cells than was needed of strain RH065A as shown by crystal violet staining 24 or 48 hours post infection with MOIs of 0.1, 0.01 and 0.001 in SK-mel-28, HT1080, MDA-MB-231, Fadu, MIA-PA-CA-2 and A549 cell lines.

Figure 5 depicts structures of HSV1 viruses modified by the deletion of ICP34.5 and ICP47 such that the US11 gene is under control of the ICP457 immediate early promoter and containing heterologous genes in the ICP34.5 locus. The viruses were constructed using the RH018A strain unless otherwise stated in the Figure.

Figure 6 shows the results of an ELISA to detect expression of human or mouse GM-CSF in supernatants from BHK cells infected with virus 16 (mGM-CSF and GALVR-), virus 17 (hGM-CSF and GALVR-) and virus 19 (mGM-CSF).

Figure 7 is a comparison between the cell-killing abilities of strain RH018A in which ICP34.5 is deleted and which expresses GALVR- and GFP (virus 10) with a virus that expresses only GFP (virus 12) as determined by crystal violet staining in three cell lines at low magnification.

Figure 8 is a comparison between the cell-killing abilities of strain RH018A in which ICP34.5 and ICP47 are deleted and which expresses GALVR- and GM-CSF (virus 17) with a prior art strain with the same modifications as determined by crystal violet staining in four cell lines.

Figure 9 shows the effectiveness of Virus 16 (ICP34.5 and ICP47 deleted expressing GALVR- and mGM-CSF) in treating mice harbouring A20 lymphoma tumors in both flanks. Tumors on the right flanks were injected with the virus or vehicle and the effects on tumor size was observed for 30 days. The virus was effective against both injected tumors and non-injected tumors.

Figure 10 demonstrates the effects of Virus 15 (ICP34.5 and ICP47 deleted expressing GALVR- and GFP) and Virus 24 (ICP34.5 and ICP47 deleted expressing GFP) on rat 9L cells *in vitro* as assessed by crystal violet staining. The virus expressing GALV (Virus 15) showed enhanced killing of rat 9L cells *in vitro* as compared to a virus which does not express GALV (Virus 24).

Figure 11 shows the antitumor effects of Virus 16 in Balb/c mice harboring mouse CT26 tumors in the left and right flanks. Groups of 10 mice were then treated with: Vehicle (3 injections into right flank tumors every other day); 5×10exp6 pfu of Virus 16 (mRP1) injected in the right flank tumor every other day; anti-mouse PD1 alone (10mg/kg i.p. every three days, BioXCell clone RMP1-14); anti-mouse CTLA-4 (3mg/kg i.p every three days, BioXCell clone 9D9); anti-mouse PD1 together with Virus 16; anti-mouse CTLA4 together with Virus 16; 1-methyl trypotophan (l-MT; IDO inhibitor (5mg/ml in drinking water)); anti-mouse PD1 together with 1-methyl trypotophan; or anti-mouse PD1 together with 1-methyl trypotophan and Virus 16. Effects on tumor size were observed for a further 30 days. Greater tumor reduction was seen in animals treated with combinations of virus and checkpoint bockade than with the single treatment groups. Figure 11A shows that using Virus 16 and anti-PD1 in combination has a better anti-tumor effect than using either anti-PD1 or the virus alone. Figure 11B shows that the anti-tumor effect of Virus 16 in combination with anti-CTLA-4 was better than the anti-tumor effect of either Virus 16 or anti-CTLA-4 alone. Figure 11C shows that enhanced tumor reduction was observed using Virus 16 together with both anti-PD1 and IDO inhibition as compared to anti-PD1 and 1-MT inhibition in the absence of the virus.

Figure 12 shows the enhanced anti-tumor activity of Virus 16 in combination with immune checkpoint blockade in mouse A20 tumors in both flanks of Balb/c mice as compared to either virus alone or checkpoint blockade alone (anti-PD1).

Figure 13 shows the structure of ICP34.5 and ICP47 deleted viruses expressing GALVR-, GM-CSF and codon optimized anti-mouse or anti-human CTLA-4 antibody constructs (secreted scFv molecules linked to human or mouse IgG1 Fc regions). The scFvs contain the linked ($[G_4S]_3$) light and heavy variable chains from antibody 9D9 (US2011044953: mouse version) and from ipilimumab (US20150283234; human version). The resulting structure of the CTLA-4 inhibitor is also shown.

Figure 14 shows anti-tumor effects of Virus 16 and Virus 19 in a human xenograft model

(A549). There were three injections of Virus 16, Virus 19 or of vehicle over one week at three different dose levels (N=10/group). The doses of the viruses used is indicated. The anti-tumor effects of Virus 16 which expresses GALV were better than those of Virus 19 which does not express GALV.

Figure 15 demonstrates the effects of viruses of the invention expressing GALVR-on 9L cells in the flanks of Fischer 344 rats. The following treatments were administered to groups of rats (ten per group), into one flank of each rat only three times per week for three weeks: 50µl of vehicle; 50µl of 10^7 pfu/ml of Virus 19 (expresses mGM-CSF but not GALV R-); or 50µl of 10^7 pfu/ml of Virus 16 (expresses both mouse GM-CSF and GALVR-). Effects on tumor growth were then observed for a further 30 days. Superior tumor control and shrinkage was observed with the virus expressing GM-CSF and GALV-R- as compared to the virus expressing GM-CSF alone.

Figure 16 shows the anti-tumor effects of viruses expressing anti-mCTLA-4 (virus 27), mCD40L (virus 32), mOX4OL (virus 35), m4-2BBL (virus 33), , each also with mGM-CSF and GALV-R- compared to virus 16 (expresses GALV and mGM-CSF).

Brief Description of the Sequence Listing

[0034]

SEQ ID NO: 1 is the nucleotide sequence of mouse GM-CSF.

SEQ ID NO: 2 is the nucleotide sequence of a codon optimized version of mouse GM-CSF.

SEQ ID NO: 3 is the nucleotide sequence of human GM-CSF.

SEQ ID NO: 4 is the nucleotide sequence of a codon optimized version of human GM-CSF.

SEQ ID NO: 5 is the amino acid sequence of mouse GM-CSF.

SEQ ID NO: 6 is the amino acid sequence of human GM-CSF.

SEQ ID NO: 7 is the nucleotide sequence of GALV-R-.

SEQ ID NO: 8 is the nucleotide sequence of a codon optimized version of GALVR- (the first three nucleotides are optional)

SEQ ID NO: 9 is the amino acid sequence of GALV-R-.

SEQ ID NO: 10 is the nucleotide sequence of a codon optimized version of a human membrane bound version of CD40L.

SEQ ID NO: 11 is the amino acid sequence of a human membrane bound version of CD40L.

SEQ ID NO: 12 is the nucleotide sequence of a codon optimized version of a multimeric secreted version of human CD40L.

SEQ ID NO: 13 is the amino acid sequence of a multimeric secreted version of human CD40L.

SEQ ID NO: 14 is the nucleotide sequence of a codon optimized version of a multimeric secreted version of mouse CD40L.

SEQ ID NO: 15 is the amino acid sequence of a multimeric secreted version of mouse CD40L.

SEQ ID NO: 16 is a codon optimized version of the nucleotide sequence of wild-type human CD40L.

SEQ ID NO: 17 is the amino acid sequence of wild-type human CD40L.

SEQ ID NO: 18 is a codon optimized version of the nucleotide sequence of wild-type mouse CD40L.

SEQ ID NO: 19 is the amino acid sequence of wild-type mouse CD40L.

SEQ ID NO: 20 is the nucleotide sequence of a codon optimized version of murine 4-1BBL.

SEQ ID NO: 21 is the nucleotide sequence of a codon optimized version of human 4-1BBL.

SEQ ID NO: 22 is the nucleotide sequence of a codon optimized version of secreted mouse 4-1BBL.

SEQ ID NO: 23 is the nucleotide sequence of a codon optimized version of human secreted 4-1BBL.

SEQ ID NO: 24 is the nucleotide sequence of a codon optimized version of murine GITRL.

SEQ ID NO: 25 is the nucleotide sequence of a codon optimized version of human GITRL.

SEQ ID NO: 26 is the nucleotide sequence of a codon optimized version of secreted murine GITRL.

SEQ ID NO: 27 is the nucleotide sequence of a codon optimized version of secreted human GITRL.

SEQ ID NO: 28 is the nucleotide sequence of a codon optimized version of murine OX40L.

SEQ ID NO: 29 is the nucleotide sequence of a codon optimized version of human OX40L.

SEQ ID NO: 30 is the nucleotide sequence of a codon optimized version of secreted murine OX40L.

SEQ ID NO: 31 is the nucleotide sequence of a codon optimized version of secreted human OX40L.

SEQ ID NO: 32 is the nucleotide sequence of a codon optimized version of murine ICOSL.

SEQ ID NO: 33 is the nucleotide sequence of a codon optimized version of human ICOSL.

SEQ ID NO: 34 is the nucleotide sequence of a murine scFv CTLA-4 antibody. The first six and last eight nucleotides are restriction sites added for cloning purposes.

SEQ ID NO: 35 is the nucleotide sequence of a murine scFv CTLA-4 antibody. The first six and last eight nucleotides are restriction sites added for cloning purposes.

SEQ ID NO: 36 is the nucleotide sequence of the CMV promoter.

SEQ ID NO: 37 is the nucleotide sequence of the RSV promoter.

SEQ ID NO: 38 is the nucleotide sequence of BGH polyA.

SEQ ID NO: 39 is the nucleotide sequence of SV40 late polyA.

SEQ ID NO: 40 is the nucleotide sequence of the SV40 enhancer promoter.

SEQ ID NO: 41 is the nucleotide sequence of rabbit beta-globulin (RBG) polyA.

SEQ ID NO: 42 is the nucleotide sequence of GFP.

SEQ ID NO: 43 is the nucleotide sequence of the MoMuLV LTR promoter.

SEQ ID NO: 44 is the nucleotide sequence of the EF1a promoter.

SEQ ID NO: 45 is the nucleotide sequence of HGH polyA.

Detailed Description of the Invention

Oncolytic Virus

[0035] The virus of the invention is oncolytic. An oncolytic virus is a virus that infects and replicates in tumor cells, such that the tumor cells are killed. Therefore, the virus of the invention is replication competent. Preferably, the virus is selectively replication competent in tumor tissue. A virus is selectively replication competent in tumor tissue if it replicates more effectively in tumor tissue than in non-tumor tissue. The ability of a virus to replicate in different tissue types can be determined using standard techniques in the art.

[0036] Viruses of the invention may be wild type (i.e. unaltered from the parental virus species) herpes simplex virus (HSV) 1 strain RH018A having the accession number ECCAC 16121904; RH004A having the accession number ECCAC 16121902; RH031A having the accession

number ECCAC 16121907; RH040B having the accession number ECCAC 16121908; RH015A having the accession number ECCAC 16121903; RH021A having the accession number ECCAC 16121905; RH023A having the accession number ECCAC 16121906; or RH047A having the accession number ECCAC 16121909. Viruses of the invention may be any of these HSV1 strains comprising a mutation that prevents the expression of functional ICP34.5, ICP6 and/or thymidine kinase by the HSV, one or more immune stimulatory molecules or one or more immune stimulatory molecule encoding genes; and/or one or more fusogenic protein-encoding genes.

[0037] The herpes simplex virus (HSV) 1 strain RH018A having the accession number ECCAC 16121904; RH004A having the accession number ECCAC 16121902; RH031A having the accession number ECCAC 16121907; RH040B having the accession number ECCAC 16121908; RH015A having the accession number ECCAC 16121903; RH021A having the accession number ECCAC 16121905; RH023A having the accession number ECCAC 16121906; and RH047A having the accession number ECCAC 16121909 are clinical isolates that were selected on the basis of their having particular advantageous properties for the treatment of cancer. The virus of the invention has surprisingly good anti-tumor effects compared to other strains of the same virus isolated from other patients, wherein a patient is an individual harbouring the virus species to be tested. HSV1 strains may be isolated from cold sores of individuals harboring HSV1, typically by taking a swab using e.g. Virocult (Sigma) brand swab/container containing transport media followed by transport to the facility to be used for further testing.

[0038] After isolation of viruses to be compared from individuals, stocks of the viruses may be prepared, for example by growing the isolated viruses on BHK or vero cells. Preferably, this is done following no more than 3 cycles of freeze thaw between taking the sample and it being grown on, for example, BHK or vero cells to prepare the virus stock for further use. More preferably the virus sample has undergone 2 or less than 2 cycles of freeze thaw prior to preparation of the stock for further use, more preferably one cycle of freeze thaw, most preferably no cycles of freeze thaw. Lysates from the cell lines infected with the viruses prepared in this way after isolation may be compared, typically by testing for the ability of the virus to kill tumor cell lines *in vitro.* Alternatively, the viral stocks may be stored under suitable conditions, for example by freezing, prior to testing. Viruses of the invention have surprisingly good anti-tumor effects compared to other strains of the same virus isolated from other individuals, preferably when compared to those isolated from >5 individuals, more preferably >10 other individuals, most preferably >20 other individuals.

[0039] The stocks of the clinical isolates identified as viruses of the invention (i.e. having surprisingly good properties for the killing of tumor cells as compared to other viral strains to which they were compared) may be stored under suitable conditions, before or after modification, and used to generate further stocks as appropriate.

[0040] A clinical isolate is a strain of a virus species which has been isolated from its natural host. The clinical isolate has preferably been isolated for the purposes of testing and

comparing the clinical isolate with other clinical isolates of that virus species for a desired property, in the case of viruses of the invention that being the ability to kill human tumor cells. Clinical isolates which may be used for comparison also include those from clinical samples present in clinical repositories, i.e. previously collected for clinical diagnostic or other purposes. In either case the clinical isolates used for comparison will preferably have undergone minimal culture *in vitro* prior to being tested for the desired property, preferably having only undergone sufficient culture to enable generation of sufficient stocks for comparative testing purposes. As such, the viruses used for comparison may also include deposited strains, wherein the deposited strain has been isolated from a patient, preferably an HSV1 strain isolated from the cold sore of a patient.

[0041] The virus of the invention is an oncolytic virus which is, or is derived from, a clinical isolate which has been selected by comparing the abilities of a panel of three or more clinical isolates of the same viral species to kill tumor cells of two or more tumor cell lines *in vitro* and selecting a clinical isolate which is capable of killing cells of two or more tumor cell lines more rapidly and/or at a lower dose *in vitro* than one or more of the other clinical isolates in the panel. Thus, the virus is a clinical isolate that kills two or more tumor cell lines more rapidly and/or at a lower dose *in vitro* than one or more reference clinical isolates of the same species of virus.

[0042] Typically, the virus of the invention will kill two or more tumor cell lines within 72 hours, preferably within 48 hours, more preferably within 24 hours, and may infect at multiplicities of infection (MOI) of less than or equal to 0.1, preferably less than or equal to an MOI of 0.01 more preferably less than or equal to an MOI of 0.001. The virus may be capable of killing a broad range of human tumor cell lines, such as 2, 3, 4, 5, 6, 7 or all of the following cell lines: HT29 (colorectal), MDA-MB-231 (breast), SK-MEL-28 (melanoma), Fadu (squamous cell carcinoma), MCF7 (breast), A549 (lung), MIAPACA-2 (pancreas), HT1080 (fibrosarcoma). Thus, the virus of the invention may be capable of killing cells from two or more, such as 3, 4, 5, 6, 7 or more, different types of tumor such as two or more, such as 3, 4, 5, 6, 7 or more, solid tumors, including but not limited to colorectal tumor cells, prostate tumor cells, breast tumor cells, ovarian tumor cells, melanoma cells, squamous cell carcinoma cells, lung tumor cells, pancreatic tumor cells, sarcoma cells and/or fibrosarcoma cells.

[0043] Tumor cell line killing can be determined by any suitable method. Typically, a sample is first isolated from a patient, preferably, in the case of HSV1, from a cold sore, is used to infect BHK cells, or another suitable cell line such as vero cells. Positive samples are typically identified by the presence of a cytopathic effect (CPE) 24-72 hours post infection, such as 48 hours post infection, and confirmed to be the target viral species by, for example, immunohistochemistry or PCR. Viral stocks are then generated from the positive samples. A sample from the viral stock is typically tested and compared to other samples generated in the same way using swabs from different patients. Testing may be carried out by determining the level of CPE achieved at a range of multiplicity of infection (MOI) and at various times post infection.

[0044] For example, cell lines at 80% confluency may be infected with the viral sample at MOI of 1, 0.1, 0.01 and 0.001 and duplicate plates incubated for 24 and 48 hours at 37°C, 5% CO_2 prior to determination of the extent of viral cell killing. This may be determined by, for example, fixing the cells with glutaraldehyde and staining with crystal violet using standard methods. The level of cell lysis may then be assessed by standard methods such as gross observation, microscopy (cell counts) and photography. The method may be repeated with the cells being incubated for shorter time periods, such as 8, 12 or 16 hours, or longer time periods, such as 72 hours, before cell killing is determined, or at additional MOIs such as 0.0001 or less.

[0045] Growth curve experiments may also be conducted to assess the abilities of different clinical isolates to replicate in tumor cell lines *in vitro.* For example, cell lines at 80% confluency may be infected with the viral sample at MOI of 1, 0.1, 0.01 and 0.001, incubated at 37°C, 5% CO_2 and the cells lysed, typically by freeze/thawing, at 0, 8, 16, 24 and 48 hours post infection prior to determination of the extent of viral cell killing. This may be determined by, for example, assessing viral titres by a standard plaque assay.

[0046] A virus of the invention can kill infected tumor cell lines more rapidly and/or at a lower MOI than the other clinical isolates to which it is compared, preferably 2, 3, 4, 5 or 10 or more, other clinical isolates of the same virus species. A virus of the invention typically kills a 10%, 25% or 50% greater proportion of the tumor cells present at a particular MOI and time point than at least one, preferably 2, 3, 4, 5 or 10 or more, other clinical isolates of the same virus type at the same MOI and time point to which it was compared. The virus of the invention typically kills the same or a greater proportion of tumor cells at a MOI that is half or less than half that of the MOI at which one or more, preferably 2, 3, 4, 5,10 or 15 or more, other clinical isolates of the same virus species used for the comparison at the same time point, typically at 12, 24 and/or 48 hours, kills the same proportion of tumor cells. Preferably, a virus of the invention typically kills the same or a greater proportion of tumor cells at a MOI that is 5 or 10 times lower than the MOI at which one or more, preferably 2, 3, 4, 5, 10 or 15 or more, other clinical isolates of the same virus used for the comparison at the same time point, typically at 12, 24 and/or 48 hours kills the same proportion of tumor cells. The improved tumor cell killing abilities of a virus of the invention are typically achieved compared to at least 50%, 75% or 90% of the other clinical isolates of the same viral species used for the comparison. The virus is preferably compared to at least 4 other virus strains, such as, for example, 7, 9, 19, 39 or 49 other virus strains of the same species.

[0047] The isolated strains may be tested in batches, for example of 4-8 viral strains at a time, on, for example, 4-8 of the tumor cell lines at a time. For each batch of experiments, the degree of killing achieved is ranked on each cell line for the best (i.e. least surviving cells at each time point/MOI) to the worst (i.e. most surviving cells for each time point/MOI) for the viruses being compared in that experiment. The virus strains from each experiment which perform the best across the range of tumor cell line tested (i.e. that consistently ranked as one of the best at killing the cell lines) may then be compared head to head in further experiments using other clinical isolates and/or other tumor cell lines to identify the best virus strains in the total of, for example, >20 virus strains sampled. Those ranked as the best overall are the

viruses of the invention.

[0048] A virus of the invention is a strain selected from:

strain RH018A having the accession number ECCAC 16121904;

strain RH004A having the accession number ECCAC 16121902;

strain RH031A having the accession number ECCAC 16121907;

strain RH040B having the accession number ECCAC 16121908;

strain RH015A having the accession number ECCAC 16121903;

strain RH021A having the accession number ECCAC 16121905;

strain RH023A having the accession number ECCAC 16121906; and

strain RH047A having the accession number ECCAC 16121909.

[0049] More preferably, the virus of the invention is a strain selected from:

strain RH018A having the accession number ECCAC 16121904;

strain RH004A having the accession number ECCAC 16121902;

strain RH031A having the accession number ECCAC 16121907;

strain RH040B having the accession number ECCAC 16121908; and

strain RH015A having the accession number ECCAC 16121903;

[0050] Most preferably, the virus of the invention is strain RH018A having the accession number EACC 16121904.

[0051] An HSV of the invention is capable of replicating selectively in tumors, such as human tumors. Typically, the HSV replicates efficiently in target tumors but does not replicate efficiently in non-tumor tissue. This HSV comprises one or more mutations in one or more viral genes that inhibit replication in normal tissue but still allow replication in tumors. The mutation may, for example, be a mutation that prevents the expression of functional ICP34.5, ICP6 and/or thymidine kinase by the HSV.

[0052] In one preferred embodiment, the ICP34.5-encoding genes are mutated to confer selective oncolytic activity on the HSV. Mutations of the ICP3 4.5-encoding genes that prevent the expression of functional ICP34.5 are described in Chou et al. (1990) Science 250:1262-

1266, Maclean et al. (1991) J. Gen. Virol. 72:631-639 and Liu et al. (2003) Gene Therapy 10:292-303. The ICP6-encoding gene and/or thymidine kinase-encoding gene may also be inactivated, as may other genes provided that such inactivation does not prevent the virus infecting or replicating in tumors.

[0053] The HSV may contain a further mutation or mutations which enhance replication of the HSV in tumors. The resulting enhancement of viral replication in tumors not only results in improved direct 'oncolytic' tumor cell killing by the virus, but also enhances the level of heterologous (i.e. a gene inserted into the virus, in the case of viruses of the invention genes encoding fusogenic protein(s) and an immune modulatory molecule(s)) gene expression and increases the amount of tumor antigen released as tumor cells die, both of which may also improve the immunogenic properties of the therapy for the treatment of cancer. For example, in a preferred embodiment of the invention, deletion of the ICP47-encoding gene in a manner that places the US11 gene under the control of the immediate early promoter that normally controls expression of the ICP47 encoding gene leads to enhanced replication in tumors (see Liu *et al.,* 2003).

