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(54) Title: RECOMBINANT MYXOMA VIRUSES AND USES THEREOF

(57) Abstract: The present disclosure provides a recombinant oncolytic virus engineered to express a soluble form of an immune checkpoint protein. In certain aspects, the oncolytic virus is a replication competent virus such as myxoma virus. Methods of cancer treatment comprising administering the recombinant oncolytic virus expressing the soluble form of the immune checkpoint protein are also provided.

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DESCRIPTION

RECOMBINANT MYXOMA VIRUSES AND USES THEREOF

[0001] This application claims the benefit of United States Provisional Patent Application Nos. 62/718,990, filed August 16, 2018; 62/741,404, filed October 4, 2018; 62/754,622, filed November 2, 2018; and 62/813,375, filed March 4, 2019, the entirety of each of which are incorporated herein by reference.

[0002] The invention was made with government support under Grant No. 5R01CA194090 awarded by the National Institute of Health. The government has certain rights in the invention.

BACKGROUND

1. Field

[0003] The present disclosure relates generally to the field of molecular biology and medicine. More particularly, it concerns oncolytic viruses expressing an immune checkpoint protein, such as PD1 or TIM3.

2. Description of Related Art

[0004] Current treatments used to treat various types of cancer tend to work by poisoning or killing the cancerous cell. Unfortunately, treatments that are toxic to cancer cells typically tend to be toxic to healthy cells as well. Moreover, the heterogeneous nature of tumors is one of the primary reasons that effective treatments for cancer remain elusive. Current mainstream therapies such as chemotherapy and radiotherapy tend to be used within a narrow therapeutic window of toxicity. These types of therapies have limited applicability due to the varying types of tumor cells and the limited window in which these treatments can be administered. Modern anticancer therapies currently being developed attempt to selectively target tumor cells while being less toxic to healthy cells, thereby being more likely to leave healthy cells unaffected.

[0005] Metastatic melanoma is an aggressive disease with a 16% 5-year survival rate and responds poorly to most standard chemotherapies. Interferon and interleukin 2 (IL-2) have both been approved by the U.S. Food and Drug Administration for the treatment of melanoma. Both mediate their benefit by stimulating an antitumor immune response.

However, toxicity and low response rates have limited their use significantly. The first immune-checkpoint inhibitor approved by the U.S. Food and Drug Administration (FDA) was ipilimumab, a fully human immunoglobulin G1 monoclonal antibody that blocks cytotoxic T-lymphocyte antigen (CTLA)-4 and consequently the PD-1 pathway for the treatment of metastatic melanoma in 2011. The finding that programmed cell death protein 1 ligand 1 (PDL1 or PD-L1) and PDL2 are expressed by melanoma cells, T cells, B cells and natural killer cells led to the development of programmed cell death protein 1 (PD1 or PD-1)-specific antibodies (*e.g.*, nivolumab and pembrolizumab).

[0006] Thus, PD1 pathway blockade has become a major focus in anticancer drug development beyond melanoma. In addition to benefiting patients with renal cell carcinoma, it has reported benefit in patients with tumors previously not considered sensitive to immunotherapies, including non–small cell lung cancer. However, there are still limitations due to toxicity associated with these immunotherapies. Thus, there is a need for an immunotherapy blocking the PD1 pathway with the best balance of high efficacy and low toxicity.

[0007] Immune checkpoint inhibition in cancer therapy has been shown to be effective for the treatment of a number of different types of cancer. However, not all cancers cells respond equally. Additionally, toxicity and the development of resistance to individual checkpoint inhibitors are problematic (Pardoll, 2012; Topalian *et al.*, 2015). Improvements for immune checkpoint inhibitors are needed to combat aforementioned drawbacks.

[0008] Another promising therapeutic approach for cancer therapy is the use of oncolytic viruses. Treatment with oncolytic viruses by themselves and combined with other therapies elicit direct tumor cytotoxicity and potentiate activation of immune cells against tumor cells. Oncolytic viruses possess novelty in that they can also be 'armed' to express proteins to make them more effective (Kaufman *et al.*, 2015).

[0009] Recent work has experimentally shown the efficacy of combining oncolytic virus with immune checkpoint inhibitor by engineering a myxoma virus to express a human extracellular portion of the PD1 checkpoint molecule (Bartee *et al.*, 2017). PD1 is a membrane protein on T-cells that binds to PDL1 on tumor cells. This interaction triggers signaling through PD1 leading to inhibition of activation of T-cells toward tumor cells, thus protecting tumor cells from immune cell elimination (Pardoll, 2012). Upon infection of tumor cells, through

direct injection in the tumor, of the myxoma virus expressing the extracellular PD1 protein (vPD1), the interaction of PDL1 on tumor and PD1 on T-cells in inhibited locally. This occurs by the extracellular PD1 protein directly binding to tumor cell PDL1 blocking T-cell PD1 from binding PDL1, leading to T-cell immune activation and anti-tumor effect.

[0010] Another major inhibitory pathway present in tumor microenvironments is the TIM3 checkpoint in which various TIM3 ligands (including GAL9, phosphatidyl serine, and HMGB1) expressed by tumor cells binds to TIM3 on anti-tumor T or NK cells resulting in immune cell exhaustion. Current methods to overcome this pathway including systemic injection of antibodies which block the TIM3-GAL9 interaction; however, these systemic treatments are costly, time consuming, and associated with low response rates and noticeable toxicities. Thus, there is an unmet need for improved methods of inhibiting immune checkpoints.

SUMMARY

[0011] Certain embodiments of the present disclosure provide methods and compositions comprising a recombinant oncolytic virus comprising one or more expression cassettes encoding (a) a soluble form of programmed cell death protein 1 (PD1 or PD-1), and optionally (b) interleukin 2 (IL-2) or interleukin 12 (IL-12) or interleukin 15 (IL-15), wherein the virus is replication competent. In some aspects, the soluble PD1 comprises an extracellular region of human PD1. In particular aspects, the extracellular region of human PD1 comprises SEQ ID NO: 4. In some aspects, the extracellular region of human PD1 comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 4. In particular aspects, human IL-12 sequence comprises SEQ ID NOs: 7 and 8. In some aspects, the human IL-12 comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 7 and 8. In particular aspects, human IL-2 sequence comprises SEQ ID NO: 6. In some aspects, the human IL-2 comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 6. In certain other aspects, a mutated or mutant version of soluble PD1 is provided to enhance the effects of combination therapies with anti-PD1 antibodies by reducing the inhibitory binding of the anti-PD1 antibody to the soluble PD1. In particular aspects, the mutant human soluble PD1 sequence comprises SEQ ID NO: 5. In some aspects, the extracellular region of human PD1 comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5.

[0012] Certain embodiments of the present disclosure provide methods and compositions comprising a recombinant oncolytic virus comprising an expression cassette encoding a mutated soluble form of PD1, wherein the virus is replication competent. In some aspects, the mutated soluble PD1 comprises an extracellular region of human PD1. In particular aspects, the extracellular region of human PD1 comprises SEQ ID NO: 5. In some aspects, the extracellular region of human PD1 comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5.

[0013] In certain aspects, IL-12 is fused to a transmembrane domain. For example, in some aspects, IL-12 can be fused to a transmembrane domain of CD28, CD8a or CD137. In further aspect, the IL-12 can be fused to a polypeptide that binds to a membrane anchored protein. Transmembrane proteins for fusions with IL-12 and methods for the same are provided, for example, in Cheng *et al.* 2008 (incorporated herein by reference).. In some

specific aspects, IL-12 is fused to a transmembrane domain that is encoded by SEQ ID NO:12. In some aspects, the oncolytic virus is encoded by a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO:13.

[0014] Further embodiments of the present disclosure provide a recombinant oncolytic virus comprising one or more expression cassettes encoding a soluble form of T-cell immunoglobulin and mucin-domain containing-3 (TIM3). In certain aspects, the soluble TIM3 comprises an extracellular region of murine or human TIM3.

[0015] In still a further embodiment there is provided a method of treating a disease in a subject in need thereof comprising (a) testing the subject for overexpression of GAL9; and (b) administering to a subject with increased expression of GAL9 a therapeutically effective amount of the oncolytic virus or a recombinant oncolytic virus comprising one or more expression cassettes encoding a soluble form of TIM3. In certain aspects, the subject has a cancer that exhibits increased GAL9 expression.

[0016] In certain aspects, the one or more expression cassettes may be under the control of a viral promoter. In some aspects, the viral promoter is synthetic early/late poxvirus promoter. For example, in some aspects, the synthetic early/late poxvirus promoter is about 80%, 85%, 90%, 95% or 100% identical to a sequence of SEQ ID NO: 20 (AAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATA).

[0017] In some aspects, the virus is selected from the group consisting of myxoma virus, reovirus, herpes simplex virus, Newcastle Disease virus, measles virus, retrovirus, poxvirus, rhabdovirus, picornavirus, coxsackievirus and parvovirus. In particular aspects, the oncolytic virus is myxoma virus. In some aspects, the soluble form of TIM3 or the extracellular region of PD1, such as mutated PD1, and/or IL-12, or IL-2 expression cassette(s) is/are incorporated into the myxoma genome at the viral *M153R* open reading frame. For example, in some aspects, an extracellular IL-12 expression construct is inserted in place of the viral *M153R*. In further aspects, a PD1 (extracellular domain) expression construct is inserted after viral *M135* or between the viral *M135* and *M136*. In further aspects, expression constructs for both PD1 and IL-12 can be inserted in place of the viral *M153R* or between the viral *M135* and *M136*.

[0018] In a further embodiment, there is provided a pharmaceutical composition of the oncolytic virus provided herein, said pharmaceutical composition comprising a recombinant

oncolytic virus comprising one or more expression cassettes encoding a soluble form of PD1, such as mutated PD1, (*e.g.*, a recombinant oncolytic virus of the above embodiments) and optionally IL-12 or IL-2. In further aspects, the recombinant oncolytic virus comprises one or more expression cassettes encoding a soluble form of TIM3.

[0019] In another embodiment, there is provided a method of treating a disease in a subject in need thereof comprising administering an effective amount of the pharmaceutical composition provided herein, said pharmaceutical composition comprising a recombinant oncolytic virus comprising one or more expression cassettes encoding a soluble form of TIM3 or a soluble form of PD1, such as mutated PD1, and optionally IL-12 or IL-2.

[0020] In some aspects, the disease is cancer, such as a metastatic cancer. In certain aspects, the cancer has increased expression of programmed death-ligand 1 (PDL1). Thus, in some aspects, a subject for treatment according to the embodiments is a subject who has been determined to have a cancer that expresses PDL1. In other aspects, the cancer does not have increased expression of PDL1. For example, the cancer can be a melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer. In particular, the cancer is melanoma. In some aspects, the melanoma is metastatic melanoma. In some aspects, the cancer has metastasized to the lungs.

[0021] In certain aspects, the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally. In some aspects, the oncolytic virus is administered two or more times.

[0022] In some aspects, administration of the oncolytic virus results in the expression of soluble form TIM3 or soluble PD1 (*e.g.* mutated PD1), such as a protein of about 20-40 kDa, such as about 30 kDa. In particular aspects, the expressed, soluble PD1 (*e.g.* mutated PD1) is glycosylated. In certain aspects, expression of the soluble PD1 (*e.g.* mutated PD1) persists long-term after administration, such as for at least 3-5 days, particularly at least 6-14 days after administration. In particular aspects, expression of soluble PD1 is essentially localized to a tumor in the subject being treated.

[0023] In particular aspects, administration of the oncolytic virus does not result in alopecia, or results in at most a minor level of alopecia.

[0024] In some aspects, the method of treatment further comprises administering at least a second anti-cancer therapy to the subject. In some aspects, the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus. For example, the second anti-cancer therapy may be an immunomodulator. In other aspects, the second anti-cancer therapy is selected from chemotherapy, immunotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy and cytokine therapy. In some aspects, the second anti-cancer therapy comprises administration of T cells, such as $CD8^+$ T cells (*e.g.*, $CD25^+/CD69^{hi}CD8^+$ T cells). In some aspects, the immunotherapy is immune checkpoint inhibitor therapy. In specific aspects, the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4. In specific aspects, the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab. In a preferred aspect, the treatment method comprises treatment with an antibody directed to PD1. In a particularly preferred aspect, the treatment comprises treatment with Pembrolizumab.

[0025] In another embodiment, the present disclosure provides a method of treating a disease in a subject in need thereof comprising (a) testing the subject for overexpression of PDL1; and (b) administering to a subject with increased expression of PDL1 a therapeutically effective amount of the oncolytic virus provided herein comprising a recombinant oncolytic virus comprising an expression cassette encoding soluble PD1 (e.g. mutated PD1).

[0026] In some aspects, the disease is cancer, such as a metastatic cancer. In certain aspects, the cancer has increased expression of programmed death-ligand 1 (PDL1). In other aspects, the cancer does not have increased expression of PDL1. For example, the cancer is melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer. In particular, the cancer is melanoma. In some aspects, the melanoma is metastatic melanoma. In some aspects, the cancer has metastasized to the lungs.

[0027] In certain aspects, the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally. In some aspects, the oncolytic virus is administered two or more times.

[0028] In some aspects, administration of the oncolytic virus results in the expression of soluble form of TIM3 or soluble PD1, such as mutant PD1, such as a protein of about 20-40 kDa, such as about 30 kDa. In particular aspects, the expressed, soluble PD1 is glycosylated.

In certain aspects, expression of the soluble TIM3 or soluble PD1 persists long-term after administration, such as for at least 3-5 days, particularly at least 6-14 days after administration. In particular aspects, expression of soluble TIM3 or PD1 is essentially localized to a tumor in the subject being treated. In particular aspects, administration of the oncolytic virus does not result in alopecia, or results in at most a minor level of alopecia.

[0029] In some aspects, the method of treatment further comprises administering at least a second anti-cancer therapy to the subject. In some aspects, the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus. For example, the second anti-cancer therapy may be an immunomodulator. In other aspects, the second anti-cancer therapy is chemotherapy, immunotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy or cytokine therapy. In some aspects, the second anti-cancer therapy comprises administration of T cells, such as $CD8^+$ T cells (*e.g.*, $CD25^+/CD69^{hi}CD8^+$ T cells). 40. In some aspects, the immunotherapy is immune checkpoint inhibitor therapy. In specific aspects, the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4. In specific aspects, the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab.

[0030] As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0031] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

[0032] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0033] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0034] Other objects, features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure. The disclosure may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0036] FIG. 1 – Schematics of recombinant viral genomic structures.

[0037] FIG. 2 - vPD1-IL2 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model.

[0038] FIG. 3 - vPD1-IL12 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model.

[0039] FIG. 4 - vPD1-IL15 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model.

[0040] FIG. 5 - vPD1-IL18 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model.

[0041] FIG. 6 - *In vivo* SC contralateral model. Starting tumor size.

[0042] FIG. 7 - *In vivo* SC contralateral model. Treatment results are shown. The studies demonstrate that vPD1/IL12 constructs were superior to other constructs tested.

[0043] FIG. 8 – Sequence alignment of the C'D loop in ectodomains of PD-1. Secondary structural elements of human PD-1 (hPD-1) are shown on top of the alignment while those of murine PD-1 (mPD-1) are shown at the bottom.

[0044] FIG. 9 - Schematic depicting therapy with soluble TIM3 myxoma virus.

[0045] FIG. 10: MYXV therapy induces TIM3 on CD8+ T cells and NK cells. Analysis of TIM3 expression on the indicated immunological subsets six days after initiation of viral treatment.

[0046] FIGS. 11A-11C: TIM3 blockade improves MYXV treatment of melanoma. SQ B16/F10 tumors were treated as indicated. **(A)** Tumor volume as a percent of starting volume.

Complete eradication of visible tumor is marked with white circles. (**B**) Overall survival of animals. (**C**) Example of alopecia observed in animals.

[0047] FIGS. 12A-12D: vTIM3 secretes soluble TIM3 from infected cells. (**A**) Schematic of the genomic structure of vGFP and vTIM3. (**B**) Production of new virus in B16/F10 cells. (**C**) MTT assay analyzing cellular viability 24 hours post infection. (**D**) Expression of TIM3 transgene.

[0048] FIGS. 13A-13C: vTIM3 duplicates efficacy of combination therapy with reduced toxicities. (**A**) Tumor volume as a percent of starting volume. Complete eradication of visible tumor is marked with white circles. (**B**) Overall survival of animals. (**C**) Average alopecia score observed in animals treated with the indicated therapy.

[0049] FIGS. 14A-14B: Generation of mutations in TIM3. (**A**) Schematic of proposed mutations for TIM3 transgenes. (**B**) Expression of mutated TIM3 out of newly generated recombinant vTIM3 mutant viruses. Note that the GAL9 mutant runs at a lower MW due to the loss of glycosylation.

[0050] FIGS. 15A-15C: (A) Schematic depicting mouse study. **(B)** Individual tumor growth over time. **(C)** Overall survival.

[0051] FIGS. 16A-B: vPD1 is effective against localized but not metastatic tumors. Single (A) or contralateral (B) B16/F10 tumors were established in syngeneic mice. Tumors on the left flank were then treated with either control virus (vGFP) or vPD1. Tumors on the right flank in contralateral model were left untreated. Responsiveness of individual tumors and overall survival were then monitored.

[0052] FIGS. 17A-17D: MYXV expressing both soluble PD1 and IL12 is highly effective against metastatic disease. (A) Genomic structure of viruses expressing both soluble PD1 and proinflammatory cytokines. Contralateral LLC tumors were established in syngeneic mice. Tumors on the left flank were then treated as indicated and tumors on the right flank were left untreated. (B) Responsiveness of individual tumors and (C) overall survival were then monitored. (D) Picture of mouse bearing bulky, contralateral LLC tumors treated as above.

[0053] FIGS. 18A-18C: vPD1/IL12 is effective against metastatic lung cancer. (A) Contralateral LLC tumors were established in syngeneic mice. Tumors on the left flank were

then treated as indicated and tumors on the right flank were left untreated. (B) Responsiveness of individual tumors and (C) overall survival were then monitored.

[0054] FIGS. 19A-19C: vPD1/IL12 is effective against metastatic melanoma. (A) Contralateral B16/F10 tumors were established in syngeneic mice. Tumors on the left flank were then treated as indicated and tumors on the right flank were left untreated. (B) Responsiveness of individual tumors and (C) overall survival were then monitored.

[0055] FIGS. 20A-20C: vPD1/IL12 is effective against spontaneously metastatic breast cancer. (A) Single 4T1 tumors were established in syngeneic mice and allowed to establish and metastasize. Primary tumors were then treated as indicated. (B) Responsiveness of individual tumors and (C) overall survival were then monitored.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0056] Two of the major inhibitory pathways present in tumor microenvironments are the PD1-PDL1 checkpoint in which PDL1 expressed on tumor cells binds to PD1 on anti-tumor T cells resulting in T cell exhaustion as well as the TIM3 checkpoint. Current methods to overcome these pathways include systemic injection of antibodies which block the PD1-PDL1 or TIM3-GAL9 interaction; however, these systemic treatments are costly, time consuming and associated with low response rates and noticeable toxicities.

[0057] Certain embodiments of the present disclosure provide compositions and methods for targeting the PD1-PDL1 or TIM3 pathway in cancer. In some aspects, a recombinant oncolytic virus is provided, which has been engineered to express the extracellular portion of the human PD1 protein or TIM3 protein along with IL-2 or IL-12. In certain aspects, the oncolytic virus is a replication competent virus such as myxoma virus. In particular, the extracellular region of PD1 or TIM3 and IL-2 or IL-12 can be encoded by one or more expression cassettes that is integrated into a region of the viral genome that is not necessary for replication. In the present studies, the oncolytic virus provided tumor inhibition that can significantly improve outcomes during oncolytic virotherapy.

[0058] Accordingly, further embodiments of the present disclosure provide methods of cancer treatment comprising administering the recombinant oncolytic virus expressing the soluble form of PD1 or TIM3 and IL-2 or IL-12 are also provided. Thus, the present aspects of the disclosure provide methods and compositions for a therapy targeting the PD1-PDL1 or TIM3-GAL9 pathway, in combination with cytokine therapy, with a low toxicity and high response rate.

I. Definitions

[0059] The term "oncolytic virus," as used herein, refers to a virus capable of selectively replicating in and slowing the growth or inducing the death of a cancerous or hyperproliferative cell, either *in vitro* or *in vivo*, while having no or minimal effect on normal cells. Exemplary oncolytic viruses include vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), herpes simplex virus (HSV), reovirus, measles virus, retrovirus, influenza virus, Sinbis virus, vaccinia virus, and adenovirus.

[0060] A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription of a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

[0061] The term "innate immunity" or "innate immune response" refers to the repertoire of host defenses, both immunological and nonimmunological, that exist prior to or independent of exposure to specific environmental antigens, such as a microorganism or macromolecule, etc. For example, the first host immune response to an antigen involves the innate immune system.

[0062] The term "immunogen" or "antigen," as used herein, refers to an agent that is recognized by the immune system when introduced into a subject and is capable of eliciting an immune response. In certain embodiments, the immune response generated is an innate cellular immune response and the recombinant oncolytic viruses of the instant disclosure are capable of suppressing or reducing the innate cellular immune response.

[0063] As employed herein, the phrase "an effective amount," refers to a dose sufficient to provide concentrations high enough to impart a beneficial effect on the recipient thereof. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated, the severity of the disorder, the activity of the specific compound, the route of administration, the rate of clearance of the compound, the duration of treatment, the drugs used in combination or coincident with the compound, the age, body weight, sex, diet, and general health of the subject, and like factors well known in the medical arts and sciences.

[0064] As used herein the term "multiplicity of infection" (MOI) means the number of infectious virus particles added per cell.

II. Oncolytic viruses

A. Oncolytic Viral Platforms

[0065] In one aspect, the present disclosure generally pertains to recombinant, replication competent, oncolytic viruses. In one embodiment, there is provided a recombinant oncolytic virus having a heterologous nucleic acid sequence encoding PD1 or TIM3. Oncolytic viruses that can be administered according to the methods of the disclosure include, without limitation, adenoviruses (*e.g.* Delta-24, Delta-24-RGD, ICOVIR-5, ICOVIR-7, Onyx-015, ColoAd1, H101, AD5/3-D24-GMCSF), reoviruses, herpes simplex virus (HSV; OncoVEX GMCSF), Newcastle Disease virus, measles viruses, retroviruses (*e.g.* influenza viruses), poxviruses (*e.g.* vaccinia virus including Copenhagen, Western Reserve, Wyeth strains), myxoma viruses, rhabdoviruses (*e..g* vesicular stomatitis virus (VSV)), picornaviruses (*e.g.* Seneca Valley virus; SVV-001), coxsackievirus and parvovirus.

[0066] In one embodiment, the recombinant oncolytic virus comprises myxoma virus. Myxoma virus (MYVX) is a member of the Poxviridae family and prototype for the genus Leporipoxvirus. It is pathogenic only for European rabbits (Oryctolagus cuniculus), in which it causes a lethal disease called myxomatosis, and for two North American species, Sylvilagus audubonni and Sylvilagus nuttalli, in which it causes a less severe disease. Myxoma virus replicates exclusively in the cytoplasm of the host cell, and its genome encodes 171 open reading frames (Smallwood *et al.*, 2010). A number of these genes encode proteins that can interfere with or modulate host defense mechanisms, and several show promise in a clinical setting.

