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(54) Titre : THERAPIE POUR LE TRAITEMENT DU CANCER PAR UNE ADMINISTRATION INTRATUMORALE ET/OU
INTRAVEINEUSE D'UN MVA RECOMBINANT CODANT POUR 4-1BBL (CD137L) ET/OU CD40L
(54) Title: THERAPY FOR TREATING CANCER WITH AN INTRATUMORAL AND/OR INTRAVENOUS
ADMINISTRATION OF A RECOMBINANT MVA ENCODING 4-1BBL (CD137L) AND/OR CD40L

(57) Abrégé/Abstract:

The invention relates to a composition and related methods for reducing tumor volume and/or increasing the survival of a cancer patient. The composition comprises a recombinant MVA encoding a Tumor Associated Antigen ("TAA") as well as 4-1BBL and/or CD40L and can be administered to a subject in any suitable manner, including by intravenous and/or intratumoral administration.

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(54) Title: THERAPY FOR TREATING CANCER WITH AN INTRATUMORAL AND/OR INTRAVENOUS ADMINISTRATION OF A RECOMBINANT MVA ENCODING 4-1BBL (CD137L) AND/OR CD40L

(57) Abstract: The invention relates to a composition and related methods for reducing tumor volume and/or increasing the survival of a cancer patient. The composition comprises a recombinant MVA encoding a Tumor Associated Antigen ("TAA") as well as 4-1BBL and/or CD40L and can be administered to a subject in any suitable manner, including by intravenous and/or intratumoral administration.

THERAPY FOR TREATING CANCER WITH AN INTRATUMORAL AND/OR INTRAVENOUS ADMINISTRATION OF A RECOMBINANT MVA ENCODING 4-1BBL (CD137L) AND/OR CD40L

FIELD OF THE INVENTION

[001] The present invention relates to a therapy for the treatment of cancers; the treatment includes an intravenously or intratumorally administered recombinant modified vaccinia Ankara (MVA) virus comprising a nucleic acid encoding 4-1BBL (CD137L). Recombinant modified vaccinia Ankara (MVA) virus as used herein (also “recombinant MVA” or “rMVA”) refers to an MVA comprising at least one polynucleotide encoding a tumor associated antigen (TAA). In a more particular aspect, the invention includes intravenously or intratumorally administered recombinant MVA comprising a nucleic acid encoding a TAA and a nucleic acid encoding 4-1BBL. In additional aspects, the invention includes an intravenously or intratumorally administered recombinant MVA comprising a nucleic acid encoding a TAA and a nucleic acid encoding CD40L. In additional aspects, the invention includes an intravenously and/or intratumorally administered recombinant MVA comprising nucleic acids encoding a TAA, 4-1BBL (CD137L), and CD40L.

BACKGROUND OF THE INVENTION

[002] Recombinant poxviruses have been used as immunotherapy vaccines against infectious organisms and, more recently, against tumors (Mastrangelo *et al.* (2000) *J Clin Invest.* 105(8):1031-1034).

[003] One poxviral strain that has proven useful as an immunotherapy vaccine against infectious disease and cancer is the Modified Vaccinia Ankara (MVA) virus (sometimes referred to simply as “MVA”). MVA was generated by 516 serial passages on chicken embryo fibroblasts of the Ankara strain of vaccinia virus (CVA) (for review see Mayr *et al.* (1975) *Infection* 3: 6-14). As a consequence of these long-term passages, the genome of the resulting MVA virus had about 31 kilobases of its genomic sequence deleted and, therefore, was described as highly host cell restricted for replication to avian cells (Meyer *et al.* (1991) *J. Gen. Virol.* 72: 1031-1038). It was shown in a variety of animal models that the resulting MVA was significantly avirulent (Mayr & Danner (1978) *Dev. Biol. Stand.* 41: 225-34). Strains of MVA having enhanced safety profiles for the development of safer products, such as vaccines or pharmaceuticals, have been described (see International PCT

publication WO2002042480; see also, *e.g.*, U.S. Pat. Nos. 6,761,893 and 6,913,752, all of which are incorporated by reference herein). Such variants are capable of reproductive replication in non-human cells and cell lines, especially in chicken embryo fibroblasts (CEF), but are replication incompetent in human cell lines, in particular including HeLa, HaCat and 143B cell lines. Such strains are also not capable of reproductive replication *in vivo*, for example, in certain mouse strains, such as the transgenic mouse model AGR 129, which is severely immune-compromised and highly susceptible to a replicating virus (see U.S. Pat. Nos. 6,761,893). Such MVA variants and its derivatives, including recombinants, referred to as "MVA-BN," have been described (see International PCT publication WO2002/042480; see also, *e.g.*, U.S. Pat. Nos. 6,761,893 and 6,913,752).

[004] The use of poxviral vectors that encode tumor-associated antigens (TAAs) have been shown to successfully reduce tumor size as well as increase overall survival rate of cancer patients (see, *e.g.*, WO 2014/062778). It has been demonstrated that when a cancer patient is administered a poxviral vector encoding a TAA, such as HER2, CEA, MUC1, and/or Brachyury, a robust and specific T-cell response is generated by the patient to fight the cancer (*Id.*; see also, Guardino *et al.* ((2009) *Cancer Res.* 69 (24), doi 10.1158/0008-5472.SABCS-09-5089), Heery *et al.* (2015) *JAMA Oncol.* 1: 1087-95).

[005] One type of TAA that was found to be expressed on many cancer and tumor cells are Endogenous Retroviral (ERV) proteins. ERVs are remnants of former exogenous forms that invaded the germ line of the host and have since been vertically transmitted through a genetic population (see Bannert *et al.* (2018) *Frontiers in Microbiology*, Volume 9, Article 178). ERV-induced genomic recombination events and dysregulation of normal cellular genes have been documented to have contributory effects to tumor formation (*Id.*). Further, there is evidence that certain ERV proteins have oncogenic properties (*Id.*). ERVs have been found to be expressed in a large variety of cancers including, *e.g.*, breast, ovarian, melanoma, prostate, pancreatic, and lymphoma. (See, *e.g.*, Bannert *et al.* (2018) *Front. Microbiol.* 9: 178; Cegolon *et al.* (2013) *BMC Cancer* 13: 4; Wang-Johanning *et al.* (2003) *Oncogene* 22: 1528-35; Wang-Johanning *et al.* (2007) *Int. J. Cancer* 120: 81-90; Wang-Johanning *et al.* (2008) *Cancer Res.* 68: 5869-77; Wang-Johanning *et al.* (2018) *Cancer Res.* 78 (13 Suppl.), AACR Annual Meeting April 2018, Abstract 1257; Contreras-Galindo *et al.* (2008) *J. Virol.* 82: 9329-36; Schiavetti *et al.* (2002) *Cancer Res.* 62: 5510-16; Maliniemi *et al.* (2013) *PLoS One* 8: e76281; Fava *et al.* (2017) *Genes Dev.* 31: 34-45, Muster *et al.* (2003) *Cancer Res.* 63: 8735-41; Buscher *et al.* (2005) *Cancer Res.* 65: 4172-80; Serafino *et al.* (2009) *Expt'l. Cell Res.* 315: 849-62;

Iramaneerat *et al.* (2011) *Int. J. Gynecol. Cancer* 21: 51-7; Ishida *et al.* (2006) *Cancer Sci.* 97: 1139-46; Goering *et al.* (2011) *Carcinogenesis* 32: 1484-92; Agoni *et al.* (2013) *Front. Oncol.* 9: 180; Li *et al.* (2017) *J. Mol. Diagn.* 19: 4-23).

[006] In addition to their effectiveness with TAAs, poxviruses such as MVA have been shown to have enhanced efficacy when combined with a CD40 agonist such as CD40 Ligand (CD40L) (see WO 2014/037124) or with a 4-1BB agonist such as 4-1BB Ligand (4-1BBL) (Spencer *et al.* (2014) *PLoS One* 9: e105520).

[007] CD40/CD40L is a member of the tumor necrosis factor receptor/tumor necrosis factor ("TNFR/TNF") superfamily. While CD40 is constitutively expressed on many cell types, including B cells, macrophages and DCs, its ligand CD40L is predominantly expressed on activated CD4+ T-cells (Lee *et al.* (2002) *J. Immunol.* 171(11): 5707-5717; Ma and Clark (2009) *Semin. Immunol.* 21(5): 265-272). The cognate interaction between DCs and CD4+ T-cells early after infection or immunization `licenses` DCs to prime CD8+ T-cell responses (Ridge *et al.* (1998) *Nature* 393: 474-478). DC licensing results in the upregulation of co-stimulatory molecules, increased survival and better cross-presenting capabilities of DCs. This process is mainly mediated via CD40/CD40L interaction (Bennet *et al.* (1998) *Nature* 393: 478-480; Schoenberger *et al.* (1998) *Nature* 393: 480-483), but CD40/CD40L-independent mechanisms also exist (CD70, LT.beta.R). Interestingly, a direct interaction between CD40L expressed on DCs and CD40 expressed on CD8+ T-cells has also been suggested, providing a possible explanation for the generation of helper-independent CTL responses (Johnson *et al.* (2009) *Immunity* 30: 218-227).

[008] 4-1BB/4-1BBL is a member of the TNFR/TNF superfamily. 4-1BBL is a costimulatory ligand expressed in activated B cells, monocytes and DCs. 4-1BB is constitutively expressed by natural killer (NK) and natural killer T (NKT) cells, Tregs and several innate immune cell populations, including DCs, monocytes and neutrophils. Interestingly, 4-1BB is expressed on activated, but not resting, T cells (Wang *et al.* (2009) *Immunol. Rev.* 229: 192-215). 4-1BB ligation induces proliferation and production of interferon gamma (IFN- γ) and interleukin 2 (IL-2), as well as enhances T cell survival through the upregulation of antiapoptotic molecules such as Bcl-xL (Snell *et al.* (2011) *Immunol. Rev.* 244: 197-217). Importantly, 4-1BB stimulation enhances NK cell proliferation, IFN- γ production and cytolytic activity through enhancement of Antibody-Dependent Cell Cytotoxicity (ADCC) (Kohrt *et al.* (2011) *Blood* 117: 2423-32).

[009] The 4-1BB/4-1BBL axis of immunity is currently being explored by different immunotherapeutic strategies. As an example, autologous transfer of Chimeric Antigen Receptor (CAR) T cells shows clinical benefit in large B cell lymphomas, being approved by the FDA in 2017. Patient autologous T cells are transduced with CARs that combine an extracellular domain derived from a tumor-specific antibody, the CD3 ζ intracellular signaling domain and the 4-1BB costimulatory motif. The addition of 4-1BB is crucial for in vivo persistence and antitumor toxicity of CAR T cells (Song *et al.* (2011) *Cancer Res.* 71: 4617e27). Antibodies targeting 4-1BB are currently being investigated.

[010] Several studies have shown that agonistic antibodies targeting 4-1BB / 4-1BBL pathway show anti-tumor activity when utilized as a monotherapy (Palazón *et al.* (2012) *Cancer Discovery* 2: 608-23). Agonistic antibodies targeting 4-1BB (Urelumab, BMS; Utolimumab, Pfizer) are currently in clinical development. In recent years, studies that have combined 4-1BBL with other therapies have shown varied success. For example, when mice with preexisting MC38 (murine adenocarcinoma) tumors, but not B16 melanoma tumors, were administered with antibodies to CTLA-4 and anti-4-1BB, significant CD8+ T cell-dependent tumor regression was observed, together with long-lasting immunity to these tumors. In another example, treatment with anti-4-1BB (Bristol-Myers Squibb (BMS)-469492) led to only modest regression of M109 tumors, but significantly delayed the growth of EMT6 tumors.

[011] The tumor microenvironment is composed of a large variety of cell types, from immune cell infiltrates to cancer cells, extracellular matrix, endothelial cells, and other cellular players that influence tumor progression. This complex and entangled equilibrium changes not only from patient to patient, but within lesions in the same subject (Jiménez-Sánchez *et al.* (2017) *Cell* 170(5): 927-938). Stratification of tumors based on Tumor Infiltrating Lymphocytes (TIL) and Programmed Death Ligand 1 (PD-L1) expression emphasizes the importance of an inflammatory environment to achieve objective responses against cancer (Teng *et al.* (2015) *Cancer Res.* 75(11): 2139-45). Pan-cancer analysis of gene expression profiles from the Cancer Genome Atlas (TCGA) support that a tumor inflammation signature correlates with objective responses to immunotherapy (Danaher *et al.* (2018) *J. Immunother. Cancer* 6(1): 63).

[012] In recent years, attempts to improve cancer therapies routes of administration of vaccines have been expanded from subcutaneous injection to an intravenous route of administration. For example, it was demonstrated that an intravenous administration of an MVA vaccine encoding a

heterologous antigen was able to induce a strong specific immune response to the antigen (see WO 2014/037124). Further, enhanced immune response were generated when the MVA vaccine included CD40L.

[013] The inoculation of bacterial-derived material (Coley's toxin) into tumor lesions achieving curative responses has long been reported, highlighting the role of local infection in promoting antitumor responses (Coley (1906) *Proc. R. Soc. Med.* 3 (Surg Sect): 1-48). The local administration of Pathogen Associated Molecular Patterns (PAMPs), bacterial products, and viruses into tumor lesions induces an antimicrobial program that results in a cascade of events following the administration, including: i) secretion of pro-inflammatory cytokines as Type I, II and III interferons and Tumor necrosis Factor alpha (TNF-alpha); ii) danger signals such as alarmins and heat-shock proteins; and iii) release of tumor antigens (Aznar *et al.* (2017) *J. Immunol.* 198: 31-39). Local administration of immunotherapy into the tumor induces systemic immune responses, as regressions have been assessed in non-treated tumor lesions ((2018) *Cancer Discov.* 8(6): 67).

[014] Intratumoral administration of MVA vaccines has been reported in the past few years. It was found that intratumoral injections of MVA expressing GM-CSF and immunization with DNA vaccine prolonged the survival of mice bearing HPV16 E7 tumors (Nemeckova *et al.* (2007) *Neoplasma* 54: 4). Other studies of intratumoral injection of MVA were unable to demonstrate inhibition of pancreatic tumor growth (White *et al.* (2018) *PLoS One* 13(2): e0193131). Intratumoral injection of heat-inactivated MVA induced antitumor immune responses dependent in the generation of danger signals, type I interferon, and antigen cross-presentation by dendritic cells (Dai *et al.* (2017) *Sci. Immunol.* 2(11): eaal1713).

[015] There is clearly a substantial unmet medical need for additional cancer treatments, including active immunotherapies and cancer vaccines. Additionally, there is a need for therapies that can induce enhanced immune responses in multiple areas of a patient's immune response. In many aspects, the embodiments of the present disclosure address these needs by providing vaccines, therapies, and combination therapies that increase and improve the cancer treatments currently available.

BRIEF SUMMARY OF THE INVENTION

[016] It was determined in the various embodiments of the present invention that a recombinant MVA encoding a tumor-associated antigen (TAA) and a 4-1BB Ligand (also referred to

herein as 41BBL, 4-1BBL, or CD137L) when administered intratumorally or intravenously increases the effectiveness of and/or enhances treatment of a cancer patient. More particularly, it was determined that the various embodiments of the present disclosure resulted in increased inflammation in the tumor, decreases in regulatory T cells (Tregs) and T cell exhaustion in the tumor, expansion of tumor-specific T cells and activation of NK cells, increases in reduction in tumor volume, and/or increases in the survival of a cancer subject as compared to an administration of a recombinant MVA by itself.

[017] It was determined in the various embodiments of the present invention that a recombinant MVA encoding a tumor-associated antigen (TAA) and a CD40 Ligand (CD40L) when administered intratumorally or intravenously enhances treatment of a cancer patient. More particularly, it was determined that the various embodiments of the present disclosure resulted in increased inflammation in the tumor, decreases in regulatory T cells (Tregs) and T cell exhaustion in the tumor, expansion of tumor-specific T cells and activation of NK cells, increases in reduction in tumor volume, and/or increases in the survival of a cancer subject as compared to an administration of a recombinant MVA by itself.

[018] In additional embodiments, the invention includes a recombinant modified vaccinia Ankara (MVA) virus comprising a nucleic acid encoding 4-1BBL (CD137L) and a nucleic acid encoding CD40L that when administered intravenously and/or intratumorally enhances treatment of a cancer patient.

[019] Accordingly, in one embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[020] In an additional embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid

encoding CD40L, wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a CD40L antigen.

[021] In an additional embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally and/or intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA), a second nucleic acid encoding CD40L, and a third nucleic acid encoding 4-1BBL (CD137L) wherein the administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to an injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA, a CD40L antigen, and a 4-1BBL antigen by a different route of injection (*i.e.*, non-intratumoral or non-intravenous injection).

[022] In an additional embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intravenous administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T-cell responses specific to the TAA as compared to a non-intravenous injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[023] In an additional embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding CD40L, wherein the intravenous administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T cell responses specific to the TAA as compared to a non-intravenous injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a CD40L antigen.

[024] In an additional embodiment, the present invention includes a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously and/or intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA), a second nucleic acid encoding CD40L, and a third nucleic acid encoding 4-1BBL, wherein the intravenous and/or intratumoral administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T cell responses specific to the TAA as compared to a non-intravenous or non-intratumoral injection of a recombinant MVA virus comprising a first nucleic acid encoding a TAA, a second nucleic acid encoding a CD40L antigen, and a third nucleic acid encoding a 4-1BBL antigen.

[025] In yet another embodiment, the present invention includes a method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA) and a second nucleic acid encoding a 4-1BBL antigen, wherein the intratumoral administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a heterologous tumor-associated antigen and a 4-1BBL antigen.

[026] In yet another embodiment, the present invention includes a method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA) and a second nucleic acid encoding a CD40L antigen, wherein the intratumoral administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a heterologous tumor-associated antigen and a CD40L antigen.

[027] In yet another embodiment, the present invention includes a method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally and/or intravenously administering to the subject a recombinant modified Vaccinia

Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA), a second nucleic acid encoding a CD40L antigen, and a third nucleic acid encoding a 4-1BBL antigen, wherein the intratumoral and/or intravenous administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral or non-intravenous injection of a recombinant MVA virus comprising a first nucleic acid encoding a heterologous tumor-associated antigen, a second nucleic acid encoding a CD40L antigen, and a third nucleic acid encoding a 4-1BBL antigen.

[028] In various additional embodiments, the present invention provides a recombinant modified Vaccinia Ankara (MVA) for treating a subject having cancer, the recombinant MVA comprising a) a first nucleic acid encoding a tumor-associated antigen (TAA) and b) a second nucleic acid encoding 4-1BBL.

[029] In various additional embodiments, the present invention includes a recombinant modified Vaccinia Ankara (MVA) for treating a subject having cancer, the recombinant MVA comprising a) a first nucleic acid encoding a tumor-associated antigen (TAA) and b) a second nucleic acid encoding CD40L.

[030] In various additional embodiments, the present invention includes a recombinant modified Vaccinia Ankara (MVA) for treating a subject having cancer, the recombinant MVA comprising: a) a first nucleic acid encoding a tumor-associated antigen (TAA); b) a second nucleic acid encoding CD40L; and c) a third nucleic acid encoding 4-1BBL.

[031] In yet another embodiment, a recombinant MVA encoding a 4-1BBL antigen, when administered intratumorally to a patient in combination with an administration of a checkpoint inhibitor antagonist enhances treatment of a cancer patient, more particularly increases reduction in tumor volume and/or increases survival of the cancer patient.

[032] In yet another embodiment, a recombinant MVA encoding a CD40L antigen, when administered intratumorally to a patient in combination with an administration of a checkpoint inhibitor antagonist enhances treatment of a cancer patient, more particularly increases reduction in tumor volume and/or increases survival of the cancer patient.

[033] In yet another embodiment, a recombinant MVA encoding a CD40L and 4-1BBL antigen, when administered intratumorally and/or intravenously to a patient in combination with an administration of a checkpoint inhibitor antagonist enhances treatment of a cancer patient, more particularly increases reduction in tumor volume and/or increases survival of the cancer patient.

[034] In another embodiment, the recombinant MVA of the present invention is administered at the same time or after administration of the antibody. In a more preferred embodiment, the recombinant MVA is administered after the antibody.

[035] In another embodiment, the recombinant MVA of the present invention is administered by the same route(s) of administration and at the same time or after administration of the antibody. In another embodiment, the recombinant MVA is administered by a different route or routes of administration or after administration of the antibody.

[036] In yet another embodiment, the present invention includes a method for enhancing antibody therapy in a cancer patient, the method comprising administering the pharmaceutical combination of the present invention to a cancer patient, wherein administering the pharmaceutical combination enhances antibody dependent cell-mediated cytotoxicity (ADCC) induced by the antibody therapy, as compared to administering the antibody therapy alone.

[037] In preferred embodiments, the first nucleic acid encodes a TAA that is an endogenous retroviral (ERV) protein. In more preferred embodiments, the ERV protein is from the human endogenous retroviral protein K (HERV-K) family. In more preferred embodiments, the ERV protein is selected from a HERV-K envelope and a HERV-K gag protein.

[038] In preferred embodiments, the first nucleic acid encodes a TAA that is an endogenous retroviral (ERV) peptide. In more preferred embodiments, the ERV peptide is from the human endogenous retroviral protein K (HERV-K) family. In more preferred embodiments, the ERV peptide is selected from a pseudogene of a HERV-K envelope protein (HERV-K-MEL).

[039] In other preferred embodiments, the first nucleic acid encodes a TAA selected from the group consisting of: carcinoembryonic antigen (CEA), mucin 1 cell surface associated (MUC-1), prostatic acid phosphatase (PAP), prostate specific antigen (PSA), human epidermal growth factor receptor 2 (HER-2), survivin, tyrosine related protein 1 (TRP1), tyrosine related protein 1 (TRP2), Brachyury, Preferentially Expressed Antigen in Melanoma (PRAME), Folate receptor 1 (FOLR1), and combinations thereof.

[040] In one or more preferred embodiments, the recombinant MVA is MVA-BN or a derivative thereof.

[041] In various additional embodiments, the recombinant MVAs and methods described herein are administered to a cancer subject in combination with either an immune checkpoint molecule antagonist or agonist. In further embodiments, the recombinant MVAs and methods described herein

are administered to a cancer subject in combination with an antibody specific for a TAA to treat a subject with cancer. In a more preferred embodiment, the recombinant MVAs and methods described herein are administered in combination with an immune checkpoint molecule antagonist or agonist selected from CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS. In most preferred embodiments, the immune checkpoint molecule antagonist or agonist comprises an antibody. In a most preferred embodiment, the immune checkpoint molecule antagonist or agonist comprises a PD-1 or PD-L1 antibody.

[042] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[043] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one or more embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[044] Figures 1A, 1B, 1C, and 1D illustrate that 4-1BBL-mediated costimulation of CD8 T cells by MVA-OVA-4-1BBL infected tumor cells influences cytokine production without the need of DC. MVA-OVA-CD40L in contrast only enhances cytokine production in the presence of DC. As described in Example 2, dendritic cells (DCs) were generated after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 cells were infected with MVA-OVA, MVA-OVA-CD40L, or MVA-OVA-4-1BBL and infected tumor cells were harvested and cocultured when indicated in the presence of DCs. Naïve OVA(257-264) specific CD8+ T cells were magnetically purified from OT-I mice and added to the coculture. Cells were cultured and the supernatant was collected for cytokine concentration analysis by Luminex. Supernatant concentration of IL-6 (Figure 1A), GM-CSF (Figure 1B), IL-2 (Figure 1C) and IFN- γ (Figure 1D) is shown. Data are shown as Mean \pm SEM.

[045] Figure 2A and Figure 2B show that MVA-OVA-4-1BBL infected tumor cells directly, i.e., without the need of DC, drive differentiation of antigen-specific CD8 T cells into activated effector T cells, whereas CD40L-mediated costimulation of MVA-OVA-CD40L infected tumor cells is dependent on the presence of DC. As described in Example 3, dendritic cells (DCs) were generated

after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 (melanoma model) cells were infected with MVA-OVA, MVA-OVA-CD40L or MVA-OVA-4-1BBL. The next day, infected tumor cells were harvested and cocultured (when indicated) in the presence of DCs. Naïve OVA(257-264)-specific CD8+ T cells were magnetically purified from OT-I mice and added to the coculture at a ratio of 1:5. Cells were cultured at 37°C 5% CO₂ for 48 hours. Cells were then stained and analyzed by flow cytometry. Figure 2A shows GMFI of T-bet on OT-I CD8+ T cells (indicated as “CD8+” in the figure); Figure 2B shows percentage of CD44+Granzyme B+ IFN γ + TNF α + of OT-I CD8+ T cells. Data are shown as Mean \pm SEM.

[046] Figures 3A, 3B, 3C, 3D, and 3E illustrate that infection with MVAs encoding either CD40L or 4-1BBL induce tumor cell death in tumor cell lines and macrophages. As described in Example 4, tumor cell lines B16.OVA (Figure 3A and 3B), MC38 (Figure 3C) and B16.F10 (Figure 3D) were infected with vectors at the indicated MOI for 20 hours. Cells were analyzed for their viability by flow cytometry; Figures 3A, 3C, 3D, and 3E show the percentage of dead cells (“Live/Dead+”). Figure 3B: HMGB1 in the supernatants from Figure 3A was quantified by ELISA. Figure 3E: Bone marrow-derived macrophages (BMDMs) were infected at the indicated MOI for 20 hours. Cells were analyzed for their viability by flow cytometry. Data are presented as Mean \pm SEM.

[047] Figures 4A and 4B show that rMVA-4-1BBL induces NK cell activation in vivo. As described in Example 5, C57BL/6 mice (n=5/group) were immunized intravenously either with saline or 5 \times 10⁷ TCID50 “rMVA” (=MVA-OVA), “rMVA-4-1BBL” (=MVA-OVA-4-1BBL) or 5 \times 10⁷ TCID50 rMVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1). 24 hours later, mice were sacrificed and spleens processed for flow cytometry analysis. Geometric Mean Fluorescence Intensity (GMFI) of CD69 (A) and CD70 (B) is shown. Data are shown as Mean \pm SEM.

[048] Figures 5A and 5B show that intravenous rMVA-4-1BBL immunization promotes serum IFN- γ secretion in vivo. As described in Example 6, C57BL/6 mice (n=5/group) were immunized intravenously either with saline or 5 \times 10⁷ TCID50 “rMVA” (=MVA-OVA), “rMVA-4-1BBL” (=MVA-OVA-4-1BBL), or 5 \times 10⁷ TCID50 rMVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1). Figure 5A: 6 hours later, mice were bled, serum was isolated from whole blood and IFN- γ concentration in serum determined by Luminex. Figure 5B: 3, 21 and 45 hours later, mice were intravenously injected with Brefeldin A to stop protein secretion. Mice were sacrificed 6, 24 and 48 hours after immunization and splenocytes analyzed by flow cytometry. Data are shown as Mean \pm SEM.

[049] Figure 6 shows that intravenous “rMVA-4-1BBL” (=MVA-OVA-4-1BBL) immunization promotes serum IFN- γ secretion in B16.OVA tumor-bearing mice. As described in Example 7, B16.OVA tumor-bearing C57BL/6 mice (n=5/group) were grouped and received i.v. (intravenous) PBS or 5×10^7 TCID50 rMVA (=MVA-OVA) or rMVA-4-1BBL at day 7 after tumor inoculation. 6 hours later, mice were bled, serum was isolated from whole blood and IFN- γ concentration in serum determined by Luminex. Data are shown as Mean \pm SEM.

[050] Figures 7A, 7B, 7C, and 7D show antigen and vector-specific CD8+ T cell expansion after intravenous “rMVA-4-1BBL” (=MVA-OVA-4-1BBL) prime and boost immunization. As described in Example 8, C57BL/6 mice (n = 4/group) received intravenous prime immunization either with saline or 5×10^7 TCID50 “rMVA” (=MVA-OVA), rMVA-4-1BBL or 5×10^7 TCID50 rMVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1) on day 0 and boost immunization on day 41. Mice were bled on days 6, 21, 35, 48 and 64 after prime immunization, and flow cytometric analysis of peripheral blood was performed. Figure 7A shows percentage of antigen (OVA)-specific CD8+ T cells among Peripheral Blood Leukocytes (PBL); Figure 7B shows the percentage of vector (B8R)-specific CD8+ T cells among PBL. Mice were sacrificed on day 70 after prime immunization. Spleens were harvested and flow cytometry analysis performed. Figure 7C shows percentage of antigen (OVA)-specific CD8+ T cells among live cells; and Figure 7D shows percentage of vector (B8R)-specific CD8+ T cells among live cells. Data are shown as Mean \pm SEM.

[051] Figure 8 shows an increased antitumor effect of intravenous injection of MVA virus encoding 4-1BBL as compared to the recombinant MVA without 4-1BBL. As described in Example 9, B16.OVA tumor-bearing C57BL/6 mice (n=5/group) were grouped and received intravenous administrations of PBS or 5×10^7 TCID50 MVA-OVA (“rMVA” in figure) or MVA-OVA-4-1BBL (“rMVA-4-1BBL” in figure) at day 7 (black dotted line) after tumor inoculation. Tumor growth was measured at regular intervals.

[052] Figures 9A, 9B, 9C, and 9D show an enhanced antitumor effect of intratumoral injection of MVA virus encoding 4-1BBL or CD40L. As described in Example 10, B16.OVA tumor-bearing C57BL/6 mice (n = 4-5/group) were grouped and received intratumoral administrations of PBS or 5×10^7 TCID50 of MVA-OVA (labelled “rMVA” in figure), MVA-OVA-CD40L (labelled “rMVA-CD40L” in figure), or MVA-OVA-4-1BBL (labelled “rMVA-4-1BBL” in figure) at days 7 (black dotted line), 12, and 15 (grey dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals.

[053] Figures 10A, 10B, and 10C show the antitumor effect of intratumoral injection of MVA virus encoded with CD40L against established colon cancer. As described in Example 11, MC38-tumor-bearing C57BL/6 mice (n = 5/group) were grouped and received intratumoral (i.t.) administrations of PBS or 5×10^7 TCID50 MVA-TAA (labelled “rMVA” in the figure) or MVA-TAA-CD40L (labelled “rMVA-CD40L” in the figure) at days 14 (black dotted line), 19, and 22 (black dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals. In these experiments, the TAA encoded by the recombinant MVAs comprised antigens AH1A5, p15E, and TRP2.

[054] Figure 11 illustrates that checkpoint blockade and tumor-targeting antibodies synergize with intratumoral (i.t.) administration of rMVA-4-1BBL (also referred to herein as “MVA-OVA-4-1BBL”). As described in Example 12, B16.OVA tumor-bearing C57BL/6 mice (n = 5/group) were grouped and received 200 μ g IgG2a, anti TRP-1, or anti PD-1 antibody intraperitoneally when indicated (ticks). Mice were immunized intratumorally (i.t.) either with PBS or with 5×10^7 TCID50 MVA-OVA-4-1BBL at days 13 (black dotted line), 18 and 21 (grey dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals.

[055] Figure 12 demonstrates that intratumoral MVA-OVA-4-1BBL injection leads to a superior anti-tumor effect when compared to anti-CD137 antibody treatment. As described in Example 13, C57BL/6 mice received 5×10^5 B16.OVA cells s.c. (subcutaneously). Seven days later, when tumors measured above 5x5 mm, mice were grouped and intratumorally injected with either PBS, 5×10^7 TCID50 MVA-OVA-4-1BBL, or 10 μ g anti-4-1BB (3H3) antibody. Tumor growth was measured at regular intervals. In Figure 12A, tumor mean volume is shown. Figure 12B: On day 12 after prime, peripheral blood lymphocytes were stained with OVA-dextramer and analyzed by FACS. Percentage OVA dextramer+ CD44+ T cells among CD8+ T cells is shown.