[0054] Other mutations that place the US11 coding sequence, which is an HSV late gene, under the control of a promoter that is not dependent on viral replication may also be introduced into a virus of the invention. Such mutations allow expression of US11 before HSV replication occurs and enhance viral replication in tumors. In particular, such mutations enhance replication of an HSV lacking functional ICP34.5-encoding genes.

[0055] Accordingly, in one embodiment the HSV of the invention comprises a US11 gene operably linked to a promoter, wherein the activity of the promoter is not dependent on viral replication. The promoter may be an immediate early (IE) promoter or a non-HSV promoter which is active in mammalian, preferably human, tumor cells. The promoter may, for example, be a eukaryotic promoter, such as a promoter derived from the genome of a mammal, preferably a human. The promoter may be a ubiquitous promoter (such as a promoter of β-actin or tubulin) or a cell-specific promoter, such as tumor-specific promoter. The promoter may be a viral promoter, such as the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter or the human or mouse cytomegalovirus (CMV) IE promoter. HSV immediate early (IE) promoters are well known in the art. The HSV IE promoter may be the promoter driving expression of ICP0, ICP4, ICP22, ICP27 or ICP47.

[0056] The genes referred to above may be rendered functionally inactive by any suitable method, for example by deletion or substitution of all or part of the gene and/or control sequence of the gene or by insertion of one or more nucleic acids into or in place of the gene and/or the control sequence of the gene. For example, homologous recombination methods, which are standard in the art, may be used to generate the virus of the invention.

[0057] As used herein, the term "gene" is intended to mean the nucleotide sequence encoding a protein, i.e. the coding sequence of the gene. The various genes referred to above may be rendered non-functional by mutating the gene itself or the control sequences flanking the gene,

for example the promoter sequence. Deletions may remove one or more portions of the gene, the entire gene or the entire gene and all or some of the control sequences. For example, deletion of only one nucleotide within the gene may be made, resulting in a frame shift. However, a larger deletion may be made, for example at least about 25%, more preferably at least about 50% of the total coding and/or non-coding sequence. In one preferred embodiment, the gene being rendered functionally inactive is deleted. For example, the entire gene and optionally some of the flanking sequences may be removed from the virus. Where two or more copies of the gene are present in the viral genome both copies of the gene are rendered functionally inactive.

[0058] A gene may be inactivated by substituting other sequences, for example by substituting all or part of the endogenous gene with a heterologous gene and optionally a promoter sequence. Where no promoter sequence is substituted, the heterologous gene may be inserted such that it is controlled by the promoter of the gene being rendered non-functional. In an HSV of the invention it is preferred that the ICP34.5 encoding-genes are rendered non-functional by the insertion of a heterologous gene or genes and a promoter sequence or sequences operably linked thereto, and optionally other regulatory elements such as polyadenylation sequences, into each the ICP34.5-encoding gene loci.

[0059] A virus of the invention may be used to express a fusogenic protein and/or an immune stimulatory protein in tumors. This is typically achieved by inserting a heterologous gene encoding the fusogenic protein and/or a heterologous gene encoding the immune stimulatory protein in the genome of a selectively replication competent virus wherein each gene is under the control of a promoter sequence. As replication of such a virus will occur selectively in tumor tissue, expression of the fusogenic protein and/or immune stimulatory protein by the virus is also enhanced in tumor tissue as compared to non-tumor tissue in the body. Enhanced expression occurs where expression is greater in tumors as compared to other tissues of the body. Accordingly, the invention provides benefits of expression of both a fusogenic protein and/or an immune stimulatory protein selectively in tumors combined with the anti-tumor effect provided by oncolytic virus replication.

Fusogenic protein

[0060] The virus of the invention may comprise a gene encoding a fusogenic protein. The fusogenic protein may be any heterologous protein capable of promoting fusion of a cell infected with the virus of the invention to another cell. A fusogenic protein, preferably a wild type or modified viral glycoprotein (i.e. modified to increase its fusogenic properties), is a protein which is capable in inducing the cell to cell fusion (syncitia formation) of cells in which it is expressed. Examples of fusogenic glycoprotiens include VSV-G, syncitin-1 (from human endogenous retrovirus-W (HERV-W)) or syncitin-2 (from HERVFRDE1), paramyxovirus SV5-F, measles virus-H, measles virus-F, RSV-F, the glycoprotein from a retrovirus or lentivirus, such as gibbon ape leukemia virus (GALV), murine leukemia virus (MLV), Mason-Pfizer monkey virus (MPMV) and equine infectious anemia virus (EIAV) with the R transmembrane peptide

removed (R- versions). In a preferred embodiment the fusogenic protein is from GALV and has the R- peptide removed (GALV-R-).

[0061] The virus of the invention may comprise multiple copies of the fusogenic protein-encoding gene, preferably 1 or 2 copies. The virus may comprise two or more different fusogenic proteins, including any of the fusogenic proteins listed above.

[0062] The fusogenic protein or proteins expressed by a virus of the invention may be identical to a naturally occurring protein, or may be a modified protein.

[0063] The fusogenic protein-encoding gene (fusogenic gene) may have a naturally occurring nucleic acid sequence or a modified sequence. The sequence of the fusogenic gene may, for example, be modified to increase the fusogenic properties of the encoded protein, or to provide codon optimisation and therefore increase the efficiency of expression of the encoded protein.

Immune stimulatory molecule

[0064] The virus of the invention may comprise one or more immune stimulatory molecules and/or one or more genes encoding an immune stimulatory molecule. Immune stimulatory molecules include proteins which may aid in the induction of an immune response, proteins which may relieve inhibitory signals to the induction or effectiveness of an immune response and RNA molecules (e.g. shRNA, antisense RNA, RNAi or micro RNA) which inhibit the expression of immune inhibitory molecules.

[0065] Examples of immune stimulatory molecules include IL-2, IL12, IL-15, IL-18, IL-21, IL-24, CD40 ligand, GITR ligand, 4-1-BB ligand, OX40 ligand, ICOS ligand, flt3 ligand, type I interferons, including interferon alpha and interferon beta, interferon gamma, type III interferon (IL-28, IL-29), other cytokines such as TNF alpha or GM-CSF, TGF beta or immune checkpoint antagonists. Immune checkpoint antagonists include antibodies, single chain antibodies and RNA1/siRNA/microRNA/antisense RNA knockdown approaches. Agonists of immune potentiating/co-stimulatory pathways include mutant or wild type, soluble, secreted and/or membrane bound ligands, and agonistic antibodies including single chain antibodies. With regard to the targeting of immune co-inhibitory or immune co-stimulatory pathways, proteins or other molecules (agonistic or antagonistic depending on the case) targeting CTLA-4 (antagonist), PD-1 (antagonist), PD-L1 (antagonist), LAG-3 (antagonist), TIM-3 (antagonist), VISTA (antagonist), CSF1R (antagonist), IDO (antagonist), CEACAM1 (antagonist), GITR (agonist), 4-1-BB (agonist), KIR (antagonist), SLAMF7 (antagonist), OX40 (agonist), CD40 (agonist), ICOS (agonist) or CD47 (antagonist) are particularly preferred. Viruses of the invention therefore preferably encode one or more of these molecules. More preferably viruses of the invention encode GM-CSF and/or a wild type or modified version of CD40L, ICOSL, 4-1-BBL, GITRL or OX40L, most preferably GM-CSF.

[0066] The inhibitor of a co-inhibitory pathway may be a CTLA-4 inhibitor. The CTLA-4 inhibitor is typically a molecule such as a peptide or protein that binds to CTLA-4 and reduces or blocks signaling through CTLA-4, such as by reducing activation by B7. By reducing CTLA-4 signalling, the inhibitor reduces or removes the block of immune stimulatory pathways by CTLA-4.

[0067] The CTLA-4 inhibitor is preferably an antibody or an antigen binding fragment thereof. The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (i.e., "antigen-binding portion") or single chains thereof. An antibody refers to a glycoprotein comprising at least two heavy (H) chains and two light (kappa)(L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (Clq) of the classical complement system.

[0068] The antibody is typically a monoclonal antibody. The antibody may be a chimeric antibody. The antibody is preferably a humanised antibody and is more preferably a human antibody.

[0069] The term "antigen-binding fragment" of an antibody refers to one or more fragments of an antibody that retain the ability to specifically bind to CTLA-4. The antigen-binding fragment also retains the ability to inhibit CTLA-4 and hence to reduce or remove the CTLA-4 blockade of a stimulatory immune response. Examples of suitable fragments include a Fab fragment, a $F(ab')_2$ fragment, a Fab' fragment, a Fd fragment, a Fv fragment, a dAb fragment and an isolated complementarity determining region (CDR). Single chain antibodies such as scFv and heavy chain antibodies such as VHH and camel antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. In a preferred embodiment, the antibody is an scFv. Examples of suitable scFv molecules are disclosed in, for example, WO2007/123737 and WO2014/066532. The scFv may be encoded by the nucleotide sequence shown in SEQ ID NO: 34 the nucleotide sequence shown in SEQ ID NO: 35.

[0070] Viruses of the invention may encode one or more immune stimulatory molecules, preferably 1, 2, 3 or 4 immune stimulatory molecules, more preferably 1 or 2 immune stimulatory molecules.

[0071] The sequence of the gene encoding the immune stimulatory molecule may be codon optimized so as to increase expression levels of the respective proteins in target cells as compared to if the unaltered sequence is used.

Modification of virus strains

[0072] Modified viruses of the invention are modified versions of deposited clinical isolates identified as having advantageous properties for killing tumor cells as compared to other virus strains used for the comparison. Modified viruses of the invention are constructed using methods well known in the art. For example plasmids (for smaller viruses and single and multiple genome component RNA viruses) or BACS (for larger DNA viruses including herpes viruses) encoding the viral genome to be packaged, including any genes encoding fusogenic and/or immune stimulating molecules under appropriate regulatory control, can be constructed by standard molecular biology techniques and transfected into permissive cells from which recombinant viruses can be recovered.

[0073] Alternatively, in a preferred embodiment plasmids containing DNA regions flanking the intended site of insertion can be constructed, and then co-transfected into permissive cells with viral genomic DNA such that homologous recombination between the target insertion site flanking regions in the plasmid and the same regions in the parental clinical isolate occur. Recombinant viruses can then be selected and purified through the loss or addition of a function inserted or deleted by the plasmid used for modification, e.g. insertion or deletion of a marker gene such as GFP or lacZ from the parental virus at the intended insertion site. In a most preferred embodiment the insertion site is the ICP34.5 locus of HSV, and therefore the plasmid used for manipulation contains HSV sequences flanking this insertion site, between which are an expression cassette encoding a fusogenic protein and an immune stimulatory molecule. In this case, the parental clinical isolate may contain a cassette encoding GFP in place of ICP34.5 and recombinant virus plaques are selected through the loss of expression of GFP. In a most preferred embodiment the US 11 gene of HSV is also expressed as an IE gene. This may be accomplished through deletion of the ICP47-encoding region, or by other means.

[0074] Fusogenic protein encoding sequences and immune stimulatory molecule encoding sequences may be inserted into the viral genome under appropriate regulatory control. This may be under the regulatory control of natural promoters of the virus species of the invention used, depending on the species and insertion site, or preferably under the control of heterologous promoters. Suitable heterologous promoters include mammalian promoters, such as the IEF2a promoter or the actin promoter. More preferred are strong viral promoters such as the CMV IE promoter, the RSV LTR, the MMLV LTR or promoters derived from SV40. Preferably each exogenous gene (i.e. encoding the fusogenic protein and immune modulatory molecule) will be under separate promoter control, but may also be expressed from a single RNA transcript, for example through insertion of an internal ribosome entry sites (IRES) between protein coding sequences. RNA derived from each promoter is typically terminated using a polyadenylation sequence (e.g. mammalian sequences such as the bovine growth hormone (BGH) poly A sequence, synthetic polyadenylation sequences, or viral sequences such as the SV40 early or late polyadenylation sequence).

[0075] The invention also provides a virus, such as a pox virus or a HSV, preferably HSV1, which expresses at least three heterologous genes, wherein each of the three heterologous genes is driven by a different promoter selected from the CMV promoter, the RSV promoter, the EF1a promoter, the SV40 promoter and a retroviral LTR promoter. The virus may, for example, express four heterologous genes, wherein each of the four heterologous genes is driven by a different promoter selected from the CMV promoter, the RSV promoter, the EF1a promoter, the SV40 promoter and a retroviral LTR promoter. The retroviral LTR promoter is preferably from MMLV (SEQ ID NO:43), also known as MoMuLV. The heterologous genes may be terminated by poly adenylation sequences. The poly adenylation sequences may be the same or different. Preferably each heterologous gene is terminated by a different poly adenylation sequence, which is preferably selected from the BGH, SV40, HGH and RBG poly adenylation sequences.

[0076] The invention also provides a virus, such as a pox virus or a HSV, preferably HSV1, which expresses at least three heterologous genes, wherein each of the three heterologous genes is terminated by a different poly adenylation sequence selected from the BGH, SV40, HGH and RBG poly adenylation sequences. The virus may, for example, express four heterologous genes terminated by each of the BGH, SV40, HGH and RBG poly adenylation sequences, respectively.

Pharmaceutical Compositions

[0077] The invention provides a pharmaceutical composition comprising a virus of the invention and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may further comprise other constituents such as sugars or proteins to improve properties such as stability of the product. Alternatively a lyophilized formulation may be used, which is reconstituted in a pharmaceutically acceptable carrier or diluent before use.

[0078] The choice of carrier, if required, is frequently a function of the route of delivery of the composition. Within this invention, compositions may be formulated for any suitable route and means of administration. Pharmaceutically acceptable carriers or diluents are those used in compositions suitable for intra-tumoral administration, intravenous/intraarterial administration, administration into the brain or administration into a body cavity (e.g. bladder, pleural cavity or by intraperitoneal administration). The composition may be administered in any suitable form, preferably as a liquid.

[0079] The virus of the invention may be comprised in a sterile vial, ampoule or syringe as a product of manufacture.

Medical Uses

[0080] The invention provides the virus of the invention for use in the treatment of the human or animal body by therapy, particularly for use in a method of treating cancer. The cancer is typically in a mammal, preferably in a human. The virus kills infected tumour cells by virus mediated toxicity, including by lysis, necrosis or apoptosis, preferably by lysis or necrosis. The virus of the invention also elicits a systemic anti-tumor immune response, augmented through the expression of the immune stimulatory molecule, which also kills cancer cells.

[0081] The virus of the invention is particularly useful in treating any solid tumor including any adenocarcinoma, carcinoma or sarcoma. For example, the virus of the invention is useful in treating head and neck, prostate, breast, ovarian, lung, liver, endometrial, bladder, gall bladder, pancreas, colon, kidney, stomach/gastric, esophageal, or cervical cancers, mesothelioma, melanoma or other skin cancer, lymphoma, glioma or other cancer of the nervous system, or sarcomas such as soft tissue sarcoma.

[0082] The virus of the invention may be used to treat malignant tumors, including tumors that have metastasised from the site of the original tumor. In this embodiment, the virus may be administered to the primary tumor or to one or more secondary tumors.

[0083] The virus of the invention may be administered in combination with other therapeutic agents, including chemotherapy, targeted therapy, immunotherapy (including immune co-inhibitory pathway blockade or immune co-stimulatory pathway activation) and/or in combination with radiotherapy and/or in combination with any combination of these. The therapeutic agent is preferably an anti-cancer agent.

[0084] The virus of the invention may be administered in combination with a second virus, such as a second oncolytic virus.

[0085] For example, the therapeutic agent may comprise an immunogen (including a recombinant or naturally occurring antigen, including such an antigen or combination of antigens delivered as DNA or RNA in which it/they are encoded), to further stimulate an immune response, such as a cellular or humoral immune response, to tumor cells, particularly tumor neoantigens. The therapeutic agent may be an agent intended to increase or potentiate an immune response, such as a cytokine, an agent intended to inhibit an immune checkpoint pathway or stimulate an immune potentiating pathway or an agent which inhibits the activity of regulatory T cells (Tregs).

[0086] The therapeutic agent may be an agent known for use in an existing cancer therapeutic treatment. The therapeutic agent may be radiotherapy or a chemotherapeutic agent. The therapeutic agent may be selected from cyclophosmamide, alkylating-like agents such as cisplatin or melphalan, plant alkaloids and terpenoids such as vincristine or paclitaxel (Taxol), antimetabolites such as 5-fluorouracil, topoisomerase inhibitors type I or II such as camptothecin or doxorubicin, cytotoxic antibiotics such as actinomycin, anthracyclines such as epirubicin, glucocorticoids such as triamcinolone, inhibitors of protein, DNA and/or RNA synthesis such as methotrexate and dacarbaxine, histone deacetylase (HDAC) inhibitors, or

any other chemotherapy agent.

[0087] The therapeutic agent may be one, or a combination of: immunotherapeutics or immunomodulators, such as TLR agonists; agents that down-regulate T-regulatory cells such as cyclophosphamide; or agents designed to block immune checkpoints or stimulate immune potentiating pathways, including but not limited to monoclonal antibodies, such as a CTLA-4 inhibitor, a PD-1 inhibitor, a PD-L1 inhibitor, a LAG-3 inhibitor, a TIM-3 inhibitor, a VISTA inhibitor, a CSF1R inhibitor, an IDO inhibitor, a CEACAM1 inhibitor, a GITR agonist, a 4-1-BB agonist, a KIR inhibitor, a SLAMF7 inhibitor, an OX40 agonist, a CD40 agonist, an ICOS agonist or a CD47 inhibitor. In a preferred embodiment, the therapeutic agent is a CTLA-4 inhibitor such as an anti-CTLA-4 antibody, a PD1 inhibitor, such as an anti-PD-1 antibody or a PD-L1 inhibitor such as an anti-PD-L1 antibody. Such inhibitors, agonists and antibodies can be generated and tested by standard methods known in the art.

[0088] Immunotherapeutic agents may also include bi-specific antibodies, cell based-therapies based on dendritic cells, NK cells or engineered T cells such CAR-T cells or T cells expressing engineered T cell receptors. Immunotherapeutic agents also include agents that target a specific genetic mutation which occurs in tumors, agents intended to induce immune responses to specific tumor antigens or combinations of tumor antigens, including neoantigens and/or agents intended to activate the STING/cGAS pathway, TLR or other innate immune response and/or inflammatory pathway, including intra-tumoral agents.

[0089] For example, a virus of the invention may be used: in combination with dacarbazine, a BRAF inhibitor and or CTLA-4, PD1 or PD-L1 blockade to treat melanoma; in combination with taxol, doxorubicin, vinorelbine, cyclophosphamide and/or gemcitabine to treat breast cancer; in combination with 5-fluorouracil and optionally leucovorin, irinoteacan and/or oxaliplatin to treat colorectal cancer; in combination with taxol, carboplatin, vinorelbine and/or gemcitabine, PD-1 or PD-L1 blockade to treat lung cancer; in combination with cisplatin and/or radiotherapy to treat head and neck cancer.

[0090] The therapeutic agent may be an inhibitor of the idoleamine 2,3-dioxygenase (IDO) pathway. Examples of IDO inhibitors include epacadostat (INCB024360), 1-methyl-tryptophan, indoximod (1-methyl-D-tryptophan), GDC-0919 or F001287.

[0091] The mechanism of action of IDO in suppressing anti-tumor immune responses may also suppress immune responses generated following oncolytic virus therapy. IDO expression is induced by toll like receptor (TLR) activation and interferon-γ both of which may result from oncolytic virus infection. One embodiment of the use of oncolytic virus therapy for cancer treatment includes combination of an oncolytic virus, including a virus expressing an immune stimulating protein or proteins and/or a fusogenic protein, with an inhibitor of the IDO pathway and optionally one or more further antagonist of an immune co-inhibitory pathway and/or one or more agonist of an immune co-stimulatory pathway, including those targeting CTLA-4, PD-1 and/or PD-L1.

[0092] Where a therapeutic agent and/or radiotherapy is used in conjunction with a virus of the invention, administration of the virus and the therapeutic agent and/or radiotherapy may be contemporaneous or separated by time. The composition of the invention may be administered before, together with or after the therapeutic agent or radiotherapy. The method of treating cancer may comprise multiple administrations of the virus of the invention and/or of the therapeutic agent and/or radiotherapy. A skilled practitioner will readily be able to determine suitable courses of administration of the virus and the therapeutic agent.

[0093] In preferred embodiments, in the case of combination with one or more antagonist of an immune co-inhibitory pathway, one or more agonist of an immune co-stimulatory pathway and/or other immune potentiating agents, the virus of the invention is administered once or multiple times prior to the concurrent administration of the antagonist of an immune co-inhibitory pathway, agonist of an immune co-stimulatory pathway and/or other immune potentiating agent or agents thereafter, or concurrent with the administration of the antagonist of an immune co-inhibitory pathway, agonist of an immune co-stimulatory pathway and/or other immune potentiating agent or agents without prior administration of the virus of the invention.

[0094] The virus of the invention may be administered to a subject by any suitable route. Typically, a virus of the invention is administered by direct intra-tumoral injection, including through the use of imaging guidance to target the tumor or tumors. The virus may be administered into a body cavity, for example into the pleural cavity, bladder or by intra-peritoneal administration. The virus may be injected into a blood vessel, preferably a blood vessel supplying a tumor.

[0095] Therapeutic agents which may be combined with a virus of the invention can be administered to a human or animal subject *in vivo* using a variety of known routes and techniques. For example, the composition may be provided as an injectable solution, suspension or emulsion and administered via parenteral, subcutaneous, oral, epidermal, intradermal, intramuscular, interarterial, intraperitoneal, intravenous injection using a conventional needle and syringe, or using a liquid jet injection system. The composition may be administered topically to skin or mucosal tissue, such as nasally, intratrachealy, intestinally, sublingually, rectally or vaginally, or provided as a finely divided spray suitable for respiratory or pulmonary administration. In preferred embodiments, the compositions are administered by intravenous infusion, orally, or directly into a tumor.