[0067] Like other members of the poxvirus family, the myxoma virus genome consists of a single double stranded DNA (dsDNA), the central part of the which encodes approximately 100 essential genes that are conserved among the members of poxvirus genera. The rest of the genes, including two copies each of the 12 genes that map within the terminal inverted repeats, encode proteins that interfere with and modulate host defense mechanisms. A number of these proteins share a sequence similarity with host cellular genes, suggesting a coevolutionary path (Johnston and McFadden, 2003). Some, called viroceptors, are secreted and able to bind specific ligands such as TNF, for example. Others, known as virokines, are also secreted, and imitate host immune inhibitors, while viromitigators function as host range factors that inhibit apoptosis (Johnston and McFadden, 2003; Kerr and McFadden, 2002). These characteristics give myxoma virus possible utility in a number of therapeutic settings. One of the myxoma

virus-encoded immunomodulatory proteins, Serp-1, is in clinical trials for acute unstable coronary syndromes (*e.g.*, unstable angina and small heart attacks). The M-T7 protein of myxoma virus, a secreted glycoprotein that inhibits rabbit gamma interferon, has also been shown to inhibit inflammatory responses in rabbit models of balloon angioplasty injury to arteries (Liu *et al.*, 2000), and it is likely that a variety of other immunomodulatory proteins can be developed as anti-inflammatory or anti-immune therapeutics.

[0068] Myxoma virus has been shown to productively infect a variety of human cancer cell lines originated from a diverse group of tissues (Sypula *et al.*, 2004), and therefore has the potential for development as an oncolytic virus useful in treatment against a variety of cancers. Wildtype myxoma virus can selectively infect and kill cells, including human cells, which have a deficient innate anti-viral response, for example, cells that are non-responsive to interferon, as described in the application PCT/CA2004/000341, which is herein fully incorporated by reference. Furthermore, myxoma virus is adept at evading and interfering with the host immune response and might serve as a source of immunomodulatory proteins that can be used as therapeutic agents in a variety of clinical settings (Lucas and McFadden, 2004). Additionally, although myxoma virus is not infectious in humans, it is able to productively infect a number of human cancer cell lines, but not normal human cells, and has also been shown to increase survival time in mouse models of human glioma. These characteristics suggest that myxoma virus could prove to be a viable therapeutic agent in a variety of clinical settings, including as an anti-inflammatory or anti-immune therapy, or as an oncolytic agent.

[0069] Myxoma virus has established oncolytic potential against a variety of malignancies including myeloma, melanoma, glioblastoma, pancreatic cancer, and others. The virus is thought to exhibit anti-tumor effects through two distinct mechanisms. First, the virus directly infects and kills tumor cells. Second, viral infection of tumor cells induces a secondary anti-tumor immune response. While the combination of these mechanisms is effective at debulking primary tumors, it often fails to produce long-term cures due to immune inhibition within the tumor microenvironment.

[0070] The myxoma virus of the present disclosure can be attenuated to enhance anti-tumor activity. For example, the myxoma virus can be genetically modified to inactivate one or more genes. In particular, myxoma virus that does not express functional M135R is useful for treatment of cells having a deficient innate anti-viral response, including for oncolytic studies, since this virus provides a safer alternative for oncolytic viral therapy as no unusual

containment strategies should be needed for patients undergoing treatment (U.S. Patent Application No. 20090035276, incorporated herein by reference). In certain aspects, the myxoma virus is an attenuated strain of myxoma virus such as the SG33 strain (U.S. Patent No. 8613915, incorporated herein by reference). An attenuated myxoma virus which can be used in accordance with the disclosure may be obtained from a virulent wild-type myxoma virus, especially by deletion of one or more of the genes M151R, M152R, M153R, M154L, M156R, and M001R, and preferably by the additional deletion of one or more of the genes M008.1R, M008R, M007R, M006R, M005R, M004.1R, M004R, M003.2R, M003.1R, and M002R.

[0071] Myxoma virus can be propagated in a number of cell lines, including adherent cells and suspension cultures, and minimal purification is required. For example, myxoma virus can grow in several cell lines, including RK13 (rabbit kidney epithelial), BHK-21 (baby hamster kidney), BGMK (Buffalo green monkey kidney), Vero (African green monkey kidney epithelial), BSC-40 (African green monkey kidney), and CV-1 (African green monkey kidney fibroblast) cells. Minimal purification is needed to provide a stock that is appropriate for both *in vitro* and *in vivo* work. Protocols for propagating, purifying, and quantifying stocks of myxoma virus are known in the art (Smallwood *et al.*, 2010, incorporated herein by reference).

B. Recombinant Oncolytic Viruses

[0072] The recombinant virus can be constructed by procedures known in the art to generate recombinant viruses. An expression cassette encoding PD1, such as mutant PD1, or TIM3 is inserted into the genome of an oncolytic virus at a region nonessential for viral replication. For example, the expression cassette can be integrated in myxoma virus at an intergenic region, such as between the M135 and M136 open reading frames. The recombinant virus can comprise an expression cassette comprising a nucleotide sequence which is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the nucleotide sequence (*e.g.*, to the entire length of the nucleotide sequence) of the extracellular portion of human PD1, which is shown in SEQ ID NO:3. The nucleotide sequence of SEQ ID NO:3 can be optimized for expression in the recombinant virus, for example, through codon optimization. The expression cassette can encode for soluble TIM3 (SEQ ID NO:11) or sequence with at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID ID NO:11).

[0073] Homologous recombination (HR), also known as general recombination, is a type of genetic recombination used in all forms of life in which nucleotide sequences are exchanged between two similar or identical strands of DNA. The technique has been the standard method for genome engineering in mammalian cells since the mid-1980s. The process involves several steps of physical breaking and the eventual rejoining of DNA. This process is most widely used to repair potentially lethal double-strand breaks in DNA. In addition, homologous recombination produces new combinations of DNA sequences during meiosis, the process by which eukaryotes make germ cells like sperm and ova. These new combinations of DNA represent genetic variation in offspring which allow populations to evolutionarily adapt to changing environmental conditions over time. Homologous recombination is also used in horizontal gene transfer to exchange genetic material between different strains and species of bacteria and viruses. Homologous recombination is also used as a technique in molecular biology for introducing genetic changes into target organisms.

[0074] Expression cassettes included in vectors useful in the disclosure preferably contain (in a 5'-to-3' direction) a eukaryotic transcriptional promoter operably linked to a protein-coding sequence. Non-limiting examples of promoters include early or late viral promoters, such as, SV40 early or late promoters, cytomegalovirus (CMV) immediate early promoters, Rous Sarcoma Virus (RSV) early promoters; eukaryotic cell promoters, such as, *e.g.*, beta actin promoter (Ng, 1989; Quitsche *et al.*, 1989), GADPH promoter (Alexander *et al.*, 1988, Ercolani *et al.*, 1988), metallothionein promoter (Karin *et al.*, 1989; Richards *et al.*, 1984); and concatenated response element promoters, such as cyclic AMP response element promoters (cre), serum response element promoter (sre), phorbol ester promoter (TPA) and response element promoters (tre) near a minimal TATA box. It is also possible to use human growth hormone promoter sequences (*e.g.*, the human growth hormone minimal promoter described at Genbank, accession no. X05244, nucleotide 283-341) or a mouse mammary tumor promoter (available from the ATCC, Cat. No. ATCC 45007). A specific example could be a synthetic early/late (sE/L) poxvirus promoter (see, e.g., the promoter of the construct to SEQ ID NO: 10).

[0075] The expression cassette is introduced to cells which are then infected with the unmodified oncolytic virus to produce the recombinant virus. Introduction of the expression cassette into cells may use any suitable methods for nucleic acid delivery for transformation of a cell, as described herein or as would be known to one of ordinary skill in the art. Such

methods include, but are not limited to, direct delivery of DNA such as by ex vivo transfection (Wilson *et al.*, 1989, Nabel *et al*, 1989), by injection (U.S. Patent Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, 1985; U.S. Patent No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent No. 5,384,253, incorporated herein by reference; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patent Nos. 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler *et al.*, 1990; U.S. Patent Nos. 5,302,523 and 5,464,765, each incorporated herein by reference); by *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,591,616 and 5,563,055, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), and any combination of such methods. Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

[0076] The recombinant virus is then purified from the cells such as by a selectable marker. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selection marker is one that confers a property that allows for selection. A positive selection marker is one in which the presence of the marker allows for its selection, while a negative selection marker is one in which its presence prevents its selection. An example of a positive selection marker is a drug resistance marker. Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selection markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes as negative selection markers such as herpes simplex virus

thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. For example, the recombinant oncolytic virus can be untagged or express fluorescent proteins such as green fluorescent protein (GFP), red fluorescent protein (RFP), tomato Red (tdRed), or other fluorescent proteins. Further examples of selection and screenable markers are well known to one of skill in the art.

[0077] The transgene expressing tomato Red fluorescent (tdTr), which serves as a fluorescent marker for myxoma replication *in vitro* and *in vivo*, has been described in Liu *et al.* (2009) *J. Virology* 83:5933-5938. Liu observed that a myxovirus expressing IL-15 fused to tdTr (vMyx-IL-15-tdTr) was significantly attenuated and failed to induce lethal myxomatosis in rabbits. The construct secreted IL-15 and supported normal virus replication. Thus, Liu concluded that vMyx-IL-15-tdTr was a safe candidate for *in vivo* animal studies of oncolytic virotherapy, and tdTr is a suitable marker for use in recombinant myxovirus.

[0078] If desired, one or more genetic elements, such as transgenes expressing fluorescent markers, can be excised from a viral transposon, using methods known in the art, such as Flp recombinase or Cre-lox recombination-based systems.

C. PD1

[0079] Programmed cell death protein 1, also known as PD-1 and CD279 (cluster of differentiation 279), is a protein found on the surface of cells that has a role in regulating the immune system's response to the cells of the human body by down-regulating the immune system and promoting self-tolerance by suppressing T cell inflammatory activity. This prevents autoimmune diseases, but it can also prevent the immune system from killing cancer cells.

[0080] The amino acid sequence of the extracellular domain of human PD-1 is found at Uniprot Accession Number Q15116, SEQ ID NO: 4, and is 168 amino acids in length, which includes a 20 amino acid signal sequence which may be replaced by a different signal sequence, or omitted from the PD-1 sequences of the present disclosure, when not needed in order to direct secretion.

[0081] PD-1 is an immune checkpoint and guards against autoimmunity through two mechanisms. First, it promotes apoptosis (programmed cell death) of antigen-specific T-cells

in lymph nodes. Second, it reduces apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells). PD-1 inhibitors, a new class of drugs that block PD-1, activate the immune system to attack tumors and are used to treat certain types of cancer.

[0082] The PD-1 protein in humans is encoded by the *PDCD1* gene. PD-1 is a cell surface receptor that belongs to the immunoglobulin superfamily and is expressed on T cells and pro-B cells. PD-1 binds two ligands, PD-L1 and PD-L2. PD-1 is a type I membrane protein of 268 amino acids. PD-1 is a member of the extended CD28/CTLA-4 family of T cell regulators. The protein's structure includes an extracellular IgV domain followed by a transmembrane region and an intracellular tail. The intracellular tail contains two phosphorylation sites located in an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif, which suggests that PD-1 negatively regulates T-cell receptor TCR signals. This is consistent with binding of SHP-1 and SHP-2 phosphatases to the cytoplasmic tail of PD-1 upon ligand binding. In addition, PD-1 ligation up-regulates E3-ubiquitin ligases CBL-b and c-CBL that trigger T cell receptor down-modulation. PD-1 is expressed on the surface of activated T cells, B cells, and macrophages, suggesting that compared to CTLA-4, PD-1 more broadly negatively regulates immune responses.

[0083] PD-1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family. PD-L1 protein is upregulated on macrophages and dendritic cells (DC) in response to LPS and GM-CSF treatment, and on T cells and B cells upon TCR and B cell receptor signaling, whereas in resting mice, PD-L1 mRNA can be detected in the heart, lung, thymus, spleen, and kidney. PD-L1 is expressed on almost all murine tumor cell lines, including PA1 myeloma, P815 mastocytoma, and B16 melanoma upon treatment with IFN-γ. PD-L2 expression is more restricted and is expressed mainly by DCs and a few tumor lines.

[0084] Several lines of evidence suggest that PD-1 and its ligands negatively regulate immune responses. PD-1 knockout mice have been shown to develop lupus-like glomerulonephritis and dilated cardiomyopathy on the C57BL/6 and BALB/c backgrounds, respectively. *In vitro*, treatment of anti-CD3 stimulated T cells with PD-L1-Ig results in reduced T cell proliferation and IFN-γ secretion. IFN-γ is a key pro-inflammatory cytokine that promotes T cell inflammatory activity. Reduced T cell proliferation was also correlated with attenuated IL-2 secretion and together, these data suggest that PD-1 negatively regulates T cell responses.

[0085] Experiments using PD-L1 transfected DCs and PD-1 expressing transgenic (Tg) $CD4^+$ and $CD8^+$ T cells suggest that $CD8^+$ T cells are more susceptible to inhibition by PD-L1, although this could be dependent on the strength of TCR signaling. Consistent with a role in negatively regulating $CD8^+$ T cell responses, using an LCMV viral vector model of chronic infection, Rafi Ahmed's group showed that the PD-1-PD-L1 interaction inhibits activation, expansion and acquisition of effector functions of virus specific $CD8^+$ T cells, which can be reversed by blocking the PD-1-PD-L1 interaction.

[0086] Expression of PD-L1 on tumor cells inhibits anti-tumor activity through engagement of PD-1 on effector T cells. Expression of PD-L1 on tumors is correlated with reduced survival in esophageal, pancreatic and other types of cancers, highlighting this pathway as a target for immunotherapy. Triggering PD-1, expressed on monocytes and up-regulated upon monocytes activation, by its ligand PD-L1 induces IL-10 production which inhibits CD4 T-cell function.

[0087] In mice, expression of this gene is induced in the thymus when anti-CD3 antibodies are injected and large numbers of thymocytes undergo apoptosis. Mice deficient for this gene bred on a BALB/c background developed dilated cardiomyopathy and died from congestive heart failure. These studies suggest that this gene product may also be important in T cell function and contribute to the prevention of autoimmune diseases. Overexpression of PD1 on CD8+ T cells is one of the indicators of T-cell exhaustion (*e.g.*, in chronic infection or cancer).

[0088] PD-L1, the primary ligand for PD1, is highly expressed in several cancers and hence the role of PD1 in cancer immune evasion is well established. Monoclonal antibodies targeting PD-1 that boost the immune system are being developed for the treatment of cancer. Many tumor cells express PD-L1, an immunosuppressive PD-1 ligand; inhibition of the interaction between PD-1 and PD-L1 can enhance T-cell responses *in vitro* and mediate preclinical antitumor activity. This is known as immune checkpoint blockade.

[0089] Combination therapy using both anti-PD1 along with anti-CTLA4 therapeutics have emerged as important tumor treatments within the field of checkpoint inhibition. A combination of PD1 and CTLA4 antibodies has been shown to be more effective than either antibody alone in the treatment of a variety of cancers. The effects of the two antibodies do not appear to be redundant. Anti-CTLA4 treatment leads to an enhanced antigen specific T

cell dependent immune reaction while anti-PD-1 appears to reactivate CD8+ T cells ability to lyse cancer cells.

[0090] In clinical trials, combination therapy has been shown to be effective in reducing tumor size in patients that are unresponsive to single co-inhibitory blockade, despite increasing levels of toxicity due to anti-CTLA4 treatment. A combination of PD1 and CTLA4 induced up to a ten-fold higher number of CD8+ T cells that are actively infiltrating the tumor tissue. The authors hypothesized that the higher levels of CD8+ T cell infiltration was due to anti-CTLA-4 inhibited the conversion of CD4 T cells to T regulator cells and further reduced T regulatory suppression with anti-PD-1. This combination promoted a more robust inflammatory response to the tumor that reduced the size of the cancer. Most recently, the FDA has approved a combination therapy with both anti-CTLA4 (ipilimumab) and anti-PD1 (nivolumab) in October 2015.

[0091] The molecular factors and receptors necessary making a tumor receptive to anti-PD1 treatment remains unknown. PD-L1 expression on the surface on cancer cells plays a significant role. PD-L1 positive tumors were twice as likely to respond to combination treatment. However patients with PD-L1 negative tumors also have limited response to anti-PD1, demonstrating that PD-L1 expression is not an absolute determinant of the effectiveness of therapy.

[0092] Higher mutational burden in the tumor is correlated with a greater effect of the anti-PD1 treatment. In clinical trials, patients who benefited from anti-PD1 treatment had cancers, such as melanoma, bladder cancer, and gastric cancer, that had a median higher average number of mutations than the patients who do did not respond to the therapy. However, the correlation between higher tumor burden and the clinical effectiveness of PD-1 immune blockade is still uncertain.

D. IL-12

[0093] Interleukin 12 (IL-12) is an interleukin that is naturally produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation. IL-12 is composed of a bundle of four alpha helices. It is a heterodimeric cytokine encoded by two separate genes, IL-12A (p35) and IL-12B (p40). The active heterodimer (referred to as 'p70'), and a homodimer of p40 are formed following protein synthesis. The amino acid sequence of human IL-12 alpha subunit is found at Uniprot

Accession Number P29459, SEQ ID NO: 7, and is 219 amino acids in length, which includes a 22 amino acid signal sequence which may be replaced by a different signal sequence, or omitted from the IL-12 alpha subunit sequences of the present invention, when not needed in order to direct secretion. The amino acid sequence of human IL-12 beta subunit is found at Uniprot Accession Number P29460, SEQ ID NO: 8, and is 328 amino acids in length, which includes a 22 amino acid signal sequence, which may be replaced by a different signal sequence, or omitted from the IL-12 beta subunit sequences of the present invention, when not needed in order to direct secretion. The nucleotide sequences encoding IL-12 alpha and beta subunits can be optimized for expression in the recombinant virus, for example, through codon optimization.

[0094] In certain embodiments, the IL-12 alpha subunit and IL-12 beta subunit may be expressed as a fusion protein from a single DNA construct. In such cases, only a single signal peptide is required, preferably at the N-terminal end of the expressed fusion protein. In such cases, a flexible linker peptide may be used to join the IL-12 alpha subunit and IL-12 beta subunits. Suitable linker peptide sequences are known in the art, and include, for example $(GGGS)_n$, where n = 1 to 4.

[0095] IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T cells. It stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ. T cells that produce IL-12 have a coreceptor, CD30, which is associated with IL-12 activity.

[0096] IL-12 plays an important role in the activities of natural killer cells and T lymphocytes. IL-12 mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes. There also seems to be a link between IL-2 and the signal transduction of IL-12 in NK cells. IL-2 stimulates the expression of two IL-12 receptors, IL-12R-β1 and IL-12R-β2, maintaining the expression of a critical protein involved in IL-12 signaling in NK cells. Enhanced functional response is demonstrated by IFN-γ production and killing of target cells.

[0097] IL-12 also has anti-angiogenic activity, which means it can block the formation of new blood vessels. It does this by increasing production of interferon gamma, which in turn

increases the production of a chemokine called inducible protein-10 (IP-10 or CXCL10). IP-10 then mediates this anti-angiogenic effect. Because of its ability to induce immune responses and its anti-angiogenic activity, there has been an interest in testing IL-12 as a possible anti-cancer drug. However, it has not been shown to have substantial activity in the tumors tested to this date. There is a link that may be useful in treatment between IL-12 and the diseases psoriasis and inflammatory bowel disease.

[0098] IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by IL-12Rβ1 and IL-12Rβ2. IL-12Rβ2 is considered to play a key role in IL-12 function, since it is found on activated T cells and is stimulated by cytokines that promote Th1 cells development and inhibited by those that promote Th2 cells development. Upon binding, IL-12R-β2 becomes tyrosine phosphorylated and provides binding sites for kinases, Tyk2 and Jak2. These kinases are important in activating critical transcription factor proteins such as STAT4 that are implicated in IL-12 signaling in T cells and NK cells. This pathway is known as the JAK-STAT pathway.

[0099] IL-12 is linked with autoimmunity. Administration of IL-12 to people suffering from autoimmune diseases was shown to worsen the autoimmune phenomena. This is believed to be due to its key role in induction of Th1 immune responses. In contrast, IL-12 gene knock-out in mice or a treatment of mice with IL-12 specific antibodies ameliorated the disease.

[00100] Interleukin 12 (IL-12) is produced by activated antigen-presenting cells (dendritic cells, macrophages). It promotes the development of Th1 responses and is a powerful inducer of IFNγ production by T and NK cells.

[00101] A child with Bacillus Calmette–Guérin and *Salmonella enteritidis* infection was found to have a large homozygous deletion within the IL-12 p40 subunit gene, precluding expression of functional IL-12 p70 cytokine by activated dendritic cells and phagocytes. As a result, IFNγ production by the child's lymphocytes was markedly impaired. This suggested that IL-12 is essential for protective immunity to intracellular bacteria such as mycobacteria and *Salmonella*.

[00102] Support is lent to this idea by the observation that a receptor for IL-12 is important for IFNγ production by lymphocytes. T and NK cells from seven unrelated patients who had severe idiopathic mycobacterial and Salmonella infections failed to produce IFNγ when stimulated with IL-12. The patients were otherwise healthy. They were found to have

mutations in the IL-12 receptor β1 chain, resulting in premature stop codons in the extracellular domain, resulting in unresponsiveness to this cytokine, again demonstrating IL-12's crucial role in host defense.

[00103] Defective Th1 and Th17 immune responses leading to chronic mucocutaneous candidiasis result from a mutation further downstream in the IL-12 signaling pathway. The trait was mapped to mutations in the STAT1 gene, which were associated with lower production of interferon-γ, IL-17, and IL-22 in response to IL-12 or IL-23 receptor associated Jak2 and Tyk2 activity.

E. IL-2

[00104] Interleukin-2 (IL-2) is an interleukin, a type of cytokine signaling molecule in the immune system. It is a protein that regulates the activities of white blood cells (leukocytes, often lymphocytes) that are responsible for immunity. IL-2 is part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self". IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes. The amino acid sequence of human IL-2 is found at Uniprot Accession Number P60568, SEQ ID NO: 6, and is 153 amino acids in length, which includes a 20 amino acid signal sequence, which may be replaced by a different signal sequence, or omitted from the IL-2 sequences of the present invention, when not needed in order to direct secretion. The nucleotide sequence encoding IL-2 can be optimized for expression in the recombinant virus, for example, through codon optimization.

[00105] In a preferred embodiment, the IL-2 useful in the present invention is the high affinity variant IL-2 amino acid sequence of SEQ ID NO: 9, which includes a 20 amino acid signal sequence, which may be replaced by a different signal sequence, and which also contains C-terminal His tag. Levin et al. (2012) *Nature* 484:529-533. Either or both of the signal sequence and His tag may be omitted, if not required for function of the IL-2.

[00106] IL-2 is a member of a cytokine family, each member of which has a four alpha helix bundle; the family also includes IL-4, IL-7, IL-9, IL-15 and IL-21. IL-2 signals through the IL-2 receptor, a complex consisting of three chains, termed alpha, beta and gamma. The gamma chain is shared by all family members.