[056] Figure 13 shows the antitumor effect of intravenous injection of MVA virus encoding the endogenous retroviral antigen Gp70. As described in Example 14, Balb/c mice received 5×10^5 CT26.wt cells s.c. (subcutaneously). When tumors measured above 5x5mm, CT26.wt tumor-bearing mice (n = 5/group) were grouped and received i.v. (intravenous) PBS or 5×10^7 TCID50 of MVA, rMVA-Gp70, or rMVA-Gp70-CD40L at day 12 after tumor inoculation. Tumor growth was measured at regular intervals. Shown are tumor mean diameter (Figure 13A) and tumor mean volume (Figure 13B). Figure 13C: 7 days after immunization, blood cells were restimulated and the percentage of CD8+ CD44+ IFN- γ + cells in blood upon stimulation is shown.

[057] Figure 14 shows the antitumor effect of intravenous injection of MVA virus encoding the endogenous retroviral antigen Gp70 plus CD40L. As described in Example 15, C57BL/6 mice received 5×10^5 B16.F10 cells s.c. (subcutaneously). Seven days later when tumors measured above 5x5 mm, B16.F10 tumor-bearing C57BL/6 mice (n = 5/group) were grouped and received i.v. (intravenous) PBS or 5×10^7 TCID50 MVA, rMVA-Gp70, or rMVA-Gp70-CD40L. Tumor growth was measured at regular intervals. Shown are tumor mean volume (Figure 14A) and percentage of CD8+ CD44+ IFN- γ + cells in blood upon stimulation with p15e peptide 7 days after immunization (Figure 14B).

[058] Figure 15: Cytokine/chemokine MVA-BN backbone responses to IT immunization can be increased by 4-1BBL adjuvantation. By “adjuvantation” herein is intended that a particular encoded protein or component of a recombinant MVA increases the immune response produced by the other encoded protein(s) or component(s) of the recombinant MVA. Here, 5×10^5 B16.OVA cells were subcutaneously (s.c.) implanted into C57BL/6 mice (see Example 23). Mice were immunized on day 10 intratumorally (i.t.) with PBS or 2×10^8 TCID50 MVA-BN, MVA-OVA, or MVA-OVA-4-1BBL (n=6 mice/group). 6 hours later, tumors were extracted and tumor lysates processed. Cytokine/chemokine profiles were analysed by Luminex. Figure 15 shows cytokine/chemokines being upregulated in immunized mice.

[059] Figure 16: Cytokine/chemokine pro-inflammatory responses to intratumoral (i.t.) immunization are increased by MVA-OVA-4-1BBL. 5×10^5 B16.OVA cells were subcutaneously (s.c.) implanted into C57BL/6 mice (see Examples 23 and 24). Mice were immunized on day 10 intratumorally (i.t.) with PBS or 2×10^8 TCID50 of MVA-BN, MVA-OVA, or MVA-OVA-4-1BBL (n=6 mice/group). 6 hours later, tumors were extracted and tumor lysates processed. Cytokine/chemokine profiles were analysed by Luminex. Figure 16 shows those cytokine/chemokines that are upregulated in MVA-OVA-4-1BBL immunized mice compared to MVA-BN.

[060] Figure 17: Quantitative and qualitative T cell analysis of the tumor microenvironment (TME) and Tumor-draining Lymph Node (TdLN) after intratumoral injection of MVA-OVA-4-1BBL. C57BL/6 mice received 5×10^5 B16.OVA cells subcutaneously (s.c.). Nine to thirteen days later when tumors measured above 5x5mm, mice were grouped and intratumorally injected with either PBS, 2×10^8 TCID50 MVA-OVA, or MVA-OVA-4-1BBL (see Example 25). One, three and seven days after immunization, mice were sacrificed and tumors as well as tumor draining lymph nodes (TdLN)

were digested with Collagenase/DNase and analyzed by flow cytometry. Number of CD45+ cells, CD8+ T cells, CD4+ T cells and OVA-specific CD8+ T cells per mg tumor and per TdLN is shown.

[061] Figure 18: Quantitative and qualitative T cell analysis of the TME and draining LN after intratumoral injection of MVA-OVA-4-1BBL. C57BL/6 mice received 5×10^5 B16.OVA cells subcutaneously (s.c.). Nine to thirteen days later when tumors measured above 5.5×5.5 mm, mice were grouped and intratumorally injected with either PBS or 2×10^8 TCID₅₀ MVA-OVA or MVA-OVA-4-1BBL (see Example 26). One, three and seven days after immunization, mice were sacrificed and tumors as well as TdLN (tumor draining lymph node) were digested with Collagenase/DNase and analyzed by flow cytometry. Figure 18A: Percentage of Ki67+ cells among OVA-specific CD8+ T cells in tumor (left panel) and TdLN (right panel) is shown. Figure 18B: GMFI of PD1 among OVA-specific CD8+ T cells in the tumor seven days after i.t. immunization is shown. Figure 18C: OVA-specific Teff/Treg ratio in the tumor seven days after i.t. immunization is shown.

[062] Figure 19: Quantitative and qualitative NK cell analysis of the TME and tumor-draining lymph node (TdLN) after intratumoral injection of MVA-OVA-4-1BBL. C57BL/6 mice received 5×10^5 B16.OVA cells subcutaneously (s.c.). Nine to thirteen days later when tumors measured above 5.5×5.5 mm, mice were grouped and intratumorally injected with either PBS or 2×10^8 TCID₅₀ MVA-OVA or MVA-OVA-4-1BBL (see Example 27). Mice were sacrificed one, three and seven days after immunization, and tumors as well as tumor-draining lymph nodes (TdLN) were digested with Collagenase/DNase and analyzed by flow cytometry. Number of NK cells per mg tumor and TdLN and GMFI of CD69, Granzyme B, and Ki67 surface markers of NK cells in tumor and TdLN is shown.

[063] Figure 20: CD8 T cell-dependency of MVA-OVA-4-1BBL mediated anti-tumor effects. C57BL/6 mice received 5×10^5 B16.OVA cells subcutaneously (s.c.). Seven days later, mice were grouped and intratumorally injected with PBS or 2×10^8 TCID₅₀ MVA-OVA-4-1BBL (see Example 28). On day 5 and day 8 following this first injection, these intratumoral (i.t.) injections were repeated (vertical dashed lines). Additionally, IgG2b isotype control antibody (left and middle panels) or anti-CD8 antibody (2.43; right panel) were injected intraperitoneally (i.p.) on day -1 before and day 1, 4, 7, 11 after the first immunization (100 μ g/mouse). Tumor growth was measured at regular intervals, and tumor mean diameter is shown.

[064] Figure 21: Batf3+ DC-dependency of MVA-OVA and MVA-OVA-4-1BBL mediated anti-tumor effects. C57BL/6 mice or Batf3-/- mice received 5×10^5 B16.OVA cells subcutaneously

(s.c.). Seven days later (vertical dashed line), mice were grouped and intratumorally injected with PBS or 2×10^8 TCID50 of MVA, MVA-OVA, or MVA-OVA-4-1BBL (see Example 29). On day 5 and day 8 following the first intratumoral injection, the i.t. injection was repeated (vertical dashed lines). Tumor growth was measured at regular intervals. Figure 21A: tumor mean diameter is shown. Figure 21B: 11 days after the first immunization blood was withdrawn and analyzed for the presence of antigen-specific T cells (*i.e.*, OVA₂₅₇₋₂₆₄-specific T cells). The percentage of OVA-specific T cells within CD8+ T cells is shown.

[065] Figure 22: Role of NK cells for intratumoral administration of MVA-OVA-4-1BBL in B16.OVA melanoma bearing mice. C57BL/6 or IL15Ra^{-/-} mice received 5×10^5 B16.OVA cells subcutaneously (s.c.). Seven days later, mice were grouped and intratumorally injected with PBS or 2×10^8 TCID50 of MVA-OVA or MVA-OVA-4-1BBL (see Example 30). Treatment was repeated on day 5 and 8 after the first injection. Tumor growth was measured at regular intervals. Tumor mean diameter (Figure 22A) and percent survival is shown (Figure 22B). 11 days after the first immunization blood was withdrawn and analyzed for the presence of antigen-specific T cells (Figure 22C). The percentage of OVA₂₅₇₋₂₆₄-dextramer+ (SIINFEKL+) CD44+ T cells within CD8+ T cells is shown.

[066] Figure 23 shows NK cell-dependent cytokine/chemokine profile in response to IT immunization with MVA-OVA-4-1BBL. 5×10^5 B16.OVA cells were subcutaneously (s.c.) implanted into C57BL/6 and IL15Ra^{-/-} mice (see Example 31). Mice were immunized intratumorally (i.t.) on day 7 with PBS or 2×10^8 TCID50 MVA-OVA or MVA-OVA-4-1BBL (n=2-3 mice/group). 6 hours later, tumors were extracted and tumor lysates processed. Cytokine/chemokine profiles were analysed by Luminex. Figure 23 shows those cytokine/chemokines that are decreased in the absence of IL15Ra after MVA-OVA-4-1BBL intratumoral (i.t.) immunization.

[067] Figure 24 shows anti-tumor efficacy of intratumoral immunization with MVA-gp70-CD40L in comparison to MVA-gp70-4-1BBL in B16.F10 melanoma bearing mice. C57BL/6 mice received 5×10^5 B16.F10 cells subcutaneously (s.c.). Seven days later, mice were grouped and intratumorally injected with PBS or 5×10^7 TCID50 of MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-4-1BBL, or MVA-CD40L (see Example 32). Treatment was repeated on day 5 and 8 after the first injection. Tumor growth was measured at regular intervals. Figure 24A shows tumor mean diameter, and Figure 24B shows the appearance of vitiligo in mice treated with MVA-gp70-4-1BBL. 11 days after the first immunization, blood was withdrawn and analyzed for the presence of

antigen-specific T cells. The percentage of IFN γ producing CD44+ T cells within CD8+ T cells upon p15E restimulation is shown in Figure 24C.

[068] Figure 25: Anti-tumor efficacy of intratumoral administration of MVA-gp70-4-1BBL-CD40L in B16.F10 melanoma bearing mice. C57BL/6 mice received 5×10^5 B16.F10 cells subcutaneously (s.c.). Seven days later, mice were grouped and intratumorally injected with PBS or 5×10^7 TCID50 of: MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-gp70-4-1BBL-CD40L, MVA-4-1BBL, MVA-CD40L, or MVA-4-1BBL-CD40L (see Example 33). Treatment was repeated on day 5 and 8 after the first injection. Tumor growth was measured at regular intervals. Tumor mean diameter is shown in Figure 25A. Eleven days after the first immunization, blood was withdrawn and restimulated with p15e peptide. The percentage of IFN γ + CD44+ T cells within CD8+ T cells is shown in Figure 25B.

[069] Figure 26: Anti-tumor efficacy of MVA-gp70 adjuvanted with CD40L or 4-1BBL in CT26 tumor-bearing mice. Balb/c mice received 5×10^5 Ct26wt cells subcutaneously (s.c.). Thirteen days later, mice were grouped and injected intratumorally with PBS or 5×10^7 TCID50: MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-gp70-4-1BBL-CD40L, MVA-4-1BBL, MVA-CD40L, and MVA-4-1BBL-CD40L (see Example 34). Treatment was repeated on day 5 and 8 after the first injection. Tumor growth was measured at regular intervals. Figure 26A shows tumor mean diameter and Figure 26B shows percent survival. Figure 26C: Eleven days after the first immunization, blood was withdrawn and restimulated with AH1 peptide; the percentage of IFN γ + CD44+ T cells within CD8+ T cells is shown.

[070] Figure 27: Quantitative and qualitative T cell analysis of the tumor microenvironment (TME) and tumor draining lymph node (TdLN) after intratumoral injection of MVA-gp70 further comprising 4-1BBL and/or CD40L. C57BL/6 mice received 5×10^5 B16.F10 cells subcutaneously (s.c.). Nine days later when tumors measured above 5x5mm, mice were grouped and injected intratumorally with either PBS or 5×10^7 TCID50 of MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, or MVA-gp70-4-1BBL-CD40L (see Example 35). Three days after immunization, mice were sacrificed and tumors as well as tumor draining lymph nodes (TdLN) were collected, digested with collagenase/DNase, and analyzed by flow cytometry. Figure 27 shows number of CD8+ T cells, p15E-specific CD8+ T cells, and Ki67+ p15E-specific CD8+ T cells per mg tumor and per TdLN. Data represent Mean \pm SEM.

[071] Figure 28 shows quantitative and qualitative T cell analysis of the tumor microenvironment (TME) and tumor draining lymph node (TdLN) after intratumoral injection of MVA-gp70 further expressing 4-1BBL and/or CD40L. C57BL/6 mice received 5×10^5 B16.F10 cells subcutaneously (s.c.) (see Example 36). Nine days later when tumors measured above 5.5×5.5 mm, mice were grouped and intratumorally injected with either PBS or 5×10^7 TCID₅₀ of: MVA-Gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, and MVA-gp70-4-1BBL-CD40L. Three days after immunization, mice were sacrificed and tumors as well as TdLN were collected and digested with collagenase/DNase and resulting individual cells analyzed by flow cytometry. Number of NK cells, Ki67⁺ NK cells and Granzyme B⁺ NK cells per mg tumor and TdLN is shown. Data are shown as Mean±SEM.

[072] Figure 29: Anti-tumor efficacy of intravenous administration of MVA-gp70 adjuvanted with 4-1BBL and/or CD40L in CT26.WT tumor-bearing mice. Balb/c mice received 5×10^5 CT26.WT cells subcutaneously (s.c.). Twelve days later, mice were grouped and intravenously injected with PBS or 5×10^7 TCID₅₀ of MVA-Gp70, MVA-Gp70-4-1BBL, MVA-Gp70-CD40L, MVA-Gp70-4-1BBL-CD40L, and MVA-4-1BBL-CD40L (see Example 37). Figure 29A shows tumor mean diameter and Figure 29B shows percent survival. Seven days after the first immunization, blood was withdrawn and restimulated with AH1 peptide; Figure 29C shows the percentage of IFN γ ⁺ CD44⁺ T cells within CD8⁺ T cells as Mean±SEM.

DETAILED DESCRIPTION OF THE INVENTION

[073] It is to be understood that both the foregoing Summary and the following Detailed Description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[074] Described and illustrated in the present application, the recombinant MVA and methods of the present invention enhance multiple aspects of a cancer patient's immune response. In various aspects, the present invention demonstrates that when a recombinant MVA comprising a tumor-associated antigen (TAA) and a 4-1BBL antigen is administered intratumorally or intravenously to a cancer subject, there is an increased anti-tumor effect realized in the subject. As described in more detail herein, this increased anti-tumor effect includes a higher reduction in tumor volume, increased overall survival rate, an enhanced CD8 T cell response to the TAA, and enhanced inflammatory responses such as increased NK cell activity, increases in cytokine production, and so forth.

[075] Described and illustrated in the present application, the recombinant MVA and methods of the present invention enhance multiple aspects of a cancer patient's immune response. In various aspects, the present invention demonstrates that when a recombinant MVA comprising a tumor-associated antigen (TAA) and a CD40L antigen is administered intratumorally or intravenously to a cancer subject, there is an increased anti-tumor effect realized in the subject. As described in more detail herein, this increased anti-tumor effect includes a higher reduction in tumor volume, increased overall survival rate, an enhanced CD8 T cell response to the TAA, and enhanced inflammatory responses such as increased NK cell activity, increases in cytokine production, and so forth.

[076] In additional aspects, various embodiments of the present invention demonstrate that when a recombinant MVA comprising a tumor-associated antigen (TAA) and a 4-1BBL antigen is administered intratumorally in combination with at least one immune checkpoint molecule antagonist/agonist there is increased tumor reduction and an increase in overall survival rate in cancer subjects.

[077] In still further aspects, various embodiments of the present invention demonstrate that when a recombinant MVA comprising a tumor-associated antigen (TAA) and a 4-1BBL antigen is administered intratumorally in combination with a tumor specific antibody there is increased tumor reduction and an increase in overall survival rate in cancer subjects.

[078] While recombinant MVA viruses have previously encoded a 4-1BBL antigen, the immunogenic benefits of an MVA encoding 4-1BBL was unclear (see, e.g., Spencer *et al.* (2014) *PLoS One* 9(8): e105520). In Spencer, co-expression of 4-1BBL and a transgenic antigen in either an MVA vector or an Adenovirus vector resulted in an increase in mouse CD8 T cell responses; however, after an intra-muscular administration with the Adenovirus vector encoding 4-1BBL, there was not any increase seen in IFN- γ responses in non-human primates (Id. at pages 2, 6). Furthermore, the immunogenic benefits of utilizing an MVA encoding 4-1BBL as part of treating cancer and destroying tumor and/or tumor cells was unknown.

[079] The various embodiments of the present disclosure demonstrate that an MVA encoding 4-1BBL and a TAA (referred to herein as MVA-TAA-4-1BBL) can be effective in treating cancer in a subject, such as a human. Shown and described herein, administration of MVA-TAA-4-1BBL can enhance multiple aspects of a cancer subject's immune response and can effectively reduce and kill tumor cells. One or more of the enhanced anti-tumor effects of the various embodiments of the present disclosure are summarized as follows.

[080] Intravenous administration of recombinant MVA encoding 4-1BBL generates an enhanced antitumor effect. In at least one aspect, the present invention includes a recombinant MVA encoding a TAA and a 4-1BBL antigen (rMVA-TAA-4-1BBL) that is administered intravenously, wherein the intravenous administration enhances an anti-tumor effect, as compared to an intravenous administration of a recombinant MVA without 4-1BBL, or as compared to a non-intravenous administration of a recombinant MVA encoding 4-1BBL (for example, such as a subcutaneous administration of a recombinant MVA encoding 4-1BBL). These enhanced antitumor effects include an enhanced NK cell response (shown in Figure 4), an enhanced inflammatory response as shown by an increase in IFN- γ secretion (shown in Figures 5 and 6), an increased antigen and vector-specific CD8 T cell expansion (shown in Figure 7), and an increased tumor reduction (shown in Figure 8).

[081] Intratumoral administration of recombinant MVA encoding 4-1BBL enhances inflammation in the tumor. In another aspect of the present invention, it was determined that infection of tumor cells with MVA-OVA-4-1BBL, but not with MVA-OVA-CD40L, activated antigen-specific CD8+ T cells to produce T cell-derived cytokines such as GM-CSF, IL-2 and IFN- γ in the absence of antigen cross-presenting DCs (Figures 1A-1D). This was unexpected in the case of GM-CSF, a growth factor produced by naïve T cells upon activation that induces maturation of dendritic cell and myeloid cell subsets (Min *et al.* (2010) *J. Immunol.* 184: 4625-4629). In the presence of antigen-cross-presenting DCs, antigen-specific CD8+ T cells stimulated by infected tumor cells with rMVA-CD40L produced IFN- γ , but not IL-2 or GM-CSF as rMVA-4-1BBL (Figures 1A-1D). Interestingly, large amounts of IL-6, a key cytokine produced by DCs, were detected (Figure 1A).

[082] In one advantageous aspect, enhanced inflammation in the tumor can result in having large numbers of TILs (tumor infiltrating lymphocytes) killing tumor cells at the site of the tumor (see, *e.g.*, Lanitis *et al.* (2017) *Annals Oncol.* 28 (suppl 12): xii18-xii32). These inflamed tumors, also known as “hot” tumors, enable enhanced tumor cell destruction in view of the increased numbers of TILs, cytokines, and other inflammatory molecules.

[083] Intratumoral administration of recombinant MVA encoding 4-1BBL reduces tumor volume and increase overall survival rate. In one aspect, the present invention includes a recombinant MVA encoding a 4-1BBL antigen (MVA-4-1BBL) that is administered intratumorally, wherein the intratumoral administration enhances anti-tumor effects in a cancer subject, as compared to an intratumoral administration of a recombinant MVA without 4-1BBL.

[084] While recombinant MVA viruses have been previously administered intratumorally (see *e.g.*, White *et al.* (2018) *PLoS One* 13: e0193131, and Nemeckova *et al.* (2007) *Neoplasma* 54: 326-33), the studies have produced diverse results. For example, in Nemeckova, it was found that intratumoral injections of vaccinia virus MVA expressing GM-CSF and immunization with DNA vaccine prolonged the survival of mice bearing HPV16 induced tumors (see Nemeckova at Abstract). Alternatively, White *et al.* were unable to demonstrate inhibition of pancreatic tumor growth following intratumoral injection of MVA (see White at Abstract).

[085] As part of the present disclosure, a recombinant MVA comprising one or more nucleic acids encoding a TAA and 4-1BBL was administered intratumorally to a subject. Shown in Figure 9, an intratumoral injection of MVA-TAA-4-1BBL demonstrated a significant decrease in tumor volume as compared to recombinant MVA TAA.

[086] Intratumoral administration of recombinant MVA encoding 4-1BBL administered in combination with an immune checkpoint molecule antagonist or agonist generates an increased anti-tumor effect. In various embodiments, the present invention includes an administration of MVA-TAA-4-1BBL in combination with an immune checkpoint antagonist or agonist. Preferably the administration of the MVA-TAA-4-1BBL is intravenous or intratumoral. The MVAs of the present invention in combination with an immune checkpoint antagonist or agonist is advantageous as the combination provides a more effective cancer treatment. For example, the combination and/or combination therapy of the present invention enhances multiple aspects of a cancer patient's immune response. In at least one aspect, the combination synergistically enhances both the innate and adaptive immune responses and, when combined with an antagonist or agonist of an immune checkpoint molecule, reduces tumor volume and increase survival of a cancer patient.

[087] The data presented in this application demonstrate that MVA-TAA-4-1BBL when combined with an immune checkpoint antagonist or agonist generates an increased anti-tumor effect. Indeed, shown in Figure 11, when an intratumoral administration of MVA-OVA-4-1BBL was combined with a PD-1 antibody intraperitoneally, there was a decrease in tumor volume as compared to PD-1 by itself.

[088] Intratumoral administration of recombinant MVA encoding 4-1BBL administered in combination with an antibody specific for a tumor associated antigen (TAA) generates an increased anti-tumor effect. In various embodiments, the present invention includes an administration of MVA-TAA-4-1BBL in combination with an antibody specific for a TAA. Preferably the administration of

the MVA-TAA-4-1BBL is intravenous or intratumoral. The MVAs of the present invention in combination with an TAA specific antibody is advantageous and can work together to provide a more effective cancer treatment.

[089] In one exemplary aspect, the enhanced NK cells response induced by the administration of the MVA-TAA-4-1BBL works synergistically with the TAA specific antibody to enhance antibody dependent cytotoxicity (ADCC) in a subject. This enhanced ADCC in a cancer subject leads to an increase in tumor cell killing and tumor destruction.

[090] The data presented in the present application demonstrate that MVA-TAA-4-1BBL when combined with an TAA specific antibody generates an increased anti-tumor effect. Indeed, shown in Figure 11, when an intratumoral administration of MVA-OVA-4-1BBL was combined with intraperitoneal TRP-1 antibody, there was a decrease in tumor volume as compared to the TRP-1 antibody by itself.

[091] Administration of MVA-TAA-4-1BBL as part of a prime and boost immunization according to the invention increases antigen and vector-specific CD8+ T cell expansion. In other aspects, the invention provides a method in which MVA-TAA-4-1BBL is administered as part of a homologous and/or heterologous prime-boost regimen. Preferably the administration of the MVA-TAA-4-1BBL is intravenous or intratumoral. Illustrated in Figure 7, antigen and vector-specific CD8+ T cell expansion was increased during a priming and boosting by intravenous administration of MVA-TAA-4-1BBL.

Definitions

[092] As used herein, the singular forms "a," "an," and "the" include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a nucleic acid" includes one or more of the nucleic acid and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[093] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[094] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise," and variations such as "comprises" and "comprising," will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes when used herein with the term "having." Any of the aforementioned terms (comprising, containing, including, having), though less preferred, whenever used herein in the context of an aspect or embodiment of the present invention can be substituted with the term "consisting of. When used herein "consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[095] As used herein, the conjunctive term "and/or" between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by "and/or," a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or" as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or."

[096] "Mutated" or "modified" protein or antigen as described herein is as defined herein any a modification to a nucleic acid or amino acid, such as deletions, additions, insertions, and/or substitutions.

[097] "Percent (%) sequence homology or identity" with respect to nucleic acid sequences described herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the reference sequence (*i.e.*, the nucleic acid sequence from which it is derived), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent nucleotide sequence identity or homology can be achieved in various ways that are within the skill in the art, for example, using publicly available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the

art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximum alignment over the full length of the sequences being compared.

[098] For example, an appropriate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman ((1981) *Advances in Applied Mathematics* 2: 482-489). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, *Atlas of Protein Sequences and Structure*, M. O. Dayhoff ed., 5 suppl. 3: 353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov ((1986) *Nucl. Acids Res.* 14(6): 6745-6763). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wisconsin, USA) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wisconsin, USA). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by Collins and Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, California, USA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+ GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found at the following internet address: blast.ncbi.nlm.nih.gov/.

[099] The term "prime-boost vaccination" or "prime-boost regimen" refers to a vaccination strategy or regimen using a first priming injection of a vaccine targeting a specific antigen followed at intervals by one or more boosting injections of the same vaccine. Prime-boost vaccination may be homologous or heterologous. A homologous prime-boost vaccination uses a vaccine comprising the same antigen and vector for both the priming injection and the one or more boosting injections. A heterologous prime-boost vaccination uses a vaccine comprising the same antigen for both the priming

injection and the one or more boosting injections but different vectors for the priming injection and the one or more boosting injections. For example, a homologous prime-boost vaccination may use a recombinant poxvirus comprising nucleic acids expressing one or more antigens for the priming injection and the same recombinant poxvirus expressing one or more antigens for the one or more boosting injections. In contrast, a heterologous prime-boost vaccination may use a recombinant poxvirus comprising nucleic acids expressing one or more antigens for the priming injection and a different recombinant poxvirus expressing one or more antigens for the one or more boosting injections.

[0100] The term "recombinant" means a polynucleotide, virus or vector of semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature. By "recombinant MVA" or "rMVA" as used herein is generally intended a modified vaccinia Ankara (MVA) that comprises at least one polynucleotide encoding a tumor associated antigen (TAA).

[0101] As used herein, reducing tumor volume or a reduction in tumor volume can be characterized as a reduction in tumor volume and/or size but can also be characterized in terms of clinical trial endpoints understood in the art. Some exemplary clinical trial endpoints associated with a reduction in tumor volume and/or size can include, but are not limited to, Response Rate (RR), Objective response rate (ORR), and so forth.

[0102] As used herein an increase in survival rate can be characterized as an increase in survival of a cancer patient, but can also be characterized in terms of clinical trial endpoints understood in the art. Some exemplary clinical trial endpoints associated with an increase in survival rate include, but are not limited to, Overall Survival rate (OS), Progression Free Survival (PFS) and so forth.

[0103] As used herein, a "transgene" or "heterologous" gene is understood to be a nucleic acid or amino acid sequence which is not present in the wild-type poxviral genome (*e.g.*, Vaccinia, Fowlpox, or MVA). The skilled person understands that a "transgene" or "heterologous gene", when present in a poxvirus, such as Vaccinia Virus, is to be incorporated into the poxviral genome in such a way that, following administration of the recombinant poxvirus to a host cell, it is expressed as the corresponding heterologous gene product, *i.e.*, as the "heterologous antigen" and\or "heterologous protein." Expression is normally achieved by operatively linking the heterologous gene to regulatory

elements that allow expression in the poxvirus-infected cell. Preferably, the regulatory elements include a natural or synthetic poxviral promoter.

[0104] A "vector" refers to a recombinant DNA or RNA plasmid or virus that can comprise a heterologous polynucleotide. The heterologous polynucleotide may comprise a sequence of interest for purposes of prevention or therapy, and may optionally be in the form of an expression cassette. As used herein, a vector needs not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors and viral vectors.

Combinations and Methods

[0105] In various embodiments, the present invention comprises a recombinant MVA comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, that when administered intratumorally induces both an inflammatory response and an enhanced T cell response as compared to an inflammatory response and a T cell response induced by a non-intratumoral administration of a recombinant MVA virus comprising a first nucleic acid encoding a TAA and a second nucleic acid encoding 4-1BBL.

[0106] In various additional embodiments, the present invention comprises a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, that when administered intratumorally induces both an enhanced intratumoral inflammatory response and an enhanced T cell response as compared to an intratumoral inflammatory response and a T cell response induced by an intratumoral administration of a recombinant MVA virus comprising a first nucleic acid encoding a TAA.

[0107] Enhanced Inflammation Response in the Tumor. In various aspects of the present disclosure it was determined that an intratumoral administration of a recombinant MVA encoding a TAA and a 4-1BBL induces an enhanced inflammatory response in a tumor, as compared to an administration of a recombinant MVA by itself. In at least one aspect, an "enhanced inflammation response" in a tumor according to present disclosure is characterized by one or more of the following: 1) an increase in expression of IFN- γ and/or 2) an increase in expression of Granzyme B (GraB) in the tumor and/or tumor cells. Thus, whether an inflammatory response is enhanced in a tumor and/or tumor cells in accordance with present disclosure can be determined by measuring to determine whether there is an increase in expression of one or more molecules which are indicative of an increased inflammatory response, including the secretion of chemokines and cytokines as is known in

the art. Exemplary inflammatory response markers include one or more of markers that are useful in measuring NK cell frequency and/or activity include one or more of: IFN- γ and/or Granzyme B (GraB). These molecules and the measurement thereof are validated assays that are understood in the art and can be carried out according to known techniques. See, e.g., Borrego *et al.* ((1999) *Immunology* 7(1): 159-165).

[0108] Enhanced NK cell response. In various additional aspects of the present disclosure it was determined that an intratumoral administration or an intravenous administration of a recombinant MVA encoding a TAA and a 4-1BBL induces an enhanced NK Cells response in a tumor or tumor environment, as compared an administration of a recombinant MVA by itself. In one aspect, an “enhanced NK cell response” according to the present disclosure is characterized by one or more of the following: 1) an increase in NK cell frequency, 2) an increase in NK cell activation, and/or 3) an increase in NK cell proliferation. Thus, whether an NK cell response is enhanced in accordance with the present disclosure can be determined by measuring the expression of one or more molecules which are indicative of an increased NK cell frequency, increased NK cell activation, and/or increased NK cell proliferation. Exemplary markers that are useful in measuring NK cell frequency and/or activity include one or more of: NKp46, IFN- γ , CD69, CD70, NKG2D, FasL, granzyme B, CD56, and/or Bcl-XL. Exemplary markers that are useful in measuring NK cell activation include one or more of IFN- γ , CD69, CD70, NKG2D, FasL, granzyme B and/or Bcl-XL. Exemplary markers that are useful in measuring NK cell proliferation include: Ki67. These molecules and the measurement thereof are validated assays that are understood in the art and can be carried out according to known techniques (see, e.g., Borrego *et al.* (1999) *Immunology* 7(1): 159-165). Additionally, assays for measuring the molecules can be found in Examples 5 and 6 of the present disclosure. At least in one aspect, 1) an increase in NK cell frequency can be defined as at least a 2-fold increase in CD3-NKp46+ cells compared to pre-treatment/baseline; 2) an increase in NK cell activation can be defined as at least a 2-fold increase in IFN- γ , CD69, CD70, NKG2D, FasL, granzyme B and/or Bcl-XL expression compared to pre-treatment/baseline expression; and/or 3) an increase in NK cell proliferation is defined as at least a 1.5 fold increase in Ki67 expression compared to pre-treatment/baseline expression.

[0109] Enhanced T Cell response. In accordance with the present application, an “enhanced T cell response” is characterized by one or more of the following: 1) an increase in frequency of CD8 T cells; 2) an increase in CD8 T cell activation; and/or 3) an increase in CD8 T cell proliferation. Thus,

whether a T cell response is enhanced in accordance with the present application can be determined by measuring the expression of one or more molecules which are indicative of 1) an increase in CD8 T cell frequency 2) an increase in CD8 T cell activation; and/or 3) an increase in CD8 T cell proliferation. Exemplary markers that are useful in measuring CD8 T cell frequency, activation, and proliferation include CD3, CD8, IFN- γ , TNF- α , IL-2, CD69 and/or CD44, and Ki67, respectively. Measuring antigen specific T cell frequency can also be measured by MHC Multimers such as pentamers or dextramers as shown by the present application. Such measurements and assays as well as others suitable for use in evaluating methods and compositions of the invention are validated and understood in the art.