[0096] The virus and/or therapeutic agent may be administered to a subject in an amount that is compatible with the dosage composition that will be therapeutically effective. The administration of the virus of the invention is for a "therapeutic" purpose. As used herein, the term "therapeutic" or "treatment" includes any one or more of the following as its objective: the prevention of any metastasis or further metastasis occurring; the reduction or elimination of symptoms; the reduction or complete elimination of a tumor or cancer, an increase in the time to progression of the patient's cancer; an increase in time to relapse following treatment; or an increase in survival time.

[0097] Therapeutic treatment may be given to Stage I, II, III, or IV cancers, preferably Stage II, III or IV, more preferably Stage III or IV, pre- or post-surgical intervention, preferably before surgical intervention (either for resection of primary or recurrent/metastatic disease), i.e. while residual tumor remains.

[0098] Therapeutic treatment may be carried out following direct injection of the virus composition into target tissue which may be the tumor, into a body cavity, or a blood vessel. As a guide, the amount of virus administered is in the case of HSV in the range of from 10^4 to 10^{10} pfu, preferably from 10^5 to 10^9 pfu. In the case of HSV, an initial lower dose (e.g. 10^4 to 10^7 pfu) may be given to patients to seroconvert patients who are seronegative for HSV and boost immunity in those who are seropositive, followed by a higher dose then being given thereafter (e.g. 10^6 to 10^9 pfu). Typically up to 20ml of a pharmaceutical composition consisting essentially of the virus and a pharmaceutically acceptable suitable carrier or diluent may be used for direct injection into tumors, or up to 50ml for administration into a body cavity (which may be subject to further dilution into an appropriate diluent before administration) or into the bloodstream. However for some oncolytic therapy applications larger or smaller volumes may also be used, depending on the tumor and the administration route and site.

[0099] The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage. The dosage may be determined according to various parameters, especially according to the location of the tumor, the size of the tumor, the age, weight and condition of the patient to be treated and the route of administration. Preferably the virus is administered by direct injection into the tumor. The virus may also be administered by injection into a blood vessel or into a body cavity. The optimum route of administration will depend on the location and size of the tumor. Multiple doses may be required to achieve an immunological or clinical effect, which, if required, will be typically administered between 2 days to 12 weeks apart, preferably 3-days to 3 weeks apart. Repeat doses up to 5 years or more may be given, preferably for up to one month to two years dependent on the speed of response of the tumor type being treated and the response of a particular patient, and any combination therapy which may also be being given.

[0100] The following Examples illustrate the invention.

Example 1. Clinical Isolates with improved anti-tumor effects

[0101] The virus species used to exemplify the invention is HSV, specifically HSV1. Cold sore swabs were taken from more than 20 otherwise healthy volunteers. A sample of each swab was used to infect BHK cells. Samples containing HSV1 were identified by the presence of a cytopathic effect (CPE) 24-72 hours post infection and by immunohistochemistry and viral stocks of the primary clinical isolates were generated from the positive samples.

[0102] The abilities of the primary clinical isolates of HSV1 to kill a panel of human tumor-derived cell lines is tested and the virus strain with the greatest ability to kill a broad range of these rapidly, and at low dose is chosen. Tumor cell lines used for this comparison are HT29 (colorectal), MDA-MB-231 (breast), SK-MEL-28 (melanoma), Fadu (squamous cell carcinoma), MCF7 (breast), A549 (lung), MIAPACA-2 (pancreas), CAP AN-1 (pancreas), HT1080 (fibrosarcoma). The cell lines are used to test for the level of CPE achieved at a range of MOI and times post infection for each of the primary clinical isolates.

[0103] More specifically, the tumor cell lines are used to seed multi-well tissue culture plates so that they are about 80% confluent on the day of infection. Representative wells from each tumor cell line are trypsinised and the number of cells in the well determined. These cell counts are used to determine the volume of each clinical isolate required to give an MOI of 1, 0.1, 0.01 and 0.001. Separate wells of a tumor cell line are infected with the clinical isolate at these MOI and overlaid with growth media and carboxymethylcellulose. All infections are carried out in quadruplicate. Duplicate wells are incubated for 24 hours and duplicate wells are incubated for 48 hours, both at 37°C, 5% CO_2, prior to fixation of the cells with glutaraldehyde and staining with crystal violet. The level of cell lysis is then assessed by gross observation, microscopy (cell counts) and photography or using a metabolic assay such as an MTT assay.

[0104] Growth curve experiments are also conducted to assess the abilities of different clinical isolates to replicate in tumor cell lines *in vitro.* The tumor cell lines are used to seed multi-well tissue culture plates so that they are about 80% confluent on the day of infection. Cell counts are determined as above and used to determine the volume of virus to give MOIs of 1, 0.1, 0.01 and 0.001. The tumor cells are infected in duplicate for MOI and time point. The infected cells are incubated at 37°C, 5% CO_2 and the cells lysed by freeze/thawing at 0, 8, 16, 24 and 48 hours post infection. Viral titres are assessed by a standard plaque assay.

Example 2. Modification of Clinical Isolates

[0105] In this example the clinical isolate selected in Example 1 (i.e. a virus if the invention) is modified by deletion of ICP47 from the viral genome using homologous recombination with a plasmid containing regions flanking HSV1 nucleotides 145300 to 145582 (HSV1 nucleotides 145300 to 145582 being the sequences to be deleted; HSV1 strain 17 sequence Genbank file NC_001806.2) between which are encoded GFP. GFP expressing virus plaques are selected, and GFP then removed by homologous recombination with the empty flanking regions and plaques which do not express GFP are selected. This results in an ICP47 deleted virus in which US11 is expressed as an IE protein as it is now under the control of the ICP47 promoter. ICP34.5 is then deleted using homologous recombination with a plasmid containing regions flanking HSV1 nucleotides 124953 to 125727 (HSV1 nucleotides 124953 to 125727 being the sequences to be deleted; HSV1 strain 17 sequence Genbank file NC 001806.2) between which GFP is encoded. GFP expressing virus plaques are again selected, and GFP then removed by homologous recombination with the same flanking regions but between which are now an

expression cassette comprising a codon optimized version of the mouse GM-CSF sequence and a codon optimized version of the GALV R- sequence driven by the CMV IE promoter and RSV promoter respectively, in a back to back orientation and again selecting virus plaques which do not express GFP. This virus construction is performed using methods which are standard in the art.

[0106] The structure of the resulting virus is shown in Figure 1 (top panel). The mGM-CSF and GALV-R- sequences are shown in SEQ ID NOs 2 and 8 respectively. The structure of the resulting virus is confirmed by restriction digestion and Southern blot, GM-CSF expression is confirmed by ELISA, and GALV-R- expression is confirmed by infection of human HT1080 tumor cells and the observation of syncitial plaques.

[0107] Viruses are also constructed using similar procedures which have no insertion into ICP34.5, or which only have inserted the gene for mouse GM-CSF or GALV-R-. The structures of these viruses are also shown in Figure 1.

[0108] For human use, hGM-CSF is used, the sequence for a codon optimised version of which is shown in SEQ ID NO 4.

Example 3. Expression of two immune stimulatory molecule from a virus expressing a fusogenic protein

[0109] A virus similar to the GALV-R- and mGM-CSF expressing virus described above is constructed, but additionally expressing versions of CD40L. Here, instead of using a plasmid containing ICP34.5 flanking regions and an expression cassette comprising GM-CSF and GALV-R- driven by a CMV and an RSV promoter, a plasmid containing ICP34.5 flanking regions and an expression cassette comprising GM-CSF, GALV and CD40L driven by a CMV, an RSV and an SV40 promoter is used for recombination with the virus containing GFP inserted into ICP34.5 and non-GFP expressing plaques again selected.

Example 4. The effect of the combined expression of a fusogenic protein and an immune stimulatory molecule from an oncolytic virus in mouse tumor models

[0110] The GALV R- protein causes cell to cell fusion in human cells but not in mouse cells because the PiT-1 receptor required for cell fusion to occur has a sequence in mice which does not allow cell fusion to occur. As a result mouse tumor cells expressing human PiT-1 are first prepared using methods standard in the art. Human PiT-1 is cloned into a lentiviral vector also comprising a selectable marker gene. The vector is transfected into target CT26 mouse colorectal cancer tumor cells and clones resistant to the selectable marker are selected to generate CT26/PiT-1 cells. PiT-1 expression is confirmed by western blotting in untransfected cells and in cells transfected with the PiT-1 expressing lentivirus and by transfection of a

plasmid expressing GALV-R- and confirmation that cell fusion occurs.

[0111] The utility of the virus is demonstrated by administering CT26/PiT-1 cells into both flanks of Balb/c mice and allowing the CT26/PiT-1 tumors to grow to approximately 0.5cm in diameter.

[0112] The following treatments are then administered to groups of mice (five per group), into one flank of each mouse only 3 times per week for two weeks:

- 50µl of saline (1 group);
- 50µl of 10^5 pfu/ml, 10^6 pfu, or 10^7 pfu/ml of the HSV with no inserted gene (3 groups);
- 50µl of 10^5 pfu/ml, 10^6 pfu/ml, or 10^7 pfu/ml of the HSV with only mouse GM-CSF inserted (3 groups);
- 50µl of 10^5 pfu/ml, 10^6 pfu/ml, or 10^7 pfu/ml of the virus with only GALVR- inserted (3 groups); or
- 50µl of 10^5 pfu/ml, 10^6 pfu/ml, or 10^7 pfu/ml of the virus with both mouse GM-CSF and GALV-R- inserted (3 groups).

[0113] Effects on tumor growth are then observed for up to one month. Superior tumor control and shrinkage in both injected and uninjected tumors with the virus expressing GM-CSF and GALV-R- as compared to the other groups is observed, including through an improved dose response curve.

Example 5. The effect of combined expression of a fusogenic protein and an immune stimulatory molecule from an oncolytic virus on the therapeutic effect of immune checkpoint blockade in mouse tumor models

[0114] The experiment in Example 3 above is repeated but mice are additionally dosed biweekly by the intra-peritoneal route with an antibody targeting mouse PD-1 (10mg/kg; Bioxcell RMP-1-14 on the same days as virus dosing) or an antibody targeting mouse CTLA-4 (10mg/kg; Bioxcell 9H10 on the same days as virus dosing). An additional group of mice is added which receive no antibody treatment. More specifically, groups of mice receive (1) saline, (2) HSV with no inserted gene, (3) HSV with both GM-CSF and GALV-R-inserted as in Example 3, (4) PD-1 antibody, (5) CTLA-4 antibody, (6) HSV with no inserted gene plus PD-1 antibody, (7) HSV with no inserted gene plus CTLA-4 antibody, (8) HSV with GM-CSF and GALV-R- and PD-1 antibody or (9) HSV with GM-CSF and GALV-R- and CTLA-4 antibody. Superior tumor control and shrinkage in both injected and uninjected tumors with the virus expressing GM-CSF and GALV-R- together with the anti-PD-1 antibody or the anti-CTLA-4 antibody as compared to the other groups is observed, including through an improved dose response curve.

Example 6. Collection of Clinical Isolates

[0115] The virus species used to exemplify the invention is HSV, specifically HSV1. To exemplify the invention, 181 volunteers were recruited who suffered from recurrent cold sores. These volunteers were given sample collection kits (including Sigma Virovult collection tubes), and used these to swab cold sores when they appeared following which these samples were shipped to Replimune, Oxford UK. From June 2015-February 2016, swabs were received from 72 volunteers. A sample of each swab was used to infect BHK cells. Of these 36 live virus samples were recovered following plating out and growth on BHK cells. These samples are detailed in Table 1.

Table 1: Details of Tested Swab Samples & Result

Sample Number	Virus retrieved
RH001A	No
RH001B	
RH002A	Yes
RH003A	No
RH004A	Yes
RH004B	
RH005A	No
RH005B	
RH006A	No
RH006B	
RH007A	Yes
RH007B	
RH007C	
RH008A	No
RH008B	
RH008C	
RH009A	No
RH009B	
RH010A	No
RH011A	No
RH011B	
RH011C	
RH012A	No
RH013A	No

Sample Number	Virus retrieved
RH014A	Yes
RH014B	
RH015A	Yes
RH016A	No
RH016B	
RH017A	Yes
RH018A	Yes
RH018B	
RH018C	
RH019A	No
RH019B	
RH019C	
RH020A	Yes- RH020A only
RH020B	
RH020C	
RH021A	Yes
RH021B	
RH022A	Yes
RH022B	
RH023A	Yes
RH024A	No
RH025A	Yes -RH025B only
RH025B	
RH026A	Yes
RH027A	No
RH027B	
RH027C	
RH028A	No
RH028B	
RH028C	
RH029A	No
RH030A	No
RH031A	Yes - RH031A to RH031D
RH031B	
RH031C	

Sample Number	Virus retrieved
RH031D	
RH031E	
RH031F	
RH032A	No
RH033A	No
RH033B	
RH033C	
RH034A	No
RH034B	
RH034C	
RH035A	No
RH036A	Yes
RH037A	Yes
RH038A	Yes
RH039A	No
RH039B	
RH039C	
RH040A	Yes
RH040B	
RH040C	
RH041A	Yes
RH042A	Yes
RH043A	No
RH043B	
RH043C	
RH044A	No
RH045A	No
RH046A	Yes
RH047A	Yes- RH047A and RH047C
RH047B	
RH047C	
RH048A	No
RH049A	No
RH049B	

Sample Number	Virus retrieved
RH049C	
RH050A	No
RH051A	Yes
RH051B	
RH052A	Yes - RH052A only
RH052B	
RH053A	No
RH054A	No
RH055A	No
RH055B	
RH056A	Yes
RH057A	No
RH058A	Yes
RH058B	
RH059A	No
RH060A	No
RH061A	Yes
RH062A	No
RH063A	No
RH064A	Yes
RH065A	Yes
RH065B	
RH066A	No
RH067A	No
RH067B	
RH068A	No - contaminated
RH069A	No
RH069A	
RH070A	Yes
RH071A	Yes
RH072A	No
RH073A	Yes
RH073B	
RH074A	No
RH074B	

Sample Number	Virus retrieved
RH075A	No
RH076A	No
RH078A	No
RH078B	
RH079B	Yes
RH079B	
RH080A	No
RH081A	Yes
RH082A	No
RH082B	
RH083A	Yes
RH083B	
RH084A	Yes
RH084B	
RH084C	
RH085A	No
RH086A	No
RH087A	Yes - RH078B only
RH087B	

Designations A, B, C etc. indicate multiple swabs from the same volunteer.

Example 7. Identification of Clinical Isolates with improved anti-tumor effects

[0116] The abilities of the primary clinical isolates of HSV1 to kill a panel of human tumor-derived cell lines was tested. The tumor cell lines used for this comparison were HT29 (colorectal), MDA-MB-231 (breast), SK-MEL-28 (melanoma), Fadu (squamous cell carcinoma), MCF7 (breast), A549 (lung), MIAPACA-2 (pancreas) and HT1080 (fibrosarcoma). The cell lines were used to test for the level of CPE achieved at a range of MOI and times post infection for each of the primary clinical isolates.

[0117] Experiments were conducted in parallel using 5 to 8 of the new viruses strains at the same time. The virus strains were plated out in duplicate at a range of MOIs (0.001-1), and the extent of CPE following crystal violet staining was assessed at 24 and 48 hours following infection. The viral strains which were most effective at killing the tumor cell lines were scored, and the most effective two or three strains from each screen of 5-8 strains were identified and compared in parallel in a further experiment to identify the top strains for further development.

[0118] The initial screens demonstrated substantial variability in the ability of the different strains to kill the different tumor cell lines. Of an initial 29 strains tested, 8 strains of interest were identified in the initial screens for further comparison. These were strains RH004A, RH015A, RH018A, RH021A, RH023A, RH31A, RH040A, and RH047A.

[0119] The 8 strains for further comparison were tested in parallel on the panel of tumor cell lines, and their relative ability to kill these tumor cell lines was assessed following crystal violet staining and observation for CPE. Figure 3 shows a representative time point and MOI for these viruses on each of the viruses on each of the cell lines demonstrating the differential ability of the viruses to kill the target tumor cell lines observed.

[0120] There was substantial variation amongst the strains, and it was found that while a particular strain may be particularly effective at killing one cell line, it is not necessarily particularly effective at killing other cell lines too, further demonstrating the degree of variability in the ability of clinical strains of HSV to kill tumor cells of different types.

[0121] Figure 3 also indicates which of the virus strains was both best and second best at killing each of the cell lines, enabling the virus strains to be rank ordered as to their overall relative ability to kill the panel of cell lines as a whole. This analysis demonstrated that strains RH004A, RH015A, RH018A, RH031A and RH040A were relatively more effective than the other strains, and these five strains were chosen for potential further development as oncolytic agents. Of these top five strains, the relative rank order based on their abilities to kill across the panel of cell lines was RH018A > RH004A > RH031A > RH040A > RH015A.

[0122] More specifically, in these experiments, the tumor cell lines were used to seed multi-well tissue culture plates so that they were about 80% confluent on the day of infection. Representative wells from each tumor cell line were trypsinised and the number of cells in the well determined. These cell counts are used to determine the volume of each clinical isolate required to give an MOI of 1, 0.1, 0.01 and 0.001. Separate wells of a tumor cell line were infected with the clinical isolate at these MOI. All infections are carried out in quadruplicate. Duplicate wells were incubated for 24 hours and duplicate wells were incubated for 48 hours, both at 37°C, 5% CO_2, prior to fixation of the cells with glutaraldehyde and staining with crystal violet. The level of cell lysis was then assessed by gross observation, microscopy (cell counts) and photography.

[0123] Strain RH018A, the strain ranked first of all the strains tested was compared to an 'average' strain from the screen (i.e. a strain which was not in the top 8, but was also not in the group of strains which were least effective and killing the panel of tumor cell lines). This comparison showed that Strain RH018A was approximately 10 fold more effective than this average strain (Strain RH065A) at killing the tumor cell lines (i.e. approximately 10 fold less of Strain RH018A was needed to kill an equal proportion of cells than was needed of Strain RH065A). This is shown in Figure 4.

Example 8. Modification of Clinical Isolates

[0124] In this Example the clinical isolates selected in Example 7 were modified by deletion of ICP34.5 from the viral genome using homologous recombination with a plasmid containing regions flanking the ICP34.5 encoding gene (nucleotides 143680-145300 and 145,582-147,083 ; HSV1 strain 17 sequence Genbank file NC_001806.2) between which are encoded GFP and the GALV-R-fusogenic glycoprotein. The structure of this virus, (Virus 10) is shown in Figure 5.

[0125] Additional viruses based on Strain RH018A were also constructed in which both ICP34.5 and ICP47 (using flanking regions containing nucleotides 123464-124953 and 125727-126781; HSV1 strain 17 sequence Genbank file NC 001806.2) were deleted (resulting in placement of US11 under the control of the ICP47 promoter). To construct these viruses, GFP expressing virus plaques, with GFP expressed in place of ICP47 were first selected. GFP was then removed by homologous recombination with the empty flanking regions, and plaques not expressing GFP were selected. This resulted in an ICP47 deleted virus in which US11 is expressed as an IE protein as it is now under the control of the ICP47 promoter. ICP34.5 was then deleted using homologous recombination with a plasmid containing regions flanking HSV1 nucleotides 143680-145300 and 145,582-147,083; HSV1 strain 17 sequence Genbank file NC 001806.2) between which GFP is encoded. GFP expressing virus plaques were again selected, and GFP then removed by homologous recombination with the same flanking regions but between which are now an expression cassette comprising the genes to be inserted. The viruses that were constructed are shown in Figures 1 and 5. These included a codon optimized version of the mouse GM-CSF sequence and a codon optimized version of the GALV R- sequence driven by the CMV IE promoter and RSV promoter respectively, in a back to back orientation and again selecting virus plaques which do not express GFP. This virus construction was performed using methods which are standard in the art.

[0126] The mGM-CSF and GALV-R- sequences are shown in SEQ ID NOs 2 and 8 respectively. The structure of the resulting virus was confirmed by PCR, GM-CSF expression was confirmed by ELISA, and GALV-R- expression was confirmed by infection of human HT1080 tumor cells and the observation of syncitial plaques.

[0127] For human use, hGM-CSF is used, the sequence for a codon optimised version of which is shown in SEQ ID NO 4. The structure of this virus is shown in Figure 5. Expression of mouse or human GM-CSF from viruses 16, 17 and 19 is shown in Figure 6.

Example 9. A virus of the invention modified for oncolytic use and expressing a fusogenic glycoprotein shows enhanced tumor cell killing *in vitro* as compared to a virus which does not express a fusogenic glycoprotein

[0128] Virus 10 (see Figure 5), based on clinical Strain RH018A in which ICP34.5 is deleted

and which expresses GALVR- and GFP, was compared *in vitro* to a virus which expresses only GFP (Virus 12). Virus 10 showed enhanced killing on a panel of human tumor cell lines as compared to Virus 12, as shown in Figure 7.

Example 10. A virus of the invention modified for oncolytic use shows enhanced tumor cell killing as compared to a similarly modified virus which is not of the invention

[0129] Virus 17 (see Figure 5), based on clinical Strain RH018A in which ICP34.5 and ICP47 are deleted and which expresses GALVR- and GM-CSF, was compared *in vitro* to a known virus which was also deleted for ICP34.5 and ICP47 but which was not derived from a strain of the invention and which expresses only GM-CSF. Virus 17 showed enhanced killing on a panel of human tumor cell lines as compared to the previous virus, as shown in Figure 8.

Example 11. A virus of the invention modified for oncolytic use effectively treats mouse tumors *in vivo*

[0130] Virus 16 was tested in mice harboring A20 lymphoma tumors in the left and right flanks. One million tumor cells were first implanted in both flanks of Balb/c mice and tumors allowed to grow to 0.5-0.7cm in diameter. Tumors on the right flank were then injected 3 times (every other day) with either vehicle (10 mice) or 5×10exp6 pfu of Virus 16 (10 mice), and effects on tumor size observed for a further 30 days. This demonstrated that both injected and uninjected tumors were effectively treated with Virus 16 (see Figure 9).

Example 12. The effect of the combined expression of a fusogenic protein and an immune stimulatory molecule from an oncolytic virus of the invention in a rat tumor model

[0131] The GALV R- protein causes cell to cell fusion in human cells but not in mouse cells. However, GALV R- does cause fusion in rat cells.