[00107] The IL-2 Receptor (IL-2R) α subunit has low affinity for its ligand but has the ability (when bound to the β and ϒ subunit) to increase the IL-2R affinity 100-fold. Heterodimerization of the β and ϒ subunits of IL-2R is essential for signaling in T cells.

[00108] Gene expression regulation for IL-2 can be on multiple levels or by different ways. One of the checkpoints is signaling through TCR receptor, antigen receptor of T-lymphocytes after recognizing MHC-peptide complex. Signaling pathway from TCR then goes through phospholipase-C (PLC) dependent pathway. PLC activates 3 major transcription factors and their pathways: NFAT, NFkB and AP-1. After costimulation from CD28 the optimal activation of expression of IL-2 and these pathways is induced.

[00109] At the same time Oct-1 is expressed. It helps the activation. Oct1 is expressed in T-lymphocytes and Oct2 is induced after cell activation. NFAT has multiple family members, all of them are located in cytoplasm and signaling goes through calcineurin, NFAT is dephosphorylated and therefore translocated to the nucleus. AP-1 is a dimer and is composed of c-Jun and c-Fos proteins. It cooperates with other transcription factors including NFkB and Oct. NFkB is translocated to the nucleus after costimulation through CD28. NFkB is a heterodimer and there are two binding sites on the IL-2 promoter.

[00110] IL-2 has essential roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells. In the thymus, where T cells mature, it prevents autoimmune diseases by promoting the differentiation of certain immature T cells into regulatory T cells, which suppress other T cells that are otherwise primed to attack normal healthy cells in the body. IL-2 also promotes the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections. Its expression and secretion is tightly regulated and functions as part of both transient positive and negative feedback loops in mounting and dampening immune responses. Through its role in the development of T cell immunologic memory, which depends upon the expansion of the number and function of antigen-selected T cell clones, it plays a key role in enduring cell-mediated immunity.

[00111] Aldesleukin is a form of recombinant interleukin-2. It is manufactured using recombinant DNA technology and is marketed as a protein therapeutic and branded as Proleukin. It has been approved by the Food and Drug Administration (FDA) and in several

European countries for the treatment of cancers (malignant melanoma, renal cell cancer) in large intermittent doses and has been extensively used in continuous doses.

[00112] Interking is a recombinant IL-2 with a serine at residue 125, sold by Shenzhen Neptunus.

[00113] Various dosages of IL-2 across the United States and across the world are used. The efficiency and side effects of different dosages is often a point of disagreement. Usually, in the U.S., the higher dosage option is used, affected by cancer type, response to treatment and general patient health. Patients are typically treated for five consecutive days, three times a day, for fifteen minutes. The following approximately 10 days help the patient to recover between treatments. IL-2 is delivered intravenously on an inpatient basis to enable proper monitoring of side effects.

[00114] A lower dose regimen involves injection of IL-2 under the skin typically on an outpatient basis. It may alternatively be given on an inpatient basis over 1–3 days, similar to and often including the delivery of chemotherapy. Intralesional IL-2 is commonly used to treat in-transit melanoma metastases and has a high complete response rate and is generally well-tolerated.

[00115] IL-2 has a narrow therapeutic window, and the level of dosing usually determines the severity of the side effects. Some common side effects include flu-like symptoms (fever, headache, muscle and joint pain, fatigue), nausea/vomiting, dry, itchy skin or rash, weakness or shortness of breath, diarrhea, low blood pressure, drowsiness or confusion, and loss of appetite. More serious and dangerous side effects sometimes are seen, such as capillary leak syndrome, breathing problems, serious infections, seizures, allergic reactions, heart problems or a variety of other possible complications.

III. Therapeutic Administration

[00116] In another aspect, the present disclosure provides methods of inhibiting the growth or promoting the killing of a tumor cell or treating cancer, such as melanoma, by administering a recombinant oncolytic virus according to the instant disclosure at a multiplicity of infection sufficient to inhibit the growth of a tumor cell or to kill a tumor cell. In certain embodiments, the recombinant oncolytic virus is administered more than once, preferably twice, three times, or up to 10 times.

[00117] Examples of tumor cells or cancers that may be treated using the methods of this disclosure include breast cancer, ovarian cancer, renal cell carcinoma (RCC), melanoma (*e.g.*, metastatic malignant melanoma), prostate cancer, colon cancer, lung cancer (including small cell lung cancer and non-small cell lung cancer), bone cancer, osteosarcoma, rhabdomyosarcoma, leiomyosarcoma, chondrosarcoma, pancreatic cancer, skin cancer, fibrosarcoma, chronic or acute leukemias including acute lymphocytic leukemia (ALL), adult T-cell leukemia (T-ALL), acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphangiosarcoma, lymphomas (*e.g.*, Hodgkin's and non-Hodgkin's lymphoma, lymphocytic lymphoma, primary CNS lymphoma, T-cell lymphoma, Burkitt's lymphoma, anaplastic large-cell lymphomas (ALCL), cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), entroblastic/centrocytic (cb/cc) follicular lymphomas cancers, diffuse large cell lymphomas of B lineage, angioimmunoblastic lymphadenopathy (AILD)-like T cell lymphoma and HIV associated body cavity based lymphomas), Castleman's disease, Kaposi's Sarcoma, hemangiosarcoma, multiple myeloma, Waldenstrom's macroglobulinemia and other B-cell lymphomas, nasopharangeal carcinomas, head or neck cancer, myxosarcoma, liposarcoma, cutaneous or intraocular malignant melanoma, uterine cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, cervical carcinoma, vaginal carcinoma, vulvar carcinoma, transitional cell carcinoma, esophageal cancer, malignant gastrinoma, small intestine cancer, cholangiocellular carcinoma, adenocarcinoma, endocrine system cancer, thyroid gland cancer, parathyroid gland cancer, adrenal gland cancer, sarcoma of soft tissue, urethral, penile cancer, testicular cancer, malignant teratoma, solid tumors of childhood, bladder cancer, kidney or ureter cancer, carcinoma of the renal pelvis, malignant meningioma, neoplasm of the central nervous system (CNS), tumor angiogenesis, spinal axis tumor, pituitary adenoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, *e.g.*, mesothelioma, and combinations of these cancers. Many cancers overexpress immune checkpoint proteins, such as PDL1 ($PDL1^+$). The methods of this disclosure may be used to treat tumors or cancers regardless of PDL1 status.

[00118] Oncolytic viruses according to the disclosure may be administered locally or systemically. For example, without limitation, oncolytic viruses according to the disclosure can be administered intravascularly (intraarterially or intravenously), intratumorally,

intramuscularly, intradermally, intraperitoneally, subcutaneously, orally, parenterally, intranasally, intratracheally, percutaneously, intraspinally, ocularly, or intracranially.

[00119] In still another embodiment, the methods involve parenteral administration of a recombinant oncolytic virus, preferably via an artery or via an in-dwelling medical device. The recombinant oncolytic virus can be administered with an immunotherapeutic agent or immunomodulator, such as an antibody that binds to a tumor-specific antigen (*e.g.*, chimeric, humanized or human monoclonal antibodies). In another embodiment, the recombinant oncolytic virus treatment may be combined with surgery (*e.g.*, tumor excision), radiation therapy, chemotherapy, or immunotherapy, and can be administered before, during or after a complementary treatment.

[00120] In other embodiments, the method involves *ex vivo* transduction of cells with a myxoma virus of the present invention, followed by administration of a composition comprising the cells into a subject. In certain embodiments, the cells may be autologous, i.e., the subject's own cells. In autologous embodiments, the cells may be obtained from the subject, transduced with a myxoma virus of the present invention, and re-administered into the subject, in a process similar to apheresis. Exemplary formulations for *ex vivo* delivery of the virus into cells may include the use of various transduction agents known in the art, such as calcium phosphate, electoporation, heat shock and various liposome formulations (i.e., lipid-mediated transfection). Liposomes, as described in greater detail below, are lipid bilayers entrapping a fraction of aqueous fluid. DNA spontaneously associates to the external surface of cationic liposomes (by virtue of its charge) and these liposomes will interact with the cell membrane.

[00121] In certain embodiments, the recombinant oncolytic virus and an immunotherapeutic agent or immunomodulator can be administered concurrently or sequentially in a way that the agent does not interfere with the activity of the virus. In certain embodiments, the recombinant oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, intratumorally, or any combination thereof. In still another embodiment, an interferon, such as interferon-α or pegylated interferon, is administered prior to administering the recombinant oncolytic virus according to the instant disclosure.

[00122] Oncolytic viruses according to the disclosure may be administered in a single administration or multiple administrations. The virus may be administered at dosage of

1×10^5 plaque forming units (PFU), 5×10^5 PFU, at least 1×10^6 PFU, 5×10^6 or about 5×10^6 PFU, 1×10^7, at least 1×10^7 PFU, 1×10^8 or about 1×10^8 PFU, at least 1×10^8 PFU, about or at least 5×10^8 PFU, 1×10^9 or at least 1×10^9 PFU, 5×10^9 or at least 5×10^9 PFU, 1×10^{10} PFU or at least 1×10^{10} PFU, 5×10^{10} or at least 5×10^{10} PFU, 1×10^{11} or at least 1×10^{11}, 1×10^{12} or at least 1×10^{12}, 1×10^{13} or at least 1×10^{13}. For example, the virus may be administered at a dosage of between about 10^7-10^{13}, between about 10^8-10^{13}, between about 10^9-10^{12}, or between about 10^8-10^{12}.

A. Combination Therapies

[00123] Additional therapies may be combined with any of the methods of the disclosure heretofore described in order to increase the killing of cancer cells, the inhibition of cancer cell growth, the inhibition of angiogenesis or otherwise improve the reverse or reduction of malignant phenotype of tumor cells. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the oncolytic virus and the other includes a second agent therapy.

[00124] Alternatively, the treatment may precede or follow the other agent or treatment by intervals ranging from minutes to weeks. In embodiments where the agents are applied separately to the cell, one would generally ensure that a significant period of time did not expire between each delivery, such that the agents would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) to several months (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[00125] It also is conceivable that more than one administration of either agent will be desired. Various combinations may be employed, *e.g.* where one or more oncolytic virus treatment is administered before the administration of a second agent; or the second agent

may be administered prior to oncolytic virus administration. Successive administration can include one or more administration of the oncolytic virus therapy or second agent. Again, to achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell. For example, the combination of the claimed PD1 + IL-2/IL-12 reagent and an immune modulator.

[00126] In accordance with certain embodiments of the present disclosure, methods for treating cancer are provided that can be used in conjunction with oncolytic virus therapy once a subject is identified as a responder or likely to respond to such therapy (*e.g.* vMYX-PD1 therapy). Such therapies may be utilized when the assays of the present disclosure indicate that a subject is unlikely to respond to treatment with a replication competent oncolytic virus such as myxoma virus. Alternatively, such therapies may be utilized in combination with replication competent oncolytic virus such as adenovirus in the case that a subject is identified by the present methods as unlikely to respond to treatment with only replication competent oncolytic virus.

[00127] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic, staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present disclosure, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

[00128] Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present disclosure may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

[00129] In certain aspects, a therapy is administered by intratumoral injection prior to surgery or upon excision of a part of or all of cancerous cells, tissue or tumor. Treatment may also be accomplished by perfusion, direct injection or local application of these areas with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages.

[00130] A wide variety of chemotherapeutic agents may be used in accordance with the present disclosure. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Most chemotherapeutic agents fall within the following categories: alkylating agents, antimetabolites, antitumor antibiotics, topoisomerase inhibitors, and mitotic inhibitors.

[00131] Alkylating agents direct interact with genomic DNA to prevent the cancer cell from proliferating. This category of drugs includes agents that affect all phases of the cell cycle and are commonly used to treat chronic leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, malignant melanoma, multiple myeloma, and particular cancers of the breast, lung, and ovary. They include nitrogen mustards such as mechlorethamine (nitrogen mustard), chlorambucil, cyclophosphamide (Cytoxan®), ifosfamide and melphalan, nitrosoureas such as streptozocin, carmustine (BCNU) and lomustine, alkyl sulfonates such as busulfan, triazines such as dacarbzine (DTIC) and temozolomide (Temodar®), ethylenimines such as thiotepa and altretamine (hexamethylmelamine), and platinum drugs such as cisplatin, carboplatin, and oxalaplatin.

[00132] Antimetabolites disrupt DNA and RNA synthesis. Unlike alkylating agents, they specifically influence the cell cycle during S phase. They have been used to combat chronic leukemias, and tumors of the breast, ovary and gastrointestinal tract. Antimetabolites include 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), capecitabine (Xeloda®), cladribine, clofarabine, cytarabine (Ara-C®), floxuridine, fludarabine, gemcitabine (Gemzar®), hydroxyruea, methotrexate, pemetrexed, pentostatin and thioguanine.

[00133] Antitumor antibiotics have both antimicrobial and cytotoxic activity. These drugs also interfere with DNA by chemically inhibiting enzymes and mitosis or altering cellular membranes. These agents work in all phases of the cell cycle and are used to treat a variety of cancers. Representative examples include daunorubicin, doxorubicin (Adriamycin®), epirubicin, idarubicin, actinomycin-D, bleomycin and mitomycin-C. Generally, these compounds are administered by bolus i.v. injections at doses ranging from 25-100 mg/kg

[00134] Topoisomerase inhibitors interfere with topoisomerases, enzymes which help separate DNA strands so they can be copied and are used to treat certain leukemias, as well as lung, ovarian, gastrointestinal and other cancers and include topotecan, irinotecan, etoposide (VP-16) and teniposide.

[00135] Mitotic inhibitors, often plant alkaloids, work during M phase of the cell cycle and prevent mitosis or inhibit enzymes from producing proteins required for cell reproduction. Representative examples include taxanes such as paclitaxel (Taxol®) and docetaxel (Taxotere®), epothilones such as ixabepilone (Ixempra®), vinca alkaloids such as vinblastine (Velban®), vincristine (Onocovin®) and vinorelbine (Navelbine®), and Estramustine (Emcyt®).

[00136] In some embodiments, immunotherapy may be treatment with an immune checkpoint inhibitor. Immune checkpoints either turn up a signal (*e.g.*, co-stimulatory molecules) or turn down a signal. Inhibitory immune checkpoints that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA).

[00137] The immune checkpoint inhibitors may be drugs such as small molecules, recombinant forms of ligand or receptors, or, in particular, are antibodies, such as human antibodies directed to the immune checkpoint proteins (*e.g.*, International Patent Publication WO2015016718; Pardoll, 2012; both incorporated herein by reference). Known inhibitors of the immune checkpoint proteins or analogs thereof may be used, in particular chimerized, humanized or human forms of antibodies may be used. As the skilled person will know, alternative and/or equivalent names may be in use for certain antibodies mentioned in the present disclosure. Such alternative and/or equivalent names are interchangeable in the context of the present disclosure. For example it is known that lambrolizumab is also known under the alternative and equivalent names MK-3475 and pembrolizumab. Exemplary immune checkpoint inhibitors include PD-1 inhibitors, such as Pembrolizumab and Nivolumab; PD-L1 inhibitors, such as Atezolizumab, Avelumab, and Durvalumab; and CTLA-4 inhibitors, such as Ipilimumab.

[00138] In certain preferred embodiments, additive anti-tumor effects can be achieved by combining myxoma vPD1 with blockade of PD1 on T-cells directly. Clinically, this may be achieved through the use of antibodies that bind PD1 blocking interaction with PDL1. It is observed clinically that combination of individual immune checkpoint inhibition achieves much better antitumor activity (Johnson and Win, 2017, incorporated herein by reference in its entirety). An additional benefit of combing myxoma vPD1 and anti-PD1 antibodies according to the present invention may be in the setting of metastatic disease. Locally administered myxoma vPD1, via intra-tumoral injection, may not be optimal for metastatic disease due to PK/PD issues.

[00139] While combining the oncolytic viruses according to the present invention with anti-PD1 antibodies is a promising approach, possible complications might occur through interaction of the anti-PD1 antibody and soluble PD1 expressed from the myxoma virus. To ameliorate this possibility, a myxoma virus was produced expressing a PD1 construct containing mutations in the CD loop that prevents antibody recognition between the two clinically approved anti-PD1 antibodies. In one embodiment, a site mutation at position D85G in the PD1 protein will completely abolish the binding of anti-PD1 antibody pembrolizumab to PD1 (Tan *et al.*, 2017, incorporated herein by reference in its entirety; and Na *et al.*, incorporated herein by reference in its entirety). Thus, in this embodiment, introducing a single point mutation or combinations of single point mutations between the CD loop in the truncated PD1 myxoma construct will decrease any inhibitory binding of anti-PD1antibody.

[00140] Other chemotherapeutic agents include targeted therapies such as imatinib (Gleevec®), gefitinib (Iressa®), sunitinib (Sutent®), sorafenib (Nexavar®), bortezomib (Velcade®), bevacizumab (Avastin®), trastuzumab (Herceptin®), cetuximab (Erbitux®), and panitumumab (Vectibix®), hormone therapies including antiestrogens such as fulvestrant (Faslodex®), tamoxifen, toremifine, aromatase inhibitors such as anastrozole, exemstane and letrozole, progestins such as megestrol acetate, and gonadotropin-releasing hormone and immunotherapies such as antibodies against tumor specific antigens (*e.g.* prostate specific antigen, carcinoembryonic antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin reeptor, erb B and p155) which may be conjugated to a drug or toxin (*e.g.* radionuclide, ricin A chain, cholera toxin, pertussis toxin).

[00141] Radiotherapy, also called radiation therapy, is the treatment of cancer and other diseases with ionizing radiation which may be used to treat localized solid tumors such as cancers of the skin, tongue, larynx, brain, breast or cervix, or may be used to treat cancers of the blood-forming cells (leukemia) and lymphatic system (lymphoma). Radiation therapy includes, without limitation, the use of y-rays, X-rays and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are contemplated such as microwaves and UV-irradiation. Dosage ranges for X-rays range from daily doses of 50-200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000-6000 roentgens.

[00142] Radiotherapy also comprises the use of radiolabeled antibodies to deliver doses of radiation directly to the cancer site (*e.g.* radioimmunotherapy, conformal radiotherapy), high resolution intensity modulated radiotherapy, and stereotactic radio-surgery. Stereotactic radio-surgery (gamma knife) for brain and other tumors employs precisely targeted beams of gamma radiotherapy from hundreds of different angles. Only one session, taking about 4-5 hours is required.

B. Pharmaceutical Compositions

[00143] The recombinant oncolytic virus described herein can be administered as a pharmaceutical or medicament formulated with a pharmaceutically acceptable carrier. Accordingly, the recombinant oncolytic virus may be used in the manufacture of a medicament or pharmaceutical composition. Pharmaceutical compositions of the disclosure may be formulated as solutions or lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable carrier prior to use. Liquid formulations may be buffered, isotonic, aqueous solutions. Powders also may be sprayed in dry form. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water, or buffered sodium or ammonium acetate solution. Such formulations are especially suitable for parenteral administration, but may also be used for oral administration or contained in a metered dose inhaler or nebulizer for insufflation. It may be desirable to add excipients such as polyvinylpyrrolidone, gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride, sodium citrate, and the like.

[00144] Alternately, therapeutic agents may be encapsulated, tableted or prepared in an emulsion or syrup for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba,

magnesium stearate or stearic acid, talc, pectin, acacia, agar or gelatin. Liquid carriers include syrup, peanut oil, olive oil, saline and water. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably, will be between about 20 mg to about 1 g per dosage unit. The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing, when necessary, for tablet forms; or milling, mixing and filling for hard gelatin capsule forms. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, or an aqueous or non-aqueous suspension. For rectal administration, the disclosure compounds may be combined with excipients such as cocoa butter, glycerin, gelatin, or polyethylene glycols and molded into a suppository.

[00145] Therapeutic agents may be formulated to include other medically useful drugs or biological agents. The therapeutic agents also may be administered in conjunction with the administration of other drugs or biological agents useful for the disease or condition to which the disclosure compounds are directed.

[00146] The biologic or pharmaceutical compositions of the present disclosure can be formulated to allow the recombinant oncolytic virus contained therein to be bioavailable upon administration of the composition to a subject. The level of recombinant oncolytic virus in serum, tumors, and other tissues after administration can be monitored by various well-established techniques, such as antibody-based assays (*e.g.*, ELISA). In certain embodiments, recombinant oncolytic virus compositions are formulated for parenteral administration to a subject in need thereof (*e.g.*, a subject having a tumor), such as a non-human animal or a human. Preferred routes of administration include intravenous, intra-arterial, subcutaneous, intratumoral, or intramuscular.

[00147] Proper formulation is dependent upon the route of administration chosen, as is known in the art. For example, systemic formulations are an embodiment that includes those designed for administration by injection, *e.g.*, subcutaneous, intra-arterial, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for intratumoral, transdermal, transmucosal, oral, intranasal, or pulmonary administration. In one embodiment, the systemic or intratumoral formulation is sterile. In embodiments for injection, the recombinant oncolytic virus compositions of the instant disclosure may be formulated in aqueous solutions, or in physiologically compatible solutions or buffers such as

Hanks's solution, Ringer's solution, mannitol solutions or physiological saline buffer. In certain embodiments, any of the recombinant oncolytic virus compositions described herein may contain formulator agents, such as suspending, stabilizing or dispersing agents. In embodiments for transmucosal administration, penetrants, solubilizers or emollients appropriate to the harrier to be permeated may be used in the formulation. For example, 1-dodecylhexahydro-2H-azepin-2-one (Azon®), oleic acid, propylene glycol, menthol, diethyleneglycol ethoxyglycol monoethyl ether (Transcutol®), polysorbate polyethylenesorbitan monolaurate (Tween®-20), and the drug 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one (Diazepam), isopropyl myristate, and other such penetrants, solubilizers or emollients generally known in the art may be used in any of the compositions of the instant disclosure.

[00148] Administration can be achieved using a combination of routes, *e.g.*, first administration using an intra-arterial route and subsequent administration via an intravenous or intratumoral route, or any combination thereof.

IV. Examples

[00149] The following examples are included to demonstrate preferred embodiments of the disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the disclosure, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

Example 1 – Generation and Characterization of vMYX-PD1 Constructs

[00150] Recombinant virus construct may be made with soluble PD1 or soluble PD1 and optionally various interleukins. See, *e.g.*, the schematics of a representative recombinant viral genomic structure in FIG. 1.

[00151] To construct the vPD1 and mutant vPD1, the extracellular region of human PD1 (amino acids 1-168) was amplified from a preconstructed template plasmid (PlasmID database, clone HsCD00345685) by PCR using the following primers.