[0110] In one aspect, an increase in CD8 T cell frequency is characterized by an at least a 2-fold increase in IFN- γ and/or dextramer⁺ CD8 T cells compared to pre-treatment/baseline. An increase in CD8 T cell activation is characterized as at least a 2-fold increase in CD69 and/or CD44 expression compared to pre-treatment/baseline expression. An increase in CD8 T cell proliferation is characterized as at least a 2-fold increase in Ki67 expression compared to pre-treatment/baseline expression.

[0111] In an alternative aspect, an enhanced T cell response is characterized by an increase in CD8 T cell expression of effector cytokines and/or an increase of cytotoxic effector functions. An increase in expression of effector cytokines can be measured by expression of one or more of IFN- γ , TNF- α , and/or IL-2 compared to pre-treatment/baseline. An increase in cytotoxic effector functions can be measured by expression of one or more of CD107a, granzyme B, and/or perforin and/or antigen-specific killing of target cells.

[0112] The assays, cytokines, markers, and molecules described herein and the measurement thereof are validated and understood in the art and can be carried out according to known techniques. Additionally, assays for measuring the T cells responses can be found in the working examples, wherein T cell responses were analyzed, including but not limited to Examples 2, 3, 8, 13 and 14.

[0113] The enhanced T cell response realized by the present invention is particularly advantageous in combination with the enhanced NK cell response, and the enhanced inflammatory response as the enhanced T cells effectively target and kill those tumor cells that have evaded and/or survived past the initial innate immune responses in the cancer patient.

[0114] In yet additional embodiments, the combinations and methods described herein are for use in treating a human cancer patient. In preferred embodiments, the cancer patient is suffering from

and/or is diagnosed with a cancer selected from the group consisting of: breast cancer, lung cancer, head and neck cancer, thyroid, melanoma, gastric cancer, bladder cancer, kidney cancer, liver cancer, melanoma, pancreatic cancer, prostate cancer, ovarian cancer, urothelial, cervical, or colorectal cancer. In yet additional embodiments, the combinations and methods described herein are for use in treating a human cancer patient suffering from and/or diagnosed with a breast cancer, colorectal cancer or melanoma, preferably a melanoma, more preferably a colorectal cancer or most preferably a colorectal cancer.

[0115] Certain Exemplary Tumor-Associated Antigens. In certain embodiments, an immune response is produced in a subject against a cell-associated polypeptide antigen. In certain such embodiments, a cell-associated polypeptide antigen is a tumor-associated antigen (TAA).

[0116] The term "polypeptide" refers to a polymer of two or more amino acids joined to each other by peptide bonds or modified peptide bonds. The amino acids may be naturally occurring as well as non-naturally occurring, or a chemical analogue of a naturally occurring amino acid. The term also refers to proteins, i.e. functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups.

[0117] Endogenous Retroviral Proteins (ERVs). Preferably, the TAA is embodied in an Endogenous Retroviral Proteins (ERVs). More preferably, the ERV is an ERV from the Human HERV-K protein family. Most preferably, the HERV-K protein is selected from a HERV-K envelope (env) protein, a HERV-K group specific antigen (gag) protein, and a HERV-K "marker of melanoma risk" (mel) protein (see, e.g., Cegolon *et al.* (2013) *BMC Cancer* 13:4).

[0118] ERVs constitute 8% of the human genome and are derived from germline infections million years ago. The majority of those elements inserted into our genome are heavily mutated and thus are not transcribed or translated. However, a small, rather recently acquired fraction of ERVs is still functional and translated and in some cases even produce viral particles. The transcription of ERVs is very restricted as the locus is usually highly methylated and consequently not transcribed in somatic cells (Kassiotis (2016) *Nat. Rev. Immunol.* 16: 207-19). Only under some circumstances such as cellular stress (chemicals, UV radiation, hormones, cytokines) ERVs can be reactivated. Importantly, ERVs are also expressed in many different types of cancer but not in normal tissues

(Cegolon *et al.* (2013) *BMC Cancer* 13: 4; Wang-Johanning *et al.* (2003) *Oncogene* 22: 1528-35). This very restricted expression pattern ensures that ERVs are not or rarely exposed to immunological tolerance mechanisms which presumably results in a competent ERV-specific T cell repertoire. In this manner, ERVs can be used in MVAs as tumor antigens (“TAAs”).

[0119] In various additional embodiments, the TAA includes, but is not limited to, HER2, PSA, PAP, CEA, MUC-1, FOLR1, PRAME, survivin, TRP1, TRP2, or Brachyury alone or in combinations. Such exemplary combination may include CEA and MUC-1, for example in an MVA also known as CV301. Other exemplary combinations may include PAP and PSA.

[0120] In still further embodiments, additional TAAs may include, but are not limited to, 5 alpha reductase, alpha-fetoprotein, AM-1, APC, April, BAGE, beta-catenin, Bcl12, bcr-abl, CA-125, CASP-8/FLICE, Cathepsins, CD19, CD20, CD21, CD23, CD22, CD33 CD35, CD44, CD45, CD46, CD5, CD52, CD55, CD59, CDC27, CDK4, CEA, c-myc, Cox-2, DCC, DcR3, E6/E7, CGFR, EMBP, Dna78, farnesyl transferase, FGF8b, FGF8a, FLK-1/KDR, folic acid receptor, G250, GAGE-family, gastrin 17, gastrin-releasing hormone, GD2/GD3/GM2, GnRH, GnTV, GP1, gp100/Pmel17, gp-100-in4, gp15, gp75/TRP1, hCG, heparanase, Her2/neu, HMTV, Hsp70, hTERT, IGFR1, IL-13R, iNOS, Ki67, KIAA0205, K-ras, H-ras, N-ras, KSA, LKLR-FUT, MAGE-family, gammaglobin, MAP17, melan-A/MART-1, mesothelin, MIC A/B, MT-MMPs, mucin, NY-ESO-1, osteonectin, p15, P170/MDR1, p53, p97/melanotransferrin, PAI-1, PDGF, uPA, PRAME, probasin, progenipoitin, PSA, PSM, RAGE-1, Rb, RCAS1, SART-1, SSX-family, STAT3, STn, TAG-72, TGF-alpha, TGF-beta, Thymosin-beta-15, TNF-alpha, TRP1, TRP2, tyrosinase, VEGF, ZAG, p16INK4, and glutathione-S-transferase.

[0121] A preferred PSA antigen comprises the amino acid change of isoleucine to leucine at position 155 (see U.S. Patent 7,247,615, which is incorporated herein by reference).

[0122] In one or more preferred embodiments of present invention, the heterologous TAA is selected from HER2 and/or Brachyury.

[0123] Any TAA may be used so long as it accomplishes at least one objective or desired end of the invention, such as, for example, stimulating an immune response following administration of the MVA containing it. Exemplary sequences of TAAs, including TAAs mentioned herein, are known in the art and are suitable for use in the compositions and methods of the invention. Sequences of TAAs for use in the compositions and methods of the invention may be identical to sequences known in the art or disclosed herein, or they may share less than 100% identity, such as at least 90%, 91%, 92%,

95%, 97%, 98%, or 99% or more sequence identity to either a nucleotide or amino acid sequence known in the art or disclosed herein. Thus, a sequence of a TAA for use in a composition or method of the invention may differ from a reference sequence known in the art and/or disclosed herein by less than 20, or less than 19, 18, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 nucleotides or amino acids, so long as it accomplishes at least one objective or desired end of the invention. One of skill in the art is familiar with techniques and assays for evaluating TAAs to ensure their suitability for use in an MVA or method of the invention.

[0124] Modified Tumor-Associated Antigens. In certain embodiments, a cell-associated polypeptide antigen is modified such that a CTL response is induced against a cell which presents epitopes derived from a polypeptide antigen on its surface, when presented in association with an MHC Class I molecule on the surface of an APC. In certain such embodiments, at least one first foreign TH epitope, when presented, is associated with an MHC Class II molecule on the surface of the APC. In certain such embodiments, a cell-associated antigen is a tumor-associated antigen.

[0125] Exemplary APCs capable of presenting epitopes include dendritic cells and macrophages. Additional exemplary APCs include any pino- or phagocytizing APC, which is capable of simultaneously presenting: 1) CTL epitopes bound to MHC class I molecules; and 2) TH epitopes bound to MHC class II molecules.

[0126] In certain embodiments, modifications to one or more of the TAAs, such as, but not limited to, HERV-K env, HERV-K gag, HERV-K mel, CEA, MUC-1, PAP, PSA, PRAME, FOLR1, HER2, survivin, TRP1, TRP2, or Brachyury, are made such that, after administration to a subject, polyclonal antibodies are elicited that predominantly react with the one or more of the TAAs described herein. Such antibodies could attack and eliminate tumor cells as well as prevent metastatic cells from developing into metastases. The effector mechanism of this anti-tumor effect would be mediated via complement and antibody dependent cellular cytotoxicity. In addition, the induced antibodies could also inhibit cancer cell growth through inhibition of growth factor dependent oligo-dimerisation and internalization of the receptors. In certain embodiments, such modified TAAs could induce CTL responses directed against known and/or predicted TAA epitopes displayed by the tumor cells.

[0127] In certain embodiments, a modified TAA polypeptide antigen comprises a CTL epitope of the cell-associated polypeptide antigen and a variation, wherein the variation comprises at least one CTL epitope or a foreign TH epitope. Certain such modified TAAs can include in one non-limiting example one or more HER2 polypeptide antigens comprising at least one CTL epitope and a variation

comprising at least one CTL epitope of a foreign TH epitope, and methods of producing the same, are described in U.S. Patent No. 7,005,498 and U.S. Patent Pub. Nos. 2004/0141958 and 2006/0008465.

[0128] Certain such modified TAAs can include in one non-limiting example one or more MUC-1 polypeptide antigens comprising at least one CTL epitope and a variation comprising at least one CTL epitope of a foreign epitope, and methods of producing the same, are described in U.S. Patent Pub. Nos. 2014/0363495.

[0129] Additional promiscuous T-cell epitopes include peptides capable of binding a large proportion of HLA-DR molecules encoded by the different HLA-DR. See, *e.g.*, WO 98/23635 (Frazer IH *et al.*, assigned to The University of Queensland); Southwood *et. al.* (1998) *J. Immunol.* 160: 3363 3373; Sinigaglia *et al.* (1988) *Nature* 336: 778 780; Rammensee *et al.* (1995) *Immunogenetics* 41: 178 228; Chicz *et al.* (1993) *J. Exp. Med.* 178: 27 47; Hammer *et al.* (1993) *Cell* 74: 197 203; and Falk *et al.* (1994) *Immunogenetics* 39: 230 242. The latter reference also deals with HLA-DQ and -DP ligands. All epitopes listed in these references are relevant as candidate natural epitopes as described herein, as are epitopes which share common motifs with these.

[0130] In certain other embodiments, the promiscuous T-cell epitope is an artificial T-cell epitope which is capable of binding a large proportion of haplotypes. In certain such embodiments, the artificial T-cell epitope is a pan DR epitope peptide ("PADRE") as described in WO 95/07707 and in the corresponding paper Alexander *et al.* (1994) *Immunity* 1: 751 761.

[0131] 4-1BBL (also referred to herein as "41BBL" or "4-1BB ligand"). As illustrated by the present disclosure, the inclusion of 4-1BBL as part of the recombinant MVA and related methods induces increased and enhanced anti-tumor effects upon an intratumoral or intravenous administration in a cancer subject. Thus, in various embodiments, in addition to encoding a TAA, there is a recombinant MVA encoding a 4-1BBL antigen.

[0132] 4-1BB/4-1BBL is a member of the TNFR/TNF superfamily. 4-1BBL is a costimulatory ligand expressed in activated B cells, monocytes and DCs. 4-1BB is constitutively expressed by natural killer (NK) and natural killer T (NKT) cells, Tregs and several innate immune cell populations, including DCs, monocytes and neutrophils. Interestingly, 4-1BB is expressed on activated, but not resting, T cells (Wang *et al.* (2009) *Immunol. Rev.* 229: 192-215). 4-1BB ligation induces proliferation and production of interferon gamma (IFN- γ) and interleukin 2 (IL-2), as well as enhances T cell survival through the upregulation of antiapoptotic molecules such as Bcl-xL (Snell *et al.* (2011) *Immunol. Rev.* 244: 197-217). Importantly, 4-1BB stimulation enhances NK cell

proliferation, IFN- γ production and cytolytic activity through enhancement of Antibody-Dependent Cell Cytotoxicity (ADCC) (Kohrt *et al.* (2011) *Blood* 117: 2423-32).

[0133] In one or more preferred embodiments, 4-1BBL is encoded by the MVA of the present invention. In one or more other preferred embodiments, 4-1BBL is a human 4-1BBL. In still more preferred embodiments, the 4-1BBL comprises a nucleic acid encoding an amino acid sequence having a sequence with at least 90%, 95%, 97% 98%, or 99% identity to SEQ ID NO:3, *i.e.*, differing from the amino acid sequence set forth in SEQ ID NO:3 by less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids. In even more preferred embodiments, the 4-1BBL comprises a nucleic acid encoding an amino acid sequence comprising SEQ ID NO: 3. In additional embodiments, a nucleic acid encoding 4-1BBL comprises a nucleic acid sequence having at least 90%, 95%, 97% 98%, or 99% identity to SEQ ID NO:4, *i.e.*, differing from the nucleic acid sequence set forth in SEQ ID NO:4 by less than 20, 10, 5, 4, 3, 2, or 1 nucleic acid in the sequence. In more preferred embodiments, the 4-1BBL comprises a nucleic acid comprising SEQ ID NO: 4.

[0134] CD40L. As illustrated by the present disclosure the inclusion of CD40L as part of the combination and related method further enhances the decrease in tumor volume, prolongs progression-free survival and increase survival rate realized by the present invention. Thus, in various embodiments, the combination further comprises administering CD40L to a cancer patient. In preferred embodiments, the CD40L is encoded as part of a recombinant MVA as described herein.

[0135] While CD40 is constitutively expressed on many cell types, including B cells, macrophages, and dendritic cells, its ligand CD40L is predominantly expressed on activated T helper cells. The cognate interaction between dendritic cells and T helper cells early after infection or immunization ‘licenses’ dendritic cells to prime CTL responses. Dendritic cell licensing results in the up-regulation of co-stimulatory molecules, increased survival and better cross-presenting capabilities. This process is mainly mediated via CD40/CD40L interaction. However, various configurations of CD40L are described, from membrane bound to soluble (monomeric to trimeric) which induce diverse stimuli, either inducing or repressing activation, proliferation, and differentiation of APCs.

[0136] In one or more preferred embodiments, CD40L is encoded by the MVA of the present invention. In one or more other preferred embodiments, CD40L is a human CD40L. In still more preferred embodiments, the CD40L comprises a nucleic acid encoding an amino acid having a sequence with at least 90%, 95%, 97% 98%, or 99% identity to SEQ ID NO:1, *i.e.*, differing from the amino acid sequence set forth in SEQ ID NO:1 by less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids.

In even more preferred embodiments, the CD40L comprises a nucleic acid encoding an amino acid comprising SEQ ID NO: 1. In additional embodiments, a nucleic acid encoding CD40L comprises a nucleic acid sequence having at least 90%, 95%, 97% 98%, or 99% identity to SEQ ID NO:2, *i.e.*, differing from the nucleic acid sequence set forth in SEQ ID NO:2 by less than 20, 10, 5, 4, 3, 2, or 1 nucleic acid in the sequence. In more preferred embodiments, the CD40L comprises a nucleic acid comprising SEQ ID NO: 2.

[0137] Antagonists of Immune Checkpoint Molecules. As described herein, at least in one aspect, the invention encompasses the use of immune checkpoint antagonists. Such immune checkpoint antagonists function to interfere with and/or block the function of the immune checkpoint molecule. Some preferred immune checkpoint antagonists include, Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Programmed Cell Death Protein 1 (PD-1), Programmed Death-Ligand 1 (PD-L1), Lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain 3 (TIM-3).

[0138] Additionally, exemplary immune checkpoint antagonists can include, but are not limited to CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, TIM-3, T cell Immunoreceptor with Ig and ITIM domains (TIGIT) and V-domain Ig Suppressor of Tcell activation (VISTA).

[0139] Such antagonists of the immune checkpoint molecules can include antibodies which specifically bind to immune checkpoint molecules and inhibit and/or block biological activity and function of the immune checkpoint molecule.

[0140] Other antagonists of the immune checkpoint molecules can include antisense nucleic acid RNAs that interfere with the expression of the immune checkpoint molecules; and small interfering RNAs that interfere with the expression of the immune checkpoint molecules.

[0141] Antagonists can additionally be in the form of small molecules that inhibit or block the function of the immune checkpoint. Some non-limiting examples of these include NP12 (Aurigene), (D) PPA-1 by Tsinghua Univ, high affinity PD-1 (Stanford); BMS-202 and BMS-8 (Bristol Myers Squibb (BMS), and CA170/ CA327 (Curis/Aurigene); and small molecule inhibitors of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3.

[0142] Antagonists can additionally be in the form of Anticalins® that inhibit or block the function of the immune checkpoint molecule. See, *e.g.*, Rothe *et al.* ((2018) *BioDrugs* 32(3): 233–243).

[0143] It is contemplated that antagonists can additionally be in the form of Affimers®. Affimers are Fc fusion proteins that inhibit or block the function of the immune checkpoint molecule.

Other fusion proteins that can serve as antagonists of immune checkpoints are immune checkpoint fusion proteins (*e.g.*, anti-PD-1 protein AMP-224) and anti-PD-L1 proteins such as those described in US2017/0189476.

[0144] Candidate antagonists of immune checkpoint molecules can be screened for function by a variety of techniques known in the art and/or disclosed within the instant application, such as for the ability to interfere with the immune checkpoint molecules function in an *in vitro* or mouse model.

[0145] Agonist of ICOS. The invention further encompasses agonists of ICOS. An agonist of ICOS activates ICOS. ICOS is a positive co-stimulatory molecule expressed on activated T cells and binding to its' ligand promotes their proliferation (Dong (2001) *Nature* 409: 97-101).

[0146] In one embodiment, the agonist is ICOS-L, an ICOS natural ligand. The agonist can be a mutated form of ICOS-L that retains binding and activation properties. Mutated forms of ICOS-L can be screened for activity in stimulating ICOS *in vitro*.

[0147] Antibodies to an Immune Checkpoint Antagonist or Agonist. In preferred embodiments, the antagonist and/or agonist of an immune checkpoint molecules each comprises an antibody. As described herein, in various embodiments, the antibodies can be synthetic, monoclonal, or polyclonal and can be made by techniques well known in the art. Such antibodies specifically bind to the immune checkpoint molecule via the antigen-binding sites of the antibody (as opposed to non-specific binding). Immune checkpoint peptides, fragments, variants, fusion proteins, *etc.*, can be employed as immunogens in producing antibodies immunoreactive therewith. More specifically, the polypeptides, fragment, variants, fusion proteins, etc. contain antigenic determinants or epitopes that elicit the formation of antibodies.

[0148] In more preferred embodiments, the antibodies of present invention are those that are approved, or in the process of approval by the government of a sovereign nation, for the treatment of a human cancer patient. Some non-limiting examples of these antibodies already approved, or in the approval process include the following: CTLA-4 (Ipilimumab® and Tremelimumab); PD-1 (Pembrolizumab, Lambrolizumab, Amplimmune-224 (AMP-224)), Amplimmune -514 (AMP-514), Nivolumab, MK-3475 (Merck), . BI 754091 (Boehringer Ingelheim)), and PD-L1 (Atezolizumab, Avelumab, Durvalumab, MPDL3280A (Roche), MED14736 (AZN), MSB0010718C (Merck)); LAG-3 (IMP321, BMS-986016, BI754111 (Boehringer Ingelheim), LAG525 (Novartis), MK-4289 (Merck), TSR-033 (Tesaro).

[0149] In one exemplary aspect, the immune checkpoint molecules CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS and peptides based on the amino acid sequence of CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS can be utilized to prepare antibodies that specifically bind to CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, or ICOS. The term "antibodies" is meant to include polyclonal antibodies, monoclonal antibodies, fragments thereof, such as F(ab')2 and Fab fragments, single-chain variable fragments (scFvs), single-domain antibody fragments (VHHs or Nanobodies), bivalent antibody fragments (diabodies), as well as any recombinantly and synthetically produced binding partners.

[0150] Antibodies Specific to a Tumor Associated Antigen (TAA). In various embodiments of the present invention the recombinant MVAs and methods described herein are combined with, or administered in combination with, an antibody specific to a TAA. In more particular embodiments, the recombinant MVAs and methods described herein are combined with or administered in combination with an antibody specific to an antigen that is expressed on the cell membrane of a tumor cell. It is understood in the art that in many cancers, one or more antigens are expressed or overexpressed on the tumor cell membrane. See, *e.g.* Durig *et al.* (2002) *Leukemia* 16: 30-5; Mocellin *et al.* (2013) *Biochim. Biophys. Acta* 1836: 187-96; Arteaga (2011) *Nat. Rev. Clin. Oncol.*, doi:10.1038/nrclinonc.2011.177; Finn (2017) *Cancer Immunol. Res.* 5: 347-54; Ginaldi *et al.* (1998) *J. Clin. Pathol.* 51: 364-9. Assays for determining whether an antigen is expressed or overexpressed on a tumor cells are readily understood in the art (*Id.*), as well as methods for producing antibodies to a particular antigen.

[0151] In more specific embodiments, the pharmaceutical combination and related methods include an antibody, wherein in the antibody is a) specific to an antigen that is expressed on a cell membrane of a tumor and b) comprises an Fc domain. In at least one aspect, the characteristics of the antibody (*e.g.*, a) and b)) enable the antibody to bind to and interact with an effector cell, such as an NK cell, macrophage, basophil, neutrophil, eosinophil, monocytes, mast cells, and/or dendritic cells, and enable the antibody to bind a tumor antigen that is expressed on a tumor cell. In a preferred embodiment, the antibody comprises an Fc domain. In an additional preferred embodiment, the antibody is able to bind and interact with an NK cell.

[0152] Some exemplary antibodies to antigens expressed on tumor cells that are contemplated by the present disclosure include, but are not limited to, Anti-CD20 (*e.g.*, rituximab; ofatumumab; tositumomab), Anti-CD52 (*e.g.*, alemtuzumab Campath®), Anti-EGFR (*e.g.*, cetuximab Erbitux®, panitumumab), Anti-CD2 (*e.g.*, Siplizumab), Anti-CD37 (*e.g.*, BI836826), Anti-CD123 (*e.g.*, JNJ-

56022473), Anti-CD30 (*e.g.*, XmAb2513), Anti-CD38 (*e.g.*, daratumumab Darzalex®), Anti-PDL1 (*e.g.*, avelumab, atezolizumab, durvalumab), Anti-GD2 (*e.g.*, 3F8, ch14.18, KW-2871, dinutuximab), Anti-CEA, Anti-MUC1, Anti-FLT3, Anti-CD19, Anti-CD40, Anti-SLAMF7, Anti-CCR4, Anti-B7-H3, Anti-ICAM1, Anti-CSF1R, anti-CA125 (*e.g.* Oregovomab), anti-FRα (*e.g.* MOv18-IgG1, Mirvetuximab soravtansine (IMGN853), MORAb-202), anti-mesothelin (*e.g.* MORAb-009), anti-TRP2, and Anti-HER2 (*e.g.*, trastuzumab, Herzuma, ABP 980, and/or Pertuzumab).

[0153] In a more preferred embodiment, the antibody included as part of present invention includes an antibody that when administered to a patient binds to the corresponding antigen on a tumor cell and induces antibody dependent cell-mediated cytotoxicity (ADCC). In an even more preferred embodiment, the antibody comprises an antibody that is approved or in pre-approval for the treatment of a cancer.

[0154] In even more preferred embodiments, the antibody is an anti-HER2 antibody, an anti-EGFR antibody, and/or an anti-CD20 antibody.

[0155] In a most preferred embodiment, an anti-HER2 antibody is selected from Pertuzumab, Trastuzumab, Herzuma, ABP 980, and Ado-trastuzumab emtansine.

[0156] In a most preferred embodiment, an anti-EGFR antibody and an anti-CD20 is cetuximab and rituximab, respectively.

[0157] As described herein, in various embodiments, the antibodies can be synthetic, monoclonal, or polyclonal and can be made by techniques well known in the art. Such antibodies specifically bind to the TAA via the antigen-binding sites of the antibody (as opposed to non-specific binding). TAA peptides, fragments, variants, fusion proteins, etc., can be employed as immunogens in producing antibodies immunoreactive therewith. More specifically, the polypeptides, fragment, variants, fusion proteins, etc. contain antigenic determinants or epitopes that elicit the formation of antibodies.

[0158] Antibodies. In various embodiments of the present invention, the recombinant MVAs and methods described herein are combined with and/or administered in combination with either 1) an immune checkpoint antagonist or agonist antibody or 2) a TAA-specific antibody.

[0159] It is contemplated that the antibodies can be synthetic, monoclonal, or polyclonal and can be made by techniques well known in the art. Such antibodies specifically bind to the immune checkpoint molecule or TAA via the antigen-binding sites of the antibody (as opposed to non-specific binding). Immune checkpoint and/or TAA peptides, fragments, variants, fusion proteins, *etc.*, can be

employed as immunogens in producing antibodies immunoreactive therewith. More specifically, the polypeptides, fragment, variants, fusion proteins, etc. contain antigenic determinants or epitopes that elicit the formation of antibodies.

[0160] These antigenic determinants or epitopes can be either linear or conformational (discontinuous). Linear epitopes are composed of a single section of amino acids of the polypeptide, while conformational or discontinuous epitopes are composed of amino acids sections from different regions of the polypeptide chain that are brought into close proximity upon protein folding (Janeway, Jr. and Travers, *Immuno Biology* 3:9 (Garland Publishing Inc., 2nd ed. 1996)). Because folded proteins have complex surfaces, the number of epitopes available is quite numerous; however, due to the conformation of the protein and steric hindrances, the number of antibodies that actually bind to the epitopes is less than the number of available epitopes (Janeway, Jr. and Travers, *Immuno Biology* 2:14 (Garland Publishing Inc., 2nd ed. 1996)). Epitopes can be identified by any of the methods known in the art.

[0161] Antibodies, including scFV fragments, which bind specifically to the TAAs or the immune checkpoint molecules such as CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, or ICOS and either block its function (“antagonist antibodies”) or enhance/ activate its function (“agonist antibodies”), are encompassed by the invention. Such antibodies can be generated by conventional means.

[0162] In one embodiment, the invention encompasses monoclonal antibodies against a TAA or immune checkpoint molecules or that either block (“antagonist antibodies”) or enhance/activate (“agonist antibodies”) the function of the immune checkpoint molecules or TAAs.

[0163] Antibodies are capable of binding to their targets with both high avidity and specificity. They are relatively large molecules (~150kDa), which can sterically inhibit interactions between two proteins (e.g. PD-1 and its target ligand) when the antibody binding site falls within proximity of the protein-protein interaction site. The invention further encompasses antibodies that bind to epitopes within close proximity to an immune checkpoint molecule ligand binding site.

[0164] In various embodiments, the invention encompasses antibodies that interfere with intermolecular interactions (e.g. protein-protein interactions), as well as antibodies that perturb intramolecular interactions (e.g. conformational changes within a molecule). Antibodies can be screened for the ability to block or enhance/activate the biological activity of an immune checkpoint molecule. Both polyclonal and monoclonal antibodies can be prepared by conventional techniques.

[0165] In one exemplary aspect, the TAAs or immune checkpoint molecules CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS and peptides based on the amino acid sequence of the TAAs or CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS can be utilized to prepare antibodies that specifically bind to the TAA or CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, or ICOS. The term "antibodies" is meant to include polyclonal antibodies, monoclonal antibodies, fragments thereof, such as F(ab')2 and Fab fragments, single-chain variable fragments (scFvs), single-domain antibody fragments (VHHs or nanobodies), bivalent antibody fragments (diabodies), as well as any recombinantly and synthetically produced binding partners. In another exemplary aspect, antibodies are defined to be specifically binding if they bind to an immune checkpoint molecule if they bind with a K_d of greater than or equal to about 10^7 M^{-1} . Affinities of binding partners or antibodies can be readily determined using conventional techniques, for example those described by Scatchard *et al.* ((1949) *Ann. N.Y. Acad. Sci.* 51: 660).

[0166] Polyclonal antibodies can be readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats, using procedures that are well known in the art. In general, purified TAAs or CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS or a peptide based on the amino acid sequence of CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS that is appropriately conjugated is administered to the host animal typically through parenteral injection. Following booster immunizations, small samples of serum are collected and tested for reactivity to CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS polypeptide. Examples of various assays useful for such determination include those described in *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures, such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immunosorbent assays (ELISA), dot blot assays, and sandwich assays. See U.S. Pat. Nos. 4,376,110 and 4,486,530.

[0167] Monoclonal antibodies can be readily prepared using well known procedures. See, for example, the procedures described in U.S. Pat. Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKeam, and Bechtol (eds.) (1980).

[0168] For example, the host animals, such as mice, can be injected intraperitoneally at least once and preferably at least twice at about 3 week intervals with isolated and purified immune checkpoint molecule. Mouse sera are then assayed by conventional dot blot technique or antibody

capture (ABC) to determine which animal is best to fuse. Approximately two to three weeks later, the mice are given an intravenous boost of the immune checkpoint molecule. Mice are later sacrificed and spleen cells fused with commercially available myeloma cells, such as Ag8.653 (ATCC), following established protocols. Briefly, the myeloma cells are washed several times in media and fused to mouse spleen cells at a ratio of about three spleen cells to one myeloma cell. The fusing agent can be any suitable agent used in the art, for example, polyethylene glycol (PEG). Fusion is plated out into plates containing media that allows for the selective growth of the fused cells. The fused cells can then be allowed to grow for approximately eight days. Supernatants from resultant hybridomas are collected and added to a plate that is first coated with goat anti-mouse Ig. Following washes, a label, such as a labeled immune checkpoint molecule polypeptide, is added to each well followed by incubation. Positive wells can be subsequently detected. Positive clones can be grown in bulk culture and supernatants are subsequently purified over a Protein A column (Pharmacia).

[0169] The monoclonal antibodies of the invention can be produced using alternative techniques, such as those described by Alting-Mees *et al.* ((1990) *Strategies in Mol. Biol.* 3: 1-9, "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas"), which is incorporated herein by reference. Similarly, binding partners can be constructed using recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specific binding antibody. Such a technique is described in Larrick *et al.* ((1989) *Biotechnology* 7: 394).

[0170] Antigen-binding fragments of such antibodies, which can be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and F(ab')² fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

[0171] The monoclonal antibodies of the present invention include chimeric antibodies, *e.g.*, humanized versions of murine monoclonal antibodies. Such humanized antibodies can be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment can comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann *et al.* ((1988) *Nature*

332: 323), Liu *et al.* ((1987) *Proc. Nat'l. Acad. Sci.* 84: 3439), Lerrick *et al.* ((1989) *Bio/Technology* 7: 934), and Winter and Harris ((1993) *TIPS* 14: 139). Procedures to generate antibodies transgenically can be found in GB 2,272,440, U.S. Pat. Nos. 5,569,825 and 5,545,806 both of which are incorporated by reference herein.