[0132] The utility of the virus was further demonstrated by administering 9L cells into the flanks of Fischer 344 rats and allowing the 9L tumors to grow to approximately 0.5cm in diameter.

[0133] The following treatments were then administered to groups of rats (ten per group), into one flank only of each rat three times per week for three weeks:

- 50µl of vehicle;
- 50µl of 10^7 pfu/ml of Virus 19 (expresses mGM-CSF but not GALV R-);
- 50µl of 10^7 pfu/ml of Virus 16 (expresses both mouse GM-CSF and GALVR-).

[0134] Effects on tumor growth were then observed for a further ≈30 days. This demonstrated superior tumor control and shrinkage with the virus expressing GALV-R- in both injected and uninjected tumors, demonstrating improved systemic effects. This is shown in Figure 15. Figure 10 shows that a virus expressing GALV (Virus 15) also shows enhanced killing of rat 91 cells *in vitro* as compared to a virus which does not express GALV (Virus 24).

Example 13. A virus of the invention modified for oncolytic use is synergistic with immune checkpoint blockade in mouse tumor models

[0135] Virus 16 was tested in mice harboring CT26 tumors in the left and right flanks. One million tumor cells were first implanted in both flanks of Balb/c mice and tumors allowed to grow to 0.5-0.6cm in diameter.

[0136] Groups of 10 mice were then treated with:

- Vehicle (3 injections into right flank tumors every other day);
- 5×10exp6 pfu of Virus 16 injected in the right flank tumor every other day;
- anti-mousePD1 alone (10mg/kg i.p. every three days, BioXCell clone RMP1-14);
- anti-mouseCTLA-4 (3mg/Kg i.p every three days, BioXCell clone 9D9);
- anti-mousePD1 together with Virus 16;
- anti-mouseCTLA4 together with Virus 16;
- 1-methyl trypotophan (IDO inhibitor (5mg/ml in drinking water));
- anti-mouse PD1 together with 1-methyl trypotophan;
- anti-mouse PD1 together with 1-methyl trypotophan and Virus 16;

[0137] Effects on tumor size were observed for a further 30 days. A greater tumor reduction in animals treated with combinations of virus and checkpoint blockade was demonstrated than in animals treated with the single treatment groups (see Figure 11). Enhanced tumor reduction with Virus 16 together with both anti-PD1 and IDO inhibition was also demonstrated as compared to Virus 16 together with only anti-PD1 (see Figure 11).

[0138] Enhanced activity of Virus 16 in combination with immune checkpoint blockade was also seen in A20 tumors (Figure 12).

Example 14. The effect of the expression of a fusogenic protein from an oncolytic virus of the invention in human xenograft models in immune deficient mice

[0139] The GALV R- protein causes cell to cell fusion in human cells but not in mouse cells. However, human xenograft tumors grown in immune deficient mice can be used to assess the

effects of GALV expression on anti-tumor efficacy.

[0140] The utility of the virus was therefore further demonstrated by administering A549 human lung cancer cells into the flanks of nude mice and allowing the tumors to grow to approximately 0.5cm in diameter.

[0141] The following treatments were then administered to groups of mice (ten per group), into tumor containing flank of each mouse three times over one week:

- 50µl of vehicle;
- 50µl of 10^7 pfu/ml of Virus 16 (expresses both mouse GM-CSF and GALV-R-);
- 50µl of 10^6 pfu/ml of Virus 16;
- 50µl of 10^5 pfu/ml of Virus 16;
- 50µl of 10^7 pfu/ml of Virus 19 (expresses only mouse GM-CSF);
- 50µl of 10^6 pfu/ml of Virus 19;
- 50µl of 10^5 pfu/ml of Virus 19.

[0142] Effects on tumor growth were then observed for a further ≈ 30 days. This experiment demonstrated superior tumor control and shrinkage with the virus expressing GALV-R- in both tumor models (see Figure 14).

Example 15. Expression of two immune stimulatory molecules from a virus expressing a fusogenic protein

[0143] Viruses similar to the GALV-R- and mGM-CSF expressing virus described above (Virus 16) were constructed, but additionally expressing mouse versions of CD40L (virus 32), ICOSL (virus 36), OX40L (virus 35), 4-1BBL (virus 33) and GITRL (virus 34). Here, instead of using a plasmid containing ICP34.5 flanking regions and an expression cassette comprising GM-CSF and GALV-R- driven by a CMV and an RSV promoter, a plasmid containing ICP34.5 flanking regions and an expression cassette comprising GM-CSF, GALV and the additional proteins driven by a CMV, an RSV and an MMLV promoter respectively were used for recombination with a virus containing GM-CSF, GALV and GFP inserted into ICP34.5. Non-GFP expressing plaques were again selected. Correct insertion was confirmed by PCR, and expression by western blotting and/or ELISA for the additional inserted gene. These viruses are shown in Figure 5. Similarly, viruses expressing anti-mouse and anti-human CTLA-4 in addition to GALV and mGM-CSF were also constructed (Viruses 27 and 31 in Figure 5 and see also Figure 13). Effects of viruses expressing anti-mouse CTLA-4 (virus 27), mCD40L (virus 32), m4-1BBL (virus 33) or mOX40L (virus 35) in addition to mGM-CSF and GALVR- *in vivo* is shown in Figure 16 which showed enhanced activity in A20 tumors as compared to virus 16 (expresses mGM-CSF and GALVR-). In these experiments tumors were induced in both flanks of mice,

and virus or vehicle injected only into the right flank tumor. The dose of virus used was 5×10^4 pfu (50ul of 1×10^6 pfu/ml in each case), given three times over one week. This dose level of virus is subtherapeutic for uninjected tumors for virus 16, which allows the benefits of the delivery of the additional molecules encoded by viruses 27, 32, 33 and 35 to clearly be seen.

Deposit Information

[0144] The following HSV1 strains were deposited at the ECACC, Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, United Kingdom on 19 December 2016 by Replimune Limited and were allocated the indicated accession numbers:

RH004A - Accession Number 16121902

RH015A - Accession Number 16121903

RH018A - Accession Number 16121904

RH021A - Accession Number 16121905

RH023A - Accession Number 16121906

RH03 1A - Accession Number 16121907

RH040B - Accession Number 16121908

RH047A - Accession Number 16121909.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO2006002394A [0013]
- US2011044953A [0033]
- US20150283234A [0033]
- WO2007123737A [0069]
- WO2014066532A [0069]

Non-patent literature cited in the description

- **LIU et al.**Gene Therapy, 2003, vol. 10, 4292-303 [0008]
- **SENZER et al.**Journal of Clinical Oncology, 2009, vol. 27, 345763-5771 [0009]
- **SIMPSON et al.**Cancer Research, 2006, vol. 66, 94853-4842 [0010]
- **PIASECKI et al.**Talilmogene laherparepvec increases the anti-tumour efficacy of the anti-PD-1 immune checkpoint blockade, 2015, [0011]
- **SOKOLOWSKI et al.**Oncolytic virotherapy, 2015, vol. 4, 207-219 [0012]
- **CHOU et al.**Science, 1990, vol. 250, 1262-1266 [0052]
- **MACLEAN et al.**J. Gen. Virol., 1991, vol. 72, 631-639 [0052]
- **LIU et al.**Gene Therapy, 2003, vol. 10, 292-303 [0052]

Onkolytisk virusstamme

PATENTKRAV

1. Onkolytisk virus, der er en herpes simplex virus- (HSV) 1 stamme

RH018A med deponeringsnummeret ECCAC 16121904;

RH004A med deponeringsnummeret ECCAC 16121902;

RH031A med deponeringsnummeret ECCAC 16121907;

RH040B med deponeringsnummeret ECCAC 16121908;

RH015A med deponeringsnummeret ECCAC 16121903;

RH021A med deponeringsnummeret ECCAC 16121905;

RH023A med deponeringsnummeret ECCAC 16121906 eller

RH047A med deponeringsnummeret ECCAC 16121909.

2. Virus ifølge krav 1, der er stamme RH018A med deponeringsnummeret EACC 16121904.

3. Onkolytisk virus, der er en modificeret herpes simplex virus- (HSV) 1 stamme

RH018A med deponeringsnummeret ECCAC 16121904;

RH004A med deponeringsnummeret ECCAC 16121902;

RH031A med deponeringsnummeret ECCAC 16121907;

RH040B med deponeringsnummeret ECCAC 16121908;

RH015A med deponeringsnummeret ECCAC 16121903;

RH021A med deponeringsnummeret ECCAC 16121905;

RH023A med deponeringsnummeret ECCAC 16121906 eller

RH047A med deponeringsnummeret ECCAC 16121909,

omfattende:

(a) en mutation, der forhindrer ekspression af funktionel ICP34.5, ICP6 og/eller thymidinkinase via HSV'et;

(b) ét eller flere immunstimulerende molekyler eller ét eller flere immunstimulerende molekylekodende gener; og/eller

(c) ét eller flere fusogene proteinkodende gener.

4. Virus ifølge krav 3, der er en modificeret stamme RH018A med deponeringsnummeret EACC 16121904 omfattende:

(a) en mutation, der forhindrer ekspression af funktionel ICP34.5, ICP6 og/eller thymidinkinase via HSV'et;

(b) ét eller flere immunstimulerende molekyler eller ét eller flere immunstimulerende molekylekodende gener; og/eller

(c) ét eller flere fusogene proteinkodende gener.

5. Virus ifølge krav 3 eller krav 4, hvor virusset:

(a) ikke udtrykker funktionel ICP34.5;

(b) ikke udtrykker funktionel ICP47; og/eller

(c) udtrykker US11-genet som et umiddelbart tidligt gen.

6. Virus ifølge et hvilket som helst af kravene 3 til 5, der omfatter:

(a) ét eller flere immunstimulerende molekyler eller ét eller flere immunstimulerende molekylekodende gener; og/eller

(b) ét eller flere fusogene proteinkodende gener.

7. Virus ifølge et hvilket som helst af kravene 3 til 6, hvor:

(a) det fusogene protein vælges fra gruppen bestående af vesikulær stomatitisvirus (VSV) G-protein, syncitin-1, syncitin-2, simianvirus 5 (SV5) F-protein, mæslingevirus (MV) H-protein, MV F-protein, respiratorisk syncytialvirus (RSV) F-protein og et glycoprotein fra gibbonabe-leukæmivirus (GALV), murint leukæmivirus (MLV), Mason-Pfizer-abevirus (MPMV) eller equin infektiøs anæmivirus (EIAV), hvorfra R-peptidet er deleteret;

og/eller

(b) det immunstimulerende molekyle er GMCSF, IL-2, IL-12, IL-15, IL-18, IL-21, IL-24, et type I-interferon, interferon gamma, et type III-interferon, TNF alpha,

en antagonist af TGF beta, en immun checkpoint-antagonist eller en agonist af en immunforstærkende bane indbefattende CD40-ligand (CD40L), ICOS-ligand, GITR-ligand, 4-1-BB-ligand, OX40-ligand eller flt3-ligand.

8. Virus ifølge et hvilket som helst af kravene 3 til 7, hvor:

(a) det fusogene protein er glycoproteinet fra gibbonabe-leukæmivirus (GALV) og har R-transmembranpeptidet muteret eller fjernet (GALV-R-); og/eller

(b) det immunstimulerende molekyle er GMCSF, GITRL, ICOSL, 4-1-BBL, OX40L eller CD40L, eller de immunstimulerende molekyler er GM-CSF og GITRL, OX40L, 4-1-BBL, ICOSL eller CD40L.

9. Virus ifølge et hvilket som helst af kravene 3 til 8, hvor:

(a) det fusogene proteinkodende gen og/eller det immunstimulerende molekylekodende gen er indsat i det for ICP34.5 kodende locus, enten ved indsætning, eller delvis eller fuld deletering, hver under separat regulatorisk styring, eventuelt i en orientering ryg mod ryg i forhold til hinanden; og/eller

(b) sekvensen af det gen, der koder for det fusogene protein, og/eller sekvensen af det gen, der koder for det immunstimulerende molekyle, er codonoptimeret for således at øge ekspressionsniveauer i målceller.

10. Virus ifølge et hvilket som helst af kravene 3 til 9, der udtrykker tre heterologe gener, hvor:

(a) hvert af de tre heterologe gener drives af en forskellig promotor valgt blandt CMV-promotoren, RSV-promotoren, SV40-promotoren og en retroviral LTR-promoter, såsom retroviral LTR fra MMLV; og/eller

(b) hvert af de tre heterologe gener er termineret af en forskellig polyadenyleringssekvens valgt blandt BGH-, SV40-, HGH- og RBG-polyadenyleringssekvenserne.

11. Virus ifølge et hvilket som helst af kravene 3 til 9, der udtrykker fire heterologe gener, der drives af henholdsvis hver af CMV-promotoren, RSV-promotoren, SV40- promotoren og en retroviral LTR-promotor, såsom den

retrovirale LTR-promotor fra MMLV, og/eller er termineret af henholdsvis hver af BGH-, SV40-, HGH- og RBG-polyadenyleringssekvenserne.

12. Farmaceutisk sammensætning omfattende et virus ifølge et hvilket som helst af kravene 1 til 11 og en farmaceutisk acceptabel bærer eller fortynder.

13. Virus ifølge et hvilket som helst af kravene 1 til 11 til anvendelse i en fremgangsmåde til behandling af menneske- eller dyrekrop ved terapi.

14. Virus ifølge et hvilket som helst af kravene 1 til 11 til anvendelse i en fremgangsmåde til behandling af cancer.

15. Virus til anvendelse ifølge krav 14, hvor fremgangsmåden omfatter indgivelse af et yderligere middel mod cancer.

16. Virus til anvendelse ifølge krav 15, hvor det yderligere middel mod cancer vælges blandt en antagonist af en immun co-hæmmende bane, såsom en CTLA-4-hæmmer, en PD-1-hæmmer, en PD-L1-hæmmer, en LAG-3-hæmmer, en TIM-3-hæmmer, en VISTA-hæmmer, en CSF1R-hæmmer, en IDO-hæmmer, en CD47-hæmmer, en KIR-hæmmer, en SLAMF7-hæmmer eller en CEACAM1-hæmmer, en agonist af en immun co-stimulerende bane, såsom en GITR-agonist, en 4-1-BB-agonist, en OX40-agonist, en CD40-agonist eller en ICOS-agonist, stråling og/eller kemoterapi, et middel, der er målrettet en specifik genetisk mutation, som opstår i tumorer, det middel, der er beregnet til at inducere et immunrespons på ét eller flere tumorantigener eller neoantigener, en celleprodukt afledt af T-celler eller NK-celler, et middel, der er beregnet til at stimulere STING-, cGAS-, TLR- eller et andet iboende immunrespons og/eller en inflammatorisk bane, et andet virus eventuelt et onkolytisk virus, en hæmmer af indoleamin 2,3-dioxygenase- (IDO) banen og en kombination deraf.

17. Virus til anvendelse ifølge krav 15 eller 16, hvor:

(a) det yderligere middel mod cancer er et antistof;

(b) fremgangsmåden omfatter indgivelse af en hæmmer af indoleamin 2,3-dioxygenase- (IDO) banen og en yderligere antagonist af en immun co-hæmmende bane, eller en agonist af en immun co-stimulerende bane;

(c) virusset og det eller de yderligere midler mod cancer indgives separat eller samtidigt;

og/eller

(d) cancergen er en solid tumor.

DRAWINGS

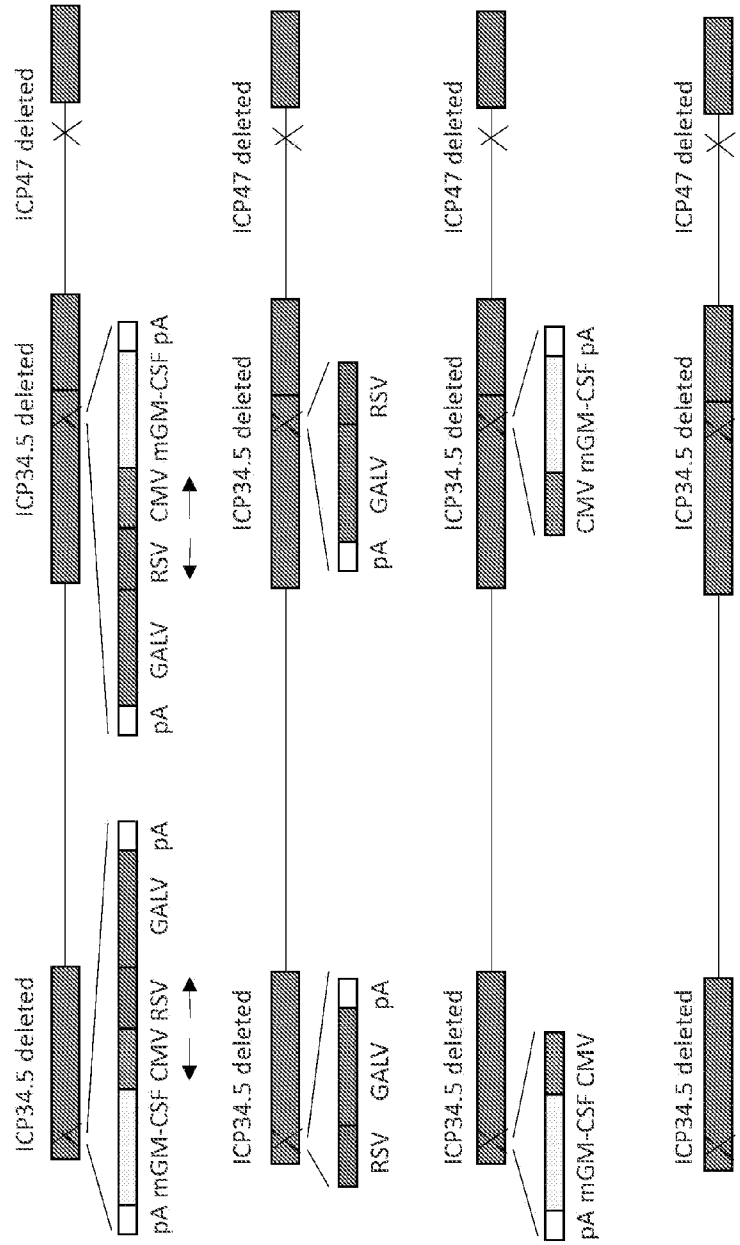


Figure 1

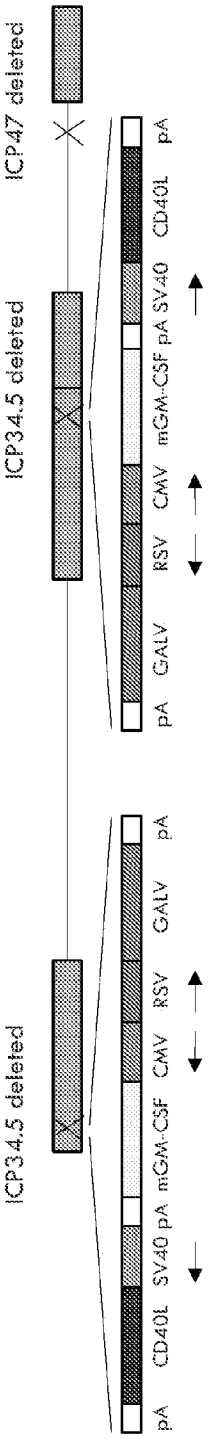

ICP34.5 deleted
ICP47 deleted
ICP34.5 deleted
pA CD40L SV40 pA mGM-CSF CMV RSV GALV pA
pA GALV RSV CMV mGM-CSF pA SV40 CD40L pA

Figure 2

Figure 3

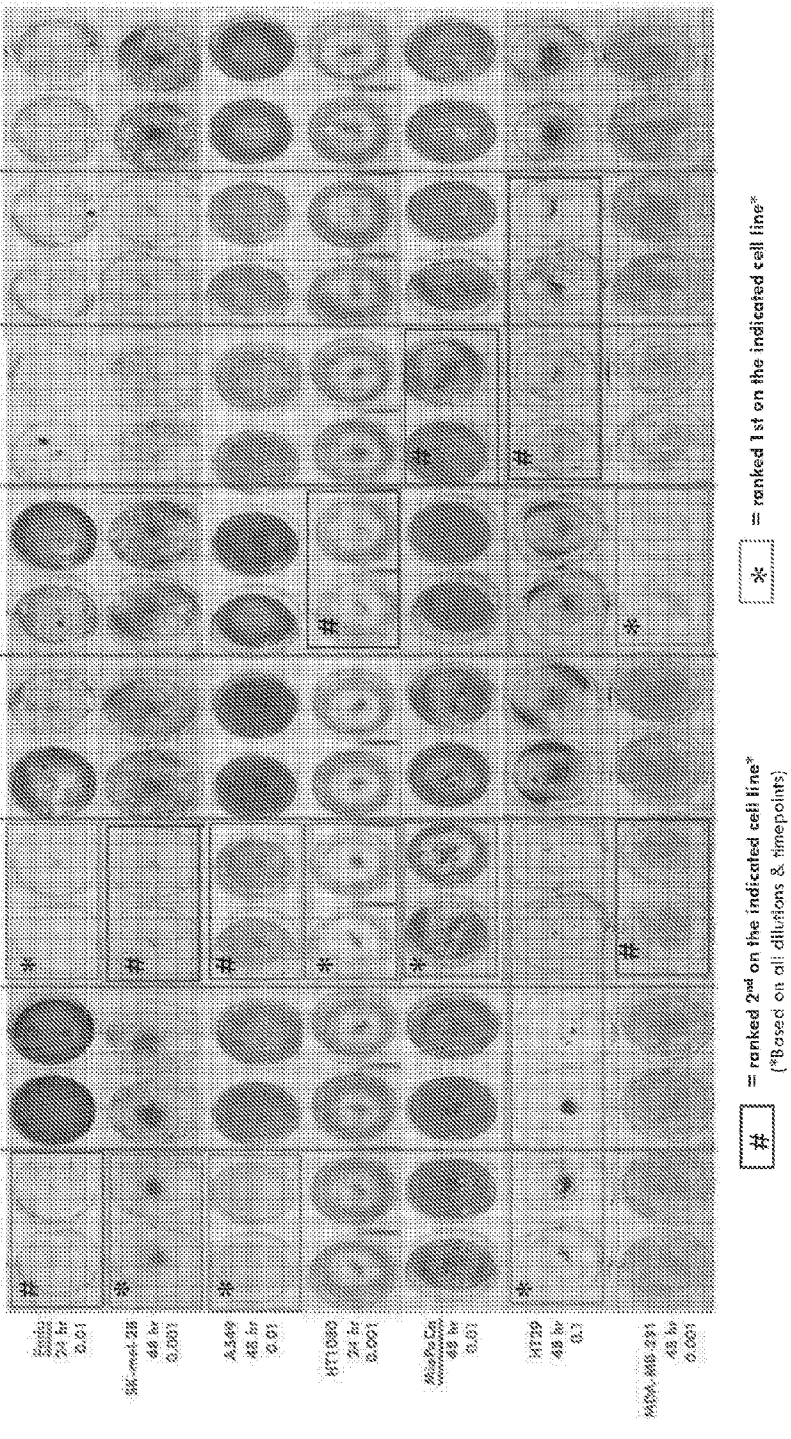

= ranked 2nd on the indicated cell line*
(*Based on all dilutions & timepoints)
= ranked 1st on the indicated cell line*