Forward Primer:
ATCGCCCGGGAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATAACCATG
CAGATCCCACAGGCGCC [SEQ ID NO: 1]

Reverse Primer:
ATCGGAATTCTCAGGTTTGGAACTGGCCGGCTG [SEQ ID NO: 2]

Soluble PD1 nucleotide sequence:
ATGCAGATCCCACAGGCGCCCTGGCCAGTCGTCTGGGCGGTGCTACAACTGGGCT
GGCGGCCAGGATGGTTCTTAGACTCCCCAGACAGGCCCTGGAACCCCCCCACCTT
CTCCCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCTGCAGC
TTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGCATGAGCCCCAGCA
ACCAGACGGACAAGCTGGCCGCTTTCCCCGAGGACCGCAGCCAGCCCGGCCAGG
ACTGCCGCTTCCGTGTCACACAACTGCCCAACGGGCGTGACTTCCACATGAGCGT
GGTCAGGGCCCGGCGCAATGACAGCGGCACCTACCTCTGTGGGGCCATCTCCCTG
GCCCCCAAGGCGCAGATCAAAGAGAGCCTGCGGGCAGAGCTCAGGGTGACAGA
GAGAAGGGCAGAAGTGCCCACAGCCCACCCCAGCCCCTCACCCAGGCCAGCCGG
CCAGTTCCAAACC [SEQ ID NO: 3]

Native Soluble PD1 amino acid sequence (1-168)
Q15116 20 amino signal peptide
MQIPQAPWPV VWAVLQLGWR PGWFLDSPDR PWNPPTFSPA LLVVTEGDNA
TFTCSFSNTS ESFVLNWYRM SPSNQTDKLA AFPEDRSQPG QDCRFRVTQL
PNGRDFHMSV VRARRNDSGT YLCGAISLAP KAQIKESLRA ELRVTERRAE
VPTAHPSPSP RPAGQFQT [SEQ ID NO: 4]

Mutated Soluble PD1 amino acid sequence (1-168) (D85G substitution abolishes the binding of pembrolizumab to PD1)
20 amino signal peptide
MQIPQAPWPV VWAVLQLGWR PGWFLDSPDR PWNPPTFSPA LLVVTEGDNA
TFTCSFSNTS ESFVLNWYRM SPSNQTDK*L*A AFP*EG*RSQP*G*___QDCRF*R*VTQL
PNGRDFHMSV VRARRNDSGT YLCGAISLAP KAQIKESLRA ELRVTERRAE
VPTAHPSPSP RPAGQFQT [SEQ ID NO: 5]

Human IL-2 amino acid sequence (1-153)
P60568 20 amino signal peptide
MYRMQLLSCI ALSLALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN
YKNPKLTRML TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHL
RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR WITFCQSIIS TLT [SEQ ID NO: 6]

Human IL-12, subunit alpha amino acid sequence (1-219)
P29459 22 amino signal peptide
MCPARSLLLV ATLVLLDHLS LARNLPVATP DPGMFPCLHH SQNLLRAVSN
MLQKARQTLE FYPCTSEEID HEDITKDKTS TVEACLPLEL TKNESCLNSR
ETSFITNGSC LASRKTSFMM ALCLSSIYED LKMYQVEFKT MNAKLLMDPK
RQIFLDQNML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLCILLH
AFRIRAVTID RVMSYLNAS [SEQ ID NO: 7]

Human IL-12, subunit beta amino acid sequence (1-328)

P29460 22 amino signal peptide
MCHQQLVISW FSLVFLASPL VA IWELKKDV YVVELDWYPD APGEMVVLTC
DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS
LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEAKNYSGRF TCWWLTTIST
DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP
AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR
QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC
RKNASISVRA QDRYYSSSWS EWASVPCS [SEQ ID NO: 8]

<u>High Affinity Human IL-2 amino acid variant (1-164)</u>
P60568 20 amino signal peptide
MYRMQLLSCI ALSLALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN
YKNPKLTRML TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHF
DPRDVVSNIN VFVLELKGSE TTFMCEYADE TATIVEFLNR WITFCQSIIS
TLTAAAHHHH HHHH [SEQ ID NO: 9]

[00152] FIG. 2 shows a vPD1-IL2 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model. FIG. 3 shows a vPD1-IL12 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model. FIG. 4 shows the results of a vPD1-IL15 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model. Likewise, FIG. 5 shows results from a vPD1-IL18 efficacy study in subcutaneous B16F10 (B16F10 PD1L-KO) contralateral xenograft model. Taken together, IL-2 and IL-15 appear to show modest reductions in tumor size, while IL-12 provides the greatest reduction. IL-18 appears to have the least effect.

[00153] As shown in FIGS. 6 and 7, an *in vivo* subcutaneous (SC) contralateral mouse model was employed to test various viral constructs. Three intratumoral injections were made two days apart on the left side (WT-B16/F10) while the right side (PDL1-KO-B16/F10) was untreated. Injected tumors (left) and contralateral non-injected tumors (right) seem to respond to vPD1/IL12 and vPD1/IL2 treatments. vPD1/IL15 showed a modest esponse as well, whereas vPD/IL18 show negligible effect. Taken together, IL-12 surprisingly shows a greater reduction in the size of both the injected and the contralateral tumors than do the other constructs tested.

TABLE 1 – RAW VIRUS TREATMENT DATA

Treatment		Animal	Admin	Day 8	Day 10	Day 12	Day 14	Day 16
Mock	1L	WT	Injected	23.52	35.96	57.6	91.18	112.11
Mock	2L	WT	Injected	22.96	32.83	43.56	113.46	151.51
Mock	3L	WT	Injected	24.44	37.12	35.99	55.2	77.7
Mock	4L	WT	Injected	22.05	26.95	49.64	69.3	123.42

Mock	5L	WT	Injected	31.92	40.8	52.65	74.25	126.26
Mock	1R	KO	Contralateral	17.22	16.92	29.15	45.14	85.36
Mock	2R	KO	Contralateral	15.58	17.63	26.5	32.76	56.21
Mock	3R	KO	Contralateral	20.16	19.68	21.12	31.92	57
Mock	4R	KO	Contralateral	17.16	23	30.09	42.78	53.13
Mock	5R	KO	Contralateral	22.05	28.05	42.48	56.7	83.66
vPD1	1L	WT	Injected	34.79	38.5	60.75	78.54	92.13
vPD1	2L	WT	Injected	14.43	11.22	11.22	6	11.55
vPD1	3L	WT	Injected	27.93	37.26	45.99	46.08	62.64
vPD1	4L	WT	Injected	28.62	34.22	58.855	33.6	55.68
vPD1	5L	WT	Injected	36.6	25.97	32.76	27.44	38.35
vPD1	1R	KO	Contralateral	22.95	23.5	36.58	63.75	105.06
vPD1	2R	KO	Contralateral	18.48	20.16	26.52	40.32	61.6
vPD1	3R	KO	Contralateral	26.52	35.4	31.8	51.84	86.49
vPD1	4R	KO	Contralateral	30.6	42.25	55.44	96.03	147.84
vPD1	5R	KO	Contralateral	29.7	36	51.12	90.25	141.52
vPD1/IL2	1L	WT	Injected	22	32.86	44.53	55.25	74.48
vPD1/IL2	2L	WT	Injected	26.5	38.86	37.8	21.5	17.22
vPD1/IL2	3L	WT	Injected	14.28	17.2	10.56	15.84	11.2
vPD1/IL2	4L	WT	Injected	22.5	23.52	34.1	39.2	49.14
vPD1/IL2	5L	WT	Injected	39.68	46.8	43.55	39.04	40.3
vPD1/IL2	1R	KO	Contralateral	25.3	29.5	48.84	66.36	88.35
vPD1/IL2	2R	KO	Contralateral	24.99	25.38	32.94	40.8	50.05
vPD1/IL2	3R	KO	Contralateral	10.88	30.09	41.58	68.06	94.05
vPD1/IL2	4R	KO	Contralateral	4	16.4	20.7	23.52	26.01
vPD1/IL2	5R	KO	Contralateral	20.7	20.68	36	61.2	63.18
vPD1/IL12	1L	WT	Injected	19.5	20.24	34.2	15.54	28.52
vPD1/IL12	2L	WT	Injected	29	29.76	23.97	36.54	22.88
vPD1/IL12	3L	WT	Injected	22.09	24.99	24.01	19.35	15.64
vPD1/IL12	4L	WT	Injected	31.27	44.1	54.72	39.53	30
vPD1/IL12	5L	WT	Injected	42.25	39.76	39.76	36.5	28.91
vPD1/IL12	1R	KO	Contralateral	4	0	14.26	0	0
vPD1/IL12	2R	KO	Contralateral	14.8	12.48	0	0	22.05
vPD1/IL12	3R	KO	Contralateral	20.4	22.96	14.35	0	0
vPD1/IL12	4R	KO	Contralateral	21.42	22.95	22.09	18.62	11.84
vPD1/IL12	5R	KO	Contralateral	10.85	7.56	7.54	7	6
vPD1/IL15	1L	WT	Injected	7.84	13.02	24.96	11.88	8.99
vPD1/IL15	2L	WT	Injected	21.12	21.56	33.06	19.8	14.04
vPD1/IL15	3L	WT	Injected	15.96	10.15	19.78	18.48	14
vPD1/IL15	4L	WT	Injected	23.46	25.5	46.8	28.6	23.22
vPD1/IL15	5L	WT	Injected	23.46	35.4	29.12	25.44	24.48
vPD1/IL15	1R	KO	Contralateral	4	9.8	18.33	23.5	40.26
vPD1/IL15	2R	KO	Contralateral	20.7	24.5	38.5	48.51	64.6
vPD1/IL15	3R	KO	Contralateral	20.24	19.27	39.65	48.3	63.99
vPD1/IL15	4R	KO	Contralateral	23	30.24	48.18	80.84	134.82
vPD1/IL15	5R	KO	Contralateral	19.27	27.5	38.4	43.55	53.25

vPD1/IL18	1L	WT	Injected	21.62	36.58	55.89	116	
vPD1/IL18	2L	WT	Injected	36.6	52.56	83.7	101.01	122.72
vPD1/IL18	3L	WT	Injected	26.95	39.04	56.07	80.64	123.12
vPD1/IL18	4L	WT	Injected	10.88	20.68	24.5	39.65	57.72
vPD1/IL18	5L	WT	Injected	9	16.81	24.08	40.87	71.2
vPD1/IL18	1R	KO	Contralateral	15.6	15.99	20.09	27.56	
vPD1/IL18	2R	KO	Contralateral	12.95	17.766	17.55	22.36	51.62
vPD1/IL18	3R	KO	Contralateral	12.24	12.58	10.56	18.92	33.63
vPD1/IL18	4R	KO	Contralateral	9.9	14.4	12.8	24.36	34.8
vPD1/IL18	5R	KO	Contralateral	22.95	27.54	50.37	49.56	108.81

[00154] As shown in FIG. 15, a mouse study was performed to assess the efficacy of vPD1 alone, vIL12 alone, and the combination of vPD1+IL12. Mice were injected with $4x10^6$ B16/F10 cells on both flanks. After tumors were established, the larger tumor was treated with 3 injections of the indicated virus over 5 days (Day 0, 2, and 4). The growth of the tumors and the body weight of the mice were monitored until the mice were euthanized when the total tumor burden exceeded 400 mm^2. It was found that the combination of vPD1+IL12 had the most significant effect on decreasing tumor growth and increasing overall survival of the mice.

[00155] Further development of the virus comprised the addition of a transmembrane domain to the IL12. The transmembrane domain prevents the IL12 from leaking into the blood.

Transmembrane and cytosolic domain

CTTGTGCTCTTTGGGGCAGGATTCGGCGCAGTAATAACAGTCGTCGTCATC
GTTGTCATCATCAAATGCTTCTGTAAGCACAGAAGCTGTTTCAGAAGAAATGAGG
CAAGCAGAGAAACAAACAACAGCCTTACCTTCGGGCCTGAAGAAGCATTAGCTG
AACAGACCGTCTTCCTT (SEQ ID NO: 12)

PD1-IL12-transmembrane domain construct

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAAT
GCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAA
TTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG
GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT
ATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGC
TGGAGCTCCACCGCGGTGGCGGCCGCATAAACGCGTTTAAACAGTCCCCCGTAC
GCGGTACATCGTACGCACACTTCACTAACGATGTCGTACATCGATTACACAAAGA
AGTAGAGTCATACGACGTACGTTTCCCTATAAAATCGGTAAACCTAGACGCGGTG
TTTCTATCCATAAACGTAACACGTGTACGTCTACGTTGGAAGATACCCTTGACCG
AACACAATCCTTATCAGACGGCCTACGGATGTTCTAACGACAGATTATACAGCTA
CAACGAGTACGCTTTTTCTCATTTAAAACAAGACCGTGTAAAGATCATAGAACTC

CCATGTGACGACGATTACAGCGTCGTGTTAATCACACACGATAGCCGTTCGACTA
TTACACCGGATAAAGTGACCGGGTGGCTGCGCACGACCCGTCTACGTTACGTAA
ACGTATCCCTACCCAAGGGTTCCACGGAAACGGGACACAACGTAACGTGTCTAA
CTCCCACACACGTCAATCTATGTCATCGTTGTCGTATAACGATTACCAAAACGGG
CGTGGACGCAACCGCGTTCTCATGCGTCGACGGCGATACATGCACCGAACACGA
CACGACCGCGTCAACGTGTACGATTATTATAAAAACGACGGGACTGGACTTTTTG
TTTATGGGGAAACTCTAAAAAAAAATTGTCAATTAAAGTAACTGCAGATCGATCGC
ATATGAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATAATGGTGAGCAAG
GGCGAGGAGGTCATCAAAGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCC
ATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAG
GGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCC
TGGGACATCCTGTCCCCCCAGTTCATGTACGGCTCCAAGGCGTACGTGAAGCACC
CCGCCGACATCCCCGATTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGA
GCGCGTGATGAACTTCGAGGACGGCGGTCTGGTGACCGTGACCCAGGACTCCTC
CCTCCAAGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAACTTCCC
CCCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGA
GCGCCTGTACCCCCGCGACGGCGTGCTGAAGGGCGAGATCCACCAGGCCCTGAA
GCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGACCATCTACATGGCCAA
GAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGGACATCAC
CTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCTCCGAGGGCCG
CCACCACCTGTTCCTGGGGCATGGCACCGGCAGCACCGGCAGCGGCAGCTCCGG
CACCGCCTCCTCCGAGGACAACAACATGGCCGTCATCAAAGAGTTCATGCGCTTC
AAGGTGCGCATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAG
GGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAG
GGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCATGTACGGCT
CCAAGGCGTACGTGAAGCACCCCGCCGACATCCCCGATTACAAGAAGCTGTCCTT
CCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGTCTGGT
GACCGTGACCCAGGACTCCTCCCTCCAAGACGGCACGCTGATCTACAAGGTGAA
GATGCGCGGCACCAACTTCCCCCCCGACGGCCCCGTAATGCAGAAGAAGACCAT
GGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAAGGG
CGAGATCCACCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTT
CAAGACCATCTACATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGT
GGACACCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACA
GTACGAGCGCTCCGAGGGCCGCCACCACCTGTTCCTGTACGGCATGGACGAGCT
GTACAAGTAACCCGGGAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAAT
AACCATGTGTCCTCAGAAGCTAACCATCTCCTGGTTTGCCATCGTTTTGCTGGTGT
CTCCACTCATGGCCATGTGGGAGCTGGAGAAAGACGTTTATGTTGTAGAGGTGG
ACTGGACTCCCGATGCCCCTGGAGAAACAGTGAACCTCACCTGTGACACGCCTG
AAGAAGATGACATCACCTGGACCTCAGACCAGAGACATGGAGTCATAGGCTCTG
GAAAGACCCTGACCATCACTGTCAAAGAGTTTCTAGATGCTGGCCAGTACACCTG
CCACAAAGGAGGCGAGACTCTGAGCCACTCACATCTGCTGCTCCACAAGAAGGA
AAATGGAATTTGGTCCACTGAAATTTTAAAAAATTTCAAAAACAAGACTTTCCTG
AAGTGTGAAGCACCAAATTACTCCGGACGGTTCACGTGCTCATGGCTGGTGCAA
AGAAACATGGACTTGAAGTTCAACATCAAGAGCAGTAGCAGTTCCCCTGACTCTC
GGGCAGTGACATGTGGAATGGCGTCTCTGTCTGCAGAGAAGGTCACACTGGACC
AAAGGGACTATGAGAAGTATTCAGTGTCCTGCCAGGAGGATGTCACCTGCCCAA
CTGCCGAGGAGACCCTGCCCATTGAACTGGCGTTGGAAGCACGGCAGCAGAATA
AATATGAGAACTACAGCACCAGCTTCTTCATCAGGGACATCATCAAACCAGACC
CGCCCAAGAACTTGCAGATGAAGCCTTTGAAGAACTCACAGGTGGAGGTCAGCT
GGGAGTACCCTGACTCCTGGAGCACTCCCCATTCCTACTTCTCCCTCAAGTTCTTT

GTTCGAATCCAGCGCAAGAAAGAAAAGATGAAGGAGACAGAGGAGGGGTGTAA
CCAGAAAGGTGCGTTCCTCGTAGAGAAGACATCTACCGAAGTCCAATGCAAAGG
CGGGAATGTCTGCGTGCAAGCTCAGGATCGCTATTACAATTCCTCATGCAGCAAG
TGGGCATGTGTTCCCTGCAGGGTCCGATCCGGTGGCGGTGGCTCGGGCGGTGGTG
GGTCGGGTGGCGGCGGATCTAGGGTCATTCCAGTCTCTGGACCTGCCAGGTGTCT
TAGCCAGTCCCGAAACCTGCTGAAGACCACAGATGACATGGTGAAGACGGCCAG
AGAAAAACTGAAACATTATTCCTGCACTGCTGAAGACATCGATCATGAAGACAT
CACACGGGACCAAACCAGCACATTGAAGACCTGTTTACCACTGGAACTACACAA
GAACGAGAGTTGCCTGGCTACTAGAGAGACTTCTTCCACAACAAGAGGGAGCTG
CCTGCCCCCACAGAAGACGTCTTTGATGATGACCCTGTGCCTTGGTAGCATCTAT
GAGGACTTGAAGATGTACCAGACAGAGTTCCAGGCCATCAACGCAGCACTTCAG
AATCACAACCATCAGCAGATCATTCTAGACAAGGGCATGCTGGTGGCCATCGAT
GAGCTGATGCAGTCTCTGAATCATAATGGCGAGACTCTGCGCCAGAAACCTCCTG
TGGGAGAAGCAGACCCTTACAGAGTGAAAATGAAGCTCTGCATCCTGCTTCACG
CCTTCAGCACCCGCGTCGTGACCATCAACAGGGTGATGGGCTATCTGAGCTCCGC
CCTTGTGCTCTTTGGGGCAGGATTCGGCGCAGTAATAACAGTCGTCGTCATCGTT
GTCATCATCAAATGCTTCTGTAAGCACAGAAGCTGTTTCAGAAGAAATGAGGCA
AGCAGAGAAACAAACAACAGCCTTACCTTCGGGCCTGAAGAAGCATTAGCTGAA
CAGACCGTCTTCCTTTGAGAATTCACGAATCGAATAAAAACCCGTGTACACACGG
ACGTTAATTTTTTTTGTGGTTTAAAAAATGACCACATTTACGCTTTTTTTTAACGC
GTTATATAAGGTATCTCGTTTGTCTATAACAAAGATCGTAACTGACC
TTTTTTATATCGAGAAAACATACGTTTAGTTCATCCTCAAACGTAACACCGTAAC
TGCCTCGGACATCCTCCTTGTTGTCGTACACAAACATACTAATCGGATGCGTGAA
ATGAGGATTCACTTTAATCGGATTGGTTTCTAGGTTAACACATGTTACACAAGAT
CCTAAGATGGTTATGGACACATCCTTGTTGTGATGTAACGAGTCGGGAAGTTGAT
TGCCGTAGTTGCCCACGTCGCCCTCCGGTTCCAGACACGTAATGGTTAGGTATAT
ATCCGAATACTTCGTCAACGGATGAGTCGTAAATAACATGATGGATAGCTTGTTC
CCATCTCCTGCACCAGCACTGGCCGCCACAAATCGTTGTACCACGTTAGTAATCG
TAATGTTTATCATAAGCCCGTACCCGGTTAATATGAGCGTGGACGTTTTATGATC
GTATCGTTCCTTCATGTGACATTCTCCCATAACCGTTTCGACGTACCGATTTAACC
CGATGGTTAGCTCGGCGGCTAAGTGCCAGTGGATCCCCCAATTCGATATCAAGCT
TATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGA
GTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCT
GGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTA
ATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATG
GCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTA
CGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTT
CTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACT
TGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGC
CCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAAC
AACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTT
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAA
CAAAATATTAACGCTTACAATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAA
CCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAA
TAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAA
CATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCT
CACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGA
GTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCC
CCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGT

ATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCT
CAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGC
ATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCG
GCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGC
ACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATG
AAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAA
CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATT
AATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCT
TCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGC
GGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCT
ACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAG
ATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATA
TACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC
GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGC
GTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGC
CGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA
GATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAAC
TCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGC
CAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGAT
AAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAG
CGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCC
ACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG
AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAG
TCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAG
GGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGG
CCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGG
ATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGAC
CGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG (SEQ ID NO:13)

Example 2 – Generation of vMYX-TIM3 Constructs

[00156] vTIM3 was generated by homologously recombining unmodified myxoma virus (strain Lausanne) with pBS-M135/M136-sE/L GFP+TIM3, a plasmid which contains the following critical elements.