[0172] Antibodies produced by genetic engineering methods, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, can be used. Such chimeric and humanized monoclonal antibodies can be produced by genetic engineering using standard DNA techniques known in the art, for example using methods described in Robinson *et al.* International Publication No. WO 87/02671; Akira *et al.* European Patent Application 0184187; Taniguchi, M., European Patent Application 0171496; Morrison *et al.* European Patent Application 0173494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Pat. No. 4,816,567; Cabilly *et al.* European Patent Application 0125023; Better *et al.*, (1988) *Science* 240: 1041-1043; Liu *et al.* (1987) *Proc. Nat'l. Acad. Sci.* 84: 3439-3443; Liu *et al.* (1987) *J. Immunol.* 139: 3521-3526; Sun *et al.* (1987) *Proc. Nat'l. Acad. Sci.* 84: 214-218; Nishimura *et al.* (1987) *Cancer Res.* 47: 999-1005; Wood *et al.* (1985) *Nature* 314: 446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80: 1553-1559; Morrison (1985) *Science* 229: 1202-1207; Oi *et al.* (1986) *BioTechniques* 4: 214; Winter U.S. Pat. No. 5,225,539; Jones *et al.* (1986) *Nature* 321: 552 525; Verhoeven *et al.* (1988) *Science* 239: 1534; and Beidler *et al.* (1988) *J. Immunol.* 141: 4053-4060.

[0173] In connection with synthetic and semi-synthetic antibodies, such terms are intended to cover but are not limited to antibody fragments, isotype switched antibodies, humanized antibodies (e.g., mouse-human, human-mouse), hybrids, antibodies having plural specificities, and fully synthetic antibody-like molecules.

[0174] For therapeutic applications, "human" monoclonal antibodies having human constant and variable regions are often preferred so as to minimize the immune response of a patient against the antibody. Such antibodies can be generated by immunizing transgenic animals which contain human immunoglobulin genes. See Jakobovits *et al.* Ann NY Acad Sci 764:525-535 (1995).

[0175] Human monoclonal antibodies against a TAA or an immune checkpoint molecule can also be prepared by constructing a combinatorial immunoglobulin library, such as a Fab phage display library or a scFv phage display library, using immunoglobulin light chain and heavy chain cDNAs prepared from mRNA derived from lymphocytes of a subject. See, e.g., McCafferty *et al.* PCT

publication WO 92/01047; Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; and Griffiths *et al.* (1993) *EMBO J.* 12: 725-734. In addition, a combinatorial library of antibody variable regions can be generated by mutating a known human antibody. For example, a variable region of a human antibody known to bind the immune checkpoint molecule can be mutated, by for example using randomly altered mutagenized oligonucleotides, to generate a library of mutated variable regions which can then be screened to bind to the immune checkpoint molecule. Methods of inducing random mutagenesis within the CDR regions of immunoglobulin heavy and/or light chains, methods of crossing randomized heavy and light chains to form pairings and screening methods can be found in, for example, Barbas *et al.* PCT publication WO 96/07754; Barbas *et al.* (1992) *Proc. Nat'l Acad. Sci. USA* 89: 4457-4461.

[0176] An immunoglobulin library can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Examples of methods and reagents particularly amenable for use in generating antibody display library can be found in, for example, Ladner *et al.* U.S. Pat. No. 5,223,409; Kang *et al.* PCT publication WO 92/18619; Dower *et al.* PCT publication WO 91/17271; Winter *et al.* PCT publication WO 92/20791; Markland *et al.* PCT publication WO 92/15679; Breitling *et al.* PCT publication WO 93/01288; McCafferty *et al.* PCT publication WO 92/01047; Garrard *et al.* PCT publication WO 92/09690; Ladner *et al.* PCT publication WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9: 1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3: 81-85; Huse *et al.* (1989) *Science* 246: 1275-1281; Griffiths *et al.* (1993) *supra*; Hawkins *et al.* (1992) *J. Mol. Biol.* 226: 889-896; Clackson *et al.* (1991) *Nature* 352: 624-628; Gram *et al.* (1992) *Proc. Nat'l. Acad. Sci.* 89: 3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9: 1373-1377; Hoogenboom *et al.* (1991) *Nucl. Acid Res.* 19: 4133-4137; and Barbas *et al.* (1991) *Proc. Nat'l. Acad. Sci.* 88: 7978-7982. Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened to identify and isolate packages that express an antibody that binds a TAA or an immune checkpoint molecule.

[0177] Recombinant MVA. In more preferred embodiments of the present invention, the one or more proteins and nucleotides disclosed herein are included in a recombinant MVA. As described and illustrated by the present disclosure, the intravenous administration of the recombinant MVAs of the present disclosure induces in various aspects an enhanced immune response in cancer patients. Thus, in one or more preferred embodiments, the invention includes a recombinant MVA comprising a first nucleic acid encoding one or more of the TAAs described herein and a second nucleic acid encoding CD40L.

[0178] Example of MVA virus strains that are useful in the practice of the present invention and that have been deposited in compliance with the requirements of the Budapest Treaty are strains MVA 572, deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom, with the deposition number ECACC 94012707 on January 27, 1994, and MVA 575, deposited under ECACC 00120707 on December 7, 2000, MVA-BN, deposited on Aug. 30, 2000 at the European Collection of Cell Cultures (ECACC) under number V00083008, and its derivatives, are additional exemplary strains.

[0179] “Derivatives” of MVA-BN refer to viruses exhibiting essentially the same replication characteristics as MVA-BN, as described herein, but exhibiting differences in one or more parts of their genomes. MVA-BN, as well as derivatives thereof, are replication incompetent, meaning a failure to reproductively replicate *in vivo* and *in vitro*. More specifically *in vitro*, MVA-BN or derivatives thereof have been described as being capable of reproductive replication in chicken embryo fibroblasts (CEF), but not capable of reproductive replication in the human keratinocyte cell line HaCat (Boukamp *et al.* (1988) *J. Cell Biol.* 106: 761-771), the human bone osteosarcoma cell line 143B (ECACC Deposit No. 91112502), the human embryo kidney cell line 293 (ECACC Deposit No. 85120602), and the human cervix adenocarcinoma cell line HeLa (ATCC Deposit No. CCL-2). Additionally, MVA-BN or derivatives thereof have a virus amplification ratio at least two-fold less, more preferably three-fold less than MVA-575 in Hela cells and HaCaT cell lines. Tests and assay for these properties of MVA-BN and derivatives thereof are described in WO 02/42480 (U.S. Patent Application No. 2003/0206926) and WO 03/048184 (U.S. Patent App. No. 2006/0159699).

[0180] The term “not capable of reproductive replication” or “no capability of reproductive replication” in human cell lines *in vitro* as described in the previous paragraphs is, for example, described in WO 02/42480, which also teaches how to obtain MVA having the desired properties as mentioned above. The term applies to a virus that has a virus amplification ratio *in vitro* at 4 days after infection of less than 1 using the assays described in WO 02/42480 or in U.S. Patent No. 6,761,893.

[0181] The term “failure to reproductively replicate” refers to a virus that has a virus amplification ratio in human cell lines *in vitro* as described in the previous paragraphs at 4 days after infection of less than 1. Assays described in WO 02/42480 or in U.S. Patent No. 6,761,893 are applicable for the determination of the virus amplification ratio.

[0182] The amplification or replication of a virus in human cell lines in vitro as described in the previous paragraphs is normally expressed as the ratio of virus produced from an infected cell (output) to the amount originally used to infect the cell in the first place (input) referred to as the “amplification ratio”. An amplification ratio of “1” defines an amplification status where the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells, meaning that the infected cells are permissive for virus infection and reproduction. In contrast, an amplification ratio of less than 1, *i.e.*, a decrease in output compared to the input level, indicates a lack of reproductive replication and therefore attenuation of the virus.

[0183] By “adjuvmentation” herein is intended that a particular encoded protein or component of a recombinant MVA increases the immune response produced by the other encoded protein(s) or component(s) of the recombinant MVA.

[0184] Expression Cassettes/Control Sequences. In various aspects, the one or more nucleic acids described herein are embodied in one or more expression cassettes in which the one or more nucleic acids are operatively linked to expression control sequences. “Operably linked” means that the components described are in relationship permitting them to function in their intended manner *e.g.*, a promoter to transcribe the nucleic acid to be expressed. An expression control sequence operatively linked to a coding sequence is joined such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to, appropriate promoters, enhancers, transcription terminators, a start codon at the beginning a protein-encoding open reading frame, splicing signals for introns, and in-frame stop codons. Suitable promoters include, but are not limited to, the SV40 early promoter, an RSV promoter, the retrovirus LTR, the adenovirus major late promoter, the human CMV immediate early I promoter, and various poxvirus promoters including, but not limited to the following vaccinia virus or MVA-derived and FPV-derived promoters: the 30K promoter, the I3 promoter, the PrS promoter, the PrS5E promoter, the Pr7.5K, the PrHyb promoter, the Pr13.5 long promoter, the 40K promoter, the MVA-40K promoter, the FPV 40K promoter, 30k promoter, the PrSynIIIm promoter, the PrLE1 promoter, and the PR1238 promoter. Additional promoters are further described in WO 2010/060632, WO 2010/102822, WO 2013/189611, WO 2014/063832, and WO 2017/021776 which are incorporated fully by reference herein.

[0185] Additional expression control sequences include, but are not limited to, leader sequences, termination codons, polyadenylation signals and any other sequences necessary for the

appropriate transcription and subsequent translation of the nucleic acid sequence encoding the desired recombinant protein (*e.g.*, HER2, Brachyury, and/or CD40L) in the desired host system. The poxvirus vector may also contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the desired host system. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel *et al.*, (1987) in “*Current Protocols in Molecular Biology*,” John Wiley and Sons, New York, N.Y.) and are commercially available.

[0186] Methods and Dosing regimens for administering the Combination. In one or more aspects, the combinations of the present invention can be administered as part of a homologous and/or heterologous prime-boost regimen. Illustrated in part by data shown in Figure 7, a homologous prime boost regimen increases a subject’s specific CD8 and CD4 T cell responses. Thus, in one or more embodiments there is a combination and/or method for a reducing tumor size and/or increasing survival in a cancer patient comprising administering to the cancer patient a combination of the present disclosure, wherein the combination is administered as part of a homologous or heterologous prime-boost regimen.

Generation of recombinant MVA viruses comprising Transgenes

[0187] The recombinant MVA viruses provided herein can be generated by routine methods known in the art. Methods to obtain recombinant poxviruses or to insert exogenous coding sequences into a poxviral genome are well known to the person skilled in the art. For example, methods for standard molecular biology techniques such as cloning of DNA, DNA and RNA isolation, Western blot analysis, RT-PCR and PCR amplification techniques are described in *Molecular Cloning, A Laboratory Manual* (2nd ed., Sambrook *et al.*, Cold Spring Harbor Laboratory Press (1989)), and techniques for the handling and manipulation of viruses are described in *Virology Methods Manual* (Mahy *et al.* (eds.), Academic Press (1996)). Similarly, techniques and know-how for the handling, manipulation and genetic engineering of MVA are described in *Molecular Virology: A Practical Approach* (Davison & Elliott (eds.), *The Practical Approach Series*, IRL Press at Oxford University Press, Oxford, UK (1993)(see, *e.g.*, “Chapter 9: Expression of genes by Vaccinia virus vectors”)) and *Current Protocols in Molecular Biology* (John Wiley & Son, Inc. (1998) (see, *e.g.*, Chapter 16, Section IV: “Expression of proteins in mammalian cells using vaccinia viral vector”)).

[0188] For the generation of the various recombinant MVA viruses disclosed herein, different methods may be applicable. The DNA sequence to be inserted into the virus can be placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA sequence to be inserted can be ligated to a promoter. The promoter-gene linkage can be positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of poxviral DNA containing a non-essential locus. The resulting plasmid construct can be amplified by propagation within *E. coli* bacteria and isolated. The isolated plasmid containing the DNA gene sequence to be inserted can be transfected into a cell culture, *e.g.*, of chicken embryo fibroblasts (CEFs), at the same time the culture is infected with MVA virus. Recombination between homologous MVA viral DNA in the plasmid and the viral genome, respectively, can generate a poxvirus modified by the presence of foreign DNA sequences.

[0189] According to a preferred embodiment, a cell of a suitable cell culture as, *e.g.*, CEF cells, can be infected with an MVA virus. The infected cell can be, subsequently, transfected with a first plasmid vector comprising a foreign or heterologous gene or genes, such as one or more of the nucleic acids provided in the present disclosure; preferably under the transcriptional control of a poxvirus expression control element. As explained above, the plasmid vector also comprises sequences capable of directing the insertion of the exogenous sequence into a selected part of the MVA viral genome. Optionally, the plasmid vector also contains a cassette comprising a marker and/or selection gene operably linked to a poxviral promoter. Suitable marker or selection genes are, *e.g.*, the genes encoding the green fluorescent protein, β -galactosidase, neomycin-phosphoribosyltransferase or other markers. The use of selection or marker cassettes simplifies the identification and isolation of the generated recombinant poxvirus. However, a recombinant poxvirus can also be identified by PCR technology. Subsequently, a further cell can be infected with the recombinant poxvirus obtained as described above and transfected with a second vector comprising a second foreign or heterologous gene or genes. In case, this gene shall be introduced into a different insertion site of the poxviral genome, the second vector also differs in the poxvirus-homologous sequences directing the integration of the second foreign gene or genes into the genome of the poxvirus. After homologous recombination has occurred, the recombinant virus comprising two or more foreign or heterologous genes can be isolated. For introducing additional foreign genes into the recombinant virus, the steps of

infection and transfection can be repeated by using the recombinant virus isolated in previous steps for infection and by using a further vector comprising a further foreign gene or genes for transfection.

[0190] Alternatively, the steps of infection and transfection as described above are interchangeable, *i.e.*, a suitable cell can at first be transfected by the plasmid vector comprising the foreign gene and, then, infected with the poxvirus. As a further alternative, it is also possible to introduce each foreign gene into different viruses, co-infect a cell with all the obtained recombinant viruses and screen for a recombinant including all foreign genes. A third alternative is ligation of DNA genome and foreign sequences *in vitro* and reconstitution of the recombined vaccinia virus DNA genome using a helper virus. A fourth alternative is homologous recombination in *E.coli* or another bacterial species between a MVA virus genome cloned as a bacterial artificial chromosome (BAC) and a linear foreign sequence flanked with DNA sequences homologous to sequences flanking the desired site of integration in the MVA virus genome.

[0191] The one or more nucleic acids of the present disclosure may be inserted into any suitable part of the MVA virus or MVA viral vector. Suitable parts of the MVA virus are non-essential parts of the MVA genome. Non-essential parts of the MVA genome may be intergenic regions or the known deletion sites 1-6 of the MVA genome. Alternatively, or additionally, non-essential parts of the recombinant MVA can be a coding region of the MVA genome which is non-essential for viral growth. However, the insertion sites are not restricted to these preferred insertion sites in the MVA genome, since it is within the scope of the present invention that the nucleic acids of the present invention (*e.g.*, HER2, Brachyury, HERV-K-env, HERV-K-gag, PRAME, FOLR1, and CD40L and/or 4-1BBL) and any accompanying promoters as described herein may be inserted anywhere in the viral genome as long as it is possible to obtain recombinants that can be amplified and propagated in at least one cell culture system, such as Chicken Embryo Fibroblasts (CEF cells).

[0192] Preferably, the nucleic acids of the present invention may be inserted into one or more intergenic regions (IGR) of the MVA virus. The term “intergenic region” refers preferably to those parts of the viral genome located between two adjacent open reading frames (ORF) of the MVA virus genome, preferably between two essential ORFs of the MVA virus genome. For MVA, in certain embodiments, the IGR is selected from IGR 07/08, IGR 44/45, IGR 64/65, IGR 88/89, IGR 136/137, and IGR 148/149.

[0193] For MVA virus, the nucleotide sequences may, additionally or alternatively, be inserted into one or more of the known deletion sites, *i.e.*, deletion sites I, II, III, IV, V, or VI of the MVA

genome. The term “known deletion site” refers to those parts of the MVA genome that were deleted through continuous passaging on CEF cells characterized at passage 516 with respect to the genome of the parental virus from which the MVA is derived from, in particular the parental chorioallantois vaccinia virus Ankara (CVA), *e.g.*, as described in Meisinger-Henschel *et al.* ((2007) *J. Gen. Virol.* 88: 3249-3259).

Vaccines

[0194] In certain embodiments, the recombinant MVA of the present disclosure can be formulated as part of a vaccine. For the preparation of vaccines, the MVA virus can be converted into a physiologically acceptable form.

[0195] An exemplary preparation follows. Purified virus is stored at -80°C with a titer of 5 x 10⁸ TCID50/ml formulated in 10 mM Tris, 140 mM NaCl, pH 7.4. For the preparation of vaccine shots, *e.g.*, 1 x 10⁸-1 x 10⁹ particles of the virus can be lyophilized in phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be prepared by stepwise, freeze-drying of the virus in a formulation. In certain embodiments, the formulation contains additional additives such as mannitol, dextran, sugar, glycine, lactose, polyvinylpyrrolidone, or other additives, such as, including, but not limited to, antioxidants or inert gas, stabilizers or recombinant proteins (*e.g.* human serum albumin) suitable for *in vivo* administration. The ampoule is then sealed and can be stored at a suitable temperature, for example, between 4°C and room temperature for several months. However, as long as no need exists, the ampoule is stored preferably at temperatures below -20°C, most preferably at about -80°C.

[0196] In various embodiments involving vaccination or therapy, the lyophilisate is dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably physiological saline or Tris buffer such as 10mM Tris, 140mM NaCl pH 7.7. It is contemplated that the recombinant MVA, vaccine or pharmaceutical composition of the present disclosure can be formulated in solution in a concentration range of 10⁴ to 10¹⁰ TCID50/ml, 10⁵ to 5x10⁹ TCID50/ml, 10⁶ to 5x10⁹ TCID50/ml, or 10⁷ to 5x10⁹ TCID50/ml. A preferred dose for humans comprises between 10⁶ to 10¹⁰ TCID50, including a dose of 10⁶ TCID50, 10⁷ TCID50, 10⁸ TCID50, 5x10⁸ TCID50, 10⁹ TCID50, 5x10⁹ TCID50, or 10¹⁰ TCID50. Optimization of dose and number of administrations is within the skill and knowledge of one skilled in the art.

[0197] In one or more preferred embodiments, as set forth herein, the recombinant MVA is administered to a cancer patient intravenously. In other embodiments, the recombinant MVA is administered to a cancer patient intratumorally. In other embodiments, the recombinant MVA is administered to a cancer patient both intravenously and intratumorally at the same time or at different times.

[0198] In additional embodiments, the immune checkpoint antagonist or agonist, or preferably antibody can be administered either systemically or locally, *i.e.*, by intraperitoneal, parenteral, subcutaneous, intravenous, intramuscular, intranasal, intradermal, or any other path of administration known to a skilled practitioner.

[0199] Kits, Compositions, and Methods of Use. In various embodiments, the invention encompasses kits, pharmaceutical combinations, pharmaceutical compositions, and/or immunogenic combination, comprising the a) recombinant MVA that includes the nucleic acids described herein and/or b) one or more antibodies described herein.

[0200] It is contemplated that the kit and/or composition can comprise one or multiple containers or vials of a recombinant poxvirus of the present disclosure, one or more containers or vials of an antibody of the present disclosure, together with instructions for the administration of the recombinant MVA and antibody. It is contemplated that in a more particular embodiment, the kit can include instructions for administering the recombinant MVA and antibody in a first priming administration and then administering one or more subsequent boosting administrations of the recombinant MVA and antibody.

[0201] The kits and/or compositions provided herein may generally include one or more pharmaceutically acceptable and/or approved carriers, additives, antibiotics, preservatives, diluents and/or stabilizers. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

CERTAIN EXEMPLARY EMBODIMENTS

[0202] Embodiment 1 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-

associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[0203] Embodiment 2 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intravenous administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T cell responses specific to the TAA as compared to a non-intravenous injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[0204] Embodiment 3 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the administration of the recombinant MVA increases tumor reduction and/or increases overall survival of the subject as compared to administration of a recombinant MVA and 4-1BBL antigen by themselves.

[0205] Embodiment 4 is a method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA) and a second nucleic acid encoding a 4-1BBL antigen, wherein the intratumoral administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a heterologous tumor-associated antigen and a 4-1BBL antigen. Such an enhanced inflammatory response is discussed elsewhere herein and can include, for example, the induction of NK cells and T cells.

[0206] Embodiment 5 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a an endogenous retroviral

antigen (ERV) and a second nucleic acid encoding 4-1BBL, wherein the administration of the recombinant MVA increases tumor reduction and/or increases overall survival of the subject as compared to administration of a recombinant MVA and 4-1BBL antigen by themselves.

[0207] Embodiment 6 is a method according to any one of embodiments 1-5, wherein the subject is human.

[0208] Embodiment 7 is a method according to any one of embodiments 1-4, wherein the TAA is an endogenous retroviral (ERV) protein.

[0209] Embodiment 8 is a method according to embodiment 7, wherein the ERV is an ERV protein expressed in a tumor cell.

[0210] Embodiment 9 is a method according to any one of embodiments 7-8, wherein the ERV is from the human endogenous retroviral protein K (HERV-K) family.

[0211] Embodiment 10 is a method according to embodiment 9, wherein the HERV-K protein is selected from a HERV-K envelope protein, a HERV-K gag protein, and a HERV-K mel protein.

[0212] Embodiment 11 is a method according to embodiment 9, wherein the HERV-K protein is selected from a HERV-K envelope protein, a HERV-K gag protein, a HERV-K mel protein, and an immunogenic fragment thereof.

[0213] Embodiment 12 is a method according to any one of embodiments 1-6, wherein the TAA is selected from the group consisting of carcinoembryonic antigen (CEA), mucin 1 cell surface associated (MUC-1), prostatic acid phosphatase (PAP), prostate specific antigen (PSA), human epidermal growth factor receptor 2 (HER-2), survivin, tyrosine related protein 1 (TRP1), tyrosine related protein 1 (TRP2), Brachyury, FOLR1, PRAME, p15, and combinations thereof.

[0214] Embodiment 13 is a method according to any one of embodiments 1-6 and 12, wherein the TAA is selected from the group consisting of carcinoembryonic antigen (CEA) and mucin 1 cell surface associated (MUC-1).

[0215] Embodiment 14 is a method according to any one of embodiments 1-6 and 12, wherein the TAA is selected from the group consisting of PAP or PSA.

[0216] Embodiment 15 is a method according to any one of embodiments 1-6, 12, and 14, wherein the TAA is PSA.

[0217] Embodiment 16 is a method according to any one of embodiments 1-6, wherein the TAA is selected from the group consisting of: 5- α -reductase, α -fetoprotein (AFP), AM-1, APC, April, B melanoma antigen gene (BAGE), β -catenin, Bcl12, bcr-abl, Brachyury, CA-125, caspase-8 (CASP-

8, also known as FLICE), Cathepsins, CD19, CD20, CD21/complement receptor 2 (CR2), CD22/BL-CAM, CD23/Fc ϵ RII, CD33, CD35/complement receptor 1 (CR1), CD44/PGP-1, CD45/leucocyte common antigen (“LCA”), CD46/membrane cofactor protein (MCP), CD52/CAMPATH-1, CD55/decay accelerating factor (DAF), CD59/protectin, CDC27, CDK4, carcinoembryonic antigen (CEA), c-myc, cyclooxygenase-2 (cox-2), deleted in colorectal cancer gene (“DCC”), DcR3, E6/E7, CGFR, EMBP, Dna78, farnesyl transferase, fibroblast growth factor-8a (FGF8a), fibroblast growth factor-8b (FGF8b), FLK-1/KDR, folic acid receptor, G250, G melanoma antigen gene family (GAGE-family), gastrin 17, gastrin-releasing hormone, ganglioside 2 (GD2)/ganglioside 3 (GD3)/ganglioside-monosialic acid-2 (“GM2”), gonadotropin releasing hormone (GnRH), UDP-GlcNAc:R1Man(α1-6)R2 [GlcNAc to Man(α1-6)] β1,6-N- α -acetylglucosaminyltransferase V (GnT V), GP1, gp100/Pme117, gp-100-in4, gp15, gp75/tyrosine-related protein-1 (gp75/TRP-1), human chorionic gonadotropin (hCG), heparanase, HER2, human mammary tumor virus (HMTV), 70 kiloDalton heat-shock protein (“HSP70”), human telomerase reverse transcriptase (hTERT), insulin-like growth factor receptor-1 (IGFR-1), interleukin-13 receptor (IL-13R), inducible nitric oxide synthase (iNOS), Ki67, KIAA0205, K-ras, H-ras, N-ras, KSA, LKLR-FUT, melanoma antigen-encoding gene 1 (MAGE-1), melanoma antigen-encoding gene 2 (MAGE-2), melanoma antigen-encoding gene 3 (MAGE-3), melanoma antigen-encoding gene 4 (MAGE-4), gammaglobin, MAP17, Melan-A/melanoma antigen recognized by T-cells-1 (MART-1), mesothelin, MIC A/B, MT-MMPs, mucin, testes-specific antigen NY-ESO-1, osteonectin, p15, P170/MDR1, p53, p97/melanotransferrin, PAI-1, platelet-derived growth factor (PDGF), μ PA, PRAME, probasin, progenipotin, prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), RAGE-1, Rb, RCAS1, SART-1, SSX-family, STAT3, STn, TAG-72, transforming growth factor-alpha (TGF- α), transforming growth factor-beta (TGF- β), Thymosin-beta-15, tumor necrosis factor-alpha (TNF- α), TP1, TRP-2, tyrosinase, vascular endothelial growth factor (VEGF), ZAG, p16INK4, and glutathione-S-transferase (GST). the group consisting of carcinoembryonic antigen (CEA), mucin 1 cell surface associated (MUC-1), prostatic acid phosphatase (PAP), prostate specific antigen (PSA), human epidermal growth factor receptor 2 (HER-2), survivin, tyrosine related protein 1 (TRP1), tyrosine related protein 1 (TRP2), Brachyury, and combinations thereof.

[0218] Embodiment 17 is a method according to any one of embodiments 1-16, wherein the recombinant MVA further comprises a third nucleic acid encoding a CD40L antigen.

[0219] Embodiment 18 is a method according to any one of embodiments 1-17, further comprising administering to the subject at least one immune checkpoint molecule antagonist or agonist.

[0220] Embodiment 19 is a method according to embodiment 18, wherein the immune checkpoint molecule antagonist or agonist is selected from CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS.

[0221] Embodiment 20 is a method according to any one of embodiments 18-19, wherein the immune checkpoint molecule antagonist is PD-1 and/or PD-L1.

[0222] Embodiment 21 is a method according to embodiment 20, wherein the immune checkpoint molecule antagonist further comprises LAG-3.

[0223] Embodiment 22 is a method according to any one of embodiments 18-21, wherein the immune checkpoint molecule antagonist comprises an antibody.

[0224] Embodiment 23 is a method according to any one of embodiments 1-17, further comprising administering to the subject an antibody specific for second TAA.

[0225] Embodiment 24 is a method according to embodiment 23, wherein the antibody specific for a second TAA is specific to an antigen that is expressed on a cell membrane of a tumor.

[0226] Embodiment 25 is a method according to embodiment 23, wherein the antibody specific for a second TAA is a) specific to an antigen that is expressed on a cell membrane of a tumor and b) comprises an Fc domain.

[0227] Embodiment 26 is a pharmaceutical composition for use in a method according to any one of embodiments 1-25.

[0228] Embodiment 27 is a vaccine for use in a method according to any one of embodiments 1-25.

[0229] Embodiment 28 is a recombinant modified Vaccinia Ankara (MVA) for treating a subject having cancer, the recombinant MVA comprising a) a first nucleic acid encoding a tumor-associated antigen (TAA) and b) a second nucleic acid encoding 4-1BBL.

[0230] Embodiment 29 is a recombinant MVA according to embodiment 28, wherein the TAA is an endogenous retroviral (ERV) protein.

[0231] Embodiment 30 is a recombinant MVA according to embodiment 29, wherein the ERV protein is from the human endogenous retroviral protein K (HERV-K) family.

[0232] Embodiment 31 is a recombinant MVA according to embodiment 30, wherein the retroviral protein K is selected from HERV-K envelope protein, a HERV-K gag protein, and a HERV-K mel protein.

[0233] Embodiment 32 is a recombinant MVA according to any one of embodiments 28-31 further comprising a third nucleic acid encoding CD40L.

[0234] Embodiment 33 is a pharmaceutical combination comprising a) a recombinant MVA of any one of embodiments 28-32 and b) at least one of least one immune checkpoint molecule antagonist or agonist.

[0235] Embodiment 34 is a pharmaceutical combination according to embodiment 33, wherein the immune checkpoint molecule antagonist or agonist is selected from CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS.

[0236] Embodiment 35 is a pharmaceutical combination according to embodiment 34, wherein the immune checkpoint molecule antagonist is PD-1 and/or PD-L1.

[0237] Embodiment 36 is a pharmaceutical combination according to embodiment 35, wherein the immune checkpoint molecule antagonist further comprises LAG-3.

[0238] Embodiment 37 is a pharmaceutical combination according to any one of embodiments 33-36, wherein the immune checkpoint molecule antagonist comprises an antibody.

[0239] Embodiment 38 is a pharmaceutical combination comprising a) a recombinant MVA of any one of embodiments 28-32 b) an antibody specific for a second TAA.

[0240] Embodiment 39 is a pharmaceutical combination according to embodiment 38, wherein the antibody specific for a second TAA is specific to an antigen that is expressed on a cell membrane of a tumor.

[0241] Embodiment 40 is a pharmaceutical combination according to embodiment 39, wherein the antibody specific for a second TAA is a) specific to an antigen that is expressed on a cell membrane of a tumor and b) comprises an Fc domain.

[0242] Embodiment 41 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in reducing tumor size and/or increasing survival in a subject having a cancerous tumor.

[0243] Embodiment 42 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a

pharmaceutical combination according to any one of embodiments 33-40, for use in method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject the recombinant MVA of embodiments 28-32, the vaccine according to embodiment 27, the pharmaceutical composition according to embodiment 26, or the pharmaceutical combination according to any one of embodiments 33-40, wherein the intratumoral administration enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[0244] Embodiment 43 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject the recombinant MVA of embodiments 28-32, the vaccine according to embodiment 27, the pharmaceutical composition according to embodiment 26, or the pharmaceutical combination according to any one of embodiments 33-40, wherein the intravenous administration increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intravenous administration of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[0245] Embodiment 44 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in method for inducing an enhanced inflammatory response in a cancerous tumor of a cancer subject, the method comprising intratumorally administering to the subject the recombinant MVA of embodiments 28-32, the vaccine according to embodiment 27, the pharmaceutical composition according to embodiment 26, or the pharmaceutical combination according to any one of embodiments 33-40, wherein the intratumoral administration enhances an inflammatory response in the cancerous tumor of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.

[0246] Embodiment 45 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a

pharmaceutical combination according to any one of embodiments 33-40, for use in method for treating cancer in subject.

[0247] Embodiment 46, is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in method for treating cancer, wherein the cancer is selected from the group consisting of: breast cancer, lung cancer, head and neck cancer, thyroid, melanoma, gastric cancer, bladder cancer, kidney cancer, liver cancer, melanoma, pancreatic cancer, prostate cancer, ovarian cancer, urothelial, cervical, or colorectal cancer.

[0248] Embodiment 47 is a recombinant MVA according to embodiment 44, wherein the enhanced inflammatory response is localized to the tumor.

[0249] Embodiment 48 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding CD40L, wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a CD40L.

[0250] Embodiment 49 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding CD40L, wherein the intravenous administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T cell responses specific to the TAA as compared to a non-intravenous injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a CD40L antigen.

[0251] Embodiment 50 is a method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding CD40L, wherein the administration of the recombinant

MVA increases tumor reduction and/or increases overall survival of the subject as compared to administration of a recombinant MVA and CD40L antigen by themselves.