Figure 4

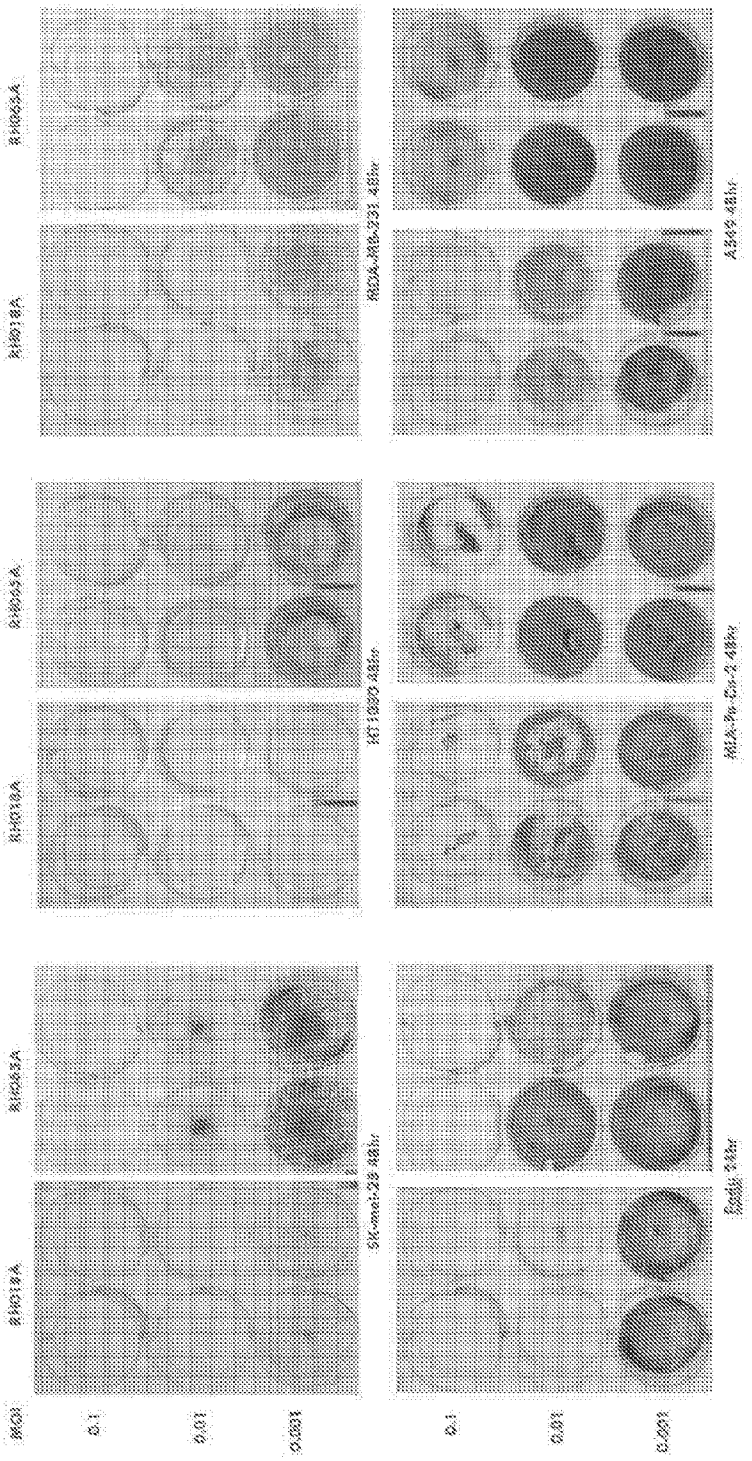

BRD4
BRD4 BD1
BRD4 BD2
0.1
0.01
0.001
SK-mel-28 48hr
MV4;11 48hr
MDA-MB-231 48hr
Raji 48hr
MIA PaCa-2 48hr
Jurkat 24hr
A549 48hr

Figure 5A

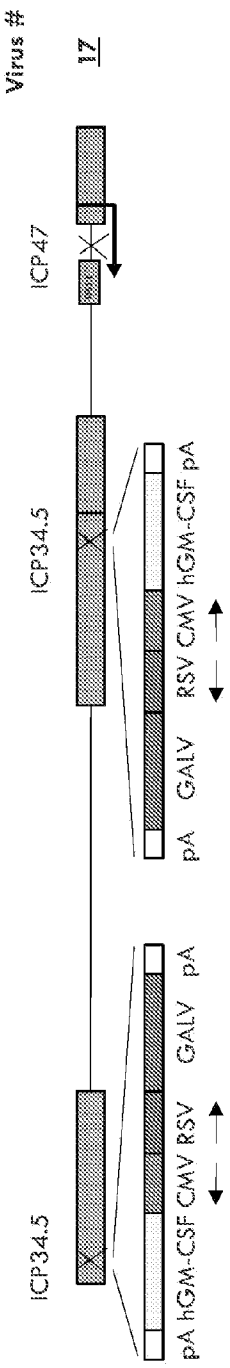
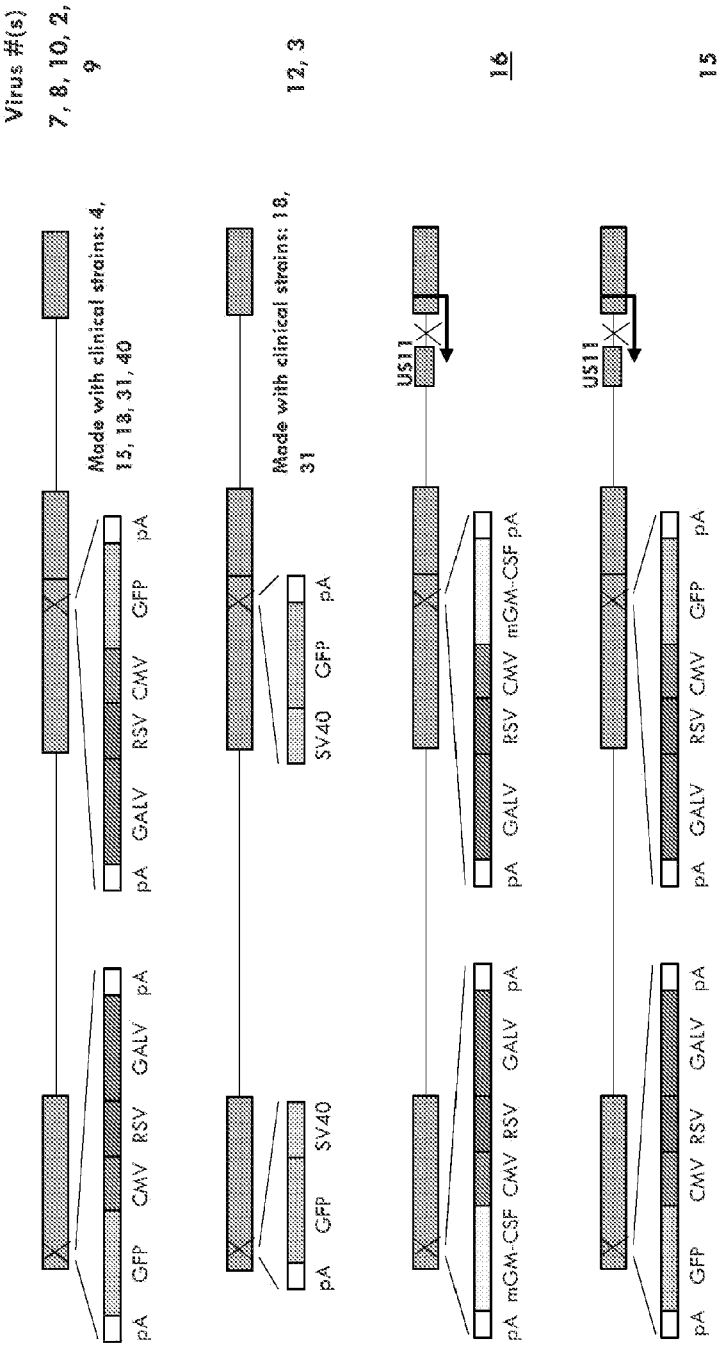

Virus #
17
ICP47
ICP34.5
RSV CMV hGM-CSF pA
pA GALV
ICP34.5
pA hGM-CSF CMV RSV
GALV pA

Figure 5B


Virus #(s)
7, 8, 10, 2, 9
Made with clinical strains: 4, 15, 18, 31, 40
pA GFP CMV RSV GALV pA
pA GALV RSV CMV GFP pA
12, 3
Made with clinical strains: 18, 31
pA GFP SV40
SV40 GFP pA
16
US11
pA mGM-CSF CMV RSV GALV pA
pA GALV RSV CMV mGM-CSF pA
15
US11
pA GFP CMV RSV GALV pA
pA GALV RSV CMV GFP pA

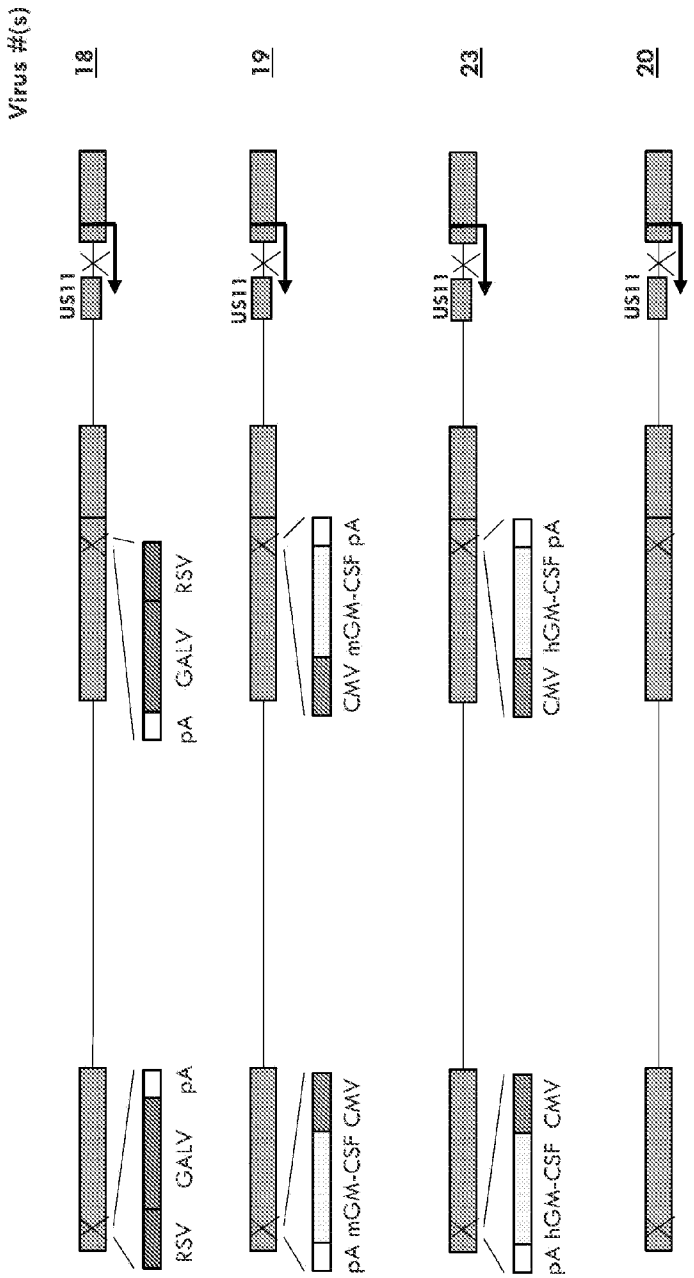

Virus #(s)
18
19
23
20
US11
RSV GALV pA
pA GALV RSV
pA mGM-CSF CMV
CMV mGM-CSF pA
pA hGM-CSF CMV
CMV hGM-CSF pA

Figure 5C

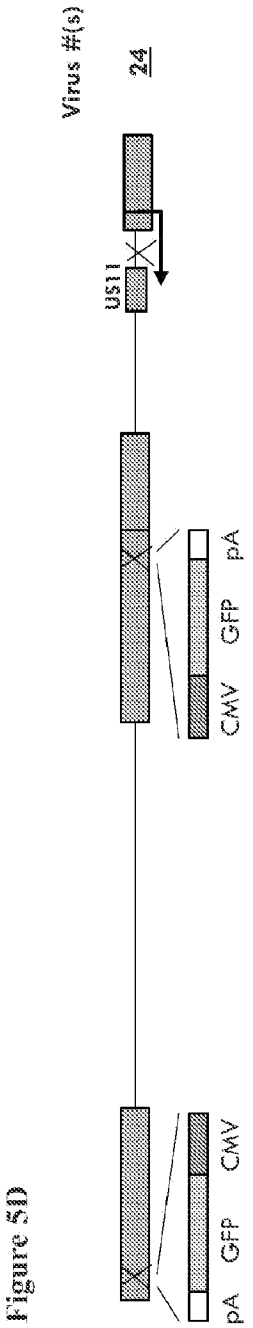


Figure 5D

Figure 5E

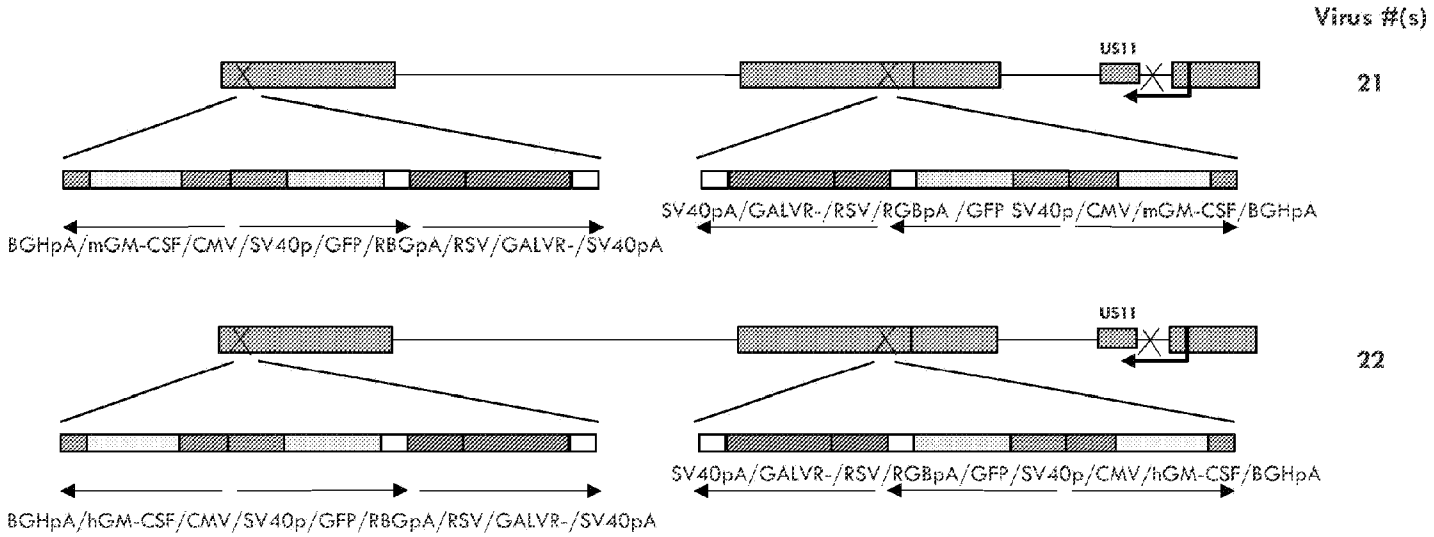


Figure 5F

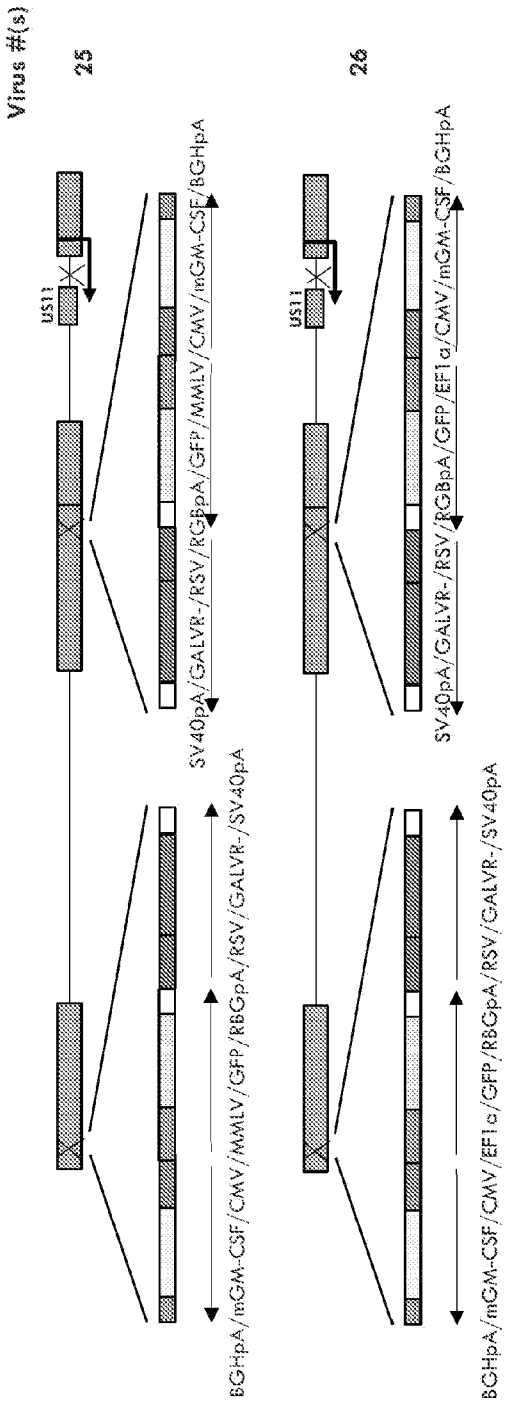

Virus #(s)
25
US11
SV40pA/GALVR-/RSV/RGBpA/GFP/MMLV/CMV/mGM-CSF/BGHpA
BGHpA/mGM-CSF/CMV/MMLV/GFP/RBGpA/RSV/GALVR-/SV40pA
26
US11
SV40pA/GALVR-/RSV/RGBpA/GFP/EF1a/CMV/mGM-CSF/BGHpA
BGHpA/mGM-CSF/CMV/EF1a/GFP/RBGpA/RSV/GALVR-/SV40pA