-pBluescript plasmid backbone
-Region of myxoma genome homologous to M134/M135
-eGFP driven by the consensus poxviral synthetic early/late promoter
-aa1-195 of murine TIM3 driven by a second consensus poxviral synthetic early/late promoter
-Region of myxoma genome homologous to M136

<u>TIM3-GFP Construct</u>

NNNNNNNNNNCNCGNGGNGGCGGCCGCTCTAGTAGGATTACCTGGTCTATATAG
ATAACAAAACCTACGTACGTATAAACGAGACCGTTGTACCGGAGAACGAGTATC
TGGCAGCGAAGGCCCCGCGAGTGACCTGTTTCCACACGGACTTGATCCCCATTAC

GGACGAAGAGACACAACGACGTTTTGAGAAAATGATTGTACAGGCGGCGTTAGA
GGACGCCCTAACGAGCATCTTTGAGGAGCACGACAATAACGTAACCGATTACTT
CGCGGAATACATGCGATCCCTCCAAATGGCGAATAAAAGTCATACGAATAATAT
TATCGCGGTCGCTTTAGCGGGGATAATCGTCATTGTAACGACCTACGTGTTTACT
AGATTACGCACTAAGCAAAAAAAAGGAAATTATAACGTACGTAATAAGATAGAT
AATTCCATACAGAAAGAGATTCAGTTGGACGGTGTATATACTACTGACAACGTTT
TTATATAAACATGGTGTTTATATTTATTATCACCTGTGTATGTTTGGTGACGAGAT
CCTGTGGGGGTGGGTTAGAAGACGATATAGATCGCATATTTCAAAAACGATACA
ACGAACTGAGCCAGCCGATTAANNNCAATATGCGTACACTGTGCAAGTTTAGAG
GAATTACCGCGACTATGTTTACGGAAGGAGAATCTTACCTTATTCAATGTCCCAT
AATTCACGATTACGTGCTACGGGCGCTGTATGACTTAGTGGAAGGAAGTTACACG
GTACGCTGGGAACGCGAAACGGAAGACGATGTTGAGTCGGTAGATCCGAAGTTA
GTCAAAGGGACGCTATTATACCTCCAACCTAACGCGTCCAGTATAGGAACGTATC
TATGTACCTTACACGATAACCGAGGTATGTGTTATCAATCTGTCGCGCACGTCAT
CCGACGTCCGAAGATGCAATGCGTGAAACATGCACATACGACATCGGACAGCAA
CCTGTGGATATACCTCGCCATTTTAGCAGTTTTGATATCCTTAGGCGTCCTGTAAA
GGAAACGCGCCAGACTCCGGAACTATGAAGGATTTATCACTGTATACAGACTCC
GACGTACGAAGGATAATCACGACGTAACTCGAACTCTGCAGGTCGACTCTAGAG
GATCTACTAGTCATATGGATTTAAAAATAGCGGAGCTTAAAAATTGAAATTTTAT
TTTTTTTTTTTGGAATATAAATAAGCTCGAAGTCGACAGATCTAGGCCTGGTACC
CGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGT
GGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGT
GTCCGGCGAGGGCGAGGGCGATGCCACNTACGGCAAGCTGACCCTGAAGTTCAT
CTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACC
TACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCT
TCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA
CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGT
GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGG
GCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAA
GCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGG
CAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCC
CGTGCTGNTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGA
CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGG
GATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGGGTAATTACCCG
GGATGTTTTCAGGTCTTACCCTCAACTGTGTCCTGCTGCTGCTGCAACTACTACTT
GCAAGGTCATTGGAAAATGCTTATGTGTTTGAGGTTGGTAAGAATGCCTATCTGC
CCTGCAGTTACACTCTATCTACACCTGGGGCACTTGTGCCTATGTGCTGGGGCAA
GGGATTCTGTCCTTGGTCACAGTGTACCAACGAGTTGCTCAGAACTGATGAAAGA
AATGTGACATATCAGAAATCCAGCAGATACCAGCTAAAGGGCGATCTCAACAAA
GGAGACGTGTCTCTGATCATAAAGAATGTGACTCTGGATGACCATGGGACCTACT
GCTGCAGGATACAGTTCCCTGGTCTTATGAATGATAAAAAATTAGAACTGAAATT
AGACATCAAAGCAGCCAAGGTCACTCCAGCTCAGACTGCCCATGGGGACTCTAC
TACAGCTTCTCCAAGAACCCTAACCACGGAGAGAAATGGTTCAGAGACACAGAC
ACTGGTGACCCTCCATAATAACAATGGAACAAAAATTTCCACATGGGCTGATGA
AATTAAGGACTCTGGAGAAACGATCAGAACTGCTATCCACTAGGAATTCTAACA
TTTTTTAAAACAATTTCGTTATGTTAAATTATGGAACGGTCGCCCACTTACACGGT
ACACGATAAACGCTTTTCTATCGTCGCACTAAACGGACAATACGACATGGTGGAC
GATTTTGGTCTTAGTTTTTCTTACACAGCGATCGACGATATTTCTAAAAATCATTC
CATCAAACACGTTTTAGAAGAATACTTTTCATGGCGCGCGTATATAGGCCGGGTA
TGTATCATACCGAATCACGTGGGAAAGCTCTACATCAAACTTACAAAGTTGGACA

CCACGGCGAAGAACAAACTAGGCAATCTAGATATATTGTTATGCGACGTGTTAA
AAATAGACGAGGACGGAGGCAACGAGAAACTGTTTCAATTCATACGGTCGCGGA
TCCCCCAATTCGATATCAAGCTTATCGATACCGTCGACCTCG (SEQ ID NO:10)

Soluble TIM3

ATGTTTTCAGGTCTTACCCTCAACTGTGTCCTGCTGCTGCTGCAACTACTACTTGC
AAGGTCATTGGAAAATGCTTATGTGTTTGAGGTTGGTAAGAATGCCTATCTGCCC
TGCAGTTACACTCTATCTACACCTGGGGCACTTGTGCCTATGTGCTGGGGCAAGG
GATTCTGTCCTTGGTCACAGTGTACCAACGAGTTGCTCAGAACTGATGAAAGAAA
TGTGACATATCAGAAATCCAGCAGATACCAGCTAAAGGGCGATCTCAACAAAGG
AGACGTGTCTCTGATCATAAAGAATGTGACTCTGGATGACCATGGGACCTACTGC
TGCAGGATACAGTTCCCTGGTCTTATGAATGATAAAAAATTAGAACTGAAATTAG
ACATCAAAGCAGCCAAGGTCACTCCAGCTCAGACTGCCCATGGGGACTCTACTA
CAGCTTCTCCAAGAACCCTAACCACGGAGAGAAATGGTTCAGAGACACAGACAC
TGGTGACCCTCCATAATAACAATGGAACAAAAATTTCCACATGGGCTGATGAAA
TTAAGGACTCTGGAGAAACGATCAGAACTGCTATCCAC (SEQ ID NO: 11)

[00157] pBS-M135/M136-sE/L GFP+TIM3 was transfected into BSC40 cells which were then infected with unmodified myxoma virus (strain Lausanne). Cells were cultured for 72 hours which produces recombinant viruses in which the untranslated region of the viral genome between M135 and M136 is replaced by a cassette expressing both eGFP and soluble TIM3 (FIG. 12A). Recombinant virus was then quadruple plaque purified on BSC40 cells by selecting GFP^+ clones. Clonality of the final virus (vTIM3) was then confirmed using PCR.

[00158] *In Vitro Characterization of vTIM3*: *vTIM3 secretes soluble PD1 from infected cells:* Secretion of soluble PD1 from virally infected cells was confirmed by western blotting supernatants from B16/F10 melanoma cells infected with either saline (mock), vGFP (control virus), or vTIM3 after 24 hours of infection. A strong band consistent with the soluble portion of TIM3 was observed specifically in the supernatant of cell infected with vTIM3 (FIG. 12D).

[00159] *vTIM3 displays normal replication and oncolytic capacity in vitro*: To determine whether insertion of the TIM3 transgene would alter MYXV replication, single step growth curves were performed on both vGFP and vTIM3 in a variety of cells. It was observed that both viruses displayed identical replication in all tested cell types (FIGS. 12B and 12C). To further test whether secretion of the TIM3 transgene would alter MYXV's ability to kill directly infected cells, it was next asked how effective both vGFP and vTIM3 were at killing B16/F10 melanoma cells. B16/F10 cells were infected with either vGFP or vTIM3 at the

indicated multiplicities of infection. After 24 hours, cellular viability was analyzed using MTT assay. It was observed that both vGFP and vTIM3 displayed an identical capacity to kill infected melanoma cell in vitro (FIG. 13).

[00160] *Oncolytic potential against melanoma in vivo*: To test whether vTIM3 displayed increased oncolytic capacity in vivo, its ability to regress established melanoma tumors was tested in mice. C57/B6 mice were implanted subcutaneously with $5x10^5$ B16/F10 melanoma cells. Treatment was initiated seven days after injection of tumor cells (when tumors are approximately 15-20 mm^2). Treatment consisted of two intratumoral injections of either saline, $1x10^7$ FFU of vGFP, or $1x10^7$ FFU of vTIM3 given on days 7 and 12. Animals were then monitored daily for tumor size and euthanized when tumors reached 150mm in any direction. Animals treated with vGFP displayed reduced tumor growth, however, the majority of tumors in these animals still progressed eventually requiring euthanasia (FIG. 13). In contrast, many mice treated with vTIM3 displayed rapid regression of established tumors resulting in complete durable remissions in 7/12 animals.

[00161] A series of viruses were constructed expressing variants of the soluble TIM3 protein in which previously validated binding sites for each TIM3 ligand have been removed through mutagenesis (FIG. 14). Each of these viruses are tested for their ability to induce αtumor immunity and eradicate established tumors *in vivo*. This identifies the mechanisms involved in vTIM3-based checkpoint blockade as well as by allowing for the construction of a next generation vTIM3 construct with improved therapeutic efficacy.

Example 3 – vMYX-therapy in metastatic disease

[00162] A recombinant MYXV which expresses a secreted form of soluble PD1 (vPD1) was also studied to determine its activity relative to metastatic cancers. Unfortunately, while vPD1 is extremely effective at eradicating localized disease (e.g., through the maintenance of anti-tumor immunity) see, *e.g.*, FIG. 16, additional experiments have indicated that it has reduced effectiveness relative to non-injected, metastatic tumors (see FIG. 16B). Due to the inability of vPD1 to effectively regress non-treated lesions, to advance the clinical potential of this virus additional modifications were studied that could be added to the vPD1 backbone which would result in improved systemic efficacy. Additional recombinant viruses which expressed both soluble PD1 and either additional soluble T cell checkpoint proteins or a series of proinflammatory cytokines (see, FIG. 17A). Each of these viruses was then purified

to clonality and tested for its ability to regress both injected and non-injected Lewis Lung Carcinomas (LLC) in a standard contralateral tumor model. The results indicated that, of the molecules tested, only inclusion of IL-12 (encoded as a p40/p35 fusion protein) significantly improved efficacy of the vPD1 backbone against non-injected lesions (FIG. 17B-C). Impressively, however, vPD1/IL12 was able to fully regress both injected and non-injected lesions in virtually all treated mice (durable complete response lasting >120 days in 10/12 animals) resulting in an effective 'cure rate' of almost 90%. Remarkably, even in animals with bulky, well-established disease (animals from the initial 'mock' cohort displaying a total tumor burden between 350-400mm^2), treatment of a single tumor with vPD1/IL12 could cause both complete elimination of the treated tumors as well as significant regression in the non-treated tumors (FIG. 17D). These studies demonstrate that vPD1/IL12 virus represented a novel therapeutic agent with strong clinical potential against even late stage, metastatic disease.

[00163] To advance on this exciting initial finding, the therapeutic breadth of the vPD1/IL12 virus was further studied. To accomplish this, a single recombinant MYXV expressing an IL12 fusion protein (vIL12) was compared the efficacy of this virus to that of both vPD1 and vPD1/IL12 in preclinical models of metastatic disease: LLC lung cancer (SQ contralateral tumor model), B16/F10 melanoma (SQ contralateral tumor model), 4T1 and triple negative breast cancer (single SQ tumor spontaneously metastatic to the lung). The results from all models clearly demonstrated that: 1) the vPD1/IL12 virus was capable of regressing both treated and non-treated lesions from a wide range of different malignancies including tumors representing both immunologically 'hot' (LLC and B16/F10) and immunologically 'cold' (4T1 and ID8) forms of disease. 2) This clinical efficacy was not observed in any model following treatment with either singly recombinant virus (vPD1 or vIL12) indicating that that vPD1/IL12's therapeutic effect is due to a unique form of combinatorial synergy (FIGS. 18-20).

[00164] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the disclosure. More specifically, it will be

apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the disclosure as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Ausubel *et al., Current Protocols in Molecular Biology*, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., MA, 1996.

Bartee *et al.*, 2017. *Cancer Research* 77, 2952–2963.

Cameron *et al., Virology*, 264:298–318, 1999.

Chen and Okayama, *Mol. Cell Biol.*, 7(8):2745-2752, 1987.

Cheng *et al.* "Membrane-Tethered Proteins for Basic Research, Imaging, and Therapy" *Medicinal Research Reviews,* Vol. 28, No. 6, 885-928, 2008.

Fechheimer *et al.*, *Proc Natl. Acad. Sci. USA,* 84:8463-8467, 1987.

Fraley *et al., Proc. Natl. Acad. Sci. USA,* 76:3348-3352, 1979.

Frankel and Pabo, *Cell*, 55(6):1189-1193, 1988.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and Van Der Eb, *Virology*, 52:456-467, 1973.

Harland and Weintraub, *J. Cell Biol.*, 101(3):1094-1099, 1985.

Johnson and Win, 2017. *Oncoimmunology*, Combination therapy with PD-1/PD-L1 blockade: An overview of ongoing clinical trials. DOI:10.1080/2162402X.2017.1408744

Johnston and McFadden, *J. Virology*, 77:6093–6100, 2003.

Kaeppler *et al., Plant Cell Rep.*, 8:415–418, 1990.

Kaneda *et al.*, *Science*, 243:375-378, 1989.

Kato *et al, J. Biol. Chem.*, 266:3361-3364, 1991.

Kaufman, *et al.,* 2015. Nature Rev. Drug Discov 14, 642–662.

Kerr and McFadden, *Viral Immunology*, 15:229–246, 2002.

Levin *et al.* (2012) *Nature* 484:529-533

Liu *et al.*, *J. Clin. Investig.*, 105:1613–1621, 2000.

Lucas and McFadden, *J. Immunol.*, 173:4765–47742004.

Na *et al.*, 2016. Cell Res 27:147-150.

Nabel *et al., Science*, 244(4910):1342-1344, 1989.

Nicolau and Sene, *Biochim. Biophys. Acta,* 721:185-190, 1982.

Nicolau *et al.*, *Methods Enzymol.*, 149:157-176, 1987.

Pardoll, 2012. *Nature Reviews: Cancer* 12:252-264.

PCT Application No. WO 04/078206.

PCT Application No. WO 94/09699.

PCT Application No. WO 95/06128.

Potrykus *et al.*, *Mol. Gen. Genet.*, 199(2):169-177, 1985.

Potter *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:7161-7165, 1984.

Rippe, *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Sambrook and Russell, *Molecular Cloning: A Laboratory Manual,* 3rd Ed. Cold Spring Harbor Lab. Press, 2001.

Sambrook *et al.*, *In:Molecular Cloning: A Laboratory Manual*, Vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (7)7:19-17.29, 1989.

Smallwood, S.E. *et al., Curr Protoc Microbiol.*, Unit-14A.1, 2010.

Sypula *et al.*, *Gene Ther Mol Biol.*, 8:103–1142004.

Tan, *et al.,* 2017. *Nature Communications* 8:14369–10.

Topalian, S.L., *et al.,* 2015. *Cancer Cell* 27:450–461.

Tosic *et al., PLoS One,* e109801, 2014.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

U.S. Patent Application No. 20090035276.

U.S. Patent No. 5,302,523

U.S. Patent No. 5,322,783

U.S. Patent No. 5,384,253

U.S. Patent No. 5,464,765

U.S. Patent No. 5,538,877

U.S. Patent No. 5,538,880

U.S. Patent No. 5,550,318

U.S. Patent No. 5,563,055

U.S. Patent No. 5,563,055

U.S. Patent No. 5,580,859

U.S. Patent No. 5,589,466

U.S. Patent No. 5,591,616

U.S. Patent No. 5,610,042

U.S. Patent No. 5,656,610

U.S. Patent No. 5,702,932

U.S. Patent No. 5,736,524

U.S. Patent No. 5,780,448

U.S. Patent No. 5,789,215

U.S. Patent No. 5,945,100

U.S. Patent No. 5,981,274

U.S. Patent No. 5,994,624

U.S. Patent No. 8,613,915

Wilson *et al.*, *Science,* 244:1344-1346, 1989.

Wong *et al.*, *Gene*, 10:87-94, 1980.

Wu and Wu, *Biochemistry*, 27: 887-892, 1988.

Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

AMENDED CLAIMS
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WHAT IS CLAIMED IS:

1. A recombinant oncolytic myxoma virus comprising one or more expression cassettes encoding (a) a soluble form of programmed cell death protein 1 (PD1), and (b) interleukin 12 (IL-12), wherein the virus is replication competent.

2. The recombinant oncolytic myxoma virus of claim 1, comprising one or more expression cassettes encoding a mutant soluble form of PD1 (mutPD1) and (b) interleukin 12 (IL-12), wherein the virus is replication competent and wherein the mutPD1 prevents recognition of mutPD1 by an anti-PD1 antibody.

3. The recombinant oncolytic myxoma virus of claim 2, wherein the mutPD1 contains a mutation in the CD loop that prevents antibody recognition by anti-PD1 antibodies.

4. The recombinant oncolytic myxoma virus of claim 3, wherein the mutPD1 contains a point mutation in the CD loop comprising D85G.

5. The recombinant oncolytic myxoma virus of claim 4, wherein the mutPD1 is not recognized by pembrolizumab.

6. The recombinant oncolytic myxoma virus of claim 1 or 2, wherein the soluble PD1 comprises an extracellular region of human PD1.

7. The recombinant oncolytic myxoma virus of claim 6, wherein the extracellular region of human PD1 and ~~IL-2 or~~ IL-12 are encoded in a single expression cassette.

8. The recombinant oncolytic myxoma virus of claim 1 or 2, wherein the expression cassette(s) is/are under the control of one or more viral promoters.

9. The recombinant oncolytic myxoma virus of claim 8, wherein the one or more viral promoters is/are synthetic early/late poxvirus promoter.

10. The recombinant oncolytic myxoma virus of claim 9, wherein the synthetic early/late poxvirus promoter is at least 90% identical to SEQ ID NO: 20.

11. The recombinant oncolytic myxoma virus of claim 1 or 2, wherein the extracellular region of PD1 and IL-12 expression cassette(s) ~~is/~~are incorporated: (i) into the myxoma genome at the viral *M153R* open reading frame; or between the *M135* and *M136* genes.

12. The recombinant oncolytic myxoma virus of claim 11, wherein the extracellular region of PD1 is incorporated between the *M135* and *M136* genes.

13. The oncolytic virus of claim 11, wherein the IL-12 is incorporated into the myxoma genome at the viral *M153R* open reading frame.

14. The oncolytic virus of claim 11, further comprising a marker gene.

15. The oncolytic virus of claim 14, wherein IL-12 is fused to a transmembrane domain.

16. The oncolytic virus of claim 15, wherein the transmembrane domain is encoded by SEQ ID NO:12.

17. The oncolytic virus of claim 16, wherein the oncolytic virus is encoded by SEQ ID NO:13.

18. A pharmaceutical composition of the oncolytic virus of claim 1 or 2.

19. A method of treating a disease in a subject in need thereof comprising administering an effective amount of the oncolytic virus of claim 1 or 2, or the pharmaceutical composition of claim 18.

20. The method of claim 19, wherein the disease is cancer.

21. The method of claim 20, wherein the cancer has increased expression of programmed death-ligand 1 (PDL1).

22. The method of claim 20, wherein the subject has been determined to have a cancer that expresses increased PDL1.

23. The method of claim 20, wherein the cancer does not have increased expression of PDL1.

24. The method of claim 20, wherein the cancer is melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer.

25. The method of claim 20, wherein the cancer is melanoma.

26. The method of claim 25, wherein the melanoma is metastatic melanoma.

27. The method of claim 19, wherein the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally.

28. The method of claim 19, wherein the oncolytic virus is administered two or more times.

29. The method of claim 19, further comprising administering at least a second anti-cancer therapy to the subject.

30. The method of claim 29, wherein the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus.

31. The method of claim 29, wherein the second anti-cancer therapy is an immunomodulator.

32. The method of claim 29, wherein the second anti-cancer therapy is immunotherapy, chemotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy or cytokine therapy.

33. The method of claim 32, wherein the immunotherapy is immune checkpoint inhibitor therapy.

34. The method of claim 33, wherein the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4.

35. The method of claim 34, wherein the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab.

36. The method of claim 51, wherein the antibody is Pembrolizumab.

37. A method of treating a disease in a subject in need thereof comprising:

(a) testing the subject for overexpression of PDL1; and

(b) administering to a subject with increased expression of PDL1 a therapeutically effective amount of the oncolytic virus of claim 1 or 2.

38. The method of claim 37, wherein the disease is cancer.

39. The method of claim 38, wherein the cancer has increased expression of programmed death-ligand 1 (PDL1).

40. The method of claim 38, wherein the cancer does not have increased expression of PDL1.

41. The method of claim 38, wherein the cancer is melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer.

42. The method of claim 38, wherein the cancer is melanoma.

43. The method of claim 42, wherein the melanoma is metastatic melanoma.

44. The method of claim 37, wherein the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally.

45. The method of claim 37, wherein the oncolytic virus is administered two or more times.

46. The method of claim 37, further comprising administering at least a second anti-cancer therapy to the subject.

47. The method of claim 46, wherein the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus.

48. The method of claim 46, wherein the second anti-cancer therapy is an immunomodulator.

49. The method of claim 46, wherein the second anti-cancer therapy is chemotherapy, immunotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy or cytokine therapy.

50. The method of claim 49, wherein the immunotherapy is immune checkpoint inhibitor therapy.

51. The method of claim 50, wherein the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4.

52. The method of claim 51, wherein the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab.

53. A recombinant oncolytic virus comprising one or more expression cassettes encoding a soluble form of T-cell immunoglobulin and mucin-domain containing-3 (TIM3).

54. The oncolytic virus of claim 53, wherein the soluble TIM3 comprises an extracellular region of murine TIM3.

55. The oncolytic virus of claim 53, wherein the expression cassette(s) is/are under the control of one or more viral promoters.

56. The oncolytic virus of claim 56, wherein the one or more viral promoters is/are synthetic early/late poxvirus promoter.

57. The oncolytic virus of claim 53, wherein the virus is selected from the group consisting of myxoma virus, reovirus, herpes simplex virus, Newcastle Disease virus, measles virus, retrovirus, poxvirus, rhabdovirus, picornavirus, coxsackievirus and parvovirus.

58. The oncolytic virus of claim 57, wherein the oncolytic virus is myxoma virus.

59. The oncolytic virus of claim 58, wherein the is/are incorporated into the myxoma genome at the viral M153R open reading frame.

60. The oncolytic virus of claim 59, further comprising a marker gene.

61. The oncolytic virus of claim 60, wherein the marker gene is enhanced green fluorescent protein (eGFP).

62. A pharmaceutical composition of the oncolytic virus of claim 53.

63. A method of treating a disease in a subject in need thereof comprising administering an effective amount of the oncolytic virus of claim 53, or the pharmaceutical composition of claim 62.

64. The method of claim 63, wherein the disease is cancer.

65. The method of claim 70, wherein the cancer is melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer.

66. The method of claim 65, wherein the cancer is melanoma.

67. The method of claim 66, wherein the melanoma is metastatic melanoma.

68. The method of claim 67, wherein the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally.

69. The method of claim 68, wherein the oncolytic virus is administered two or more times.

70. The method of claim 69, further comprising administering at least a second anti-cancer therapy to the subject.

71. The method of claim 70, wherein the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus.

72. The method of claim 71, wherein the second anti-cancer therapy is an immunomodulator.

73. The method of claim 72, wherein the second anti-cancer therapy is immunotherapy, chemotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy or cytokine therapy.

74. The method of claim 73, wherein the immunotherapy is immune checkpoint inhibitor therapy.

75. The method of claim 74, wherein the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4.

76. The method of claim 75, wherein the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab.

77. A method of treating a disease in a subject in need thereof comprising:

(a) testing the subject for overexpression of GAL9; and

(b) administering to a subject with increased expression of GAL9 a therapeutically effective amount of the oncolytic virus of claim 53.

78. The method of claim 77, wherein the disease is cancer.

79. The method of claim 86, wherein the cancer is melanoma, kidney cancer, colorectal cancer, breast cancer, lung cancer, head and neck cancer, brain cancer, leukemia, prostate cancer, bladder cancer, and ovarian cancer.

80. The method of claim 79, wherein the cancer is melanoma.

81. The method of claim 80, wherein the melanoma is metastatic melanoma.

82. The method of claim 77, wherein the oncolytic virus is administered intra-arterially, intravenously, intraperitoneally, or intratumorally.

83. The method of claim 77, wherein the oncolytic virus is administered two or more times.

84. The method of claim 77, further comprising administering at least a second anti-cancer therapy to the subject.

85. The method of claim 84, wherein the second anti-cancer therapy is administered concurrently or sequentially with the recombinant virus.

86. The method of claim 84, wherein the second anti-cancer therapy is an immunomodulator.

87. The method of claim 84, wherein the second anti-cancer therapy is chemotherapy, immunotherapy, radiotherapy, gene therapy, surgery, hormonal therapy, anti-angiogenic therapy or cytokine therapy.