[0252] Embodiment 51 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously and/or intratumorally administering to the subject the recombinant MVA of embodiments 28-32, the vaccine according to embodiment 27, the pharmaceutical composition according to embodiment 26, or the pharmaceutical combination according to any one of embodiments 33-40, wherein said intravenous and/or intratumoral administration increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intravenous or non-intratumoral administration of any MVA selected from the group of: 1) a recombinant MVA virus comprising a first nucleic acid encoding a TAA and second nucleic acid encoding a 4-1BBL antigen; 2) a recombinant MVA virus comprising a first nucleic acid encoding a TAA and second nucleic acid encoding a CD40L antigen; or 3) a recombinant MVA virus comprising a first nucleic acid encoding a TAA, a second nucleic acid encoding a 4-1BBL antigen, and a third nucleic acid encoding a CD40L antigen.

[0253] Embodiment 52 is a recombinant MVA according to any one of embodiments 28-32, a vaccine according to embodiment 27, a pharmaceutical composition according to embodiment 26, a pharmaceutical combination according to any one of embodiments 33-40, for use in method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously and intratumorally administering to the subject the recombinant MVA of embodiments 28-32, the vaccine according to embodiment 27, the pharmaceutical composition according to embodiment 26, or the pharmaceutical combination according to any one of embodiments 33-40, wherein said intravenous and intratumoral administration increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intravenous or non-intratumoral administration of any MVA selected from the group of: 1) a recombinant MVA virus comprising a first nucleic acid encoding a TAA and second nucleic acid encoding a 4-1BBL antigen; 2) a recombinant MVA virus comprising a first nucleic acid encoding a TAA and second nucleic acid encoding a CD40L antigen; or 3) a recombinant MVA virus comprising a first nucleic acid encoding a TAA, a second nucleic acid encoding a 4-1BBL antigen, and a third nucleic acid encoding a CD40L antigen.

antigen. Said intravenous and intratumoral administration can be performed at the same time or at different times, as is evident to one of skill in the art.

EXAMPLES

[0254] The following examples illustrate the invention but should not be construed as in any way limiting the scope of the claims.

Example 1: Construction of Recombinant MVA-TAA-4-1BBL and MVA-TAA-CD40L

[0255] Generation of recombinant MVA viruses that embody elements of the present disclosure was done by insertion of the indicated transgenes with their promoters into the vector MVA-BN. Transgenes were inserted using recombination plasmids containing the transgenes and a selection cassette, as well as sequences homologous to the targeted loci within MVA-BN. Homologous recombination between the viral genome and the recombination plasmid was achieved by transfection of the recombination plasmid into MVA-BN infected CEF cells. The selection cassette was then deleted during a second step with help of a plasmid expressing CRE-recombinase, which specifically targets loxP sites flanking the selection cassette, therefore excising the intervening sequence.

[0256] For construction of MVA-OVA and MVA-OVA-4-1BBL the recombination plasmid included the transgenes OVA or OVA and 4-1BBL, each preceded by a promoter sequence, as well as sequences which are identical to the targeted insertion site within MVA-BN to allow for homologous recombination into the viral genome.

[0257] For construction of MVA-OVA-CD40L the recombination plasmid included the transgenes OVA and CD40L, each preceded by a promoter sequence, as well as sequences which are identical to the targeted insertion site within MVA-BN to allow for homologous recombination into the viral genome.

[0258] For the construction of MVA-gp70-4-1BBL the recombination plasmid includes two transgenes gp70 and 4-1BBL, each preceded by a promoter sequence, as well as sequences which are identical to the targeted insertion site within MVA-BN to allow for homologous recombination into the viral genome. Similarly, for the construction of MVA-gp70-CD40L the recombination plasmid includes two transgenes gp70 and CD40L, each preceded by a promoter sequence, and appropriate changes are made to the recombination plasmids when construction of MVA-gp70-4-1-BBL-CD40L is desired.

[0259] For the construction of MVA-HERV-K, MVA-HERV-K-4-1BBL, and MVA-HERV-K-4-1BBL-CD40L, the recombination plasmid included the HERV-K, HERV-K and 4-1BBL, and HERV-K, 4-1BBL, and CD40L transgenes, respectively. Each transgene or set of transgenes was preceded by a promoter sequence, as well as sequences which are identical to the targeted insertion site within MVA-BN to allow for homologous recombination into the viral genome.

[0260] For generation of the above described mBN MVAs, CEF cell cultures were each inoculated with MVA-BN and transfected each with the corresponding recombination plasmid. In turn, samples from these cell cultures were inoculated into CEF cultures in medium containing drugs inducing selective pressure, and fluorescence-expressing viral clones were isolated by plaque purification. Loss of the fluorescent-protein-containing selection cassette from these viral clones was mediated in a second step by CRE-mediated recombination involving two loxP sites flanking the selection cassette in each construct. After the second recombination step only the transgene sequences (e.g., OVA, 4-1BBL, gp70, HERV-K, and/or CD40L) with their promoters inserted in the targeted loci of MVA-BN were retained. Stocks of plaque-purified virus lacking the selection cassette were prepared.

[0261] Expression of the identified transgenes is demonstrated in cells inoculated with the described construct.

[0262] Generation of the constructs described herein was carried out by using a cloned version of MVA-BN in a bacterial artificial chromosome (BAC). Recombination plasmids contained the described transgene sequences, each downstream of a promoter. The plasmids included sequences that are also present in MVA and therefore allow for specific targeting of the integration site. Briefly, infectious viruses were reconstituted from BACs by transfecting BAC DNA into BHK-21 cells and superinfecting them with Shope fibroma virus as a helper virus. After three additional passages on CEF cell cultures, helper-virus-free versions of the constructs were obtained. An exemplary MVA generation is also found in Baur *et al.* ((2010) *Virol.* 84: 8743-52, "Immediate-early expression of a recombinant antigen by modified vaccinia virus Ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses").

Example 2: 4-1BBL-mediated costimulation of CD8 T cells by MVA-OVA-4-1BBL infected tumor cells influences cytokine production without the need of DCs

[0263] Dendritic cells (DCs) were generated after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 (melanoma model) cells were infected with MVA-OVA, MVA-OVA-CD40L, or MVA-OVA-4-1BBL at a MOI of 10 and cultured overnight at 37°C with 5% CO₂. The next day, infected tumor cells were harvested and cocultured when indicated in the presence of DCs at a 1:1 ratio for 4 hours at 37°C with 5% CO₂. Naïve OVA(257-264) specific CD8+ T cells were magnetically purified from OT-I mice and added to the coculture at a ratio of 1:5. Cells were cultured at 37°C with 5% CO₂ for 48 hours. Then, culture supernatant was collected for cytokine concentration analysis by Luminex. Results are shown in Figure 1 as supernatant concentration of: IL-6 (Figure 1A); GM-CSF (Figure 1B); IL-2 (Figure 1C); and IFN-γ (Figure 1D). Data are represented as Mean ± SEM.

[0264] In line with what has been previously reported, MVA-OVA-CD40L had a great impact on the activation of DC and their antigen presentation capabilities. Thus, MVA-OVA-CD40L-infected FLDC produced large amounts of IL-6 (Figure 1A). Importantly, OVA-specific T cell responses could be exclusively induced in the presence of DC but not directly by MVA-CD40L infected B16.F10 cells themselves (Figure 1B and 1C). These results show a clear requirement of DC to unfold the benefits of MVA-OVA-CD40L. In contrast, MVA-OVA-4-1BBL did not induce IL-6 production in DC, but MVA-OVA-4-1BBL-infected B16.F10 cells elicited the secretion of T cell activation cytokines IFN-γ, IL-2 and GM-CSF in a DC-independent manner (Figure 1A-1D).

Example 3: MVA-OVA-4-1BBL infected tumor cells directly (i.e., without the need of DC) drive differentiation of antigen-specific CD8 T cells into activated effector T cells

[0265] Dendritic cells (DCs) were generated after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 (melanoma model) cells were infected with MVA-OVA, MVA-OVA-CD40L, or MVA-OVA-4-1BBL at a MOI of 10 and cultured overnight at 37°C with 5% CO₂. The next day, infected tumor cells were harvested and cocultured when indicated in the presence of DCs at a 1:1 ratio for 4 hours at 37°C with 5% CO₂. Meanwhile, naïve OVA(257-264) specific CD8+ T cells were magnetically purified from OT-I mice and added to the coculture at a ratio of 1:5. Cells were cultured at 37°C 5% CO₂ for 48 hours. Cells were then stained and analyzed by flow cytometry. Results are shown in Figure 2 as GMFI of T-bet on OT-I

CD8+ T cells (Figure 2A) and percentage of CD44+Granzyme B+ IFN- γ + TNF α + of OT-I CD8+ T cells (Figure 2B). Data are shown as Mean \pm SEM.

[0266] The results show that in the absence of cross-presenting DC, the induction of Granzyme B+ and IFN γ + cytotoxic effector T cells was dependent on 4-1BBL (Figure 2B). Collectively with the results presented in Figure 1, these findings document that, in contrast to MVA-encoded CD40L, which operates through the activation of DCs, 4-1BBL encoded by MVA acts directly on T cells in a DC-independent manner.

Example 4: Infection with MVAs encoding either CD40L or 4-1BBL induce tumor cell death in tumor cell lines and macrophages

[0267] Tumor cell lines B16.OVA (Figure 3A and 3B), MC38 (Figure 3C) and B16.F10 (Figure 3D) were infected at the indicated MOI for 20 hours. Then, cells were analyzed for their viability by flow cytometry. Serum HMGB1 in the samples from Figure 3A was quantified by ELISA (Figure 3B). Bone-marrow-derived macrophages (BMDMs) were infected at the indicated MOI for 20 hours. Cells were then analyzed for their viability by flow cytometry. Results are shown in Figures 3A-3E. Data are presented as Mean \pm SEM.

[0268] As shown in Figures 3A and 3B, infection with MVA-OVA or MVA-OVA-CD40L resulted in mild induction of cell death compared to PBS-treated tumor cells. Interestingly, infection with MVA-OVA-4-1BBL significantly enhanced tumor cell death 18 hours post infection.

[0269] To further confirm these results in non-antigenic cell lines, we performed similar assays using MC38 (Figure 3C) and B16.F10 (Figure 3D) tumor cells infected with MVA, MVA-CD40L, and MVA-4-1BBL (none of which encoded TAAs). Consistently, infection with these MVAs induced cell death in these tumor cell lines and efficiently killed bone marrow-derived macrophages (BMDMs) (Figure 3E). Altogether, these data demonstrated that MVA infection resulted in tumor cell and macrophage death that were increased when CD40L or 4-1BBL were expressed by the recombinant MVA.

[0270] Oncolytic virus infection of tumor cells results in the induction of so-called immunogenic cell death (ICD) (Workenhe *et al.* (2014) *Mol. Ther.* 22: 251-56). ICD comprises the release of intracellular proteins such as calreticulin, ATP, or HMGB1 that act as alarmins to the immune system, leading to enhanced antigen-presentation and thereby inducing antitumor immunity. We tested whether MVA infection would result in induction of ICD by means of secreted HMGB1.

Unexpectedly, we found that MVA-OVA-4-1BBL and MVA-OVA-CD40L induced a significant increase of HMGB1 in comparison to MVA-OVA (Fig. 3B).

Example 5: MVA encoding 4-1BBL induces NK cell activation *in vivo*

[0271] C57BL/6 mice (n = 5/group) were immunized intravenously either with saline or 5×10^7 TCID50 MVA-OVA (“rMVA” in Figure 4), 5×10^7 TCID50 MVA-OVA-4-1BBL (“rMVA-4-1BBL” in Figure 4), or 5×10^7 TCID50 MVA-OVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1). 24 hours later, mice were sacrificed and spleens processed for flow cytometry analysis. Results are shown in Figure 4A and Figure 4B. Geometric Mean Fluorescence Intensity (GMFI) of CD69 (Figure 4A) and CD70 (Figure 4B) is shown. Data are shown as Mean \pm SEM, representative of two independent experiments.

[0272] The results showed that the quality of the NK cell response was enhanced by the addition of 4-1BBL to MVA-OVA as compared to the IV administration of MVA-OVA without 4-1BBL, and both NK cell activation markers, CD69 and CD70, were strongly upregulated as compared to MVA-OVA (Figure 4A and B). Coinjection of blocking 4-1BBL antibody showed that MVA-OVA-induced NK cell activation was completely 4-1BBL-independent, but could be enhanced when excessive 4-1BBL signal was delivered by MVA-OVA-4-1BBL.

Example 6: Intravenous immunization with MVA encoding 4-1BBL promotes serum IFN- γ secretion *in vivo*

[0273] C57BL/6 mice (n = 5/group) were immunized intravenously either with saline or 5×10^7 TCID50 “rMVA” (=MVA-OVA), 5×10^7 TCID50 “rMVA-4-1BBL” (=MVA-OVA-4-1BBL), or 5×10^7 TCID50 MVA-OVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1). Results are shown in Figures 5A and 5B. Data are shown as Mean \pm SEM. Figure 5A: 6 hours later, mice were bled, serum was isolated from whole blood and IFN- γ concentration in serum determined by Luminex. Figure 5B: 3, 21, and 45 hours later, mice were intravenously injected with Brefeldin A to stop protein secretion. Mice were sacrificed 6, 24 and 48 hours after immunization and splenocytes analyzed by flow cytometry.

[0274] The 4-1BB-mediated NK cell activation coincided with increased serum levels of the NK effector cytokine IFN γ (Figure 5A). NK cells are known to produce high amounts of IFN- γ upon activation. To determine whether the increased IFN- γ levels in the serum could have originated from

NK cells, the proportion of IFN- γ -producing NK cells was determined at different timepoints after intravenous injection of the indicated recombinant MVA vectors. 6h after injection, when high serum levels of IFN- γ were measured, the percentage of IFN- γ + NK cells was highest and slowly decreased thereafter (Figure 5B). The highest frequency of IFN- γ positive NK cells was observed when MVA-OVA-4-1BBL was used. Taken together, these data show that intravenous immunization of rMVA-4-1BBL leads to the strong activation of NK cells and increased production of the NK cell effector cytokine IFN- γ .

Example 7: Intravenous rMVA-4-1BBL immunization promotes serum IFN- γ secretion in B16.OVA tumor-bearing mice

[0275] B16.OVA tumor-bearing C57BL/6 mice (n = 5/group) were grouped and received i.v. (intravenous) PBS or 5×10^7 TCID50 MVA-OVA (“rMVA” in the figure) or MVA-OVA-4-1BBL (“rMVA-4-1BBL” in the figure) at day 7 after tumor inoculation. 6 hours later, mice were bled, serum was isolated from whole blood, and IFN- γ concentration in serum was determined by Luminex. Results are shown in Figure 6. Data are shown as Mean \pm SEM.

[0276] The data shown in Figure 6 demonstrate that similar effects on NK cells to those reported in other experiments could be also obtained in a melanoma tumor model. 6h after immunization, serum IFN- γ levels were highly increased in MVA-OVA-4-1BBL immunized tumor-bearing mice, indicating strong NK cell activation (Figure 6).

Example 8: Intravenous rMVA-4-1BBL prime and boost immunizations enhances antigen- and vector-specific CD8+ T cell expansion

[0277] Figures 7A-7D show antigen and vector-specific after intravenous rMVA-4-1BBL prime and boost immunization. C57BL/6 mice (n = 4/group) received intravenous prime immunization either with saline or 5×10^7 TCID50 rMVA (= MVA-OVA), 5×10^7 TCID50 rMVA-4-1BBL (=MVA-OVA-4-1BBL), or 5×10^7 TCID50 rMVA combined with 200 μ g anti 4-1BBL antibody (clone TKS-1) on day 0 and boost immunization on day 41. Mice were bled on days 6, 21, 35, 48, and 64 after prime immunization, and flow cytometric analysis of peripheral blood was performed. Mice were sacrificed on day 70 after prime immunization. Spleens were harvested and flow cytometry analysis performed.

[0278] Results are shown in Figures 7A-7D. Figure 7A shows percentage of antigen (OVA)-specific CD8+ T cells among Peripheral Blood Leukocytes (PBL) and Figure 7B shows percentage of vector (B8R)-specific CD8+ T cells among PBL. Figure 7C illustrates percentage of antigen (OVA)-specific CD8+ T cells among live cells. Figure 7D shows percentage of vector (B8R)-specific CD8+ T cells among live cells. Data are shown as Mean \pm SEM.

[0279] The results show that B8- and OVA-specific CD8 T cells reached a maximum on day 7 after the first immunization and were further expanded after the second immunization on day 41 (Figure 7A and B). At the day 41 timepoint, there was a clear benefit of rMVA-4-1BBL in terms of antigen-specific T cell response when compared to rMVA, both for B8 and OVA. Interestingly, co-injection of blocking 4-1BBL antibody showed that rMVA-induced T cell responses were completely 4-1BBL-independent, but could be enhanced when excessive 4-1BBL signal was delivered by rMVA-4-1BBL (Figure 7A and B). In line with these results, rMVA-4-1BBL prime/boost immunization also resulted in an improved OVA- and B8-specific T cell response in the spleen 70 days after the first immunization (Figure 7C and D).

Example 9: Increased antitumor effect of intravenous injection of MVA virus encoding a TAA and 4-1BBL

[0280] B16.OVA tumor-bearing C57BL/6 mice (n=5/group) were grouped and received i.v. (intravenous) PBS or 5×10^7 TCID50 MVA-OVA or 5×10^7 TCID50 MVA-OVA-4-1BBL at day 7 (black dotted line) after tumor inoculation. Tumor growth was measured at regular intervals. Shown in Figure 8, an intravenous administration of MVA virus encoding 4-1BBL resulted in a reduction in tumor volume as compared to MVA or the control (PBS) as a consequence of prolonged delay in growth of the tumors.

Example 10: Enhanced antitumor effect of intratumoral injection of MVA virus encoding 4-1BBL or CD40L

[0281] B16.OVA tumor-bearing C57BL/6 mice (n= 4-5/group) were grouped and received intratumoral (i.t.) PBS or 5×10^7 TCID50 of MVA-OVA (“rMVA” in the figure), MVA-OVA-CD40L (“rMVA-CD40L” in the figure), or MVA-OVA-4-1BBL (“rMVA-4-1BBL” in the figure) at days 7 (black dotted line), 12 and 15 (grey dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals. Shown in Figures 9A-9D, an enhanced antitumor effect was realized via

an intratumoral injection of MVA virus encoding a TAA and either 4-1BBL or CD40L. More particularly, shown in Figure 9D, a significantly greater reduction in tumor growth was seen with MVA virus encoding 4-1BBL. While the invention is not bound by any particular mechanism or mode of action, one hypothesis for the differences observed between 4-1BBL and CD40L is that 4-1BBL aims to activate NK cells and T cells, whereas CD40L aims to activate DCs. B16 melanoma tumors are more infiltrated with T cells (Mosely *et al.* (2016) *Cancer Immunol. Res.* 5(1): 29-41); therefore an MVA encoding 4-1BBL is more effective than an MVA encoding CD40L in this setting.

[0282] Regardless of the exact mechanism or pathway by which 4-1BBL and CD40L exert their effects on tumor growth or diameter, the data in Figure 9 showed that intratumoral injection of MVA encoding 4-1BBL resulted in prolonged tumor growth control and in some cases even in complete rejection of the tumor.

Example 11: Enhanced antitumor effect of intratumoral injection of MVA virus encoded with a TAA and CD40L against established colon cancer

[0283] MC38 tumor-bearing C57BL/6 mice (n = 5/group) were grouped and received intratumoral (i.t.) PBS or 5×10^7 TCID50 MVA-TAA (“rMVA” in figure) or MVA-TAA-CD40L (“rMVA-CD40L” in figure) at days 14 (black dotted line), 19, and 22 (black dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals. Results are shown in Figures 10A, 10B, and 10C for the non-antigenic, established MC38 colon carcinomas. The TAAs expressed by the MVAs in these experiments comprised the antigens AH1A5, p15E, and TRP2.

[0284] These results show that intratumoral injection of an MVA encoding CD40L in addition to one or more TAAs can significantly delay tumor growth in an MC38 colon carcinoma model.

Example 12: Immune checkpoint blockade and tumor antigen specific antibodies synergize with intratumoral administration of MVA-OVA-4-1BBL

[0285] B16.OVA melanoma cells (5×10^5) were subcutaneously injected into C57BL/6 mice. When tumors reached about 5mm in diameter, mice were grouped (n = 5/group) and received when indicated (ticks) 200 μ g IgG2a, anti TRP-1 or anti PD-1 intraperitoneally (i.p.). Mice were immunized intratumorally (i.t.) either with PBS or with 5×10^7 TCID50 MVA-OVA-4-1BBL at days 13 (black dotted line), 18 and 21 (grey dashed lines) after tumor inoculation. Tumor growth was measured at

regular intervals. Results are shown in Figure 11. When an antibody specific for the Tumor Associated Antigen (TAA) Trp1 (anti-Trp1) was combined with an intratumoral administration of MVA-OVA-4-1BBL, there was an increased reduction in tumor volume as compared to anti PD-1 by itself (Figure 11, middle row). When the immune checkpoint molecule antibody PD-1 was combined with an intratumoral administration of MVA-OVA-4-1BBL there was an increased reduction in tumor volume as compared to anti PD-1 by itself (Figure 11, bottom row).

[0286] These experiments demonstrate that anti-PD-1 and anti-TRP-1 antibodies enhanced tumor growth control as single agents, while the combination of either antibody with MVA-OVA-4-1BBL improved the therapeutic effect exerted by MVA-OVA-4-1BBL. Here, combination therapies of intratumoral MVA 4-1BBL with either checkpoint blockade or TAA-targeting antibodies had greater therapeutic activity than any of the monotherapies. This data also indicates that tumor-specific antibodies that potentially induce ADCC may be combined with intratumoral injections of MVA expressing 4-1BBL for a synergistic effect.

Example 13: Superior anti-tumor effect of intratumoral MVA-OVA-4-1BBL injection as compared to agonistic anti-CD137 antibody treatment

[0287] B16.OVA tumor-bearing C57BL/6 mice (n = 5/group) were grouped and were intratumorally injected with either PBS, 5×10^7 TCID50 MVA-OVA-4-1BBL, or 10 μ g anti-4-1BB (3H3, BioXcell) on day 7, 12, and 15 (black dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals.

[0288] Figure 12A shows a superior anti-tumor effect of MVA-OVA-4-1BBL as compared to the agonistic anti-4-1BBL antibody (3H3). Figure 12B shows that intratumoral immunization with MVA-OVA-4-1BBL exclusively induced an OVA-specific T cell response in the blood whereas the agonistic anti-4-1BBL antibody did not induce any OVA-specific T cells in the blood.

[0289] Thus, these data show that intratumoral MVA-OVA-4-1BBL treatment is more potent than agonistic anti-CD137 antibodies, both in terms of tumor-specific T cells responses as well as tumor growth control.

Example 14: Increased antitumor effect of intravenous injection of MVA encoding the Endogenous Retroviral (ERV) antigen Gp70 encoded with CD40L in the CT26 tumor model

[0290] CT26 tumor-bearing Balb/c mice (n = 5/group) were grouped and received intravenous (i.v.) PBS or 5×10^7 TCID50 MVA-BN, MVA-Gp70, or MVA-Gp70-CD40L at day 12 (black dotted line) after introduction of tumors into the mice. Tumor growth was measured at regular intervals. Shown in Figure 13A and 13B, intravenous administration of MVA virus encoding the endogenous retroviral antigen Gp70 resulted in a reduction in tumor volume as compared to MVA or the control (PBS). The anti-tumor effect was further improved when CD40L was additionally encoded by MVA-Gp70-CD40L.

[0291] Figure 13C shows the induction of Gp70 specific CD8 T cells in the blood upon intravenous injection of MVA-Gp70 or MVA-Gp70-CD40L.

[0292] Thus, in these experiments, an MVA was constructed encoding a model ERV that is the murine protein gp70 (envelope protein of the murine leukemia virus) (“MVA-gp70”). An MVA further comprising the costimulatory molecule CD40L was also generated (“MVA-gp70-CD40L”). The anti-tumor potential of these new constructs was tested using the CT26.wt colon carcinoma model. CT26.wt cells have been shown to express high levels of gp70 (see, *e.g.*, Scrimieri (2013) *Oncoimmunol* 2: e26889). CT26.wt tumor bearing mice were generated and, when tumors were at least 5mm x 5mm, were immunized intravenously as indicated above. Immunization with MVA alone induced a mild delay in tumor growth. In contrast, immunization with MVA-gp70 caused the complete rejection of 3/5 tumors (Figure 13A and B). Even more striking results were obtained with immunization with MVA-Gp70-CD40L, which caused the rejection of 4/5 tumors (Figure 13 A and B).

[0293] To determine whether these anti-tumor responses correlated with the induction of gp70-specific T cells following immunization, a blood re-stimulation was performed using the H-2Kd-restricted gp70 epitope AH1. These results (Figure 13C) show a strong induction of gp70-specific CD8 T cell responses in MVA-Gp70 and MVA-Gp70-CD40L treated mice (Figure 13 C).

Example 15: Increased antitumor effect of intravenous injection of MVA encoding the endogenous retroviral antigen Gp70 encoded with CD40L in the B16.F10 tumor model

[0294] B16.F10 tumor-bearing C57BL/6 mice (n=5/group) were grouped and received intravenous (i.v.) PBS or 5×10^7 TCID50 of MVA-BN, MVA-Gp70, or MVA-Gp70-CD40L at day 7 (black dotted line) after tumor inoculation when tumors measured approximately 5 x 5 mm. Tumor growth was measured at regular intervals. Shown in Figure 14A, intravenous administration of MVA virus encoding the endogenous retroviral antigen Gp70 and the CD40L resulted in a reduction in tumor volume as compared to MVA or the control (PBS).

[0295] Figure 14B shows the induction of Gp70 specific CD8 T cells in the blood upon intravenous injection of MVA-Gp70 or MVA-Gp70-CD40L.

[0296] Thus, in these experiments, the efficacy of treatment with MVA-Gp70 and MVA-Gp70-CD40L were demonstrated in an additional independent tumor model. B16.F10 is a melanoma cell line derived from C57BL/6 and expresses high levels of Gp70 (Scrimieri (2013) *Oncoimmunol* 2: e26889). Treatment with MVA alone (“MVA-BN”) led to some tumor growth delay of B16.F10 tumors, comparable to the effect of non-adjuvanted MVA-Gp70 (Figure 14A). However, MVA-Gp70-CD40L resulted in a stronger anti-tumor effect than the MVA backbone control alone (Figure 14A). Additional experiments demonstrated that both groups receiving Gp70-antigen-encoding MVAs exhibited CD8 T cell responses specific for the H-2Kb-restricted gp70 epitope p15e, but no dramatic increase in peripheral T cell responses was observed when CD40L was also encoded by the MVA (Figure 14B).

Example 16: Increased antitumor effect of intravenous injection of MVA virus encoding gp70 and 4-1BBL /Prophetic example/

[0297] B16.OVA tumor-bearing C57BL/6 mice (n=5/group) are grouped and receive intravenously PBS or 5×10^7 TCID50 MVA-OVA or MVA-gp70-4-1BBL at day 7 (black dotted line) after tumor inoculation. Tumor growth is measured at regular intervals. Because the mouse homologs of human endogenous retroviral (ERV) proteins are neither highly expressed in normal mouse tissues nor predominantly expressed in mouse tumor tissues, the efficacy of human ERVs cannot be studied effectively in a mouse model. Gp70 is a mouse ERV protein that has been well studied (see, e.g., Bronte *et al.* (2003) *J Immunol.* 171 (12): 6396-6405; Bashratyan *et al.* (2017) *Eur. J. Immunol.* 47: 575–584; and Nilsson *et al.* (1999) *Virus Genes* 18: 115-120). Accordingly, the study of

a gp70-specific cancer vaccine in mice is very likely to have strong predictive value regarding the efficacy of an ERV-specific cancer vaccine in humans.

Example 17: Enhanced antitumor effect of intratumoral injection of MVA virus encoding gp70 and either 4-1BBL or CD40L [Prophetic example]

[0298] B16.OVA tumor-bearing C57BL/6 mice (n=4-5/group) are grouped and receive intratumoral (i.t.) PBS or 5×10^7 TCID50 of MVA-OVA, MVA-OVA-CD40L, or MVA-OVA-4-1BBL at days 7 (black dotted line), 12 and 15 (grey dashed lines) after tumor inoculation. Tumor growth was measured at regular intervals.

Example 18: Administration with rMVA-HERV-K-4-1BBL influences cytokine production by direct antigen presentation of infected tumor cells [Prophetic example]

[0299] Dendritic cells (DCs) are generated after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 cells are infected with MVA-HERV-K, MVA-HERV-K-CD40L, MVA-HERV-K-4-1BBL, or MVA-HERV-K-4-1BBL-CD40L at a MOI 10 and left overnight. The next day, infected tumor cells are harvested and cocultured when indicated in the presence of DCs at a 1:1 ratio for 4 hours at 37°C 5% CO₂. HERV-K specific CD8+ T cells are magnetically purified from HERV-K immunized mice, and added to the coculture at a ratio of 1:5. Cells are cultured at 37°C 5% CO₂ for 48 hours. Then, culture supernatant is collected for cytokine concentration analysis by Luminex. Cytokine levels measure include (A) IL-6, (B) GM-CSF, (C) IL-2, and (D) IFN γ . Data are represented as Mean \pm SEM.

Example 19: Administration with rMVA-HERV-K-4-1BBL directs antigen-specific CD8+ T cells towards activated effector T cells by direct antigen presentation of infected tumor cells [Prophetic example]

[0300] Dendritic cells (DCs) are generated after culturing bone marrow cells from C57BL/6 mice in the presence of recombinant Flt3L for 14 days. B16.F10 cells are infected with MVA-HERV-K, MVA-HERV-K-CD40L, MVA-HERV-K-4-1BBL, or MVA-HERV-K-4-1BBL-CD40L at a MOI 10 and left overnight. The next day, infected tumor cells are harvested and cocultured when indicated in the presence of DCs at a 1:1 ratio for 4 hours at 37°C 5% CO₂. Meanwhile, HERV-K specific CD8+ T cells are magnetically purified from HERV-K immunized mice, and added to the coculture at

a ratio of 1:5. Cells are cultured at 37°C 5% CO₂ for 48 hours. Cells are then stained and analyzed by flow cytometry. Cytokine analysis is done for (A) GMFI of T-bet on OT-I CD8+ T cells and (B) percentage of CD44+Granzyme B+ IFN γ + TNF α + of OT-I CD8+ T cells. Data are shown as Mean \pm SEM.

Example 20: Infection with rMVA-HERV-K encoded either with CD40L or 4-1BBL induce tumor cell death in tumor cell lines and macrophages [Prophetic example]

[0301] Tumor cell lines B16.OVA (A and B), MC38 (C) and B16.F10 (D) are infected at the indicated MOI for 20 hours. Then, cells are analyzed for their viability by flow cytometry. Serum HMGB1 in the samples from (A) is quantified by ELISA. Bone marrow derived macrophages (BMDMs) are infected at the indicated MOI for 20 hours. Cells are then analyzed for their viability by flow cytometry. Data are presented as Mean \pm SEM.

Example 21: Intratumoral administration of recombinant MVA encoding 4-1BBL results a decrease in Treg cells and a decrease in Tcell exhaustion in the tumor [Prophetic example]

[0302] B16.OVA tumor-bearing C57BL/6 mice (n=5/group) are grouped and receive intratumoral (i.t.) PBS or 5x10⁷ TCID50 of MVA-OVA or MVA-OVA-4-1BBL at days 7 (black dotted line) after tumor inoculation. Five days later, mice are sacrificed, spleens and tumors harvested and stained to assess Treg infiltration and T cell exhaustion with fluorochrome conjugated antibodies. (A) Percentage of CD4+ FoxP3+ T cells among CD45+ tumor-infiltrating leukocytes; Geometric Mean Fluorescence Intensity of PD-1 (B) and Lag-3 (C) on tumor infiltrating CD8 T cells. Data are presented as Mean \pm SEM.