Figure 5G

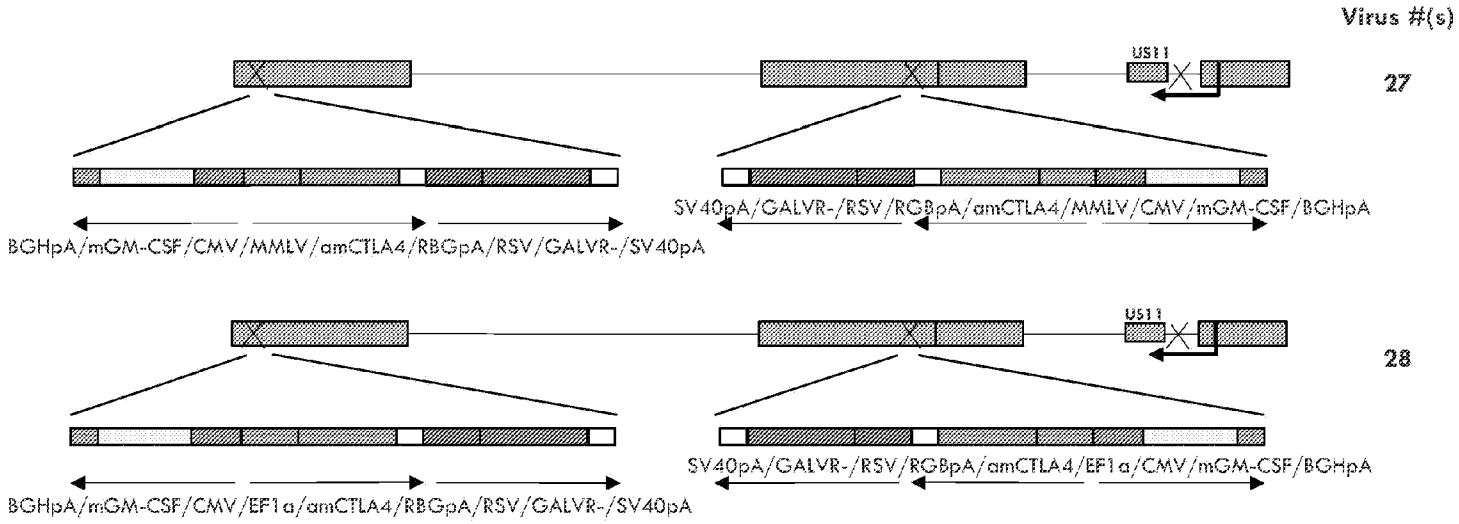


Figure 5H

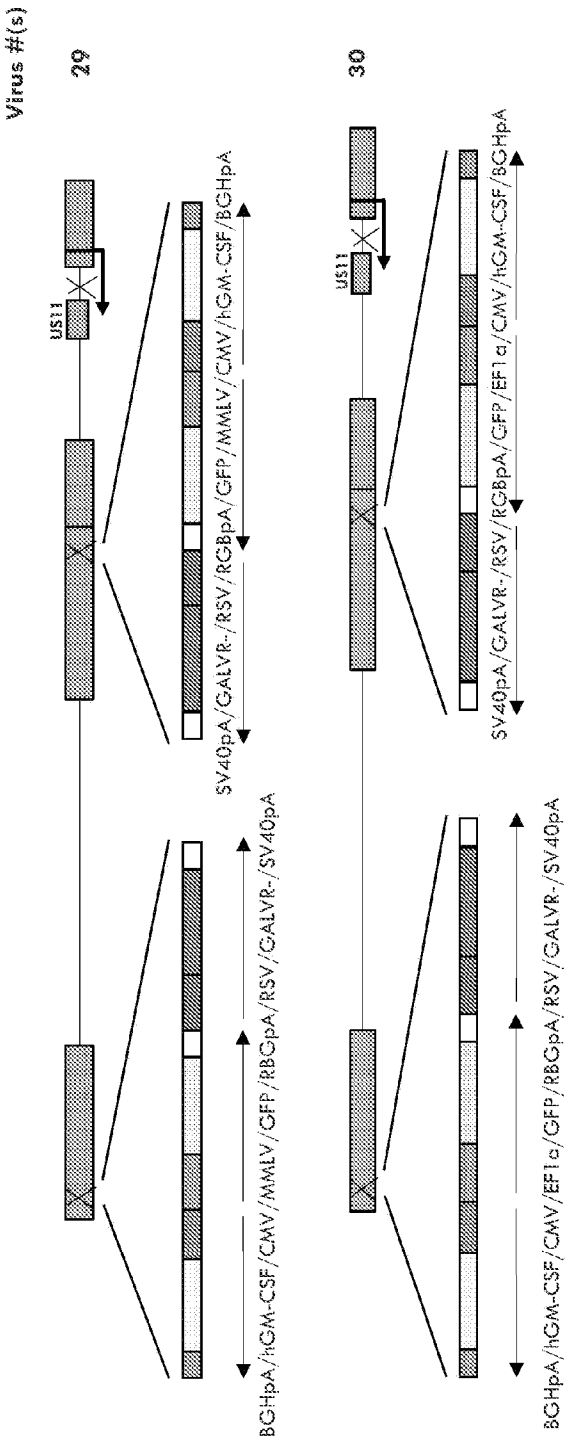

Virus #(s)
29
US11
SV40pA/GALVR-/RSV/RGBpA/GFP/MMLV/CMV/hGM-CSF/BGHpA
BGHpA/hGM-CSF/CMV/MMLV/GFP/RBGpA/RSV/GALVR-/SV40pA
30
US11
SV40pA/GALVR-/RSV/RGBpA/GFP/EF1α/CMV/hGM-CSF/BGHpA
BGHpA/hGM-CSF/CMV/EF1α/GFP/RBGpA/RSV/GALVR-/SV40pA

Figure 51

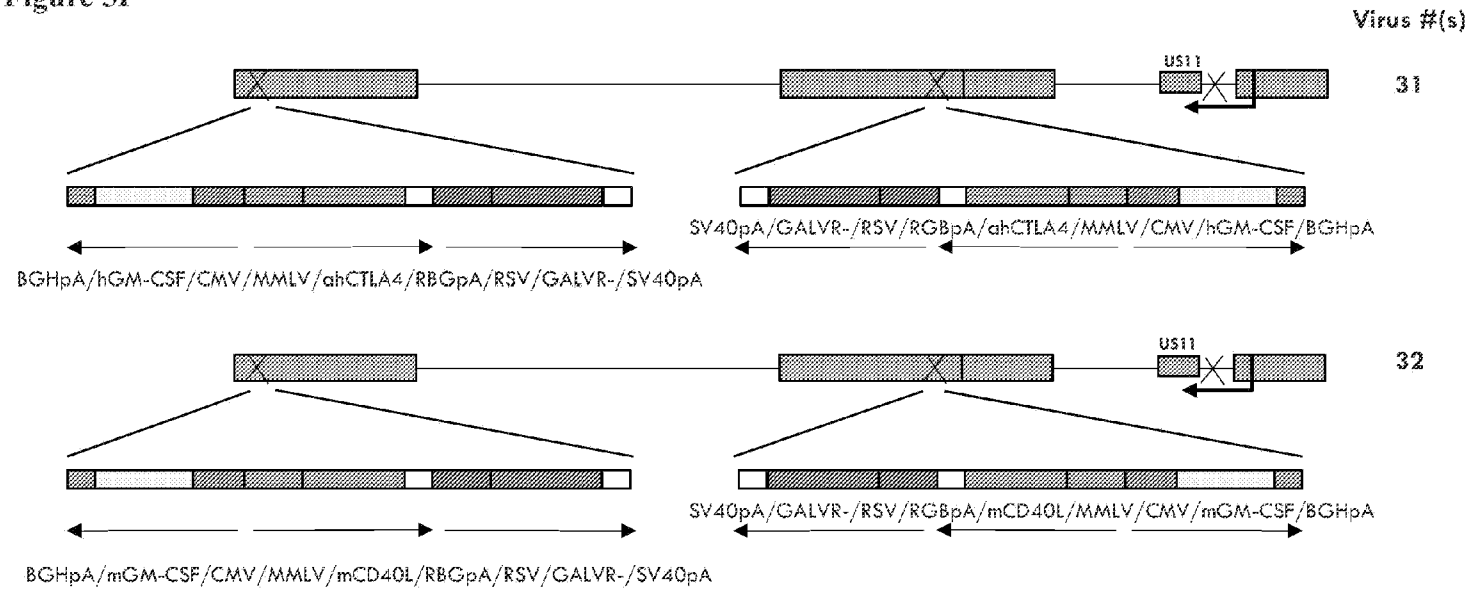


Figure 5J

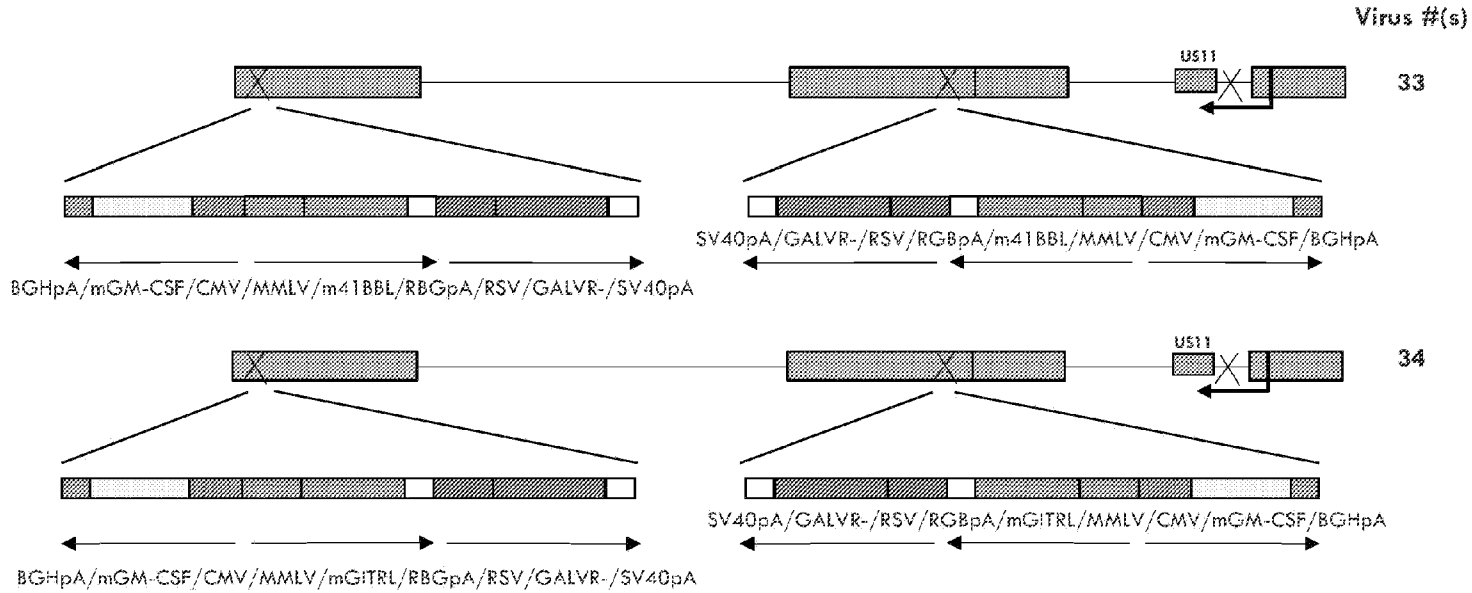


Figure 5K

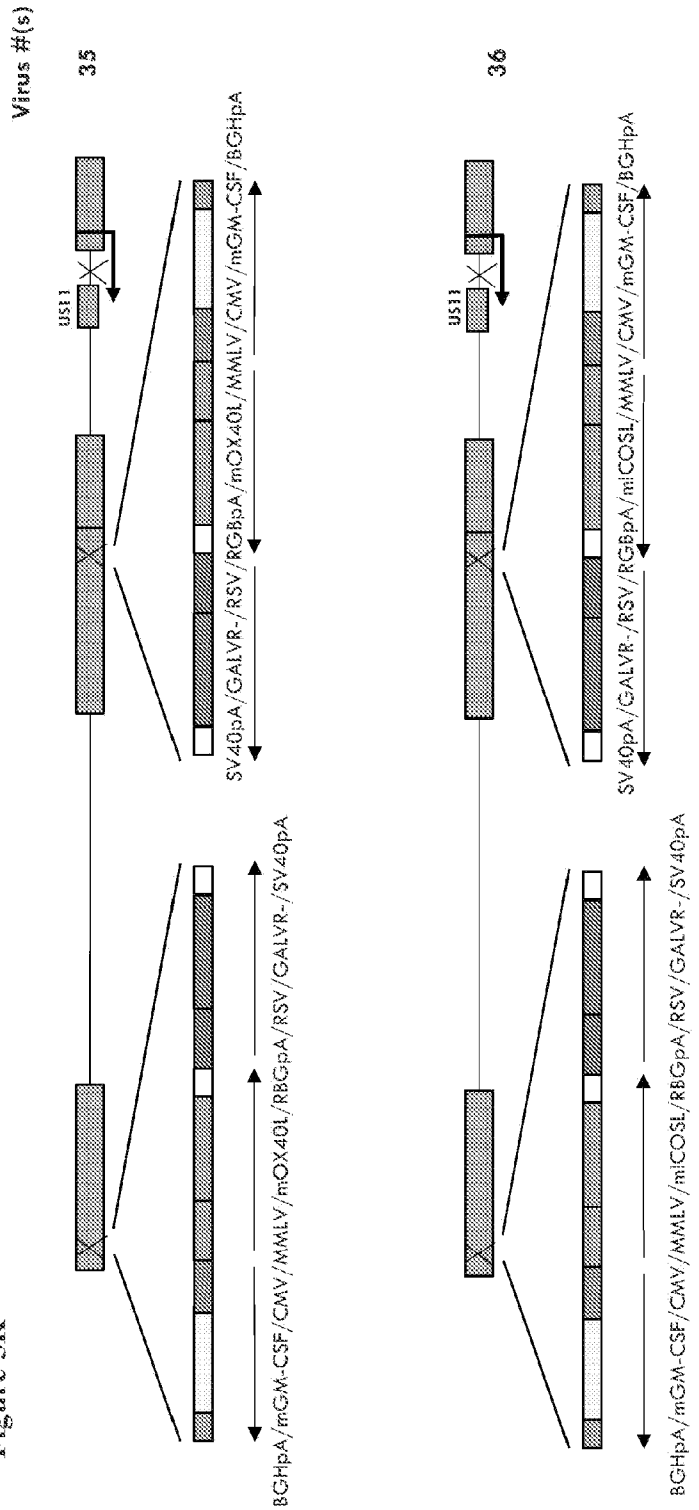

Virus #(s)
35
36
US11
SV40pA/GALVR-/RSV/RGBpA/mOX40L/MMLV/CMV/mGM-CSF/BGHpA
BGHpA/mGM-CSF/CMV/MMLV/mOX40L/RBGpA/RSV/GALVR-/SV40pA
SV40pA/GALVR-/RSV/RGBpA/miCOSL/MMLV/CMV/mGM-CSF/BGHpA
BGHpA/mGM-CSF/CMV/MMLV/miCOSL/RBGpA/RSV/GALVR-/SV40pA

Figure 6

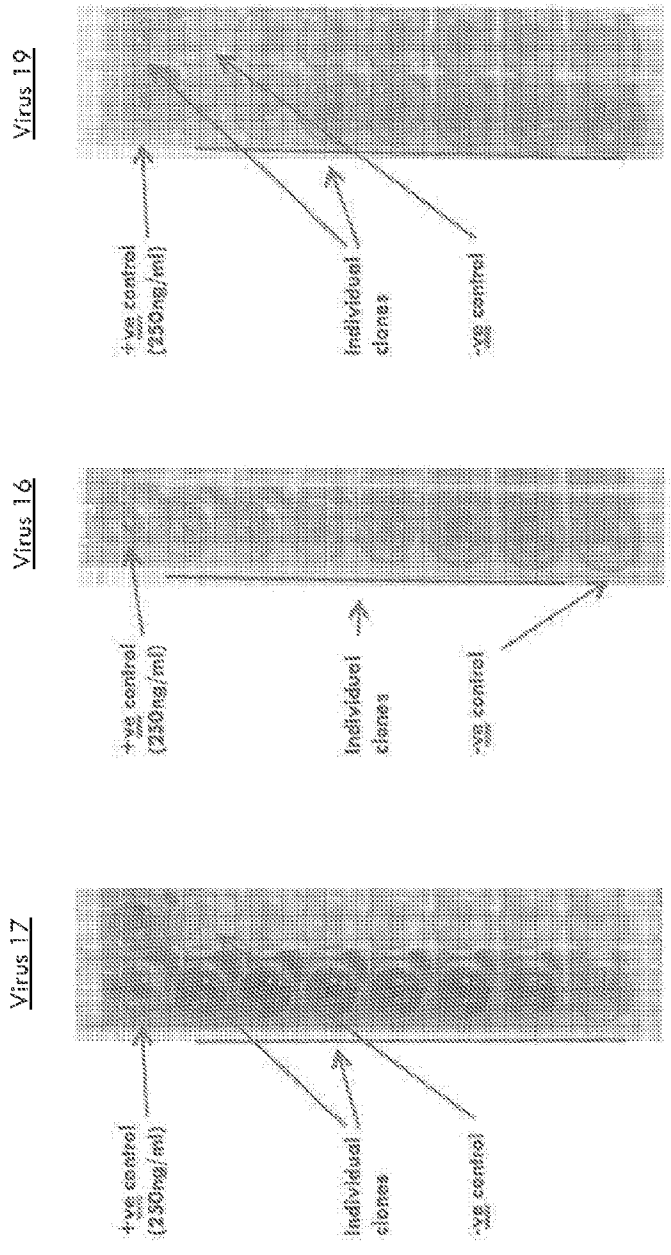

Virus 19
+ve control (250ng/ml)
Individual clones
-ve control
Virus 16
+ve control (250ng/ml)
Individual clones
-ve control
Virus 17
+ve control (250ng/ml)
Individual clones
-ve control

Figure 7A

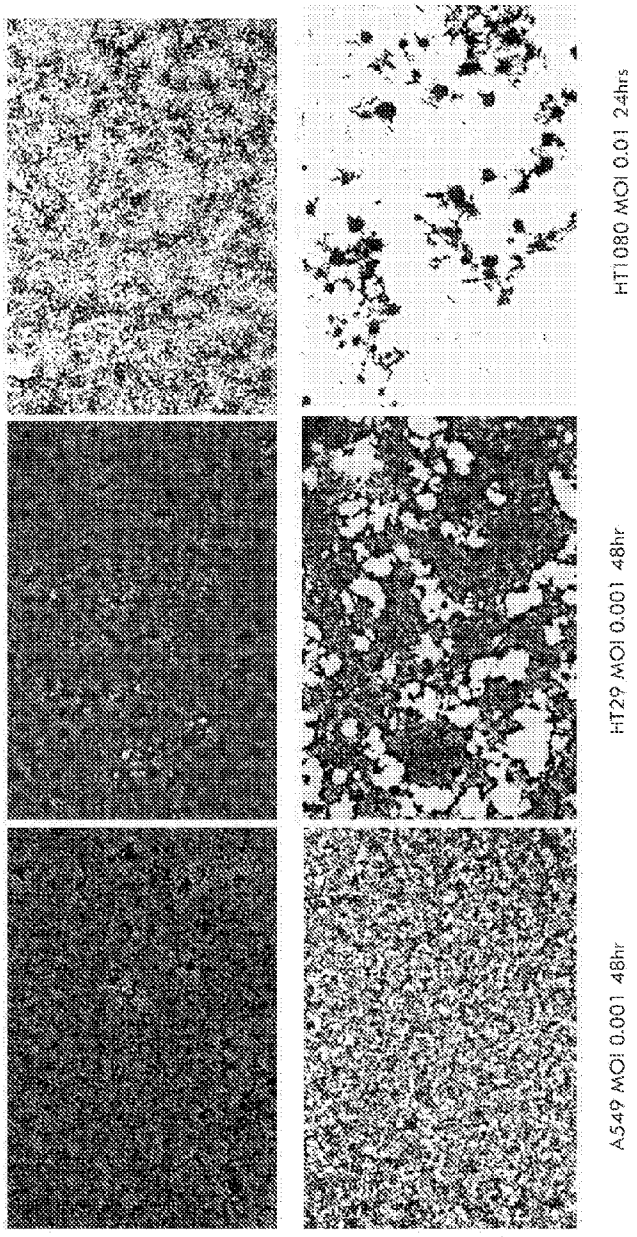
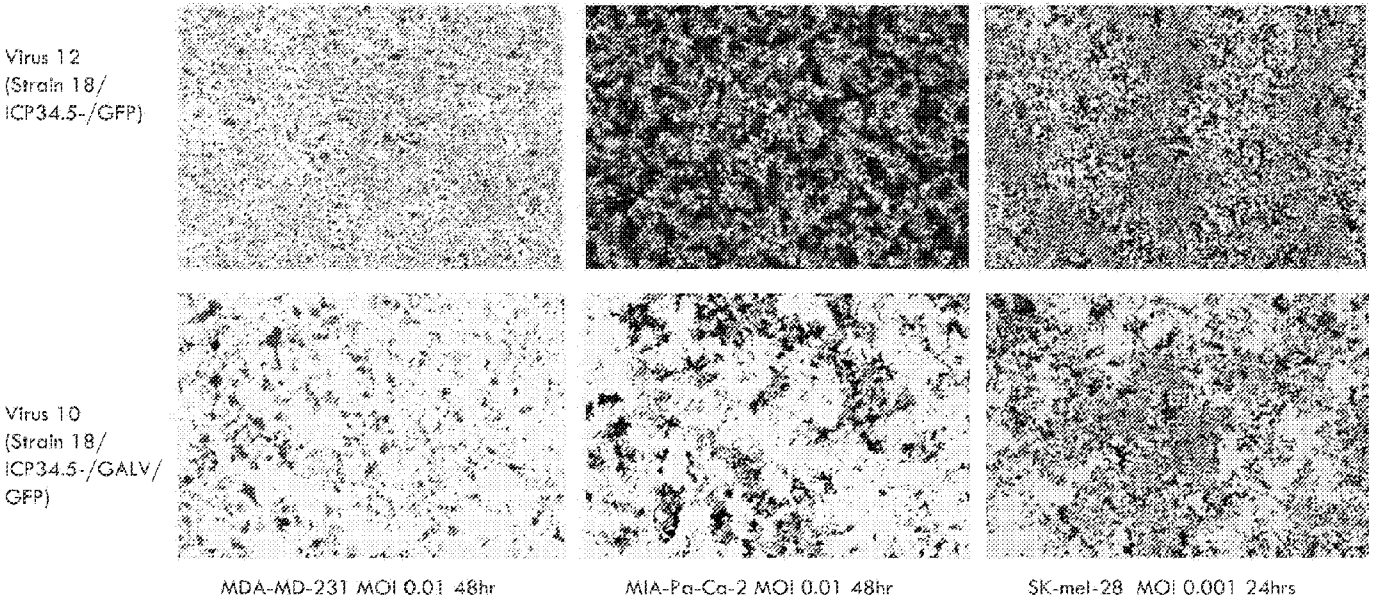

Virus 12 (Strain 18/ ICP34.5-/GFP)
Virus 10 (Strain 18/ ICP34.5-/GALV/ GFP)
A549 MOI 0.001 48hr
HT29 MOI 0.001 48hr
HT1080 MOI 0.01 24hrs

Figure 7B



Cell death assessed by crystal violet staining: low magnification

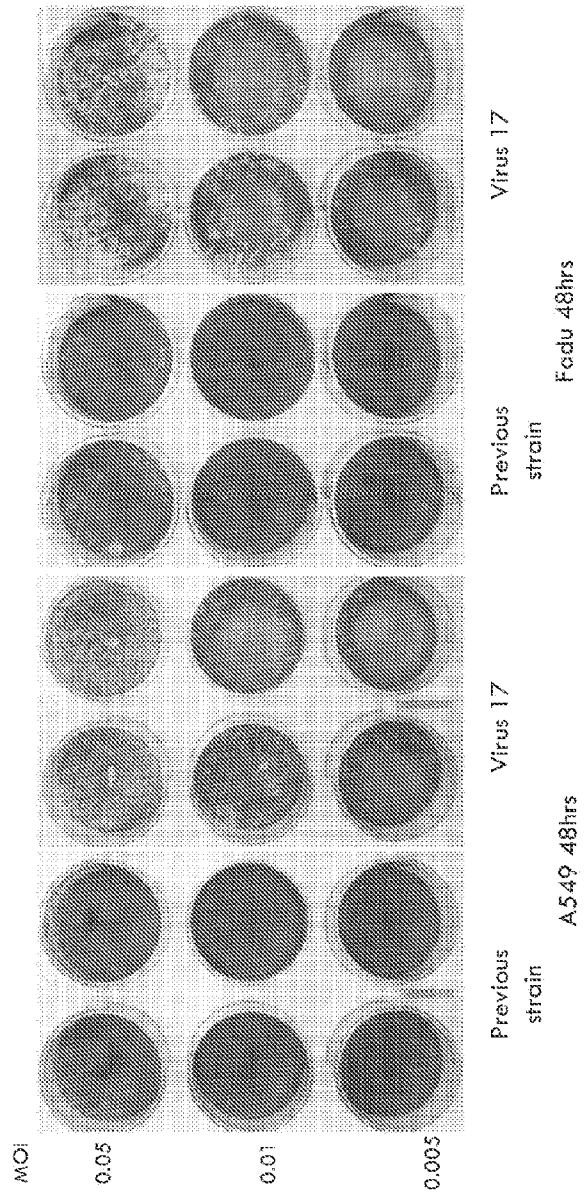

MOI
0.05
0.01
0.005
Previous strain
Virus 17
Previous strain
Virus 17
A549 48hrs
Fadu 48hrs