88. The method of claim 87, wherein the immunotherapy is immune checkpoint inhibitor therapy.

89. The method of claim 88, wherein the immune checkpoint inhibitor therapy comprises treatment with an antibody directed to PD1, PDL1, or CTLA4.

90. The method of claim 89, wherein the antibody is Pembrolizumab, Nivolumab, Atezolizumab, Avelumab, Durvalumab, or Ipilimumab.

91. The method of claim 89, wherein the antibody is Pembrolizumab.

92. The recombinant oncolytic myxoma virus of claim 6, wherein the extracellular region of human PD1 and IL-12 are encoded in more than one expression cassette.

93. The recombinant oncolytic myxoma virus of claim 92, wherein IL-12 is encoded as fusion protein, wherein IL-12 alpha subunit and IL-12 beta subunit are linked via a flexible linker.

94. The recombinant oncolytic myxoma virus of claim 93, wherein the flexible linker has the sequence $(GGGGS)_n$.

95. The recombinant oncolytic myxoma virus of claim 92, wherein the IL-12 alpha subunit and IL-12 beta subunit are expressed from two separate DNA constructs.

96. The recombinant oncolytic myxoma virus of claim 92, wherein the IL-12 alpha subunit and IL-12 beta subunit are expressed from a single DNA construct under the control of separate promoters.

97. The recombinant oncolytic myxoma virus of claim 92, wherein the IL-12 alpha subunit and IL-12 beta subunit are expressed from a single DNA construct under the control of separate promoters.

98. The recombinant oncolytic myxoma virus of claim 1, wherein the IL-12 comprises a sequence at least 90% homologous to SEQ ID NOs: 7 and 8.

99. The recombinant oncolytic myxoma virus of claim 2, wherein the mutant soluble form of PD1 comprises a sequence at least 90% homologous to SEQ ID NO: 5.

100. The recombinant oncolytic myxoma virus of claim 95, wherein the IL-12 alpha subunit comprises a sequence at least 90% homologous to SEQ ID NO: 7, and the IL-12 beta subunit a comprises a sequence at least 90% homologous to SEQ ID NO 8.

STATEMENT UNDER ARTICLE 19(1)

Claim 1 and dependent claims have been amended to focus the claims on recombinant oncolytic myxoma viruses that comprising one or more expression cassettes encoding (a) a soluble form of programmed cell death protein 1 (PD1), and (b) interleukin 12 (IL-12). Furthermore additional claims dependent on claim 1 have been added. These are the only claims that were subject to search by the International Search Authority. Claims not subject to the search have merely been amended to reflect changes in the numbering.

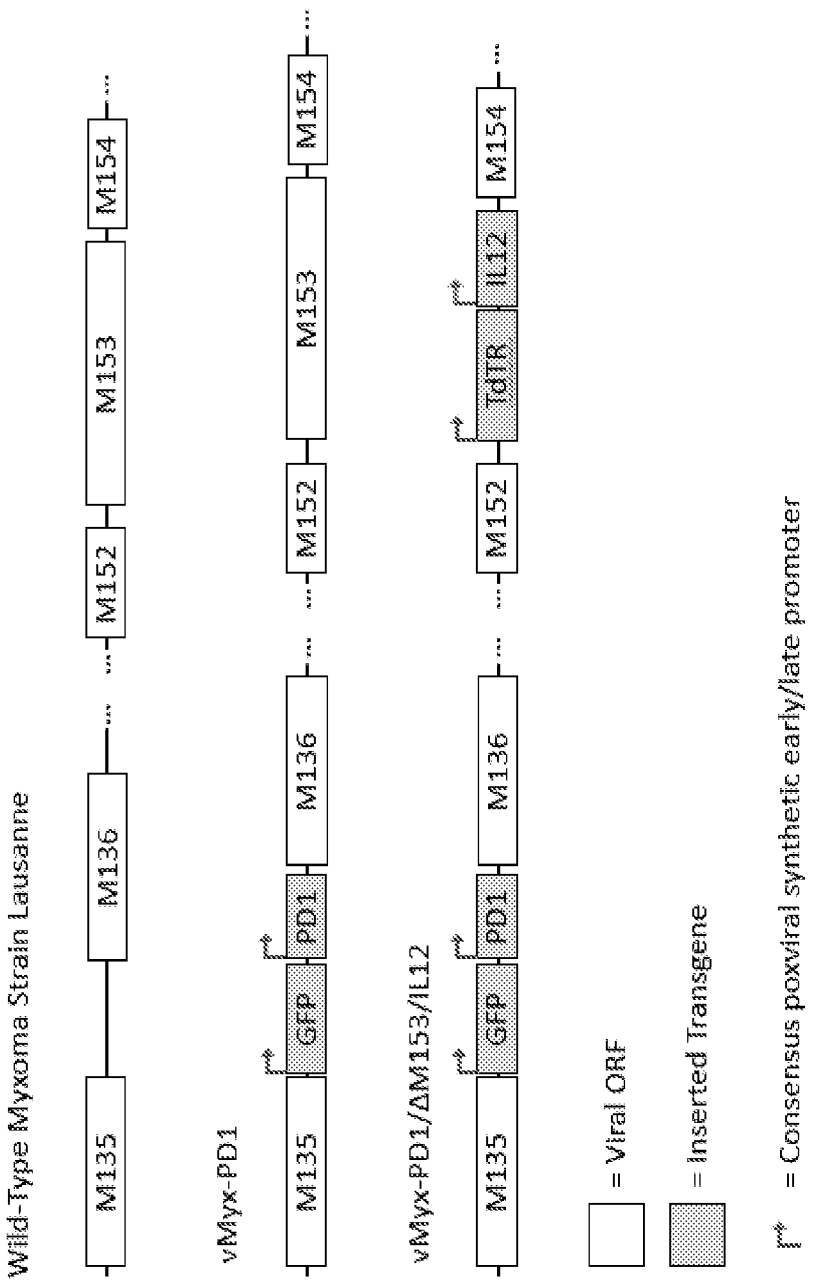

Wild-Type Myxoma Strain Lausanne
M135
M136
M152
M153
M154
vMyx-PD1
M135
GFP
PD1
M136
M152
M153
M154
vMyx-PD1/ΔM153/IL12
M135
GFP
PD1
M136
M152
TdTR
IL12
M154
= Viral ORF
= Inserted Transgene
= Consensus poxviral synthetic early/late promoter

FIG. 1

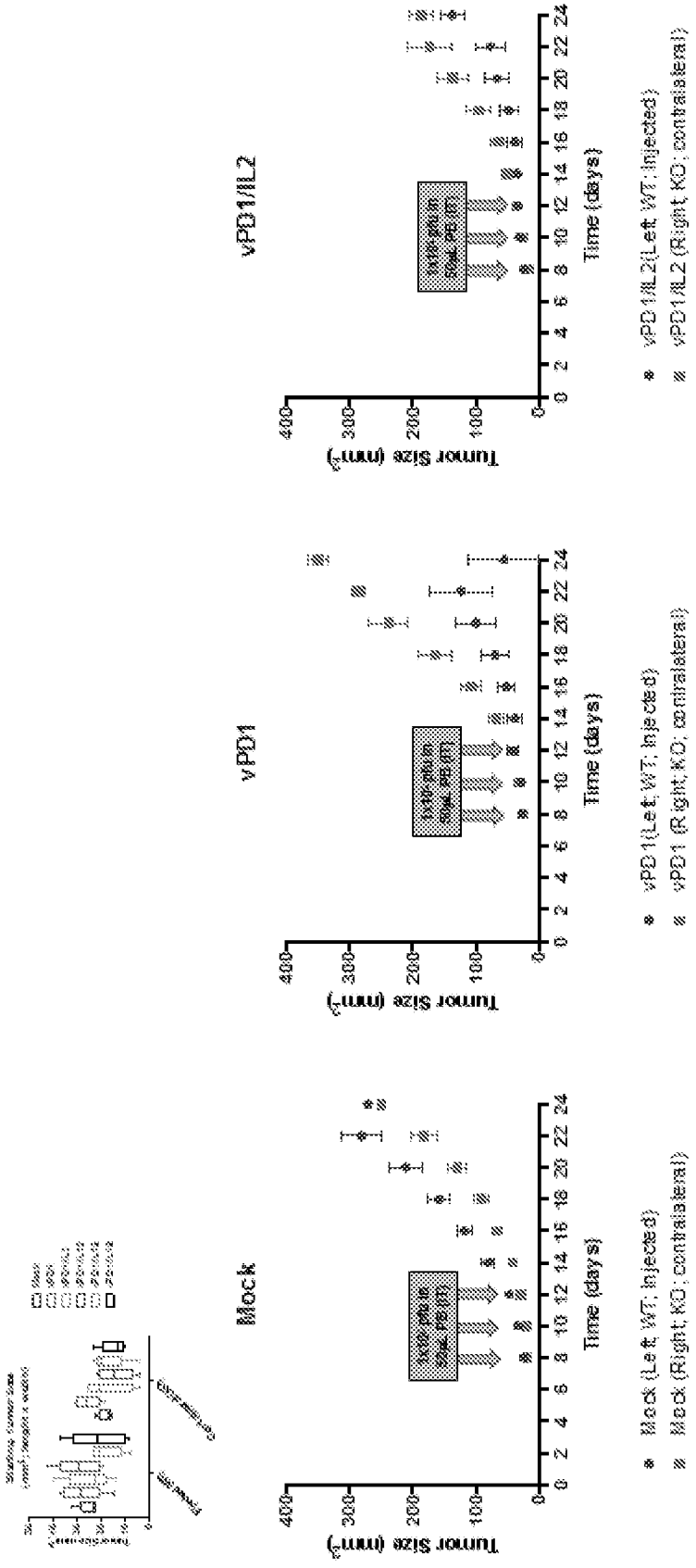

Mock
vPD1
vPD1/IL2
Tumor Size (mm²)
Time (days)
Mock (Left, WT; injected)
Mock (Right, KO; contralateral)
vPD1 (Left, WT; injected)
vPD1 (Right, KO; contralateral)
vPD1/IL2 (Left, WT; injected)
vPD1/IL2 (Right, KO; contralateral)

FIG. 2

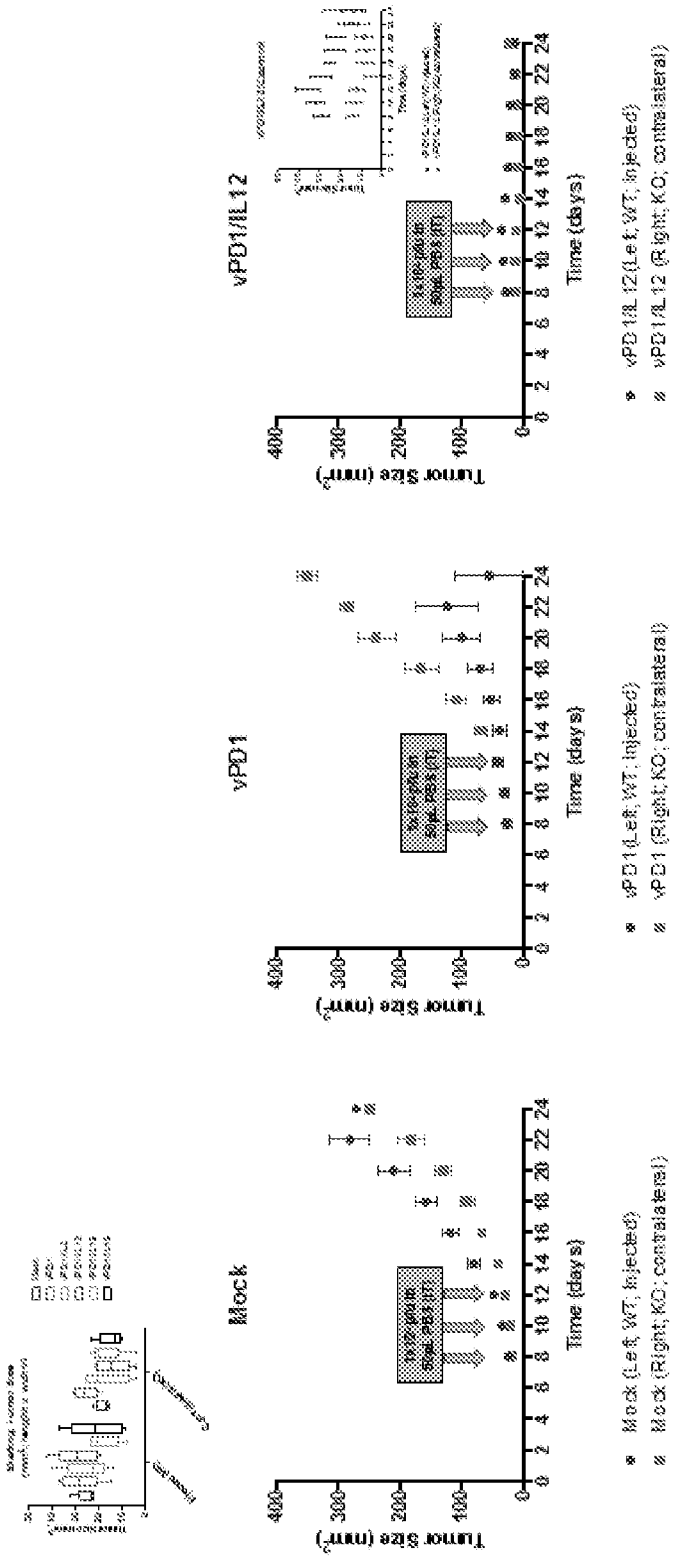

Mock
vPD1
vPD1/IL12
Tumor Size (mm²)
Time (days)
Mock (Left, WT; injected)
Mock (Right, KO; contralateral)
vPD1 (Left, WT; injected)
vPD1 (Right, KO; contralateral)
vPD1/IL12 (Left, WT; injected)
vPD1/IL12 (Right, KO; contralateral)

FIG. 3

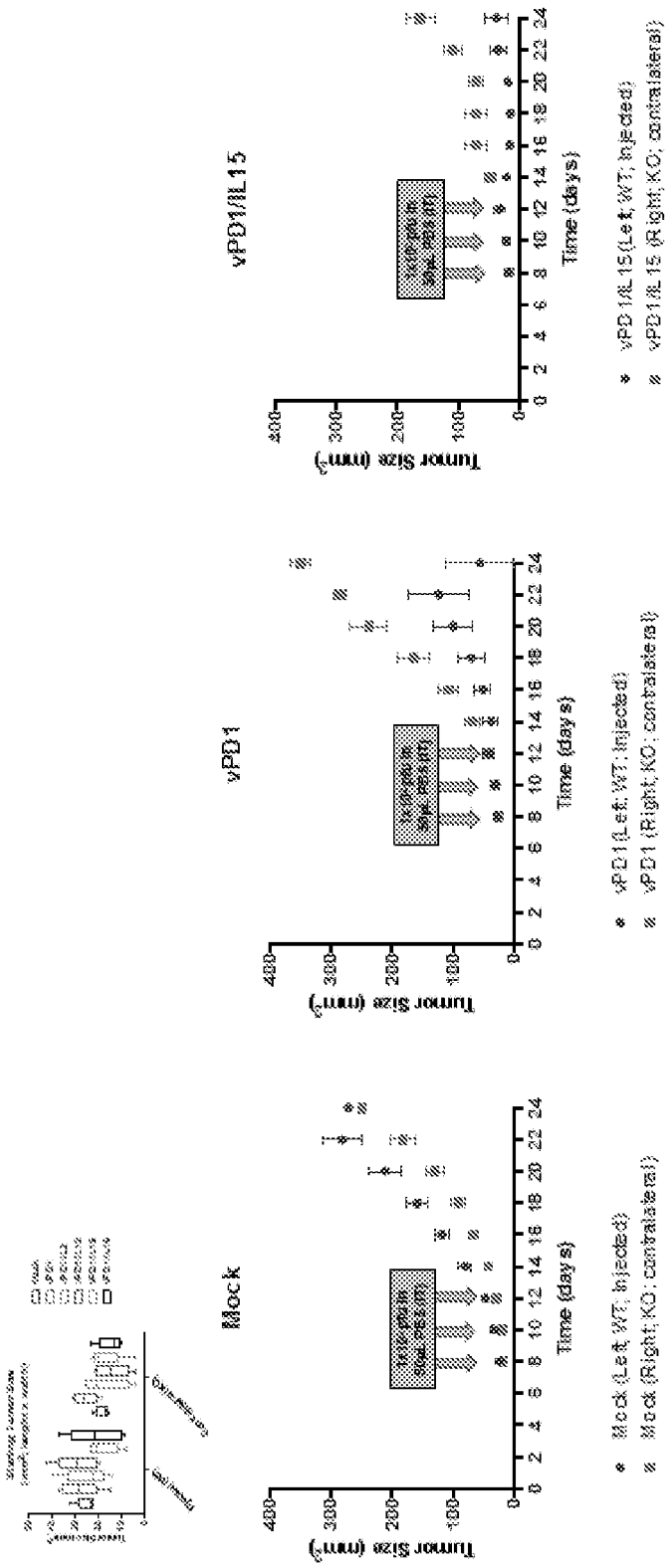

vPD1/IL15
vPD1
Mock
Tumor Size (mm³)
Time (days)
vPD1/IL15 (Left; WT; injected)
vPD1/IL15 (Right; KO; contralateral)
vPD1 (Left; WT; injected)
vPD1 (Right; KO; contralateral)
Mock (Left; WT; injected)
Mock (Right; KO; contralateral)

FIG. 4

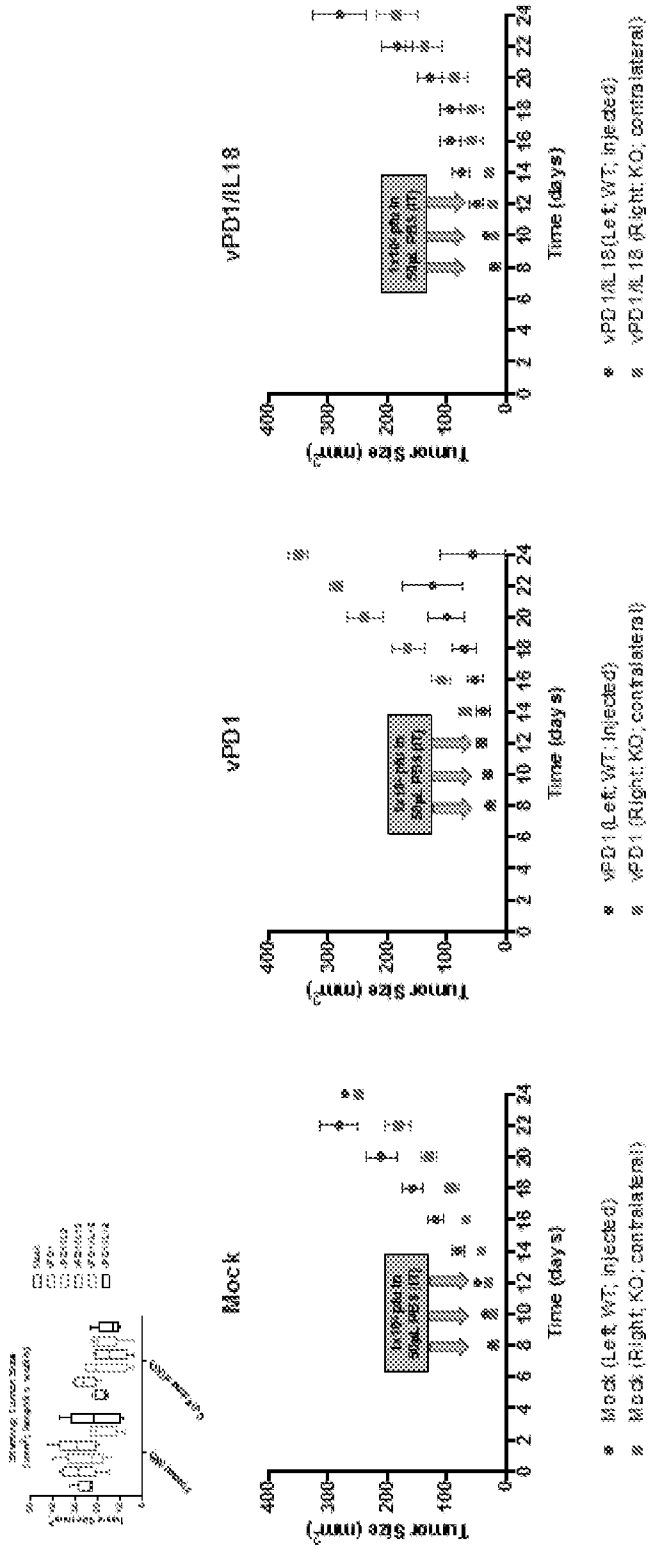

Mock
vPD1
vPD1/IL18
Tumor Size (mm²)
Time (days)
Mock (Left, WT; injected)
Mock (Right; KO; contralateral)
vPD1 (Left; WT; injected)
vPD1 (Right; KO; contralateral)
vPD1/IL18 (Left; WT; injected)
vPD1/IL18 (Right; KO; contralateral)

FIG. 5

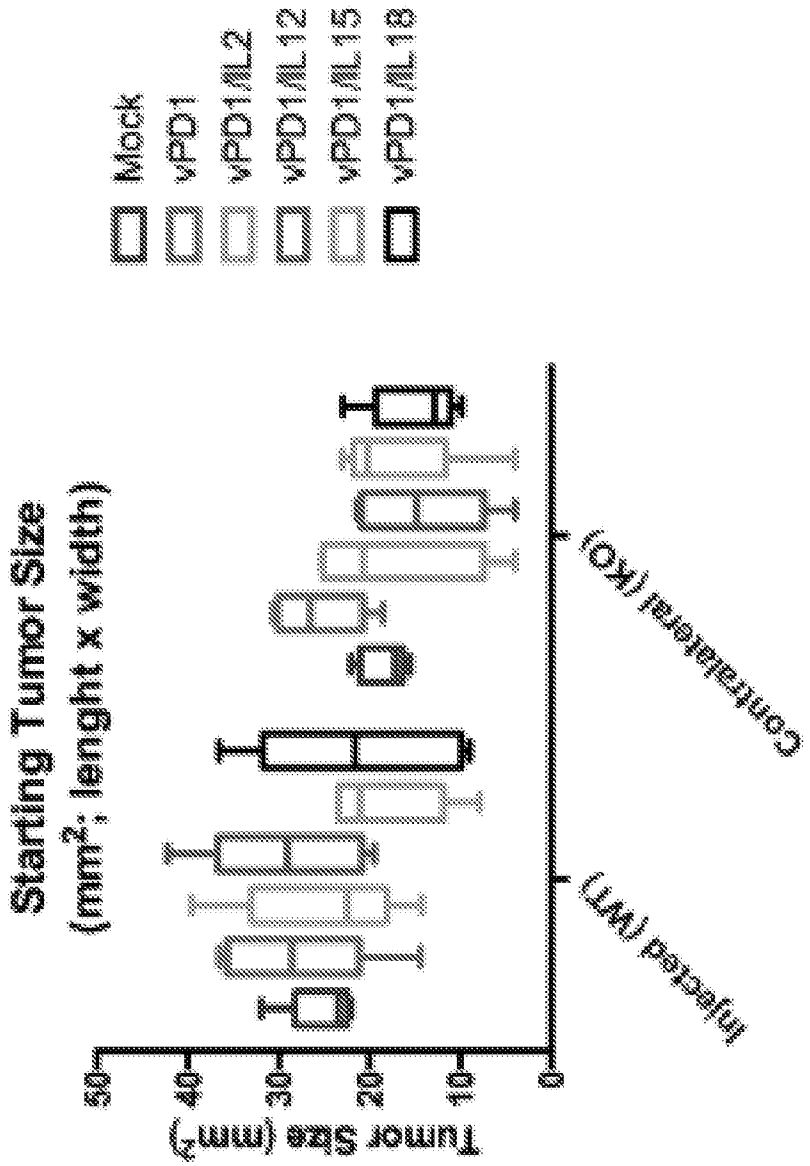

Mock
vPD1
vPD1/IL2
vPD1/IL12
vPD1/IL15
vPD1/IL18
Starting Tumor Size
(mm²; lenght x width)
Tumor Size (mm²)
50
40
30
20
10
0
Injected (IM)
Contralateral (KO)