Example 22: Immune checkpoint blockade and tumor antigen specific antibodies synergize with intratumoral administration of rMVA gp-70-4-1BBL [Prophetic example]

[0303] B16.OVA tumor-bearing C57BL/6 mice (n=5/group) are grouped and receive when indicated (ticks) 200 μ g IgG2a, anti TRP-1 or anti PD-1. Mice are immunized intratumorally either with PBS or with 5x10⁷ TCID50 MVA-gp70-4-1BBL at days 13 (black dotted line), 18 and 21 (grey dashed lines) after tumor inoculation. Tumor growth is measured at regular intervals.

Example 23: Cytokine/chemokine MVA-BN backbone responses to IT immunization can be increased by 4-1BBL adjuvantation

[0304] To assess the potential of recombinant MVAs to induce inflammation within the Tumor MicroEnvironment (TME), cytokines and chemokines were analyzed in tissue from B16.OVA tumors. First, 5×10^5 B16.OVA cells were subcutaneously (s.c.) implanted into C57BL/6 mice. On day 10, mice were immunized intratumorally (i.t.) with PBS or 2×10^8 TCID₅₀ MVA-BN, MVA-OVA, or MVA-OVA-4-1BBL (n=5 to 6 mice/group).

[0305] Six hours after injection, cytokine and chemokine expression was measured (Figure 15). Cytokine/chemokine expression in tissue treated with PBS represents the basal inflammatory profile induced by insertion of the needle into the tumor and saline shear pressure. Cytokines including IL-6, IFN- α , IL-15, and TNF- α , as well as chemokines such as CXCL1, CCL2, and MIP2 were upregulated (Figure 15). IL-25 (also known as IL-17E), which is induced by NF- κ B activation and stimulates the production of IL-8 in humans, was also detected (Lee *et al.* (2001) *J. Biol. Chem.* 276: 1660-64). Interestingly, tumors injected with MVA-OVA-4-1BBL exhibited a significant increase in pro-inflammatory cytokines such as IL-6, IFN- α , or IL-15/IL15R α compared to tumors injected with MVA-BN or MVA-OVA injected tumor lesions.

Example 24: Cytokine/chemokine pro-inflammatory responses to intratumoral (i.t.) immunization are increased by MVA-OVA-4-1BBL

[0306] Mice and tumors were treated as described in Example 23. Strikingly, several pro-inflammatory cytokines, including IFN- γ and GM-CSF, were only produced following intratumoral immunization with MVA-OVA-4-1BBL (Figure 16). Production of other pro-inflammatory cytokines including IL-18, CCL5, CCL3, and IL-22 was enhanced by intratumoral (i.t.) immunization with either MVA-OVA or MVA-OVA-4-1BBL, but not MVA-BN or PBS alone.

[0307] Altogether, this data demonstrates that intratumoral (i.t.) MVA immunization can induce an inflammatory cytokine/chemokine shift in the tumor microenvironment (TME), thereby enhancing the inflammatory response. Increased effects were observed for intratumoral immunization with MVA-OVA-4-1BBL compared to MVA or MVA-OVA. In this manner, the addition of 4-1BBL can be said to have “adjuvanted” the recombinant MVA.

Example 25: Quantitative and qualitative T-cell analysis of the TME and draining LN after intratumoral injection of MVA-OVA-4-1BBL

[0308] To better understand the cellular processes induced by inflammation following intratumoral (i.t.) injection of MVA-OVA and MVA-OVA-4-1BBL, an in-depth analysis of innate and adaptive immune infiltrates at different time points after intratumoral (i.t.) injection was performed. B16.OVA tumor-bearing mice were injected intratumorally (i.t.) with either PBS or 2×10^8 TCID₅₀ MVA-OVA or MVA-OVA-4-1BBL. Mice were sacrificed 1, 3, and 7 days after prime immunization. Tumors and tumor-draining lymph nodes (TdLN) were removed and treated with collagenase and DNase, and single cells were analyzed by flow cytometry. Immune cell populations were analyzed to determine their size, proliferative behavior, and functional state.

[0309] Results showed that injection of B16.OVA tumors either with MVA-OVA or MVA-OVA-4-1BBL induced infiltration of CD45⁺ leukocytes into the tumor 7 days after intratumoral (i.t.) immunization (Fig. 17, top row, left histogram). Interestingly, an expansion of CD45⁺ leukocyte numbers in the TdLN was already observed 3 days after the i.t. (intratumoral) immunization (Fig 17, top row, right histogram), especially following injection of MVA expressing 4-1BBL. This difference was further enlarged in the TdLN seven days after intratumoral (i.t.) immunization, suggesting that MVA immune -mediated antitumor effects start in the TdLN as soon as day 3 after immunization.

[0310] One aspect of vaccination-based antitumor therapy is the expansion and reinvigoration of tumor-specific CD8⁺ and CD4⁺ T cells and their enrichment in the tumor. Both CD4⁺ T cells and CD8⁺ T cells increased in the tumor one week after immunization (Fig. 17, second and third row respectively, left histograms). CD4⁺ T cells increased in the tumors by day 7 as well as in the TdLN starting at day 3 and peaking at day 7 following i.t. immunization with MVA-OVA-4-1BBL. CD8⁺ T cells largely contributed to the increase in CD45⁺ cells in the tumor by day 7. Injection of MVA-OVA-4-1BBL further expanded the CD8⁺ T cell population as compared to injection of MVA-OVA in both tumor (day 7) and dLN (days 3 and 7).

[0311] Quantification of OVA-specific CD8⁺ T cells revealed an increase within the tumor microenvironment 7 days after intratumoral (i.t.) immunization, particularly in the group treated with MVA-OVA-4-1BBL (Fig. 17, lower left). Strikingly, the expansion of OVA-specific CD8⁺ T cells in the TdLN peaked on day 3 after immunization, being higher in the MVA-OVA-4-1BBL treated group (Fig. 17, lower right). Altogether, these data indicate that intratumoral immunization with MVA-OVA, especially MVA-OVA-4-1BBL, enhances the generation of adaptive immune responses starting

3 days after treatment in the tumor draining lymph node, resulting in a significant increase of antigen-specific CD8⁺ T cells in the tumor microenvironment by day 7.

Example 26: Induction of antigen-specific CD8+ T cells by intratumoral injection of MVA-OVA-4-1BBL

[0312] OVA-specific CD8⁺ T cells in the tumor draining lymph node (TdLN) induced by intratumoral injection of MVA-OVA-4-1BBL exerted a high proliferative capacity. The percentage of OVA-specific CD8⁺ T cells expressing Ki67 (an indicator of cell proliferation) was higher in the TdLN after MVA-OVA treatment compared to PBS and was further increased in mice immunized with MVA-OVA-4-1BBL (Fig. 18A). Moreover, OVA-specific CD8 T cells in the tumor downregulated the exhaustion marker PD-1 by day 7 after immunization with MVA-OVA as well as MVA-OVA-4-1BBL, suggesting a regain in functionality (Fig. 18B).

[0313] Treg cells (also, “regulatory T cells”) are potent inhibitors of anti-tumor immune responses (see, *e.g.*, Tanaka *et al.* (2017) *Cell Res.* 27: 109-118). Intratumoral injection of MVA-OVA increased the OVA-specific Teff/ Treg ratio in the tumor (*i.e.*, the ratio of “Teff” cells, or “effector T cells” to Treg cells), and further increases were seen on day 7 after treatment with MVA-OVA-4-1BBL (Fig. 18C). Thus, intratumoral treatment with MVA-OVA and particularly with MVA-OVA-4-1BBL reduced the frequency of intratumoral Treg in favor of CD8⁺ T effector cells which is beneficial for anti-tumor immune responses.

Example 27: Quantitative and qualitative NK cell analysis of the TME and draining LN after intratumoral injection of MVA-OVA-4-1BBL

[0314] Quantification of NK cells after i.t. immunization with MVA-OVA showed a decrease of NK cells in the tumor on day 1 after intratumoral immunization (Fig.19, top row, left histogram). These changes were more pronounced when MVA-OVA-4-1BBL was used. Concurrently, NK cells in the tumor draining lymph node (TdLN) were increased at 3 and 7 days after immunization with both MVA-OVA and MVA-OVA-4-1BBL (Fig. 19, top row, right histogram), although MVA-OVA-4-1BBL induced the highest increase of NK cells in the TdLN.

[0315] CD69 is a marker of early NK cell activation. Both viral vectors, MVA-OVA and MVA-OVA-4-1BBL, led to the immediate upregulation of the activation marker CD69 in the tumor as well as in the draining lymph node (TdLN; Figure 19, second row). Furthermore, i.t. immunization

resulted in the induction of Granzyme B in NK cells at various timepoints both in tumors and TdLNs, which is indicative of enhanced cytotoxic NK cell function (Figure 19, third row).

[0316] Finally, the proliferative capacity of NK cells by means of Ki67 expression was analyzed. On day 3, Ki67 expression on NK cells was significantly increased in the tumor and the TdLN of mice that were treated intratumorally with either MVA-OVA or MVA-OVA-4-1BBL (Figure 19, last row).

[0317] These results demonstrate that 4-1BBL-adjuvanted MVA-OVA (*i.e.*, MVA-OVA-4-1BBL) further increased the expression of CD69, Granzyme B, and Ki67 surface markers on NK cells following intratumoral injection in comparison to MVA-OVA. These experiments also reveal a significant role of the draining lymph nodes (TdLNs) in mounting anti-tumor T cell and NK cell responses after intratumoral immunotherapy.

[0318] While the invention is not bound by any particular mechanism of operation, the expansion of T cells in the TdLN on day 3 and the delayed infiltration of T cells in the tumor on day 7 (see Figure 17) speaks in favor of a scenario in which tumor-specific T cells are primed and expanded in the TdLN and thereafter migrate to the tumor to kill tumor cells. Intratumoral injection of viral vectors might also lead to NK cell activation directly in the TdLN, thereby inducing further DC activation.

Example 28: Role of CD8 T cells in intratumoral MVA cancer therapy

[0319] The analysis of T cell responses in the tumor and the TdLN (*e.g.*, in Figure 17) showed an expansion of tumor-specific T cells at both sites after intratumoral (i.t.) treatment. Experiments were conducted to examine the contribution of T cells to MVA-OVA-4-1BBL mediated anti-tumor effects. In these experiments, C57BL/6 mice were injected with B16.OVA melanoma cells (5×10^5 cells) and tumor growth was monitored following one of several treatments. Treatments included intratumoral (i.t.) injection of PBS or MVA-OVA-4-1BBL in the presence or absence of 100 μ g CD8-T-cell-depleting antibodies (“ α CD8,” clone 2.43) or isotype control antibodies. Injection of MVA-OVA-4-1BBL was performed (i.t.) when tumors reached 5mm in diameter and was repeated twice within a week. One day before the first injection with MVA-OVA-4-BBL, mice were injected i.p. with either anti-CD8 or IgG2b antibodies, and this treatment was repeated four times within the following two weeks. Data presented in Figure 20 shows that CD8 T cells were essential for effective

MVA tumor therapy. Together, these data indicate that MVA-induced activation and expansion of tumor-specific CD8 T cell in the tumor and TdLN are important events for tumor growth control.

Example 29: Batf3+ DC-dependency of MVA-OVA and MVA-OVA-4-1BBL mediated anti-tumor effects

[0320] In order to elucidate the underlying cellular and molecular entities that contribute to anti-tumor immune responses induced by MVA-OVA-4-1BBL, we investigated the role of various immune cell players. Dendritic cells (DCs), with their ability to potently sample and present antigens and co-stimulatory signals to cells of the adaptive immune system, are considered a critical factor in antitumor immunity. Various subtypes of DCs have been implicated in the activation of potent immune responses against tumors, including CD8 α + DCs (also known as “cDC1”). This DC subset has the unique ability to cross-present antigens during immune responses, and CD8 α + DCs are the main producers of IL-12 in response to infection (Hochrein *et al.* (2001) *J. Immunol.* 166: 5448-55; Martínez-López *et al.* (2014) *Eur. J. Immunol.* 45: 119-29) and cancer (Broz *et al.* (2014) *Cancer Cell* 26: 638-52). CD8 α + DCs are also potent inducers of antitumor CD8+ T cells by cross-presentation of tumor-associated antigens (Sánchez-Paulete *et al.*, (2015) *Cancer Discovery* 6: 71-79; Salmon *et al.* (2016) *Immunity* 44: 924-38). CD8 α + DC development is crucially dependent on the transcription factor Batf3 (Hildner *et al.* (2008) *Science* 322: 1097-1100).

[0321] In order to assess the importance of this DC subset for intratumoral MVA cancer therapy, we utilized wildtype and Batf3-deficient (Batf3 $^{-/-}$) B16.OVA tumor-bearing mice. Figure 21A shows that B16.OVA tumors grew dramatically faster in the absence of cross-presenting DC (Batf3 $^{-/-}$), which indicates an important role of this Antigen Presenting Cell (APC) subset in the induction of tumor-directed immune responses. In line with previous experiments, in wildtype mice, intratumoral injection of MVA-OVA led to tumor growth delay and in one case to the complete clearance of the tumor. This effect was improved when mice were injected with MVA-OVA-4-1BBL; 3 out of 5 mice treated with MVA-OVA-4-1BBL rejected the tumor (Fig. 21A). Intriguingly, in the absence of cross-presenting DC (Batf3 $^{-/-}$), intratumoral MVA immunotherapy was not at all impaired as compared to the WT groups (Fig. 21A). However, Batf3-DC seem to participate in the 4-1BBL induced antitumor responses (Fig. 21A, bottom).

[0322] Flow cytometry analysis of CD8+ T lymphocyte populations in peripheral blood 11 days after the first immunization (Fig. 21B) showed that OVA-specific CD8+ T cell frequencies were

only mildly diminished in MVA-OVA-4-1BBL immunized *Batf3*^{-/-} tumor bearers compared to wildtype counterparts. While the invention is not bound by or dependent on any particular mechanism of operation, these data suggest that *Batf3*-dependent DC play a redundant role for intratumoral cancer therapy with MVA.

Example 30: Role of NK cells for intratumoral administration of MVA-OVA-4-1BBL

[0323] NK cells are known to express 4-1BB, and ligation of 4-1BB on NK cells has been shown to result in increased proliferation and cytotoxicity of these cells (Muntasell *et al.* (2017) *Curr. Opin. Immunol.* 45: 73-81). In earlier experiments (see Figure 19), we found that intratumoral injection of MVA-OVA-4-1BBL strongly upregulated the activation marker CD69 as well as the cytotoxicity marker granzyme B on NK cells concomitant with enhanced proliferation.

[0324] To explore the role of NK cells in the 4-1BBL-induced anti-tumor immune response, we utilized IL15R α ^{-/-} mice. The IL-15 receptor alpha subunit (IL-15R α) mediates high-affinity binding of IL-15, a pleiotropic cytokine shown to be crucial for the development of NK cells (Lodolce *et al.* (1998) *Immunity* 9: 669-76). Wildtype and IL15R α -deficient (*IL15R α* ^{-/-}) B16.OVA tumor-bearing mice were generated and intratumorally immunized with either MVA-OVA or MVA-OVA-4-1BBL. Mice treated with MVA-OVA showed a similar therapeutic efficacy irrespective of the presence or absence of IL-15R α (Fig. 22A). Intriguingly, the benefits that were observed in wildtype mice when using MVA-OVA-4-1BBL (in which 3 of 5 mice rejected the tumor) were completely lost in IL15R α -deficient tumor bearing mice treated with MVA-OVA-4-1BBL (in which 1 of 5 mice rejected the tumor; see Fig. 22A). These results were also reflected in the survival of the mice following tumor inoculation (Fig. 22B).

[0325] It is known that the absence of IL15R α not only affects the development of NK cells but also diminishes T cell homeostasis and LN migration, and selectively reduces CD8 memory T cells in mice (Lodolce *et al.* (1998) *Immunity* 9: 669-76). Therefore, we also investigated T cell responses to these treatments. In line with our previous data, we observed an induction of OVA-specific CD8 T cells upon MVA-OVA intratumoral (i.t.) immunization in wildtype animals which was further increased with MVA-OVA-4-1BBL (Fig. 22C). However, OVA-specific T cell responses in IL15R α ^{-/-} mice were similar to the responses found in wildtype mice.

[0326] While the invention is not bound by any particular mechanism or mode of operation, these findings indicate that IL15R α ^{-/-} tumor bearing mice can mount tumor-specific T cell responses

and thus support the notion that 4-1BBL-enhanced NK cell activation and function contributes to the therapeutic efficacy of intratumoral MVA-OVA-4-1BBL treatment.

Example 31: NK cell-dependent cytokine/chemokine profile in response to intratumoral immunization with MVA-OVA-4-1BBL

[0327] To identify cytokines that were selectively induced by 4-1BBL – 4-1BB interaction on NK cells, cytokines and chemokines were analyzed in tumor tissue from B16.OVA tumor bearing wildtype or IL15Ra^{-/-} mice treated intratumorally with PBS or 5x10⁷ TCID₅₀ MVA-OVA or MVA-OVA-4-1BBL.

[0328] Previous experiments showed that a large number of cytokines and chemokines increased six hours after intratumoral injection of recombinant MVA (Fig. 15 and 16). In these experiments, injection of tumors with MVA-OVA-4-1BBL exhibited a significant increase over injection with MVA-OVA in the production of pro-inflammatory cytokines or chemokines such as IFN-γ, CCL3, and CCL5 known to be produced by NK cells upon stimulation with 4-1BBL (Fig. 23). This 4-1BBL-induced increase was completely abrogated in IL15Ra^{-/-} mice, demonstrating that intratumoral injection of rMVA-OVA-4-1BBL induces a distinct cytokine and chemokine profile in the tumor microenvironment 6h after injection that emanates from NK cells.

Example 32: Anti-tumor efficacy of intratumoral immunization with MVA-gp70-CD40L in comparison to MVA-gp70-4-1BBL

[0329] Gp70 is a tumor self-antigen expressed in a number of syngeneic tumor models (B16.F10, CT26, MC38, 4T1, EL4, *etc.*) all representing distinct tumor microenvironments (TMEs) in terms of stroma and immune cell composition. Here, we tested the potency of MVA encoding the tumor antigen gp70 in addition to either CD40L or 4-1BBL in intratumoral immunization of B16.F10 tumor-bearing mice.

[0330] B16.F10 melanoma cells were subcutaneously injected into C57BL/6 mice. When tumors reached ~50 mm³ in size, mice were immunized intratumorally with PBS, MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-4-1BBL, or MVA-CD40L; results are shown in Figure 24.

[0331] Immunization with MVA-gp70 induced transient and mild tumor growth control. This anti-tumor effect could be enhanced when the virus expressed CD40L. However, intratumoral

immunization with MVA-gp70-4-1BBL produced the strongest therapeutic effects, resulting in the complete tumor clearance in 2 out of 5 animals treated (Fig. 24A).

[0332] Strikingly, the mice that were cured of tumors after treatment with MVA-gp70-4-1BBL exhibited a loss of pigmentation at the spot where the tumor had been (Fig. 24B). This depigmentation is indicative of the autoimmune condition vitiligo and is a result of melanocyte destruction by self-reactive T cells. This destruction of melanocytes suggests that the activation of the immune system by a recombinant MVA is not restricted to the TAA encoded by the MVA (here, gp70). Rather, this expanded activation of the immune system against other antigens, a phenomenon known as epitope spreading, results in a broader immune response that might provide a better therapeutic outcome.

[0333] To assess antigen-specific T cell responses induced by immunization, blood was withdrawn 11 days after the first immunization and analyzed for the presence of antigen-specific T cells. Immunization with both MVA-gp70 and MVA-gp70-CD40L, as well as with MVA-CD40L and MVA-4-1BBL induced a measurable p15E-specific T cell response which ranged between 1-2% (Figure 24C). Importantly, this response was drastically increased (>5 fold) in mice that received MVA-gp70-4-1BBL. This antigen-specific T cell response to p15E peptide restimulation correlated with the therapeutic efficacy in the different treatment groups.

Example 33: Anti-tumor efficacy of intratumoral immunization of MVA-gp70-4-1BBL-CD40L

[0334] A recombinant MVA was generated expressing the tumor antigen gp70 together with 4-1BBL and CD40L and was tested intratumorally in the B16 melanoma model. B16.F10 melanoma cells were subcutaneously injected into C57BL/6 mice. When tumors reached ~50 mm³, mice were immunized intratumorally with PBS, MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-gp70-4-1BBL-CD40L, or corresponding MVA constructs not expressing gp70.

[0335] Immunization with MVA-gp70 induced transient and significant tumor growth control (Figure 25A). This anti-tumor effect could be enhanced when the virus expressed CD40L or 4-1BBL. However, intratumoral immunization with MVA-gp70-4-1BBL-CD40L led to the strongest therapeutic effects--complete tumor clearance in 4 out of 5 treated animals (Fig. 25A). Strikingly, three of the four cured mice that were treated with the MVA-gp70-4-1BBL-CD40L showed a loss of pigmentation where the tumor used to be, indicative of the autoimmune condition vitiligo, as discussed above in Example 32.

[0336] In addition, gp70-specific T cell responses were measured in the blood 11 days after the first immunization. Immunization with MVA-gp70 and MVA-gp70-CD40L as well as with MVA-CD40L and MVA-4-1BBL induced a measurable tumor-specific T cell response which ranged between 1-2%; this response was dramatically increased (>5-fold) in mice that received MVA-gp70-4-1BBL (Fig. 25B).

[0337] Taken together, in the B16.F10 melanoma model, anti-tumor efficacy could be enhanced when MVA-gp70 was adjuvanted with either CD40L or 4-1BBL, but even stronger effects were observed when 4-1BBL and CD40L were expressed together in MVA-gp70-4-1BBL-CD40L.

Example 34: Intratumoral immunotherapy with MVA-gp70-4-1BBL-CD40L in CT26.WT tumors

[0338] Constructs were then tested using the CT26 colon carcinoma model, described to be rich in T cells and myeloid cells and considered immunogenic (see, e.g., Mosely *et al.* (2016) *Cancer Immunol. Res.* 5: 29-41). Balb/c mice were injected subcutaneously (s.c.) with CT26.wt colon carcinoma cells. When tumors reached ~60 mm³, mice were immunized intratumorally with PBS, MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L, MVA-gp70-4-1BBL-CD40L, or MVA-4-1BBL-CD40L.

[0339] Immunization i.t. with MVA-gp70 induced transient and significant tumor growth control. This anti-tumor effect was not enhanced when the MVA expressed CD40L, but strikingly, immunization with MVA-gp70-4-1BBL led to the strongest therapeutic effects resulting in complete tumor clearance in all treated animals (Fig. 26A). However, treatment with MVA-gp70-4-1BBL-CD40L did not result in a better therapeutic efficacy. Of note, the viruses that only contained the co-stimulatory molecule but not gp70 also resulted in significant tumor growth delay, however could not compete with MVA-gp70-4-1BBL. These findings were reflected in the overall survival of treated mice (Fig. 26B).

[0340] Gp70-specific T cell responses against the H2-Ld CD8+ T cell epitope AH-1 were readily detected in the blood of animals treated with MVA-gp70 and MVA-gp70-CD40L (Fig. 26C). This response was dramatically increased (>10 fold) in mice that received MVA-gp70-4-1BBL, which correlated with the therapeutic efficacy shown in Figures 26A and 26B. Treatment with MVA-gp70-4-1BBL-CD40L also enhanced AH-1-specific T cell responses in the blood (Fig. 26C).

Example 35: Comprehensive analysis of the tumor microenvironment and the tumor draining LN after IT injection of MVA-gp70-4-1BBL-CD40L into B16.F10 tumor bearing mice

[0341] Data presented above showed that intratumoral treatment of B16.F10 tumor-bearing mice with MVA-gp70-4-1BBL-CD40L resulted in tumor rejection in 80% of treated mice (see Figure 26). To study the tumor microenvironment (TME) and TdLN in this tumor model, B16.F10 tumor-bearing mice received either PBS or 5×10^7 TCID50 of MVA-gp70, MVA-gp70-4-1BBL, MVA-gp70-CD40L or MVA-gp70-4-1BBL-CD40L intratumorally (i.t.). Mice were sacrificed 3 days after prime immunization. Day 3 was selected based on previous experiments in the OVA system which showed changes in both, innate and adaptive components of the immune system at that timepoint (see Figure 17). Tumors and TdLN were removed and digested with collagenase/DNase in order to analyze single cells using flow cytometry. The abundance of immune cell populations as well as their proliferative behavior and functional state were assessed.

[0342] Intratumoral injection of 4-1BBL- and CD40L-adjuvanted MVAs did not confer an advantage at the day 3 timepoint in number of CD8 T cells or p15E-specific T cells in the tumor as determined by pentamer staining. However, in the TdLN, MVA-gp70 and MVA-gp70-CD40L produced an expansion of CD8 T cells, while the addition of 4-1BBL produced an even larger effect (Fig. 27, upper right). The increase produced by the addition of 4-1BBL was even more pronounced for p15E-specific CD8 T cells in the TdLN, for which i.t. immunization with either MVA-gp70-4-1BBL or MVA-gp70-4-1BBL-CD40L increased tumor-specific CD8 T cells (Fig. 27, middle right). The number of p15E-specific CD8 T cells also correlated with the proliferative state of those cells; for example, the addition of 4-1BBL along with gp70 and optionally CD40L to the MVA induced the highest numbers of Ki67+ gp70-p15E CD8 T cells in the TdLN (Fig. 27, lower right).

[0343] These data demonstrate that intratumoral (i.t.) immunization with MVA-gp70 enhances the generation of adaptive immune responses on day 3 after treatment in the tumor and in the tumor draining lymph node, while adjuvantation with 4-1BBL or 4-1BBL plus CD40L specifically increased p15E-specific CD8 T cell responses in the TdLN.

Example 36: Induction of NK cells in tumor and TdLN after intratumoral injection of MVAs

[0344] Intratumoral (i.t.) injection of MVA-OVA produced an activation and expansion of NK cells on day 1 and day 3, respectively (Fig. 19). We then examined NK cell infiltration, activation and expansion on day 3 after injection with different MVA constructs. Quantification of NK cells after i.t. immunization with recombinant MVAs showed an increase in NK cells infiltrating the tumor (Fig. 28, upper left) and the TdLN (Fig. 28, upper right). Infiltration was increased when the MVA encoded 4-1BBL (*e.g.*, MVA-gp70-4-1BBL and MVA-gp70-4-1BBL-CD40L). Intratumoral (i.t.) injection of MVA-gp70 induced proliferation of NK cells (Ki67+) in the tumor (see Fig. 28, middle left) and the TdLN (Fig. 28, middle right), and adjuvantation with 4-1BBL or 4-1BBL and CD40L enhanced this effect in the TdLN.

[0345] Granzyme B is a marker for cytotoxicity of NK cells (see, *e.g.*, Ida *et al.* (2005) *Mod. Rheumatol.* 15: 315-22). Granzyme B+ NK cells were induced in the tumor and TdLN following intratumoral injection with recombinant MVAs (Fig. 28, lower left). Again, the addition of 4-1BBL or 4-1BBL-CD40L to the recombinant MVA mildly increased the number of cytotoxic NK cells in the TdLN (Fig. 28, lower right).

[0346] Altogether, these data highlight a significant role of MVA-encoded 4-1BBL-CD40L in the expansion and function of NK cells and TAA-specific T cells after intratumoral (i.t.) immunotherapy. Thus, intratumoral treatment with recombinant MVAs encoding gp70 and 4-1BBL or gp70, 4-1BBL, and CD40L can enhance T cell responses to an endogenous retroviral self-antigen such as gp70.

Example 37: Intravenous immunotherapy with MVA-gp70-4-1BBL-CD40L in CT26.WT tumor-bearing mice

[0347] Experiments discussed above showed that the novel MVA construct encoding the tumor antigen gp70 together with the costimulatory molecules 4-1BBL and CD40L was highly potent when applied intratumorally (Figures 25 and 26). In addition, Lauterbach *et al.* ((2013) *Front. Immunol.* 4: 251) found that MVA-encoded CD40L enhances innate and adaptive immune responses when given intravenously. Here, we asked whether intravenous (i.v.) immunization with MVA-gp70-4-1BBL-CD40L can also provide tumor growth control.

[0348] CT26.WT colon carcinoma cells were subcutaneously injected into Balb/c mice. When tumors reached ~60 mm³, mice were immunized intravenously with PBS or MVA-Gp70, MVA-Gp70-4-1BBL, MVA-Gp70-CD40L, MVA-gp70-4-1BBL-CD40L, and MVA-4-1BBL-CD40L (which lacks gp70). I.v. immunization with MVA-gp70 led to tumor clearance in 2/5 animals (Fig. 29A). Mice that were treated with gp70-expressing virus either containing 4-1BBL or CD40L showed a strongly improved anti-tumor response which resulted in 3/5 and 4/5 cured mice, respectively. Importantly, i.v. treatment with MVA-gp70-4-1BBL-CD40L led to a prolonged tumor growth control in all treated mice with 3/5 mice rejecting the tumor (Fig.29A). Of note, the recombinant MVAs that only contained the co-stimulatory molecule but not gp70 also resulted in significant tumor growth delay, but did not lead to the same tumor rejection as observed with MVA-gp70-4-1BBL, MVA-gp70-CD40L or MVA-gp70-4-1BBL-CD40L (Fig. 29A). These findings were reflected in the overall survival of treated mice (Fig. 29B).

[0349] Analysis of tumor-directed CD8 T cell responses in the blood by peptide restimulation of PBLs revealed a significant induction of AH1-specific CD8 T cells in all MVA treatment groups, whereby this could be further increased in the presence of CD40L (*i.e.*, MVA-gp70-CD40L and MVA-gp70-4-1BBL-CD40L) (Fig. 29C).

Example 38: Recombinant MVA comprising HERV-K antigens

[0350] An MVA-based vector (“MVA-mBN489,” also referred to as “MVA-HERV-Prame-FOLR1-4-1-BBL-CD40L”) is designed comprising TAAs that are proteins of the K superfamily of human endogenous retroviruses (HERV-K), specifically, ERV-K-env and ERV-K-gag. The MVA also is designed to encode human FOLR1 and PRAME, and to express h4-1BBL or to express hCD40L; or to express both h4-1BBL and hCD40L.

Example 39: Intratumoral immunization with MVA encoding brachyury antigen

[0351] The highly attenuated, non-replicating vaccinia virus MVA-BN-Brachyury has been designed to consist of four human transgenes to elicit a specific and robust immune response to a variety of cancers. The vector co-expresses the brachyury human TAA and three human costimulatory molecules: B7.1 (also known as CD80), intercellular adhesion molecule-1 (ICAM-1, also known as CD54), and leukocyte function-associated antigen-3 (LFA-3, also known as CD58). The three

costimulatory molecules (or TRIad of COstimulatory Molecules, TRICOMTM) are included to maximize the immune response to the brachyury human TAA.

[0352] Brachyury is a transcription factor in the T-box family and is a driver of EMT, a process associated with cancer progression. It is overexpressed in cancer cells compared with normal tissue and has been linked to cancer cell resistance to several treatment modalities and metastatic potential. Cancers known to express brachyury include lung, breast, ovarian, chordoma, prostate, colorectal and pancreatic adenocarcinoma.

[0353] *In vitro* and clinical studies were conducted to demonstrate the safety and potential therapeutic efficacy of MVA encoding brachyury; see, e.g., Hamilton *et al.* (2013) *Oncotarget* 4: 1777-90 (“*Immunological targeting of tumor cells undergoing an epithelial-mesenchymal transition via a recombinant brachyury-yeast vaccine*”); Heery *et al.* (2015a) *J. Immunother. Cancer* 3: 132 (“*Phase I, dose escalation, clinical trial of MVA-brachyury-TRICOM vaccine demonstrating safety and brachyury-specific T cell responses*”); Heery *et al.* (2015b) *Cancer Immunol. Res.* 3: 1248-56 (“*Phase I trial of a yeast-based therapeutic cancer vaccine (GI-6301) targeting the transcription factor brachyury*”))

[0354] A GLP-compliant repeat-dose toxicity study is performed to evaluate any potential toxicity of MVA-BN-Brachyury (MVA-mBN240B) in NHP (cynomolgus macaques) in support of the use of the intravenous route in the Phase 1 clinical development. The toxicity study includes a biodistribution part evaluating spatial and temporal distribution of MVA-BN-Brachyury in NHP.