Figure 8A

Figure 8B

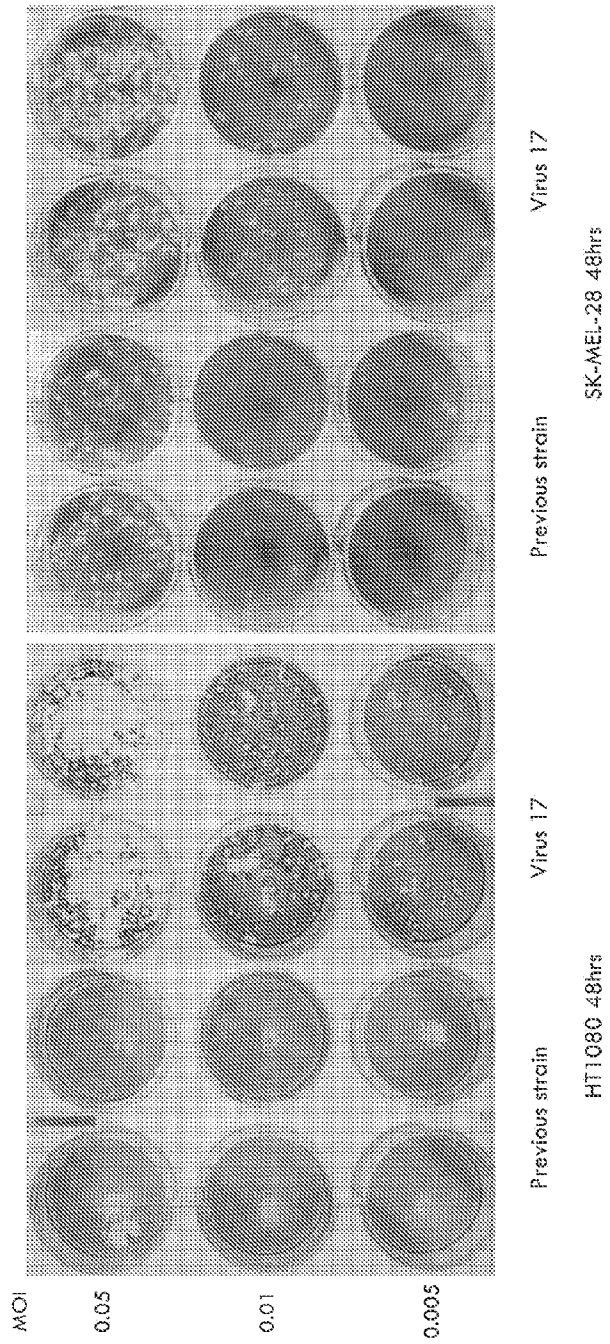
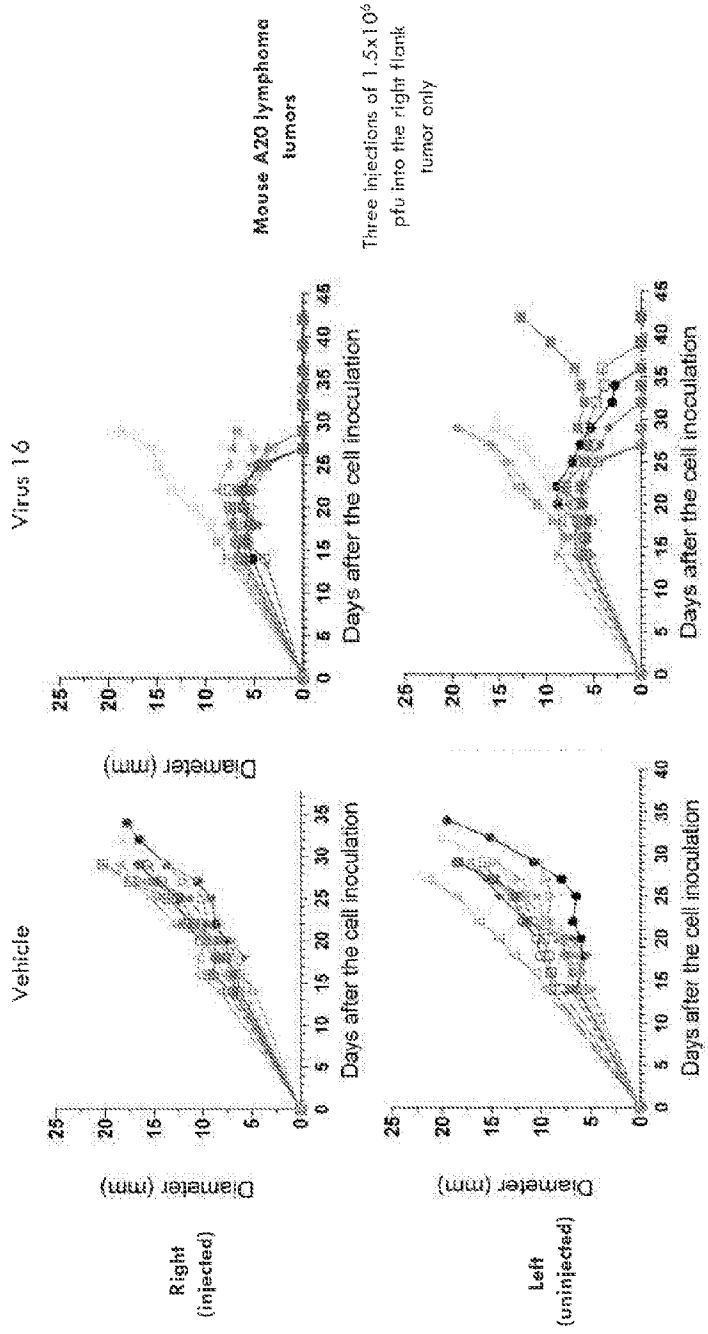

MOI
0.05
0.01
0.005
Previous strain
Virus 17
HT1080 48hrs
Previous strain
Virus 17
SK-MEL-28 48hrs

Figure 9


Mouse A20 lymphoma tumors
Three injections of 1.5x10⁶ pfu into the right flank tumor only
Vehicle
Virus 16
Right (injected)
Left (uninjected)
Diameter (mm)
Days after the cell inoculation

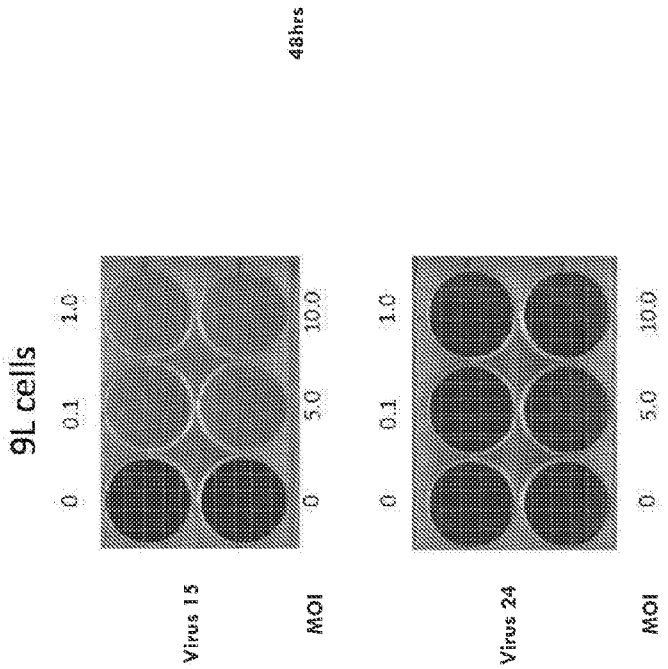
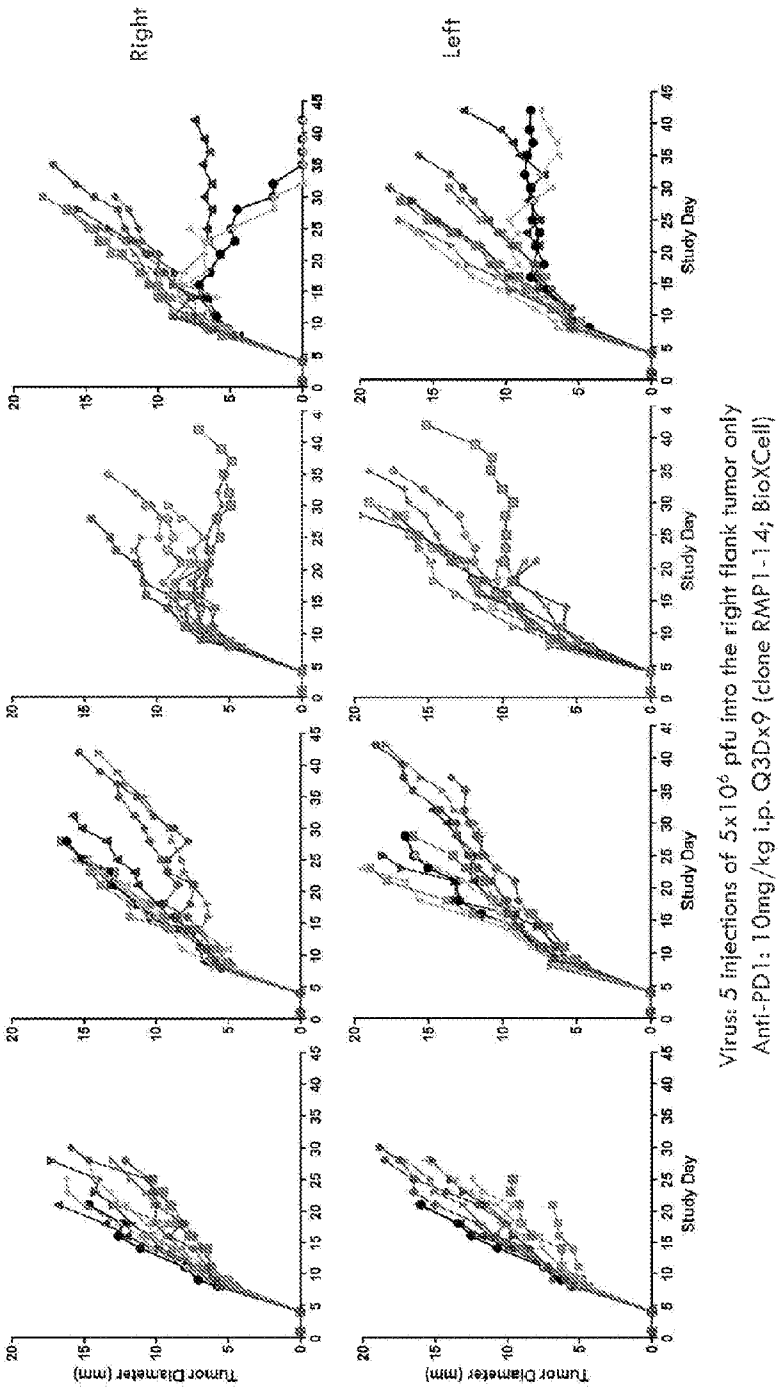


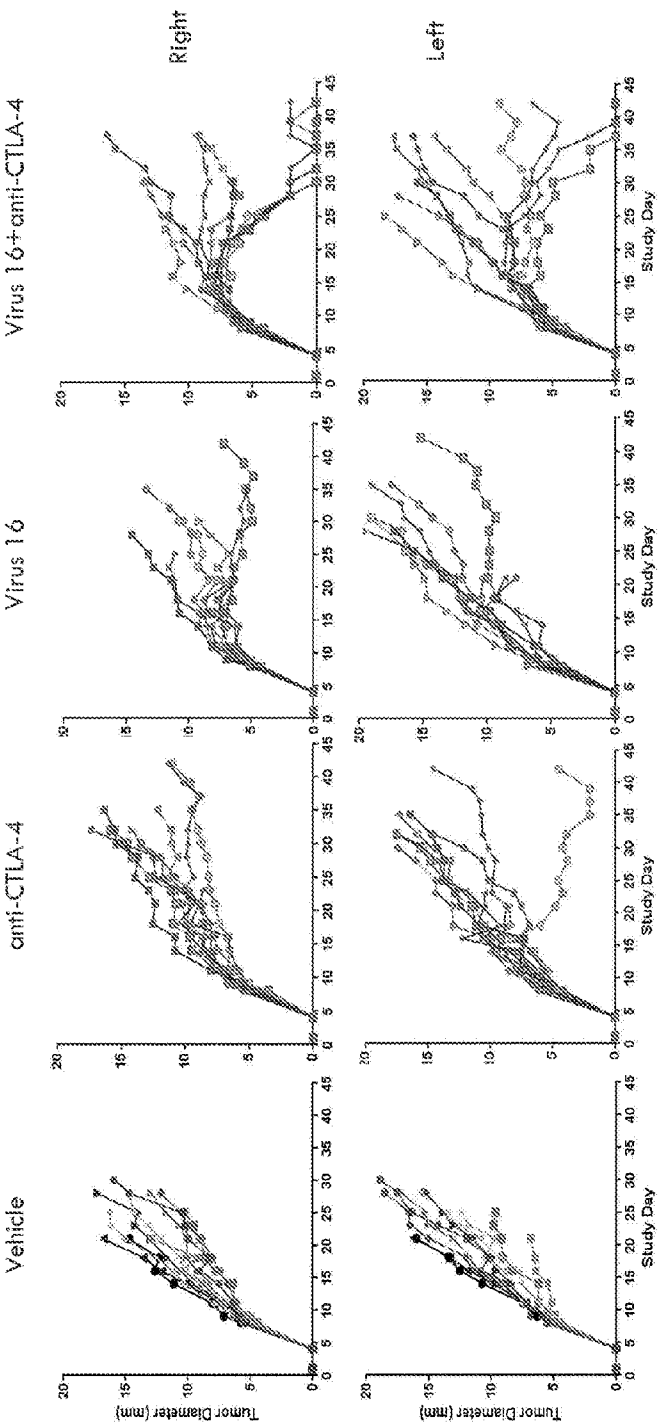
Figure 10

Figure 11A



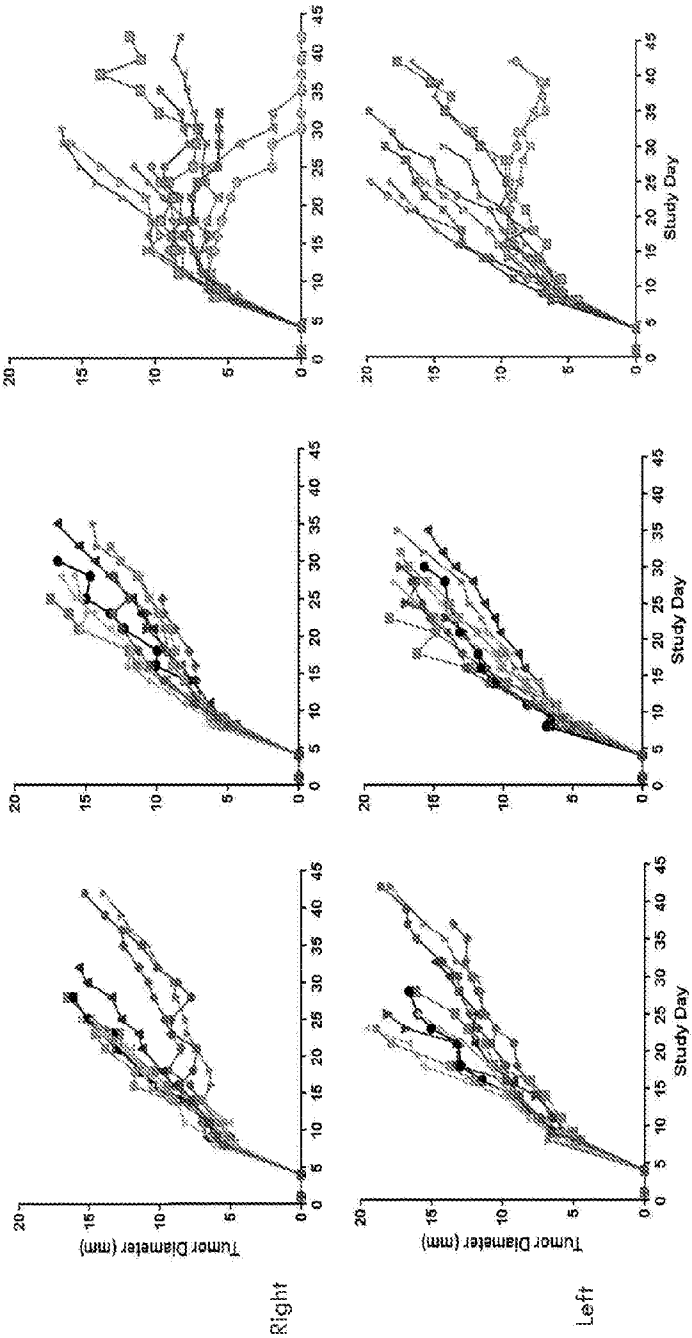
Virus: 5 injections of $5x10^6$ pfu into the right flank tumor only
Anti-PD1: 10mg/kg i.p. Q3Dx9 (clone RMP1-14; BioXCell)

Figure 11B



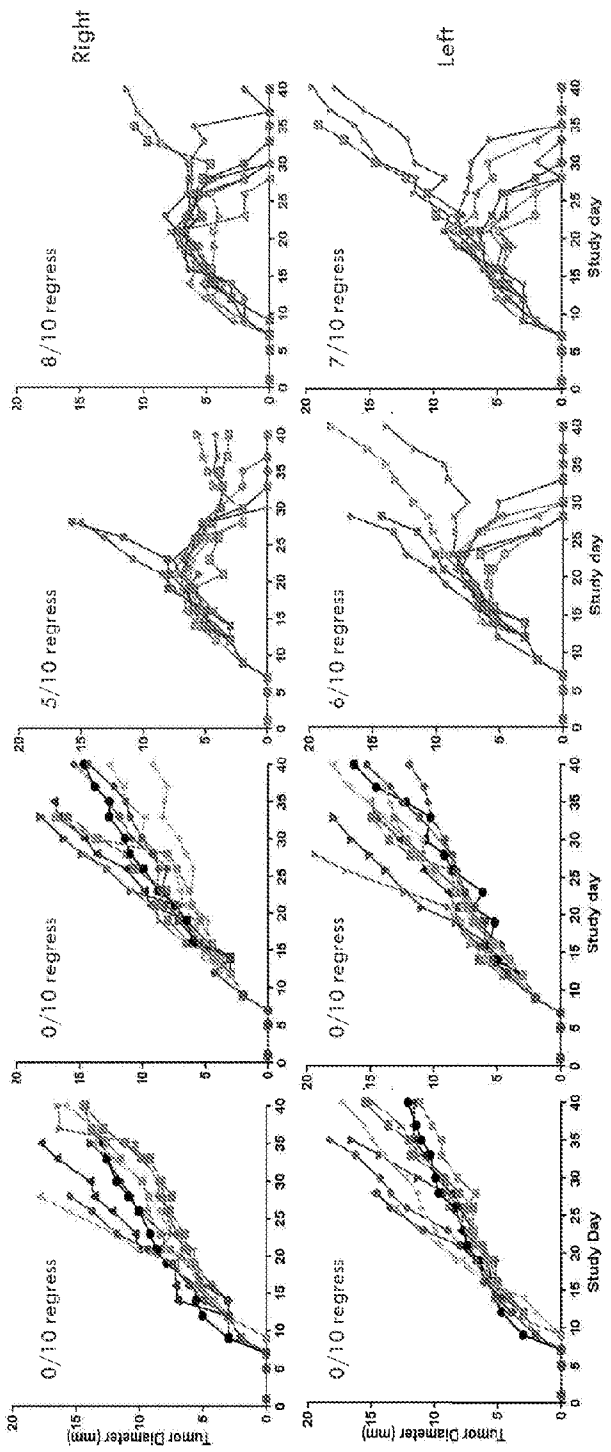
Virus: 5 injections of 5×10^6 pfu into the right flank tumor only
Anti-CTLA-4: 3mg/kg i.p. Q3Dx9 (clone 9D9; BioXCell)