FIG. 6

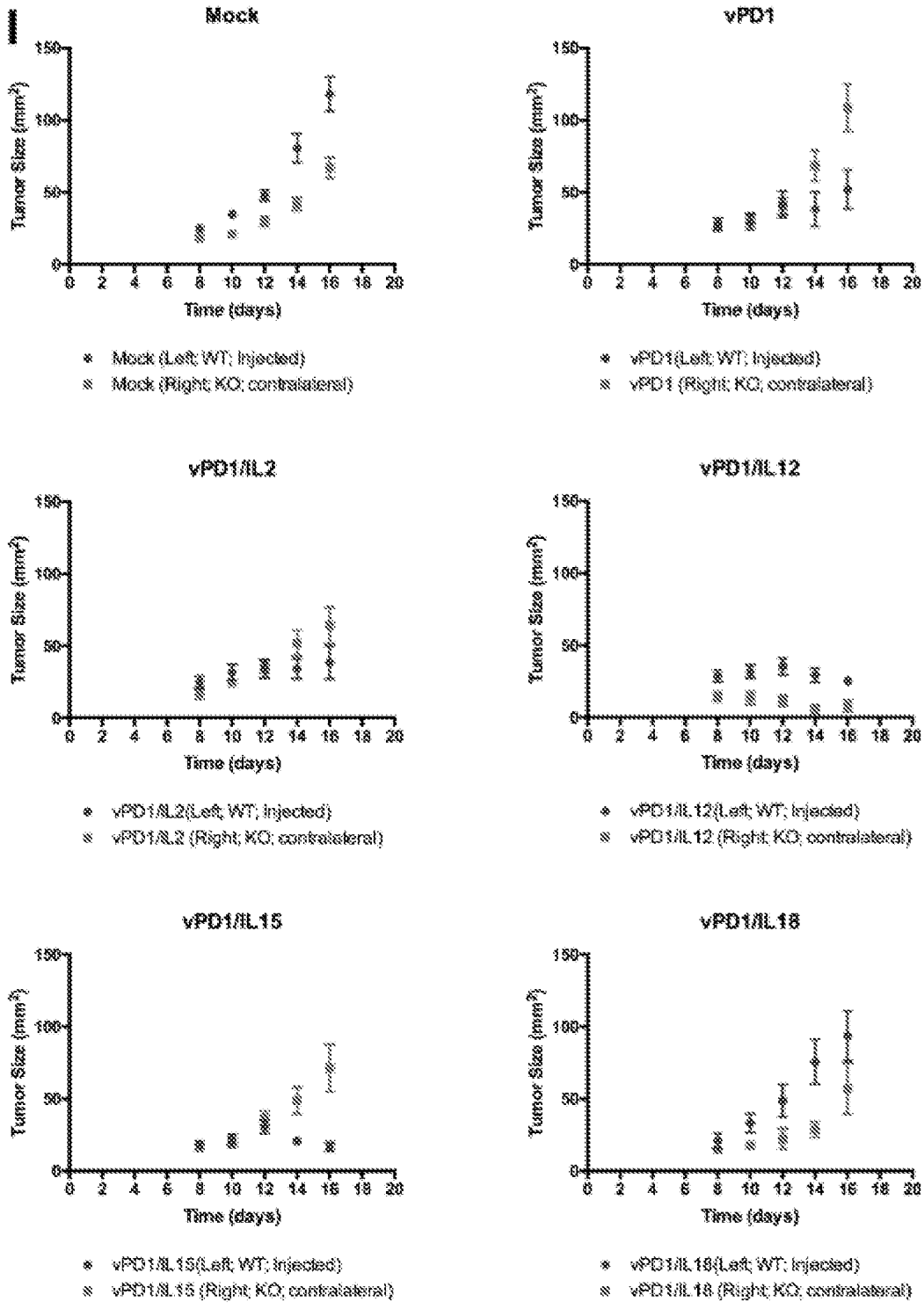
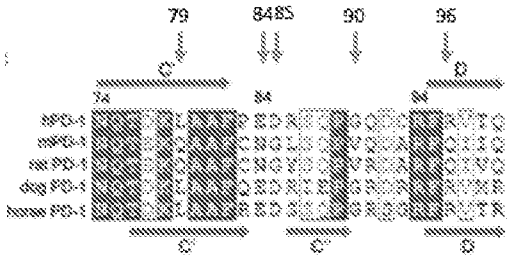

Mock
Tumor Size (mm²)
Time (days)
Mock (Left; WT; Injected)
Mock (Right; KO; contralateral)
vPD1
Tumor Size (mm²)
Time (days)
vPD1(Left; WT; Injected)
vPD1 (Right; KO; contralateral)
vPD1/IL2
Tumor Size (mm²)
Time (days)
vPD1/IL2(Left; WT; Injected)
vPD1/IL2 (Right; KO; contralateral)
vPD1/IL12
Tumor Size (mm²)
Time (days)
vPD1/IL12(Left; WT; Injected)
vPD1/IL12 (Right; KO; contralateral)
vPD1/IL15
Tumor Size (mm²)
Time (days)
vPD1/IL15(Left; WT; Injected)
vPD1/IL15 (Right; KO; contralateral)
vPD1/IL18
Tumor Size (mm²)
Time (days)
vPD1/IL18(Left; WT; Injected)
vPD1/IL18 (Right; KO; contralateral)

FIG. 7



Amino acid	Wild type	Mutation
79	Leucine (L)	Glutamine (Q)
84	Glutamic acid (E)	Asparagine (N)
85	Aspartic acid (D)	Glycine (G)
90	Glycine (G)	Valine (V)
96	Arginine (R)	Glutamine (Q)

FIG. 8

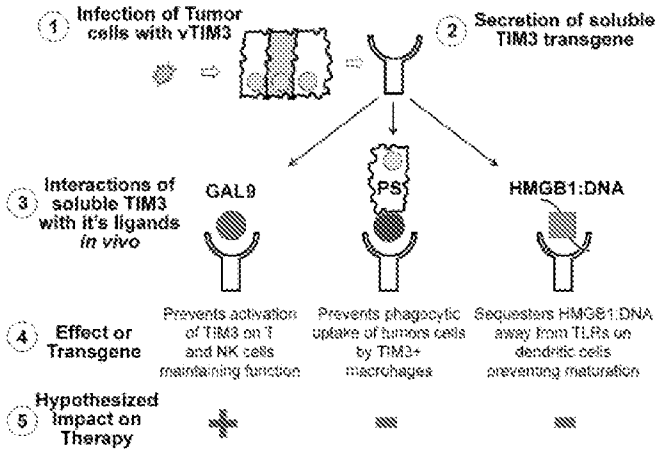


FIG. 9

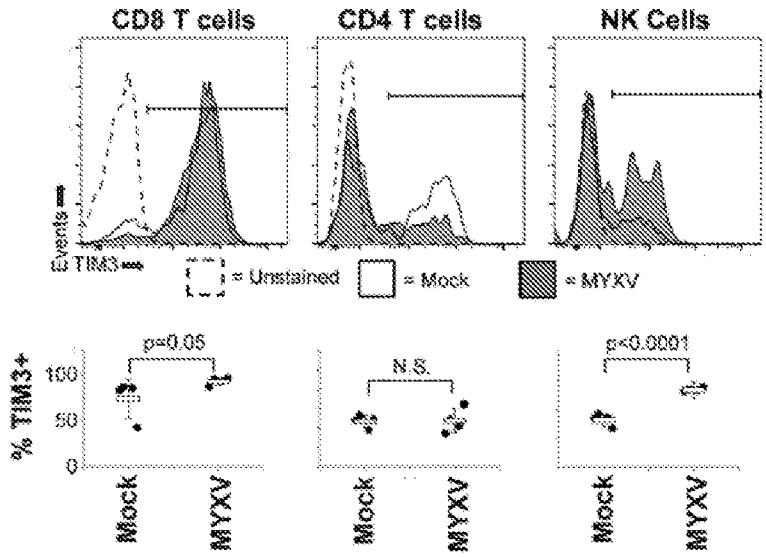
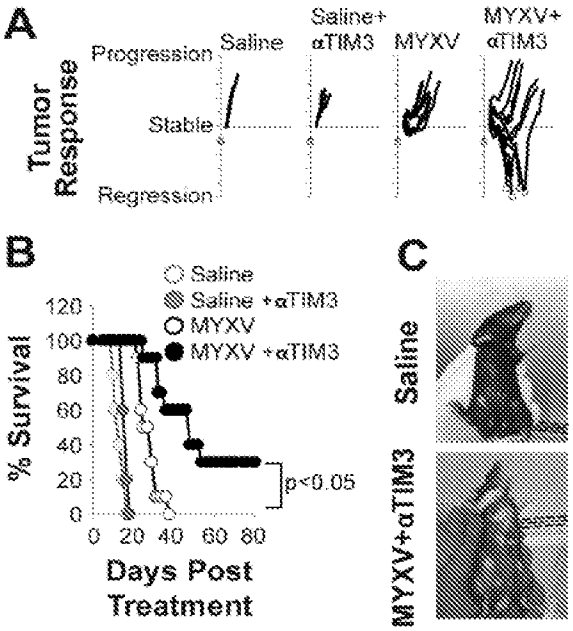
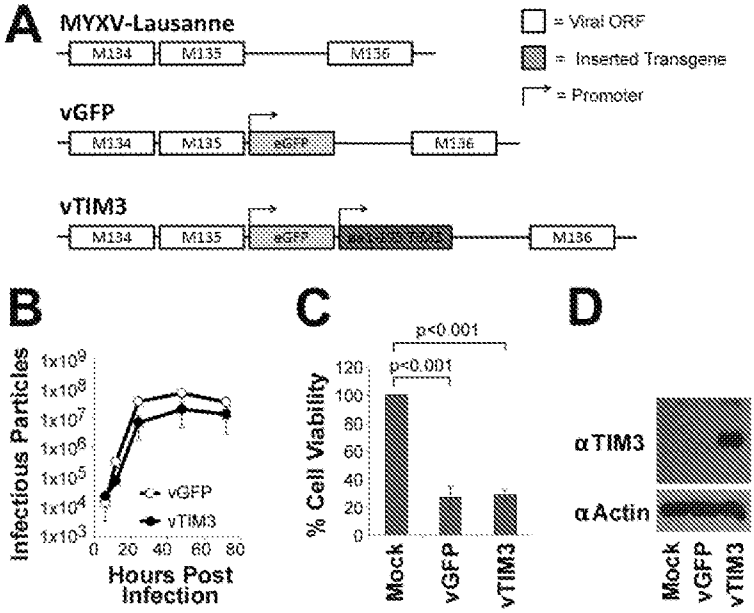


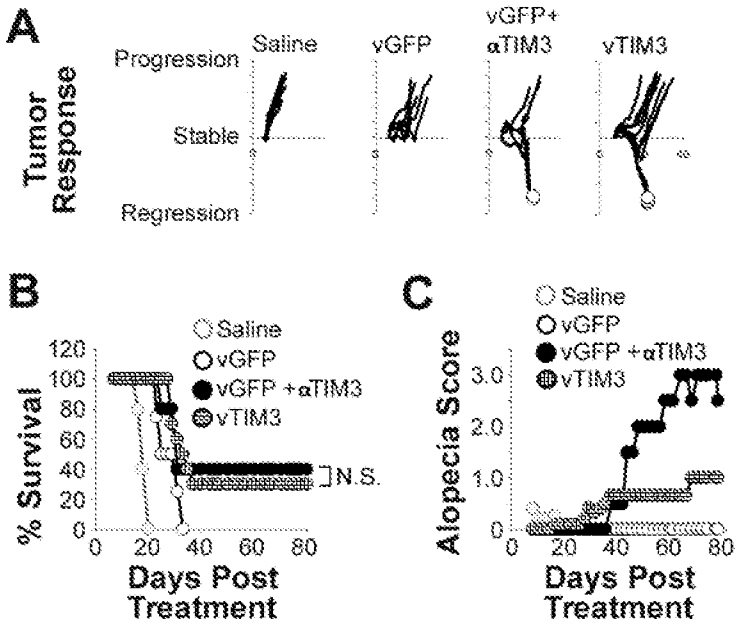
FIG. 10



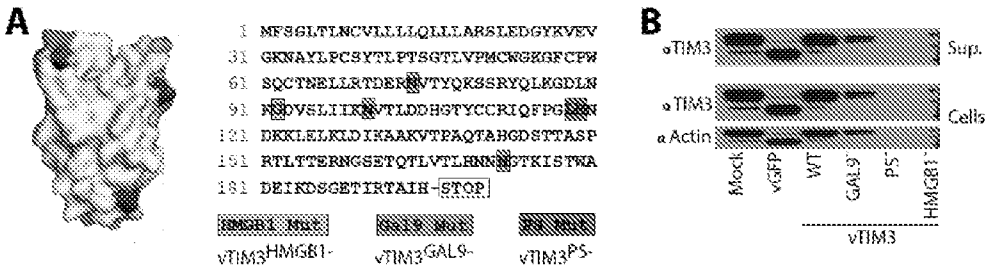
FIGS. 11A-11C



FIGS. 12A-12D



FIGS. 13A-13C



FIGS. 14A-14B

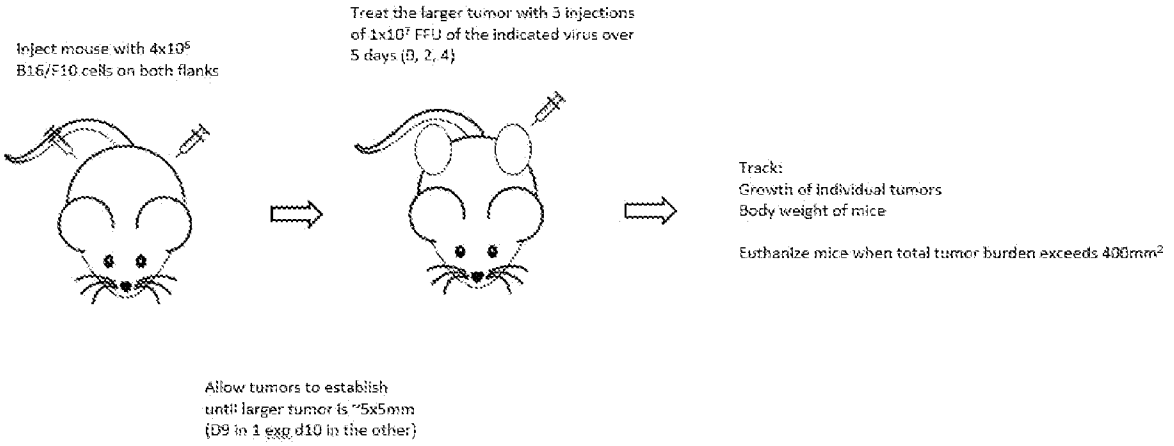


FIG. 15A

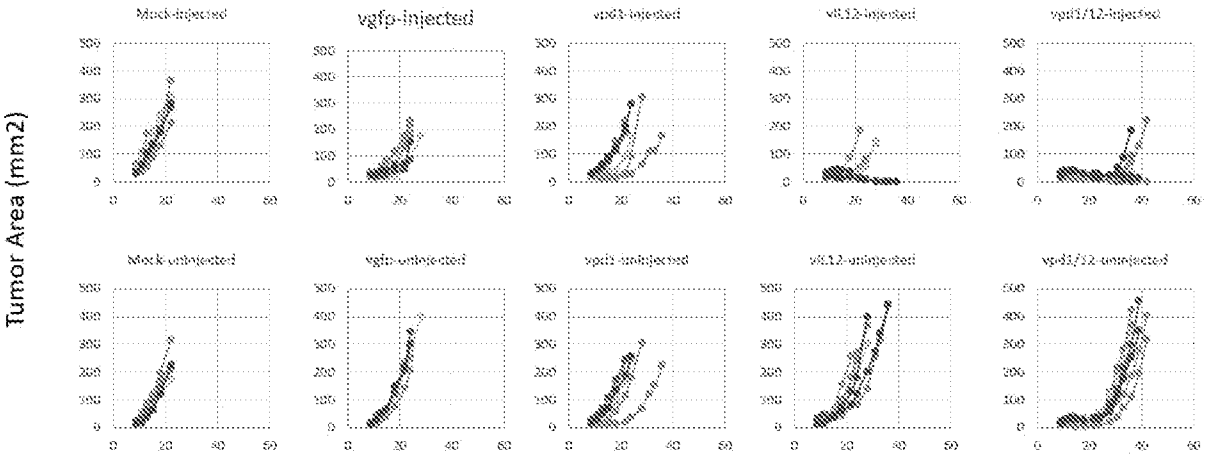


FIG. 15B

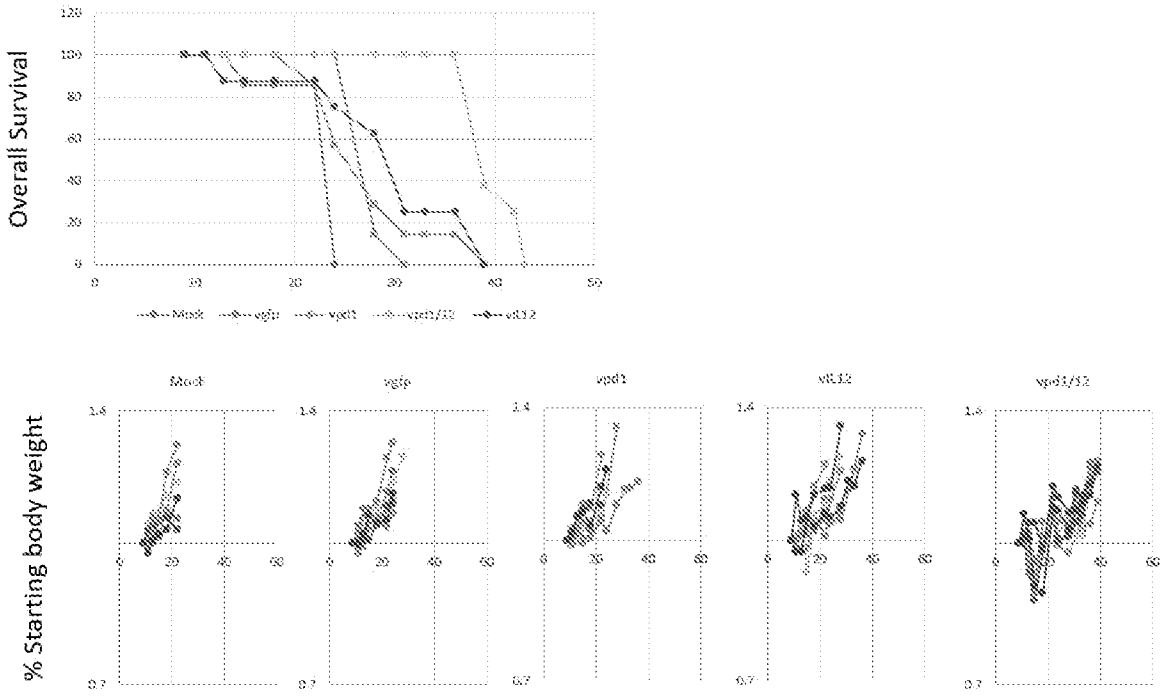
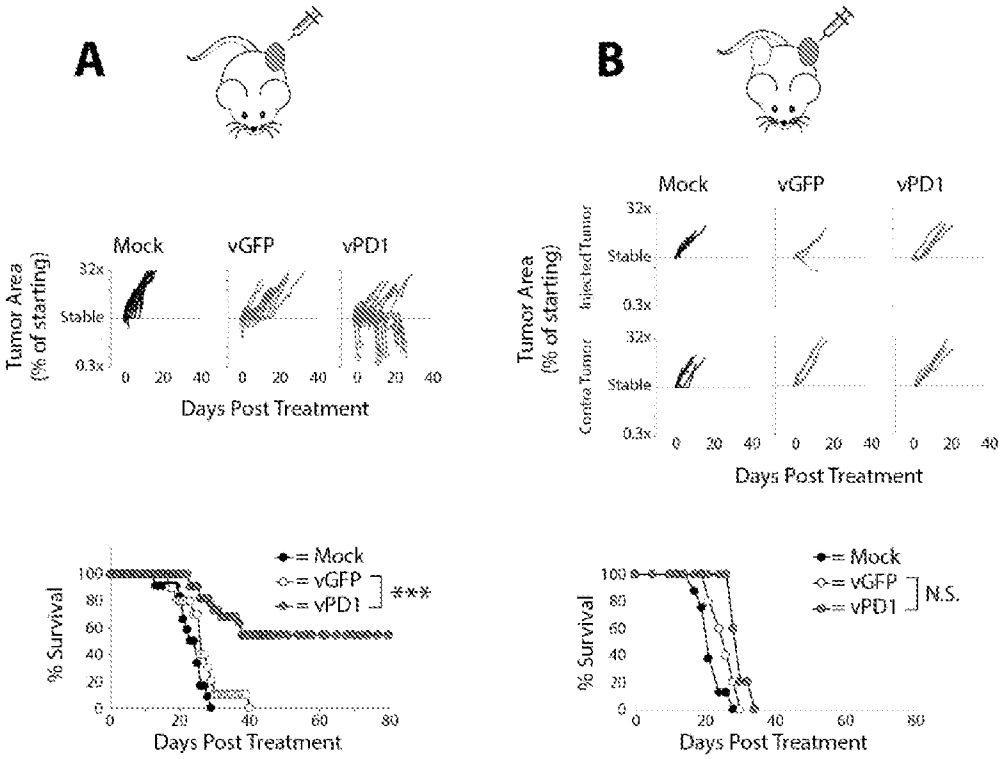
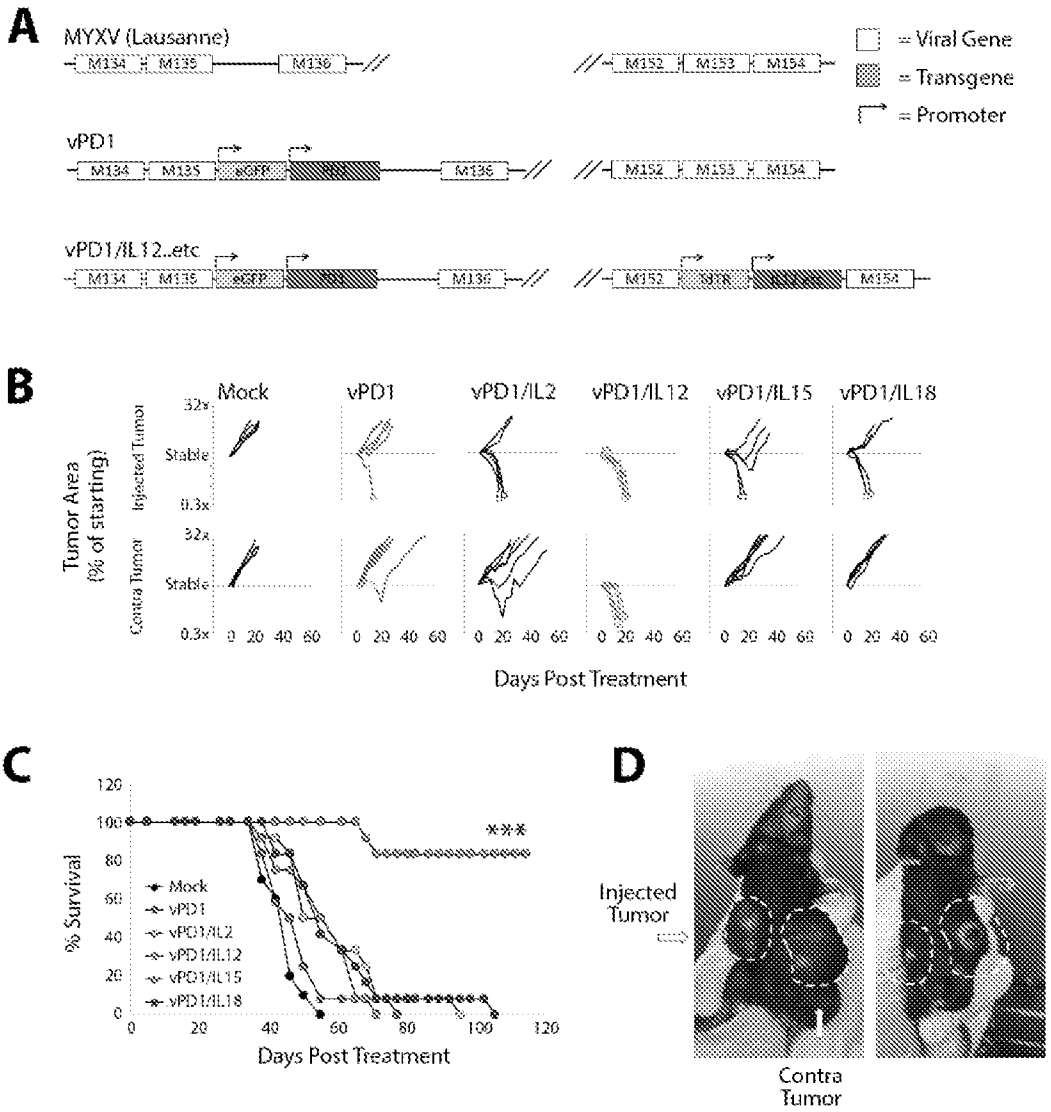