[0355] MVA-BN-Brachyury is used in a phase III trial in which cancer patients are treated with intratumoral injection of the MVA, optionally in conjunction with another treatment such as, for example, radiation and/or checkpoint inhibitors.

[0356] It will be apparent that the precise details of the methods or compositions described herein may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

SEQUENCE LISTING

[0357] The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and either one letter code or three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

Sequences in sequence listing:

SEQ ID NO:1: hCD40L amino acid sequence from NCBI RefSeq NP_000065.1. (261 amino acids)

SEQ ID NO:2: hCD40L from NCBI RefSeq NP_000065.1 (792 nucleotides)

SEQ ID NO:3: h4-1BBL from NCBI RefSeq NP_003802.1 (254 amino acids)

SEQ ID NO:4: h4-1BBL from NCBI RefSeq NP_003802.1

SEQ ID NO:1

hCD40L from NCBI RefSeq NP_000065.1. (261 amino acids)

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VTDPSQVSHGTGFTSFGLLKL

SEQ ID NO:2

hCD40L from NCBI RefSeq NP_000065.1. (792 nucleotides)

nt-Sequence:

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We claim:

1. A method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in the cancerous tumor, increases tumor reduction, and/or increases overall survival of the subject as compared to a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.
10
2. A method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising intravenously administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the intravenous administration of the recombinant MVA enhances Natural Killer (NK) cell response and enhances CD8 T cell responses specific to the TAA as compared to a non-intravenous injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a TAA and a 4-1BBL antigen.
15
3. A method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA) and a second nucleic acid encoding 4-1BBL, wherein the administration of the recombinant MVA increases tumor reduction and/or increases overall survival of the subject as compared to administration of a recombinant MVA and 4-1BBL antigen by themselves.
20
4. A method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA) and a second nucleic acid encoding a 4-
25

1BBL antigen, wherein the intratumoral administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral injection of a recombinant MVA virus comprising a first and second nucleic acid encoding a heterologous tumor-associated antigen and a 4-1BBL antigen.

5. The method of claim 1, wherein the subject is human.
6. The method of claim 1, wherein the TAA is an endogenous retroviral (ERV) protein expressed in a tumor cell.
7. The method of claim 6, wherein the ERV protein is from the human endogenous retroviral K (HERV-K) family and is selected from a HERV-K envelope protein and a HERV-K gag protein.
8. The method of claim 1, wherein the TAA is selected from the group consisting of carcinoembryonic antigen (CEA), mucin 1 cell surface associated (MUC-1), prostatic acid phosphatase (PAP), prostate specific antigen (PSA), human epidermal growth factor receptor 2 (HER-2), survivin, tyrosine related protein 1 (TRP1), tyrosine related protein 1 (TRP2), Brachyury, PRAME, FOLR1, HERV-K-env, HERV-K-gag, p15, MEL, and combinations thereof.
9. A method of reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding an endogenous retroviral (ERV) protein and a second nucleic acid encoding 4-1BBL, wherein the administration of the recombinant MVA increases tumor reduction and/or increases overall survival of the subject as compared to administration of a recombinant MVA and 4-1BBL antigen by themselves.
- 25 10. The method of claim 9, wherein the ERV protein is from the human endogenous retroviral protein K (HERV-K) family.

11. The method of claim 10, wherein the retroviral protein K is selected from HERV-K envelope protein and HERV-K-gag protein.

12. The method of claim 1, wherein the recombinant MVA further comprises a third nucleic acid encoding a CD40L antigen.

5 13. The method of claim 1, further comprising administering to the subject at least one immune checkpoint molecule antagonist or agonist.

14. The method of claim 13, wherein the immune checkpoint molecule antagonist or agonist is selected from an antagonist or agonist of CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS.

10 15. The method of claim 14, wherein the immune checkpoint molecule antagonist is an antibody.

15 16. A recombinant modified Vaccinia Ankara (MVA) for use in the treatment of cancer comprising: a) a first nucleic acid encoding a tumor-associated antigen (TAA); and b) a second nucleic acid encoding 4-1BBL; wherein the intratumoral administration of the recombinant MVA enhances an inflammatory response in a cancerous tumor, reduces the size of the cancerous tumor, and/or increases overall survival of the human patient as compared to a non-intratumoral injection of said recombinant MVA.

17. The recombinant MVA for use of claim 16, further comprising c) a third nucleic acid encoding CD40L.

20 18. The recombinant MVA for use of claim 16, wherein the TAA is an endogenous retroviral (ERV) protein.

19. The recombinant MVA for use of claim 18, wherein the ERV protein is from the human endogenous retroviral K (HERV-K) family and is selected from HERV-K-env and HERV-K-gag.

20. The recombinant MVA for use of claim 16, wherein the TAA is selected from the group consisting of carcinoembryonic antigen (CEA), mucin 1 cell surface associated (MUC-1), prostatic acid phosphatase (PAP), prostate specific antigen (PSA), human epidermal growth factor receptor 2 (HER-2), survivin, tyrosine related protein 1 5 (TRP1), tyrosine related protein 1 (TRP2), Brachyury, FOLR1, PRAME, HERV-K-env, HERV-K-gag, p15, MEL, and combinations thereof.

21. A recombinant modified Vaccinia Ankara (MVA) for treating a subject having cancer, the recombinant MVA comprising: (a) a first nucleic acid encoding a tumor-associated antigen (TAA) and (b) a second nucleic acid encoding 4-1BBL.

10 22. The recombinant MVA of claim 21, wherein said TAA is an ERV protein from the human endogenous retroviral K (HERV-K) family.

23. The recombinant MVA of claim 22, wherein the ERV protein is selected from HERV-K env protein and HERV-K gag protein.

15 24. The recombinant MVA of claim 23 further comprising c) a third nucleic acid encoding CD40L.

25. A pharmaceutical combination comprising: (a) the recombinant MVA of claim 24; and (b) at least one immune checkpoint molecule antagonist or agonist.

20 26. The pharmaceutical combination of claim 25, wherein the immune checkpoint molecule antagonist or agonist is selected from an antagonist or agonist of CTLA-4, PD-1, PD-L1, LAG-3, TIM-3, and ICOS.

25 27. A method for reducing tumor size and/or increasing survival in a subject having a cancerous tumor, the method comprising administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a tumor-associated antigen (TAA), a second nucleic acid encoding 4-1BBL, and a third nucleic acid encoding CD40L, wherein the administration of the recombinant MVA increases tumor reduction and/or increases overall survival of the subject as compared to

administration of a recombinant MVA, 4-1BBL antigen, and CD40L antigen by themselves.

28. A method of inducing an enhanced inflammatory response in a cancerous tumor of a subject, the method comprising intratumorally administering to the subject a recombinant modified Vaccinia Ankara (MVA) comprising a first nucleic acid encoding a first heterologous tumor-associated antigen (TAA), a second nucleic acid encoding a 4-1BBL antigen, and a third nucleic acid encoding a CD40L antigen, wherein the intratumoral administration of the recombinant MVA generates an enhanced inflammatory response in the tumor as compared to an inflammatory response generated by a non-intratumoral injection of a recombinant MVA virus comprising a first nucleic acid encoding a heterologous tumor-associated antigen, a second nucleic acid encoding a 4-1BBL antigen, and a third nucleic acid encoding a CD40L antigen.

29. The method of claim 27 comprising administering to the subject said recombinant modified Vaccinia Ankara (MVA) by intravenous administration.

15 30. The method of claim 27 comprising administering to the subject said recombinant modified Vaccinia Ankara (MVA) by intratumoral administration.

31. The method of claim 27 comprising administering to the subject said recombinant modified Vaccinia Ankara (MVA) by intravenous administration and intratumoral administration.

20 32. The method of claim 31 wherein said intravenous administration and said intratumoral administration are at different times.

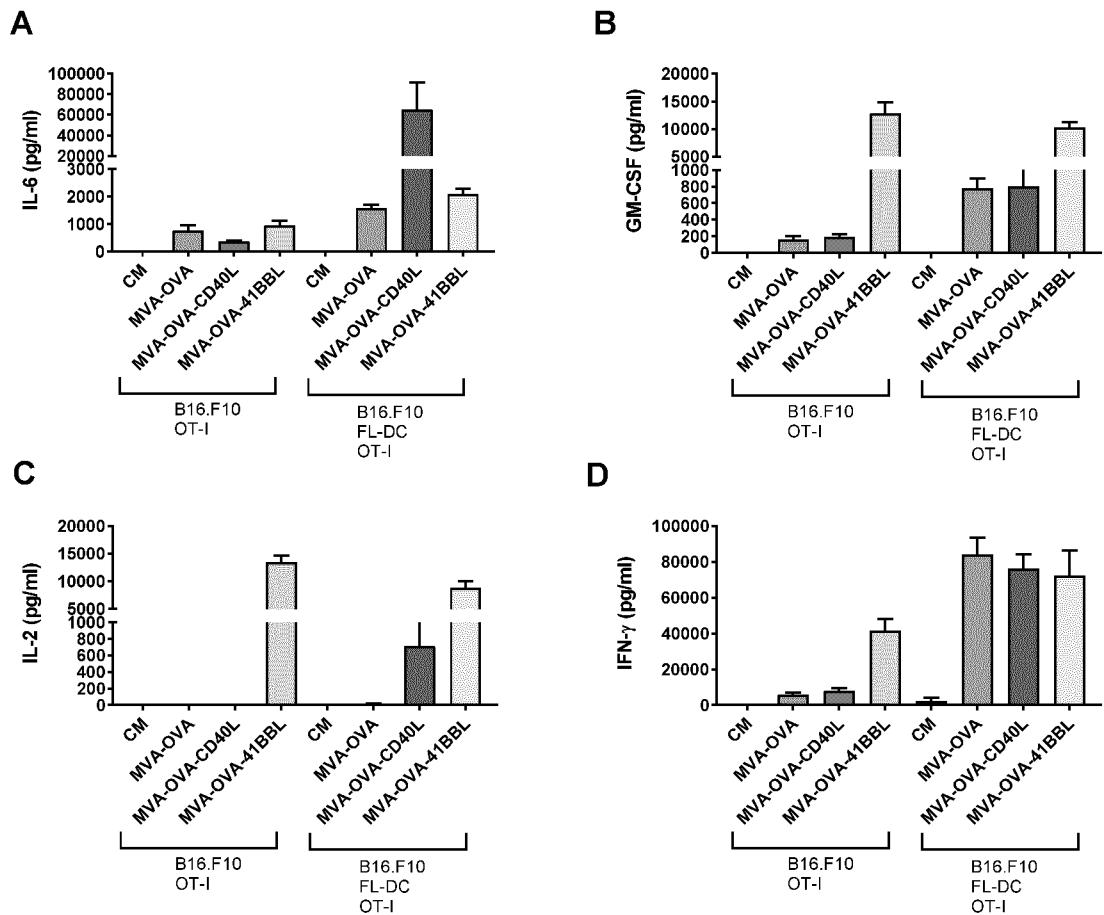


Figure 1

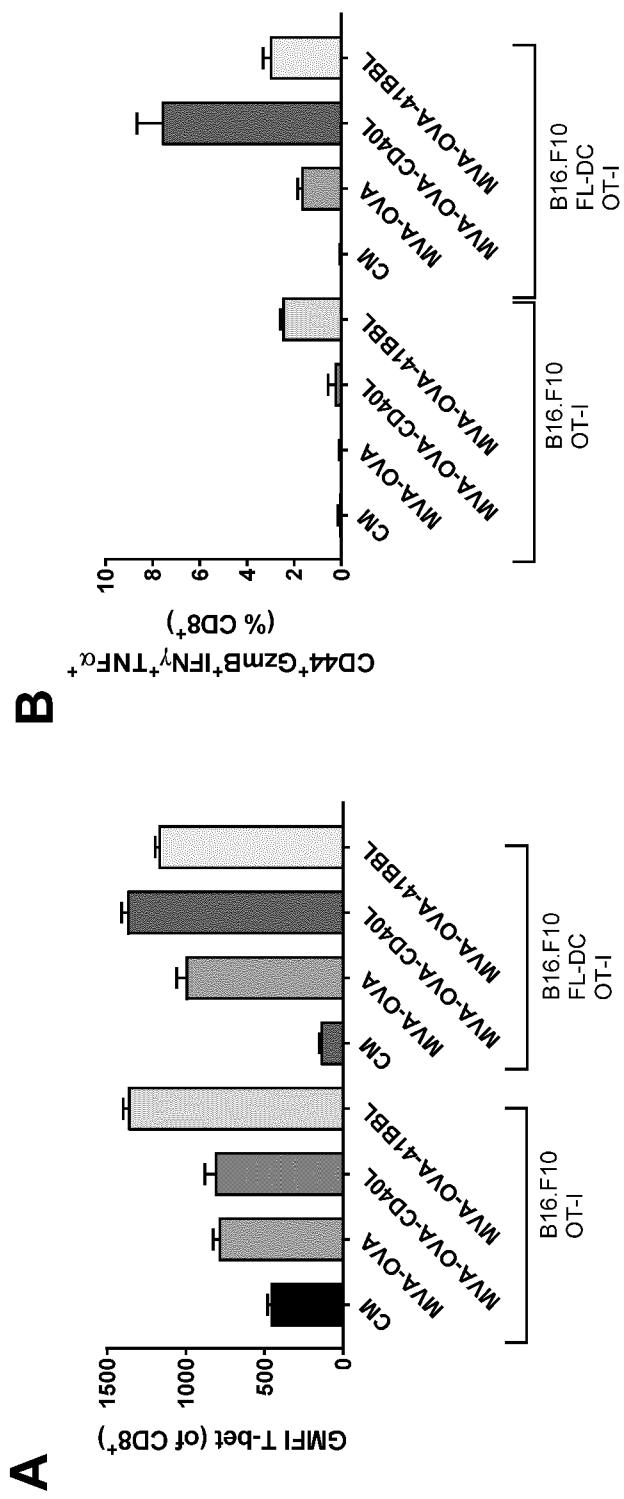


Figure 2

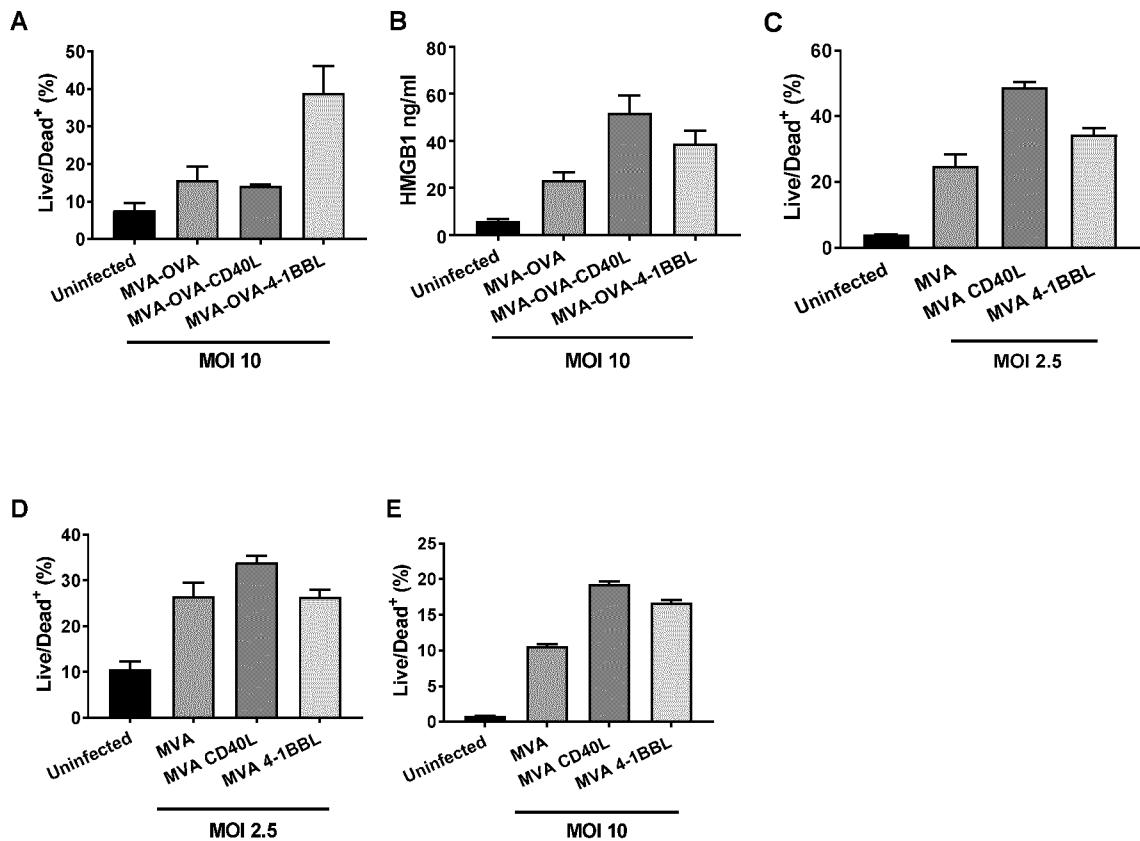


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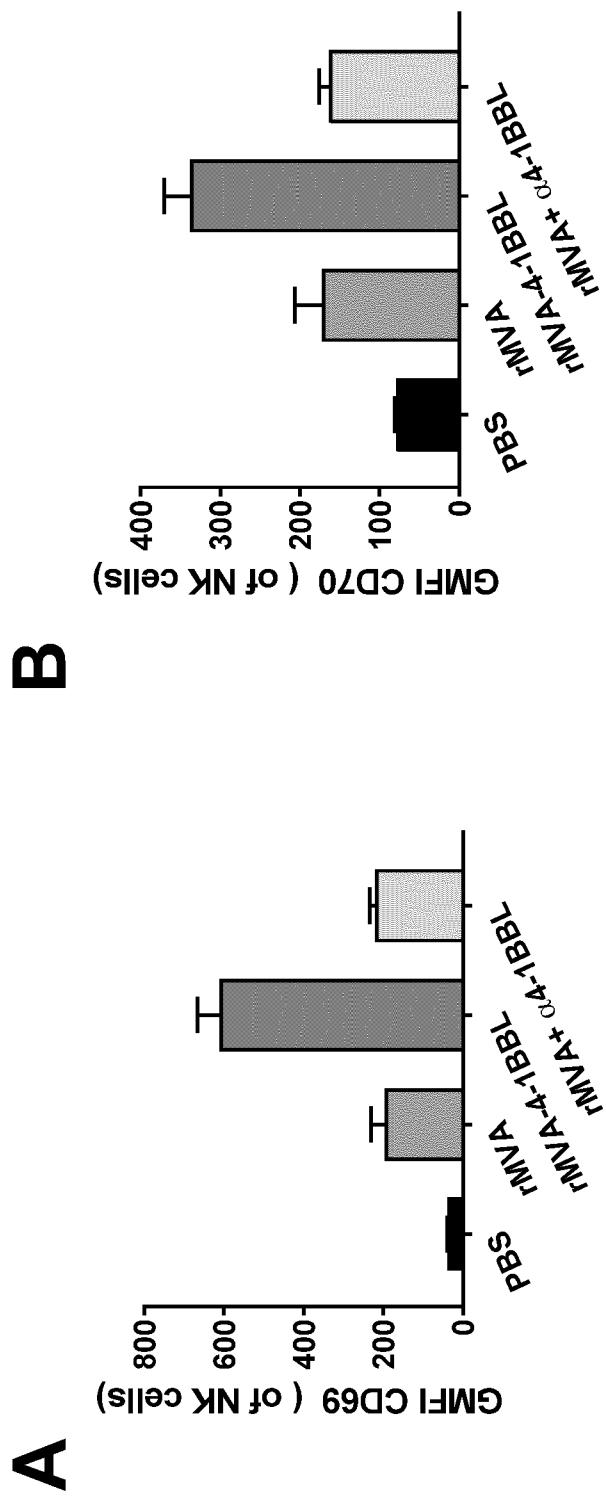


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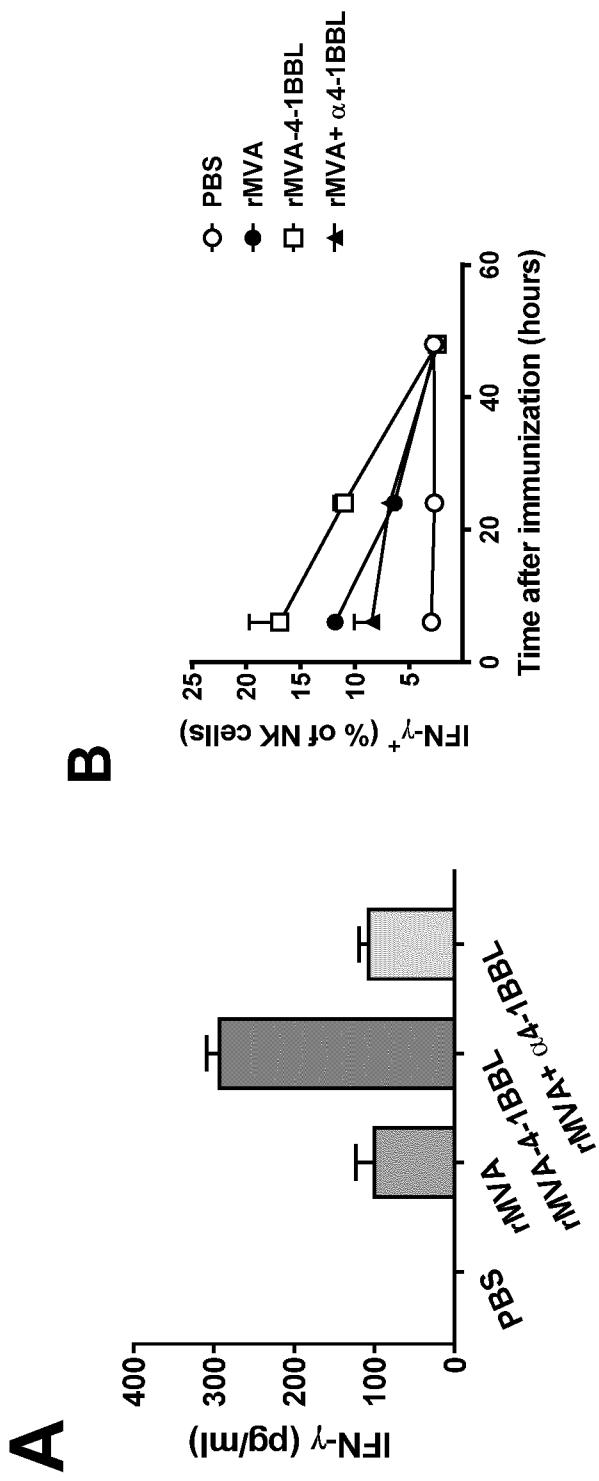


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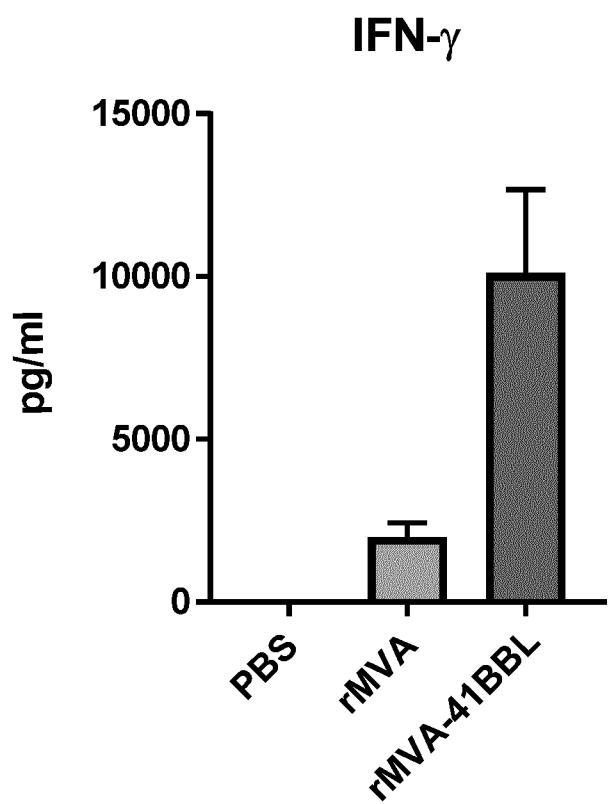


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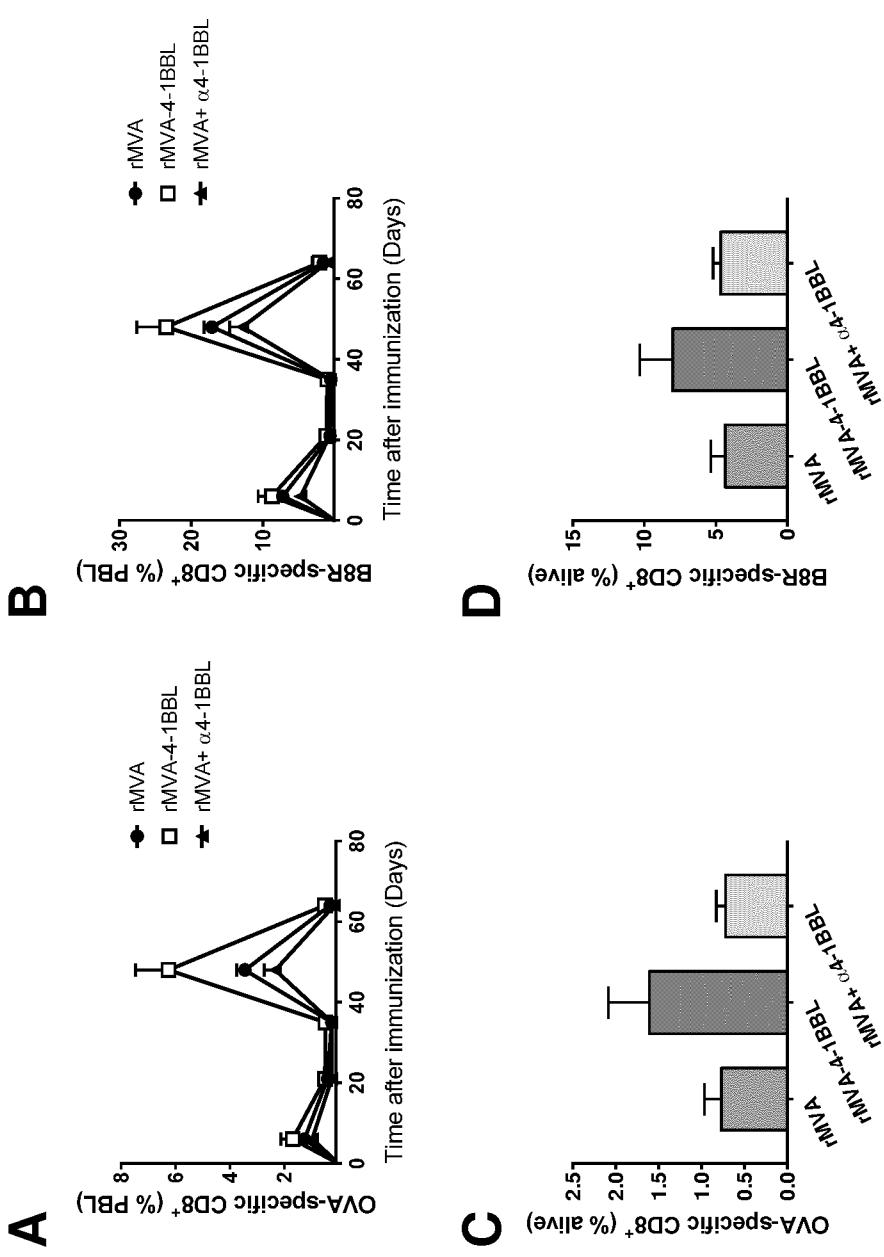


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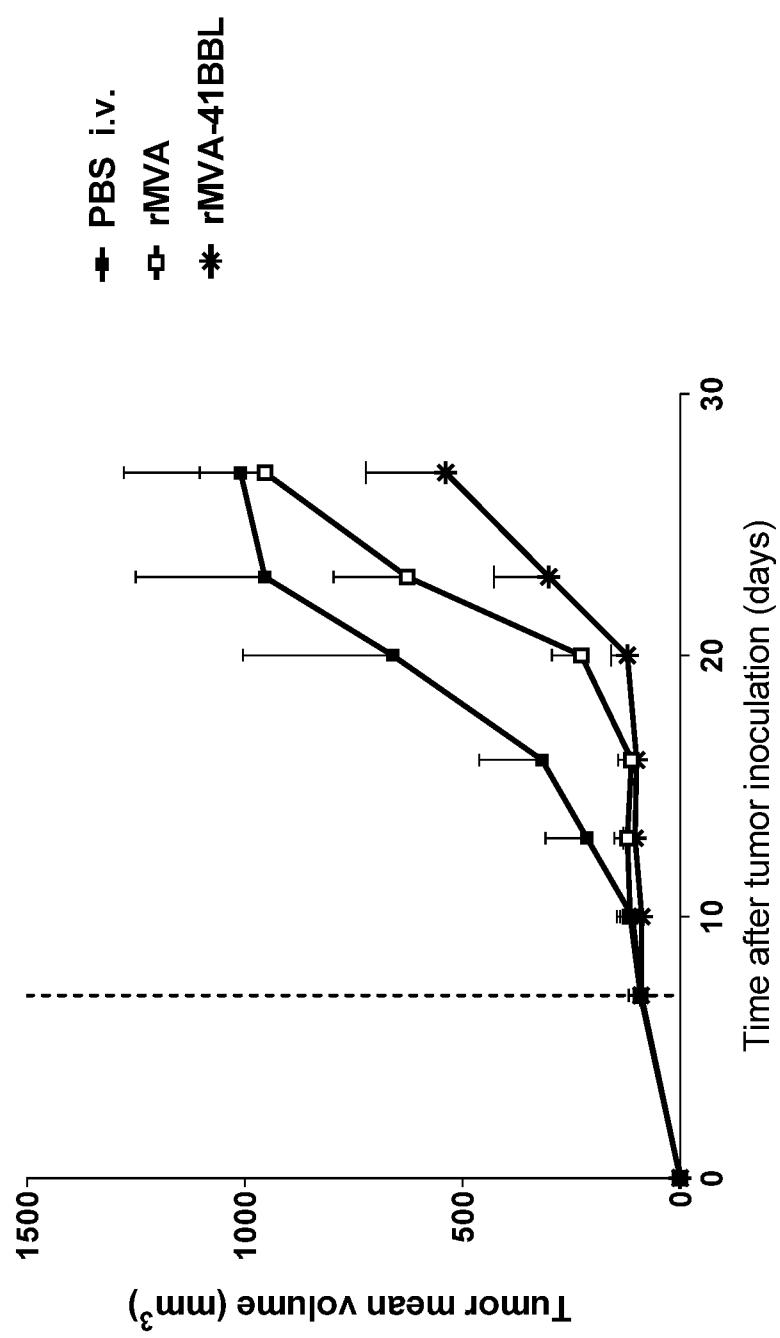