Figure 11C



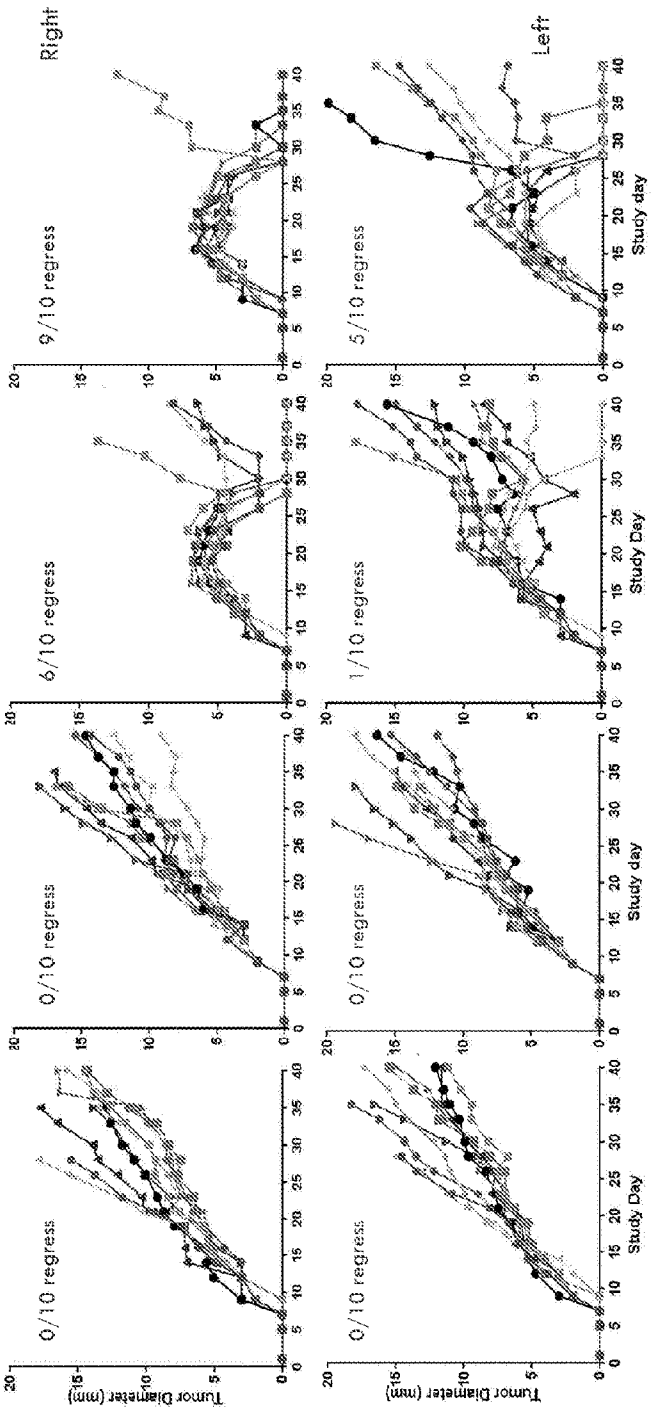
Virus: 5 injections of $5x10^6$ pfu into the right flank tumor only
Anti-PD1: 10mg/kg i.p. Q3Dx9 (clone RMP1-14; BioXCell)
1-MT: 5mg/ml in drinking water (1-MT alone has no effect)

Figure 12A



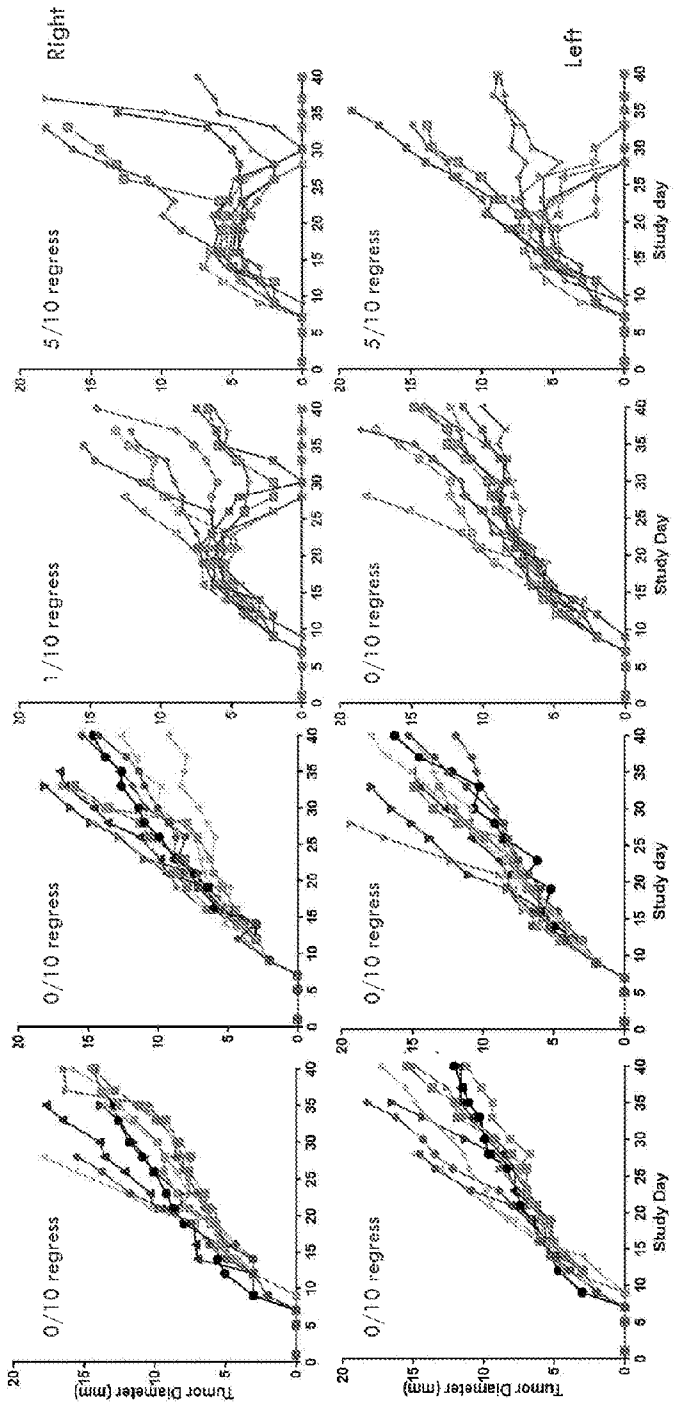
Virus: 3 injections of 5×10^6 pfu into the right flank tumor only
Anti-PD1: 10mg/kg i.p. Q3Dx9 (clone RMP1-14; BioXCell)

Figure 12B



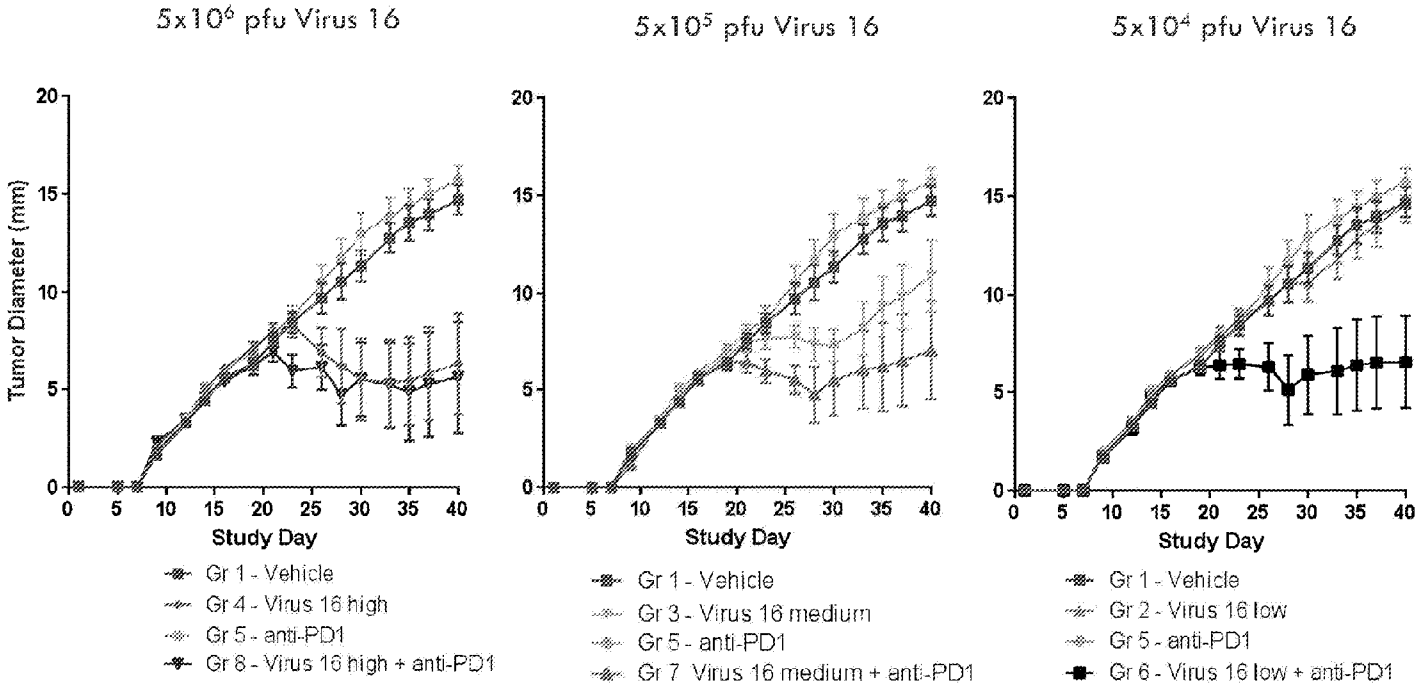
Virus: 3 injections of 5×10^5 pfu into the right flank tumor only
Anti-PD1: 10mg/kg i.p. Q3Dx9 (clone RMP1-14; BioXCell)

Figure 12C



Virus: 3 injections of 5×10^4 pfu into the right flank tumor only
Anti-PD1: 10mg/kg i.p. Q3Dx9 (clone RMP1-14; BioXCell)

Figure 12D



LEFT FLANK (NOT INJECTED WITH RP1) TUMORS BY GROUP

N=10/group

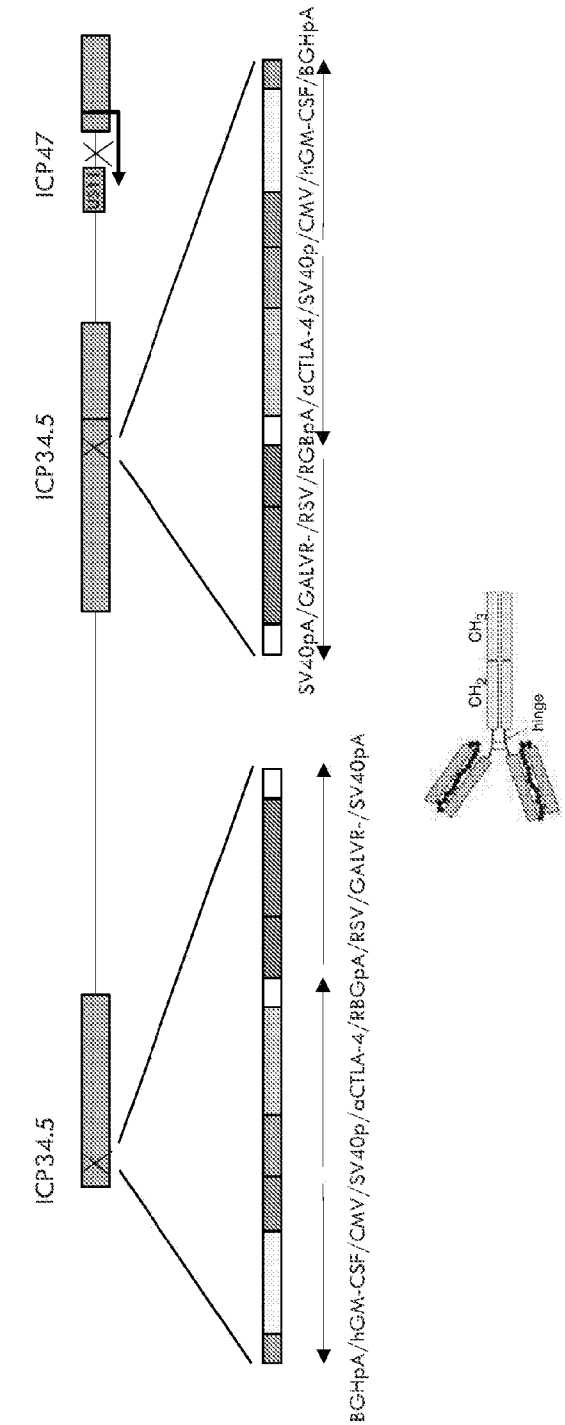
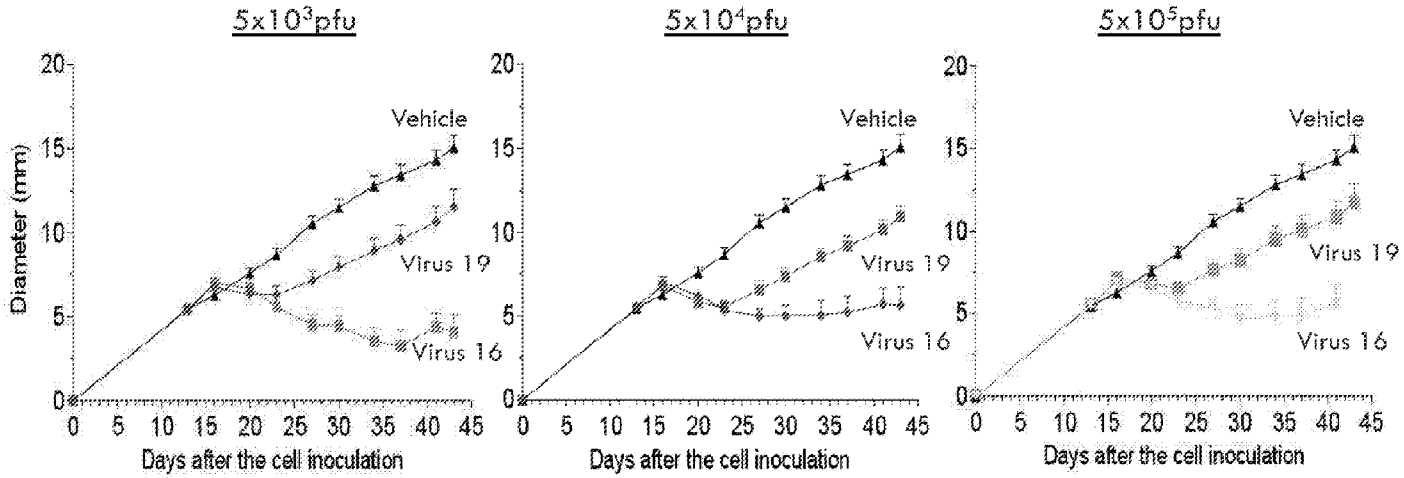

ICP47
ICP34.5
ICP34.5
SV40pA/GALVR-/RSV/RGBpA/aCTLA-4/SV40p/CMV/hGM-CSF/BGHpA
BGHpA/hGM-CSF/CMV/SV40p/aCTLA-4/RBGpA/RSV/GALVR-/SV40pA
CH1
CH2
hinge

Figure 13

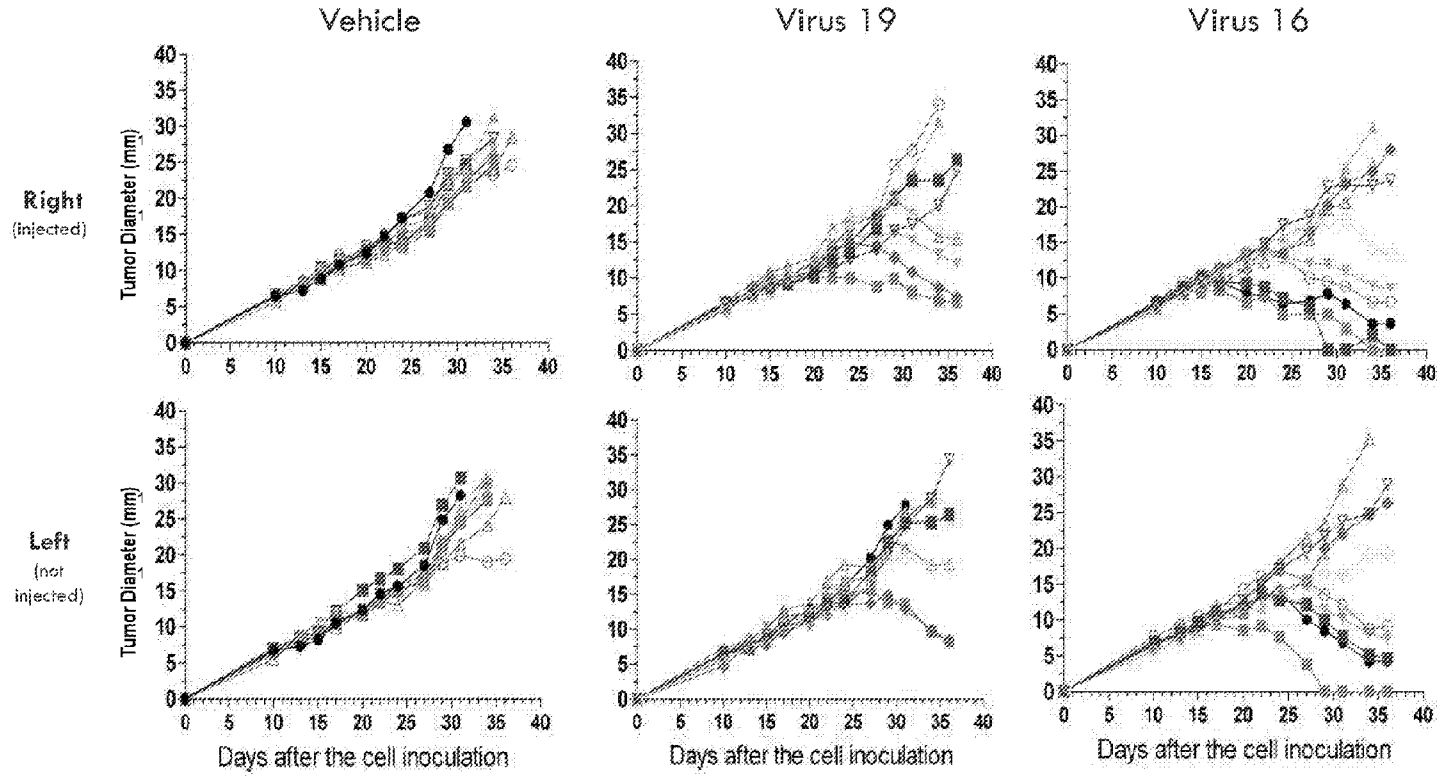
Figure 14



Human A549 lung cancer tumors in nude mice (no immune effect)

3 injections of Virus 16 or Virus 19 over 1wk of vehicle or the indicated dose of virus (N=10/group)

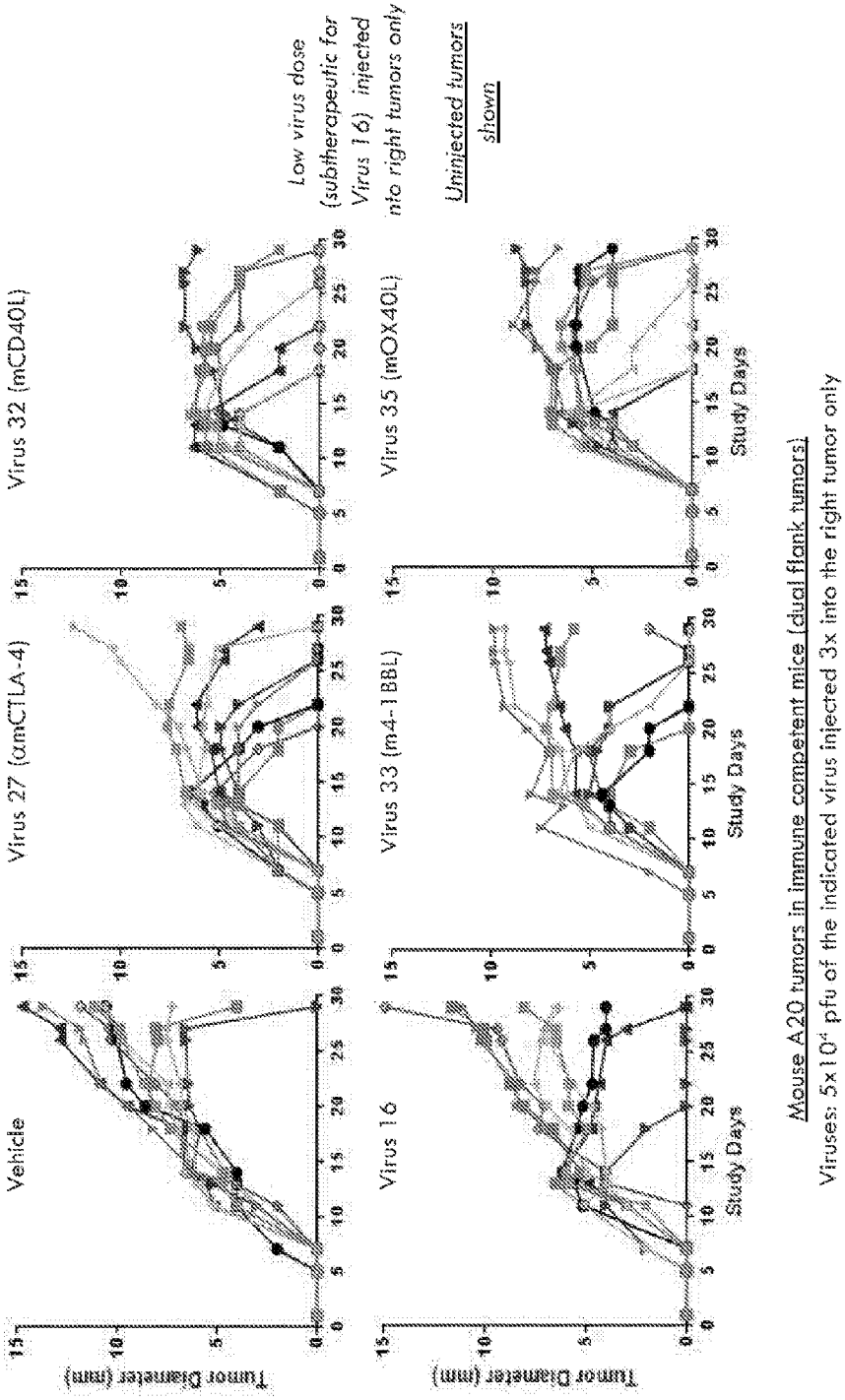
Figure 15



Rat 9L glioma tumors in immune competent rats

Virus: $5x10^5$ pfu injected 3x/wk into the right tumor only for 3 wks

Figure 16



Mouse A20 tumors in immune competent mice (dual flank tumors)

Viruses: 5×10^4 pfu of the indicated virus injected 3x into the right tumor only

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.