Overall Survival
Mock
vgfp
vgd1
vgd1/12
vd12
% Starting body weight

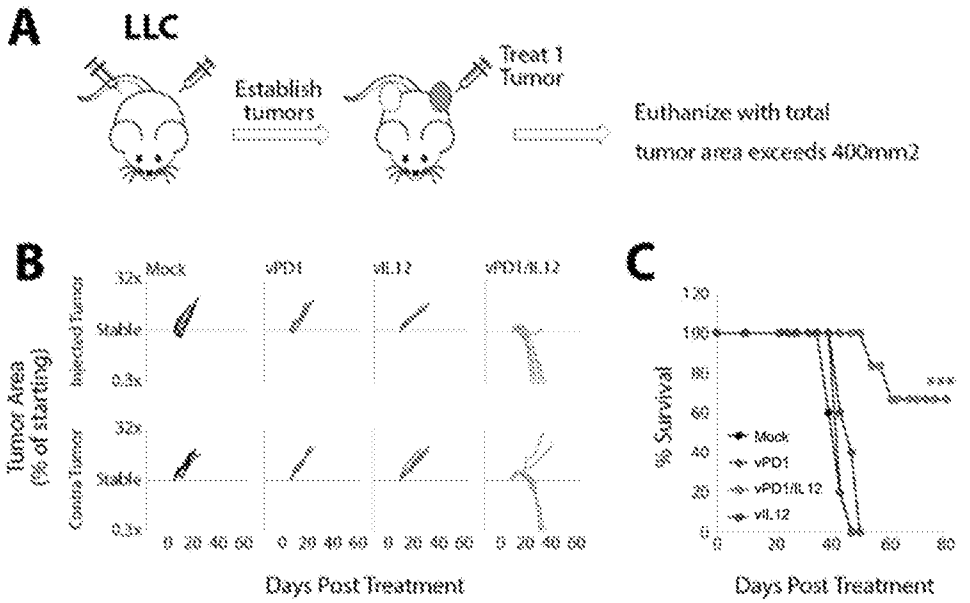
FIG. 15C


A
B
Mock
vGFP
vPD1
Tumor Area (% of starting)
32x
Stable
0.3x
Injected Tumor
Contra Tumor
0
20
40
Days Post Treatment
% Survival
= Mock
= vGFP
= vPD1
***
N.S.
60
80
100

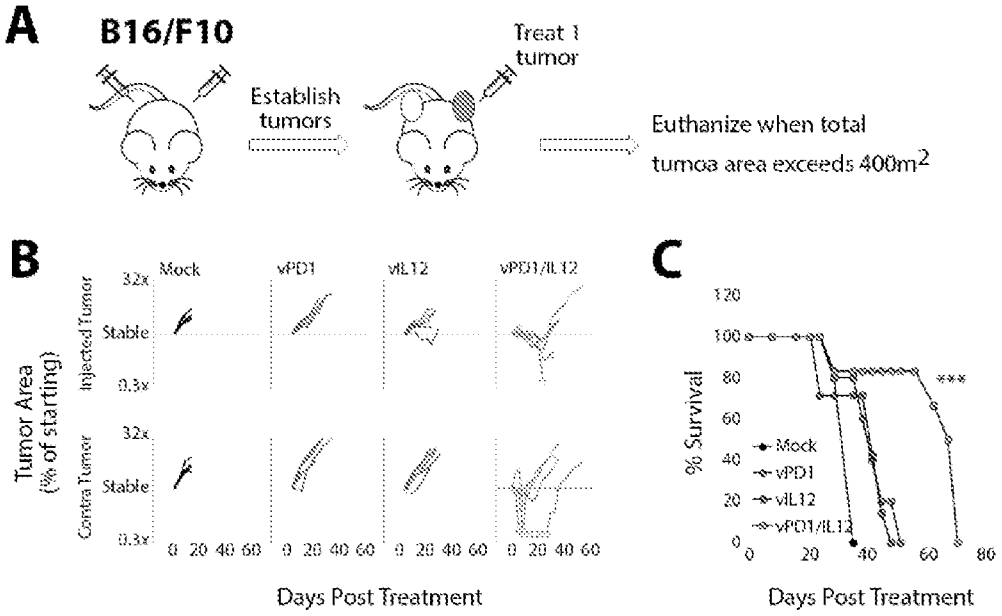
FIGS. 16A-B


A
MYXV (Lausanne)
M134
M135
M136
M152
M153
M154
= Viral Gene
= Transgene
= Promoter
vPD1
eGFP
vPD1/IL12..etc
B
Mock
vPD1
vPD1/IL2
vPD1/IL12
vPD1/IL15
vPD1/IL18
Tumor Area (% of starting)
Injected Tumor
Contra Tumor
32x
Stable
0.3x
0 20 40 60
Days Post Treatment
C
% Survival
***
Mock
vPD1
vPD1/IL2
vPD1/IL12
vPD1/IL15
vPD1/IL18
Days Post Treatment
D
Injected Tumor
Contra Tumor

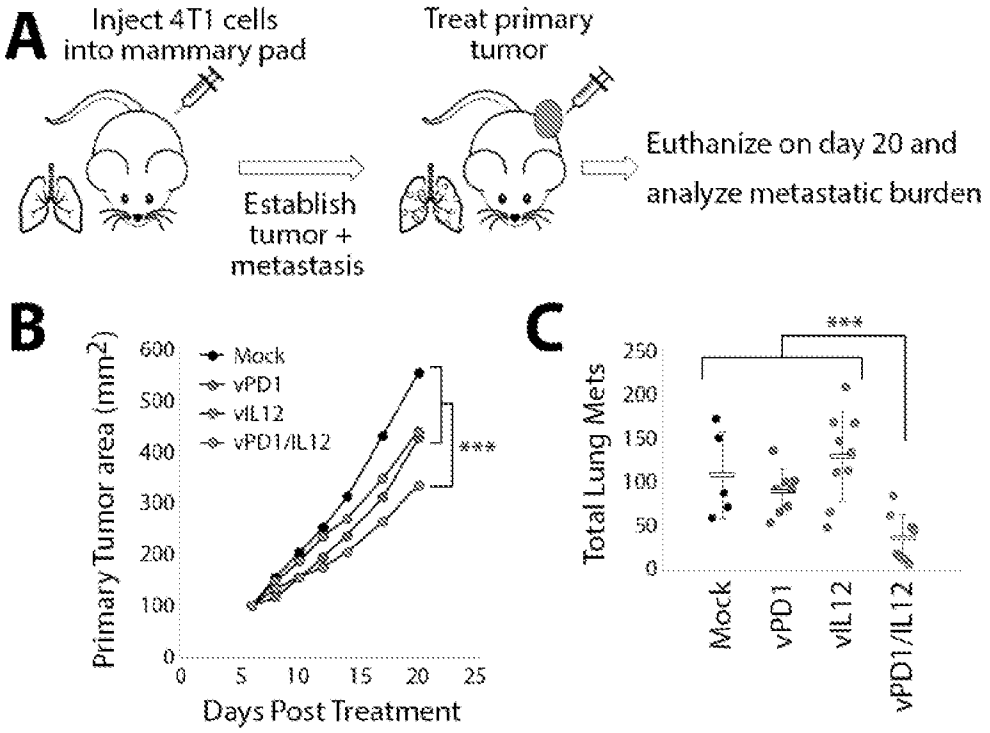
FIGS. 17A-D



FIGS. 18A-C



FIGS. 19A-C


A
Inject 4T1 cells into mammary pad
Establish tumor + metastasis
Treat primary tumor
Euthanize on day 20 and analyze metastatic burden
B
Primary Tumor area (mm2)
Mock
vPD1
vIL12
vPD1/IL12
***
Days Post Treatment
C
Total Lung Mets
***
Mock
vPD1
vIL12
vPD1/IL12

FIGS. 20A-C

SEQUENCE LISTING

<110> MEDICAL UNIVERSITY OF SOUTH CAROLINA
SYSTEMS ONCOLOGY, LLC

<120> RECOMBINANT MYXOMA VIRUSES AND USES THEREOF

<130> MESC.P0113WO

<150> US 62/718,990
<151> 2018-08-16

<150> US 62/741,404
<151> 2018-10-04

<150> US 62/754,622
<151> 2018-11-02

<150> US 62/813,375
<151> 2019-03-04

<160> 20

<170> PatentIn version 3.5

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<220>
<223> Synthetic oligonucleotide

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tcccacaggc gcc 73

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ctgctcgtgg tgaccgaagg ggacaacgcc accttcacct gcagcttctc caacacatcg 180

gagagcttcg tgctaaactg gtaccgcatg agccccagca accagacgga caagctggcc 240

gctttccccg aggaccgcag ccagcccggc caggactgcc gcttccgtgt cacacaactg 300

cccaacgggc gtgacttcca catgagcgtg gtcagggccc ggcgcaatga cagcggcacc 360

tacctctgtg gggccatctc cctggccccc aaggcgcaga tcaaagagag cctgcgggca 420

gagctcaggg tgacagagag aagggcagaa gtgcccacag cccaccccag cccctcaccc 480

aggccagccg gccagttcca aacc 504

<210> 4
<211> 168
<212> PRT
<213> Artificial sequence

<220>
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Met Gln Ile Pro Gln Ala Pro Trp Pro Val Val Trp Ala Val Leu Gln
1 5 10 15

Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp Ser Pro Asp Arg Pro Trp
20 25 30

Asn Pro Pro Thr Phe Ser Pro Ala Leu Leu Val Val Thr Glu Gly Asp
35 40 45

Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn Thr Ser Glu Ser Phe Val
50 55 60

Leu Asn Trp Tyr Arg Met Ser Pro Ser Asn Gln Thr Asp Lys Leu Ala
65 70 75 80

Ala Phe Pro Glu Asp Arg Ser Gln Pro Gly Gln Asp Cys Arg Phe Arg
85 90 95

Val Thr Gln Leu Pro Asn Gly Arg Asp Phe His Met Ser Val Val Arg
100 105 110

Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu Cys Gly Ala Ile Ser Leu
115 120 125

Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu Arg Ala Glu Leu Arg Val
130 135 140

Thr Glu Arg Arg Ala Glu Val Pro Thr Ala His Pro Ser Pro Ser Pro
145 150 155 160

Arg Pro Ala Gly Gln Phe Gln Thr
165

<210> 5
<211> 168
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<213> Artificial sequence

<220>
<223> Synthetic polypeptide

<400> 5

Met Gln Ile Pro Gln Ala Pro Trp Pro Val Val Trp Ala Val Leu Gln
1 5 10 15

Leu Gly Trp Arg Pro Gly Trp Phe Leu Asp Ser Pro Asp Arg Pro Trp
20 25 30

```
Asn Pro Pro Thr Phe Ser Pro Ala Leu Leu Val Val Thr Glu Gly Asp
        35                  40                  45

Asn Ala Thr Phe Thr Cys Ser Phe Ser Asn Thr Ser Glu Ser Phe Val
    50                  55                  60

Leu Asn Trp Tyr Arg Met Ser Pro Ser Asn Gln Thr Asp Lys Leu Ala
65                  70                  75                  80

Ala Phe Pro Glu Gly Arg Ser Gln Pro Gly Gln Asp Cys Arg Phe Arg
                85                  90                  95

Val Thr Gln Leu Pro Asn Gly Arg Asp Phe His Met Ser Val Val Arg
            100                 105                 110

Ala Arg Arg Asn Asp Ser Gly Thr Tyr Leu Cys Gly Ala Ile Ser Leu
        115                 120                 125

Ala Pro Lys Ala Gln Ile Lys Glu Ser Leu Arg Ala Glu Leu Arg Val
    130                 135                 140

Thr Glu Arg Arg Ala Glu Val Pro Thr Ala His Pro Ser Pro Ser Pro
145                 150                 155                 160

Arg Pro Ala Gly Gln Phe Gln Thr
                165
```

<210> 6
<211> 153
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide

<400> 6

```
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1               5                   10                  15
```

```
Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu
            20                  25                  30

Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile
        35                  40                  45

Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe
    50                  55                  60

Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu
65                  70                  75                  80

Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys
                85                  90                  95

Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile
            100                 105                 110

Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala
        115                 120                 125

Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe
    130                 135                 140

Cys Gln Ser Ile Ile Ser Thr Leu Thr
145                 150
```

<210> 7
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<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide

<400> 7

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Met Cys Pro Ala Arg Ser Leu Leu Leu Val Ala Thr Leu Val Leu Leu
1               5                   10                  15
```

```
Asp His Leu Ser Leu Ala Arg Asn Leu Pro Val Ala Thr Pro Asp Pro
            20                  25                  30

Gly Met Phe Pro Cys Leu His His Ser Gln Asn Leu Leu Arg Ala Val
        35                  40                  45

Ser Asn Met Leu Gln Lys Ala Arg Gln Thr Leu Glu Phe Tyr Pro Cys
    50                  55                  60

Thr Ser Glu Glu Ile Asp His Glu Asp Ile Thr Lys Asp Lys Thr Ser
65                  70                  75                  80

Thr Val Glu Ala Cys Leu Pro Leu Glu Leu Thr Lys Asn Glu Ser Cys
                85                  90                  95

Leu Asn Ser Arg Glu Thr Ser Phe Ile Thr Asn Gly Ser Cys Leu Ala
            100                 105                 110

Ser Arg Lys Thr Ser Phe Met Met Ala Leu Cys Leu Ser Ser Ile Tyr
        115                 120                 125

Glu Asp Leu Lys Met Tyr Gln Val Glu Phe Lys Thr Met Asn Ala Lys
    130                 135                 140

Leu Leu Met Asp Pro Lys Arg Gln Ile Phe Leu Asp Gln Asn Met Leu
145                 150                 155                 160

Ala Val Ile Asp Glu Leu Met Gln Ala Leu Asn Phe Asn Ser Glu Thr
                165                 170                 175

Val Pro Gln Lys Ser Ser Leu Glu Glu Pro Asp Phe Tyr Lys Thr Lys
            180                 185                 190

Ile Lys Leu Cys Ile Leu Leu His Ala Phe Arg Ile Arg Ala Val Thr
        195                 200                 205

Ile Asp Arg Val Met Ser Tyr Leu Asn Ala Ser
```

210 215

<210> 8
<211> 328
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide

<400> 8

Met Cys His Gln Gln Leu Val Ile Ser Trp Phe Ser Leu Val Phe Leu
1 5 10 15

Ala Ser Pro Leu Val Ala Ile Trp Glu Leu Lys Lys Asp Val Tyr Val
20 25 30

Val Glu Leu Asp Trp Tyr Pro Asp Ala Pro Gly Glu Met Val Val Leu
35 40 45

Thr Cys Asp Thr Pro Glu Glu Asp Gly Ile Thr Trp Thr Leu Asp Gln
50 55 60

Ser Ser Glu Val Leu Gly Ser Gly Lys Thr Leu Thr Ile Gln Val Lys
65 70 75 80

Glu Phe Gly Asp Ala Gly Gln Tyr Thr Cys His Lys Gly Gly Glu Val
85 90 95

Leu Ser His Ser Leu Leu Leu Leu His Lys Lys Glu Asp Gly Ile Trp
100 105 110

Ser Thr Asp Ile Leu Lys Asp Gln Lys Glu Pro Lys Asn Lys Thr Phe
115 120 125

Leu Arg Cys Glu Ala Lys Asn Tyr Ser Gly Arg Phe Thr Cys Trp Trp
130 135 140

Leu Thr Thr Ile Ser Thr Asp Leu Thr Phe Ser Val Lys Ser Ser Arg

145 150 155 160

Gly Ser Ser Asp Pro Gln Gly Val Thr Cys Gly Ala Ala Thr Leu Ser
165 170 175

Ala Glu Arg Val Arg Gly Asp Asn Lys Glu Tyr Glu Tyr Ser Val Glu
180 185 190

Cys Gln Glu Asp Ser Ala Cys Pro Ala Ala Glu Glu Ser Leu Pro Ile
195 200 205

Glu Val Met Val Asp Ala Val His Lys Leu Lys Tyr Glu Asn Tyr Thr
210 215 220

Ser Ser Phe Phe Ile Arg Asp Ile Ile Lys Pro Asp Pro Pro Lys Asn
225 230 235 240

Leu Gln Leu Lys Pro Leu Lys Asn Ser Arg Gln Val Glu Val Ser Trp
245 250 255

Glu Tyr Pro Asp Thr Trp Ser Thr Pro His Ser Tyr Phe Ser Leu Thr
260 265 270

Phe Cys Val Gln Val Gln Gly Lys Ser Lys Arg Glu Lys Lys Asp Arg
275 280 285

Val Phe Thr Asp Lys Thr Ser Ala Thr Val Ile Cys Arg Lys Asn Ala
290 295 300

Ser Ile Ser Val Arg Ala Gln Asp Arg Tyr Tyr Ser Ser Ser Trp Ser
305 310 315 320

Glu Trp Ala Ser Val Pro Cys Ser
325

<210> 9
<211> 164
<212> PRT

<213> Artificial sequence

<220>
<223> Synthetic polypeptide

<400> 9

```
Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
1               5                  10                  15


Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu
            20                  25                  30


Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile
        35                  40                  45


Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe
    50                  55                  60


Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu
65                  70                  75                  80


Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys
                85                  90                  95


Asn Phe His Phe Asp Pro Arg Asp Val Val Ser Asn Ile Asn Val Phe
            100                 105                 110


Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala
        115                 120                 125


Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe
    130                 135                 140


Cys Gln Ser Ile Ile Ser Thr Leu Thr Ala Ala Ala His His His His
145                 150                 155                 160


His His His His
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<210> 10
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<220>
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<223> n is a, c, g, or t

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ccccgcgagt gacctgtttc cacacggact tgatccccat tacggacgaa gagacacaac 180

gacgttttga gaaaatgatt gtacaggcgg cgttagagga cgccctaacg agcatctttg 240

aggagcacga caataacgta accgattact tcgcggaata catgcgatcc ctccaaatgg 300

cgaataaaag tcatacgaat aatattatcg cggtcgcttt agcggggata atcgtcattg 360

taacgaccta cgtgtttact agattacgca ctaagcaaaa aaaaggaaat tataacgtac 420

gtaataagat agataattcc atacagaaag agattcagtt ggacggtgta tatactactg 480

acaacgtttt tatataaaca tggtgtttat atttattatc acctgtgtat gtttggtgac 540

gagatcctgt gggggtgggt tagaagacga tatagatcgc atatttcaaa aacgatacaa 600

cgaactgagc cagccgatta annncaatat gcgtacactg tgcaagttta gaggaattac 660

cgcgactatg tttacggaag gagaatctta ccttattcaa tgtcccataa ttcacgatta 720

cgtgctacgg gcgctgtatg acttagtgga aggaagttac acggtacgct gggaacgcga 780

aacggaagac gatgttgagt cggtagatcc gaagttagtc aaagggacgc tattatacct 840

ccaacctaac gcgtccagta taggaacgta tctatgtacc ttacacgata accgaggtat 900

gtgttatcaa tctgtcgcgc acgtcatccg acgtccgaag atgcaatgcg tgaaacatgc 960

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atacagactc cgacgtacga aggataatca cgacgtaact cgaactctgc aggtcgactc 1140

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ttttttttt tggaatataa ataagctcga agtcgacaga tctaggcctg gtacccgatc 1260

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ccgaccacat gaagcagcac gacttcttca agtccgccat gcccgaaggc tacgtccagg 1560

agcgcaccat cttcttcaag gacgacggca actacaagac ccgcgccgag gtgaagttcg 1620

agggcgacac cctggtgaac cgcatcgagc tgaagggcat cgacttcaag gaggacggca 1680

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gaggttggta agaatgccta tctgccctgc agttacactc tatctacacc tggggcactt 2160

gtgcctatgt gctggggcaa gggattctgt ccttggtcac agtgtaccaa cgagttgctc 2220

agaactgatg aaagaaatgt gacatatcag aaatccagca gataccagct aaagggcgat 2280

ctcaacaaag gagacgtgtc tctgatcata aagaatgtga ctctggatga ccatgggacc 2340

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gacatcaaag cagccaaggt cactccagct cagactgccc atggggactc tactacagct 2460

tctccaagaa ccctaaccac ggagagaaat ggttcagaga cacagacact ggtgaccctc 2520

cataataaca atggaacaaa aatttccaca tgggctgatg aaattaagga ctctggagaa 2580

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aaattatgga acggtcgccc acttacacgg tacacgataa acgcttttct atcgtcgcac 2700

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