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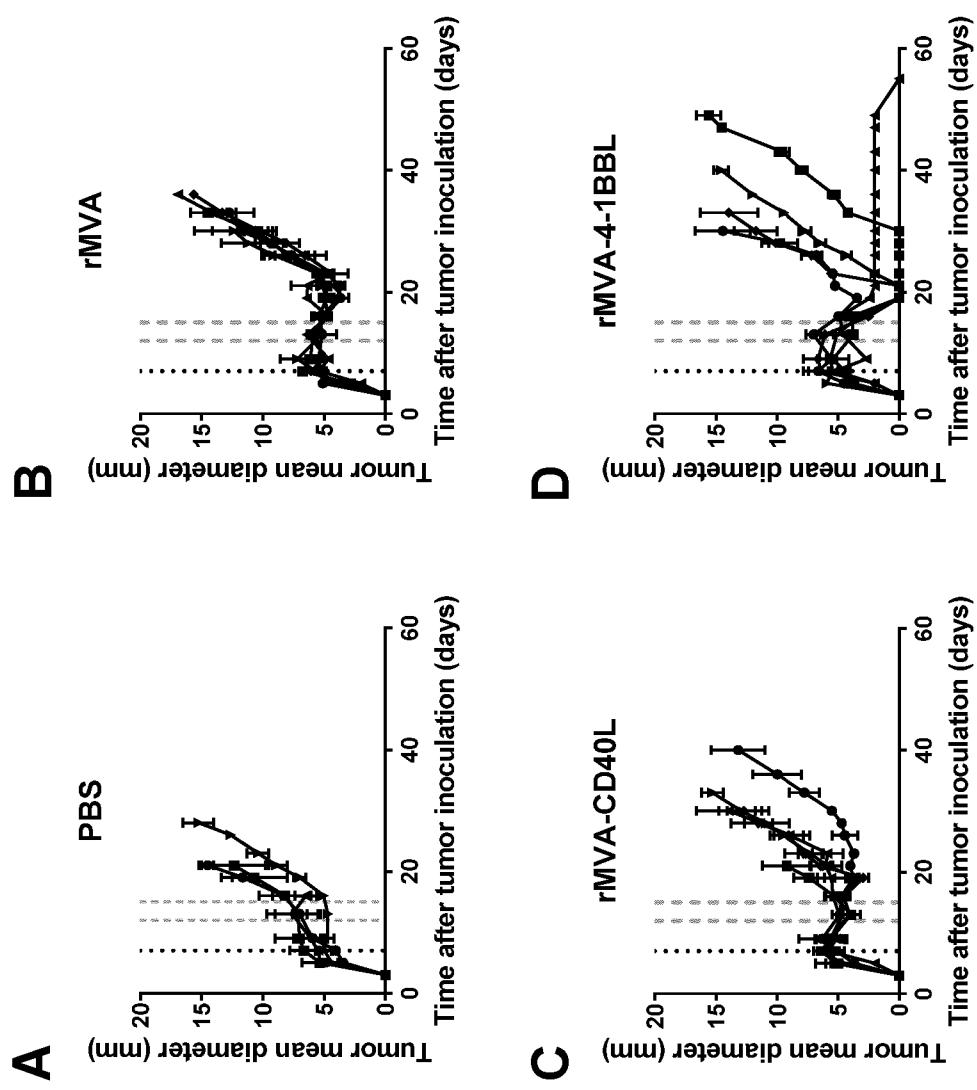


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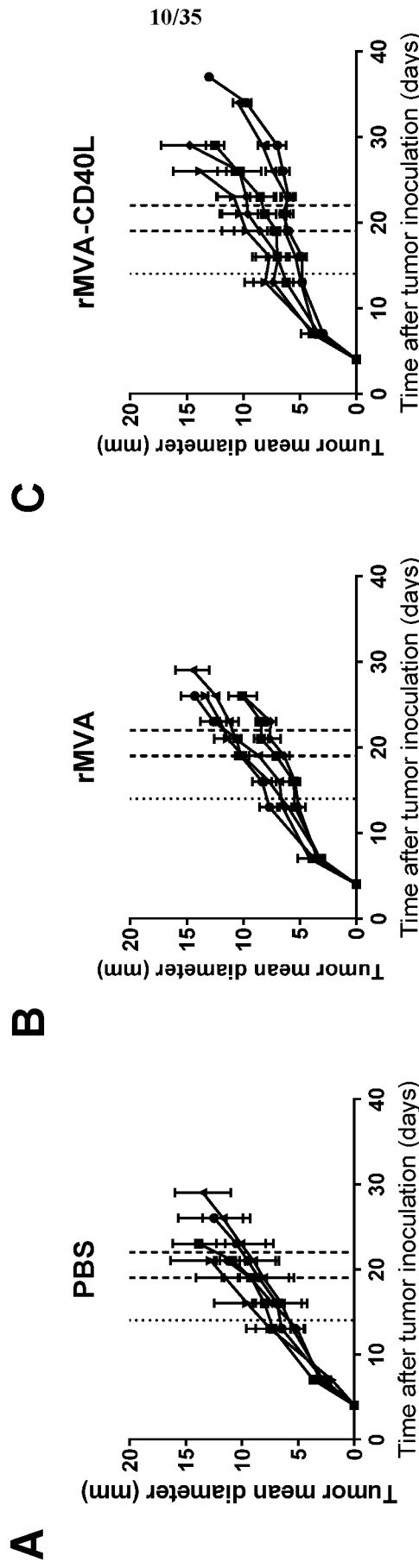


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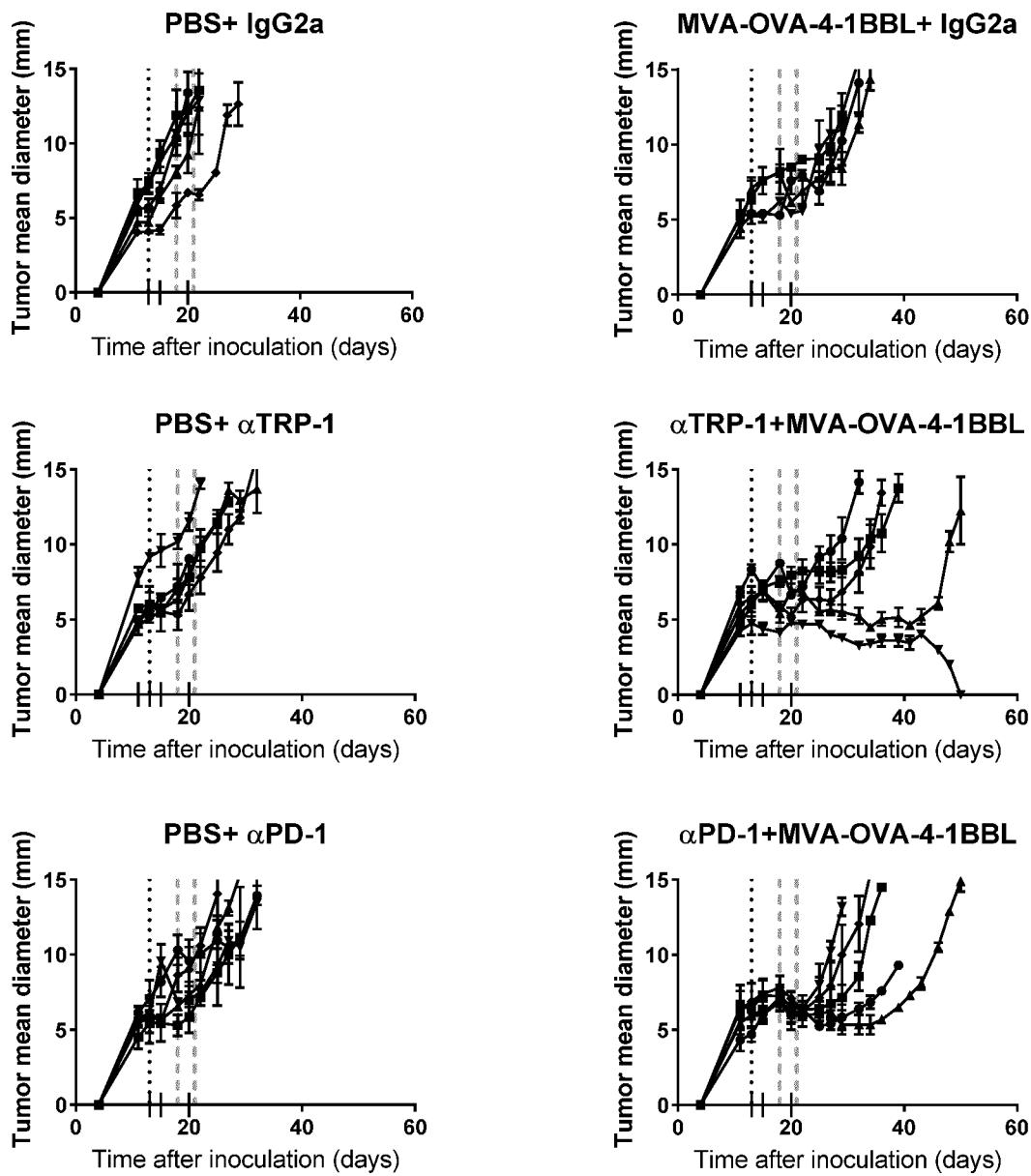


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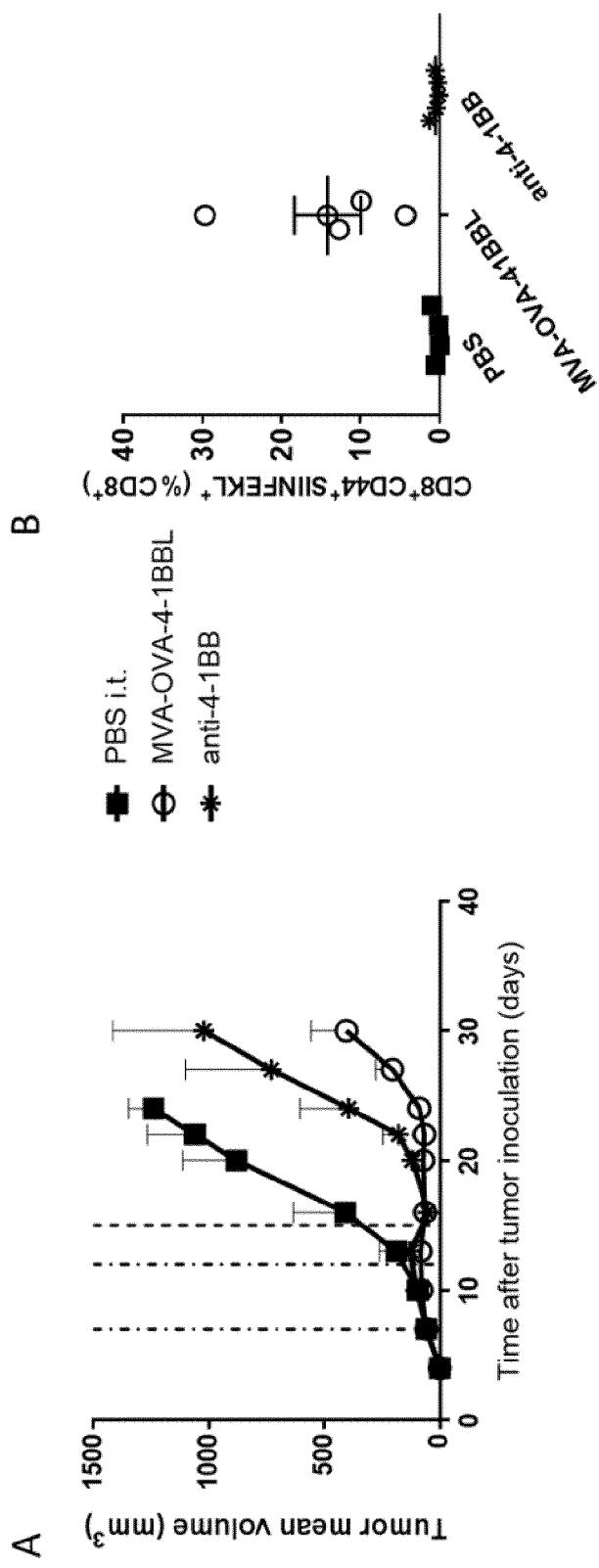
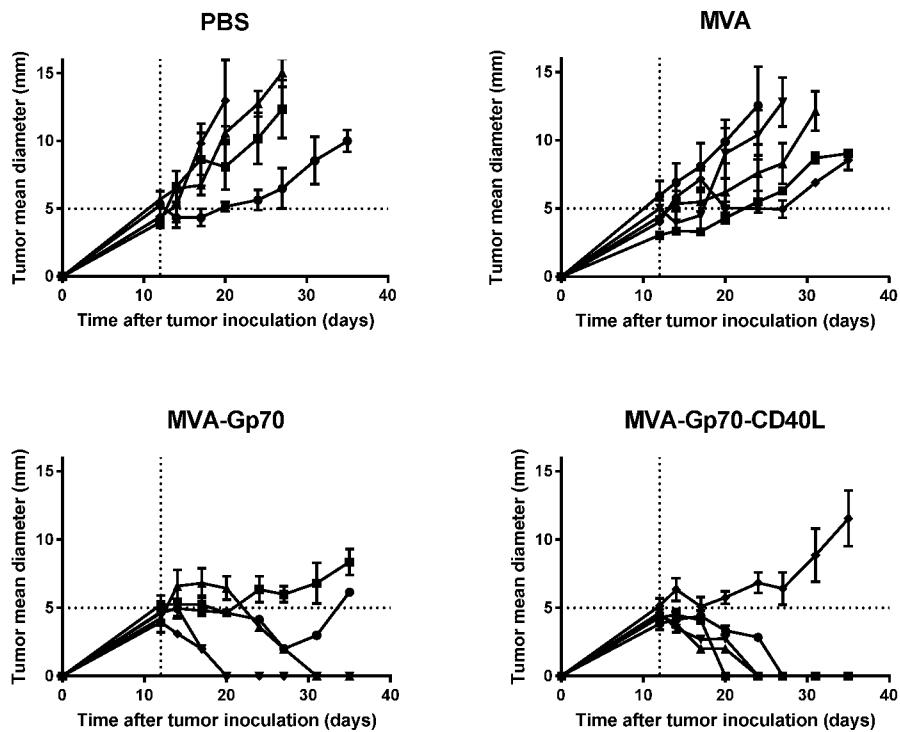
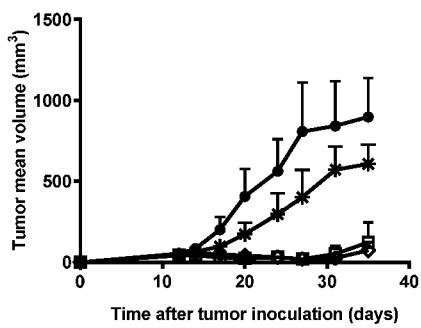


Figure 12

A



B



C

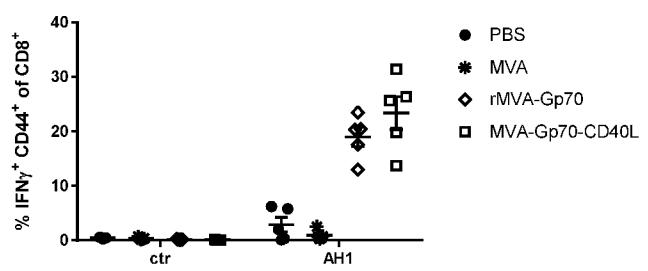
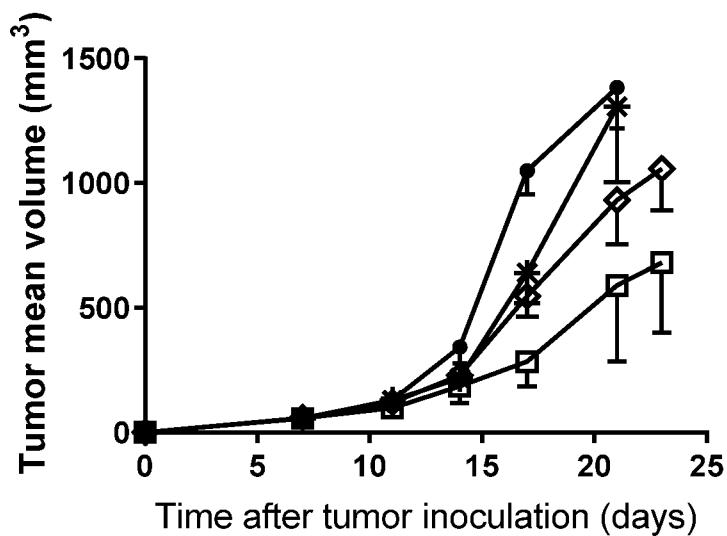


Figure 13

A



B

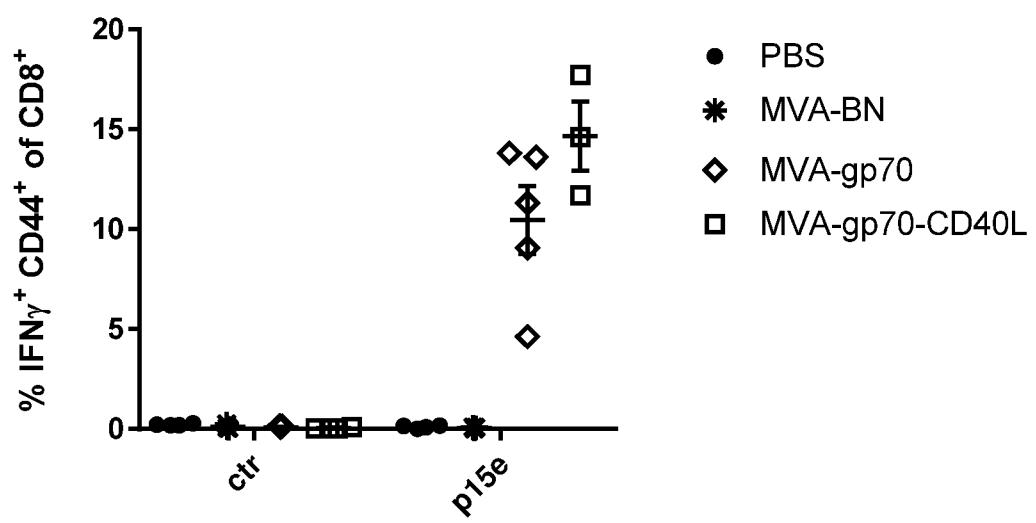


Figure 14

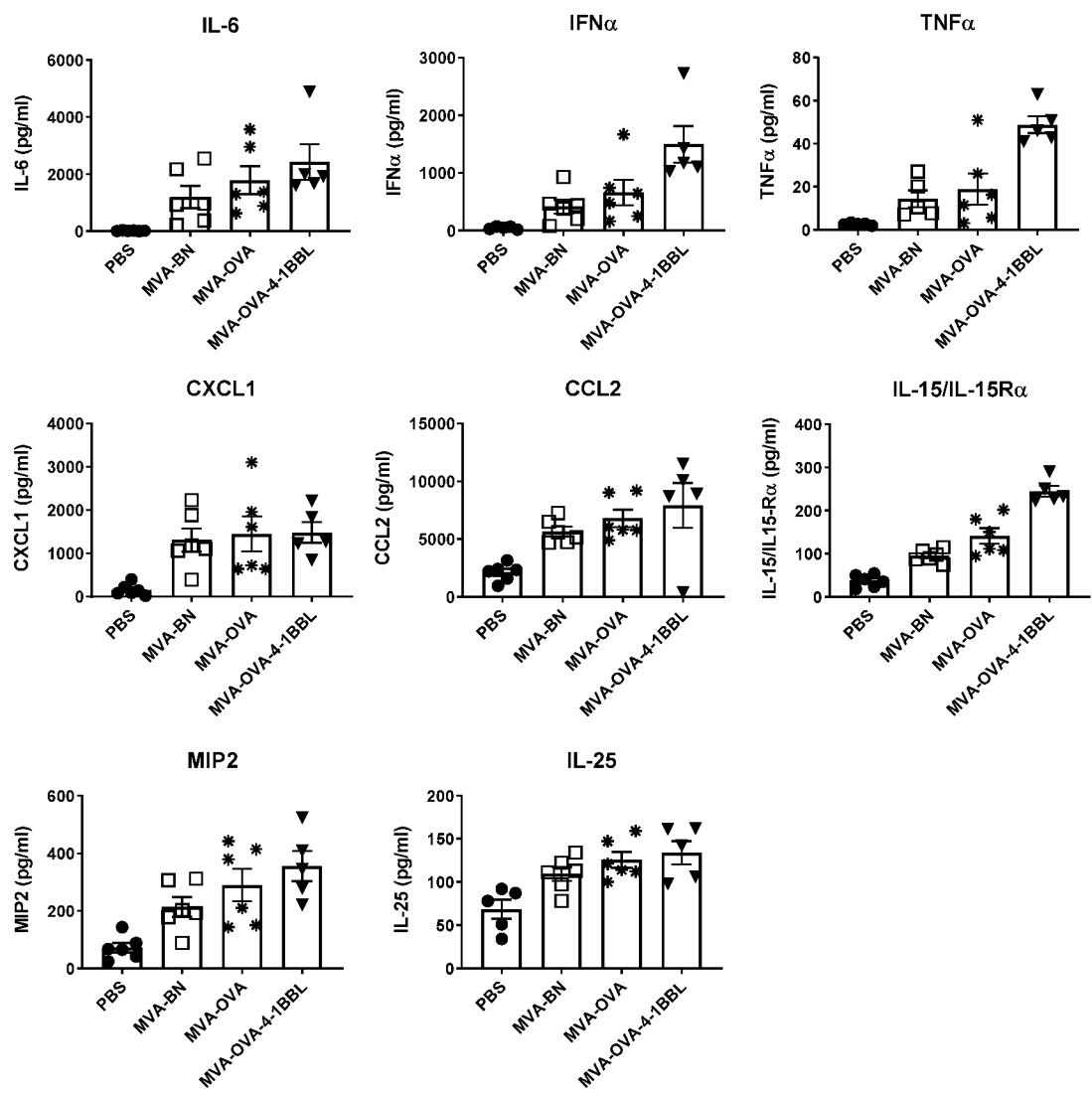


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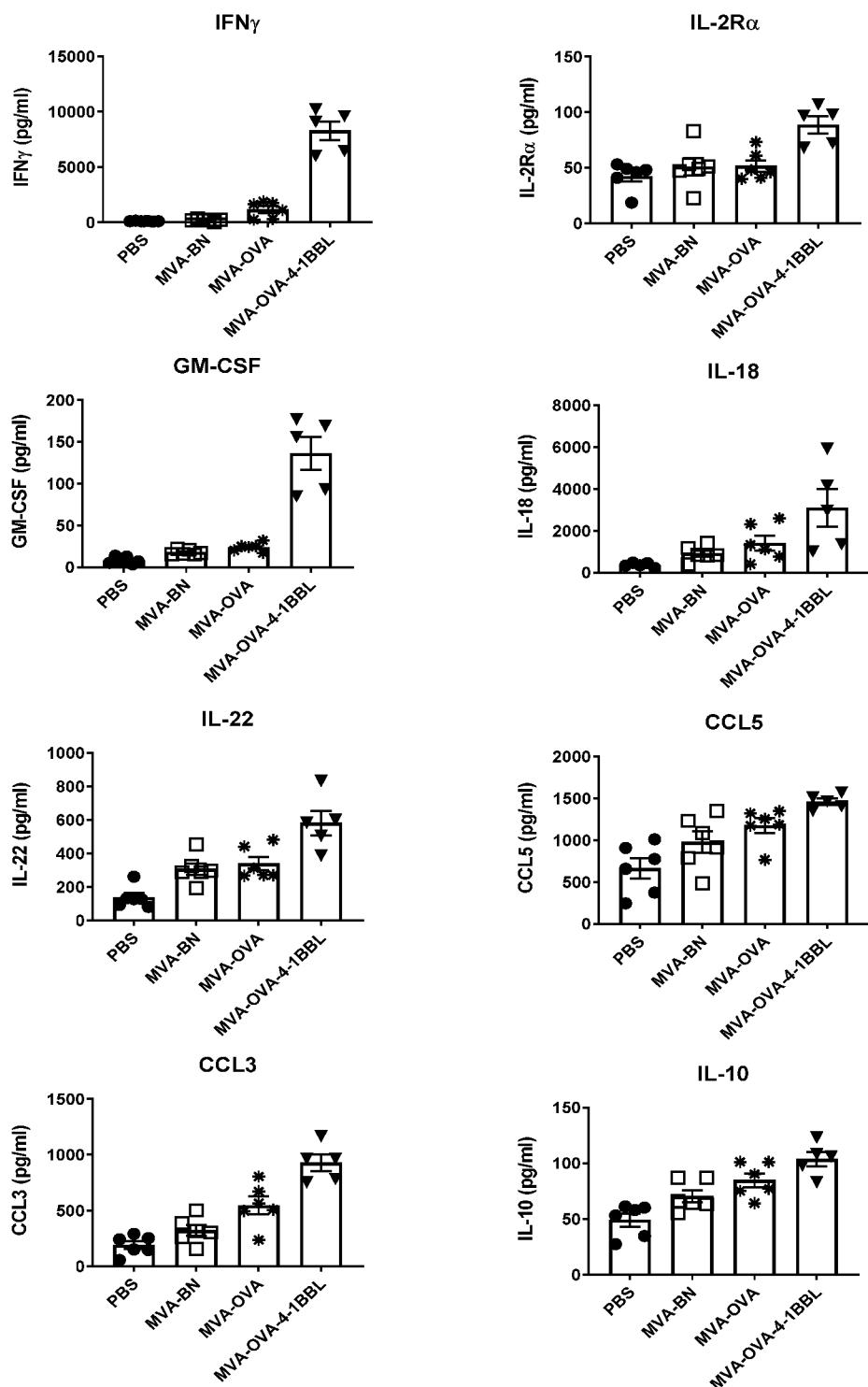


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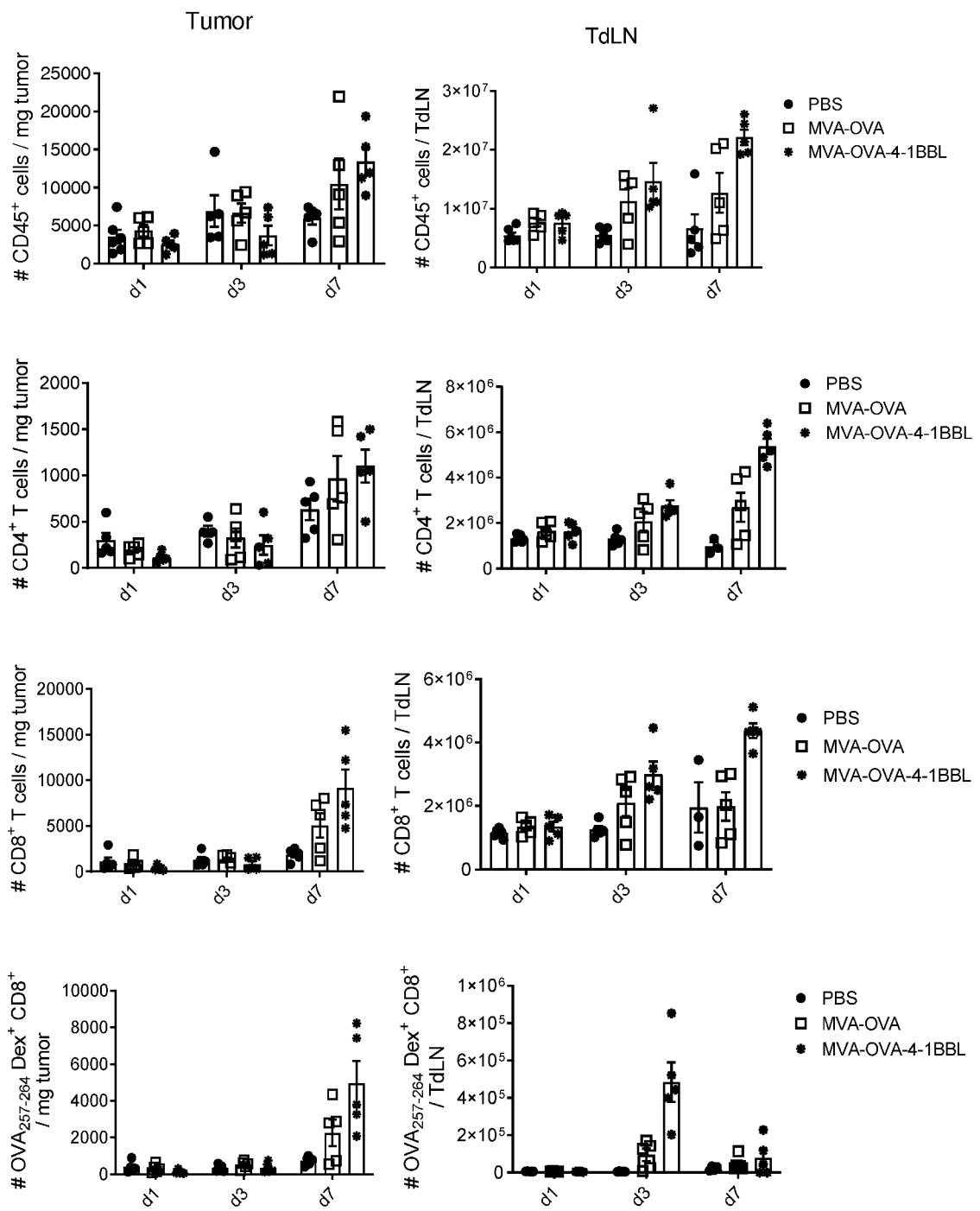
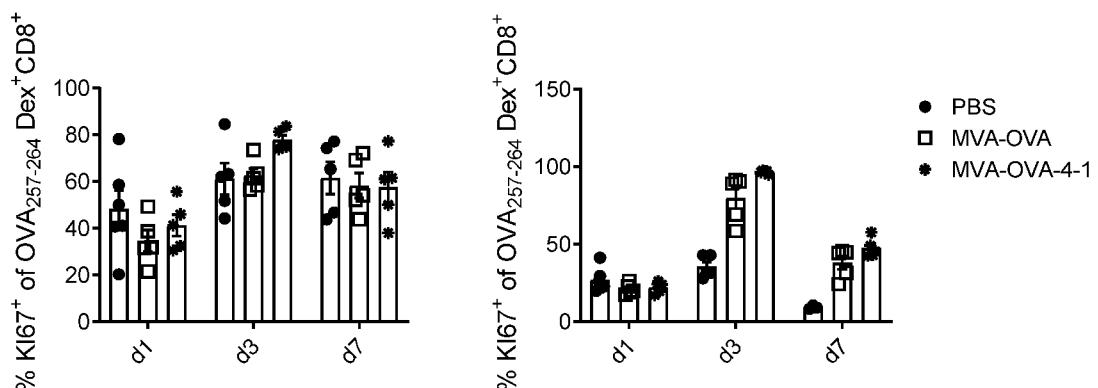
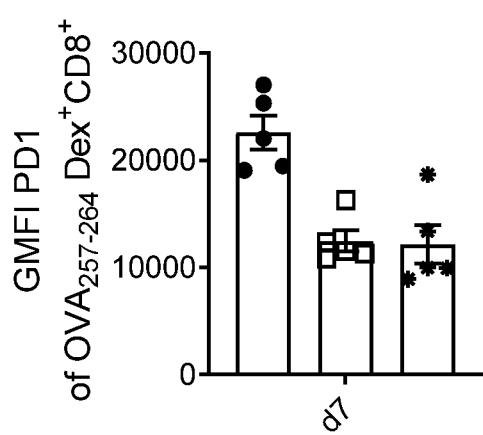
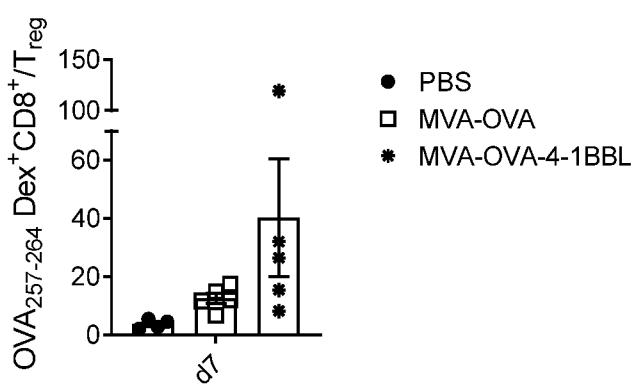


Figure 17

A**B****C****Figure 18**

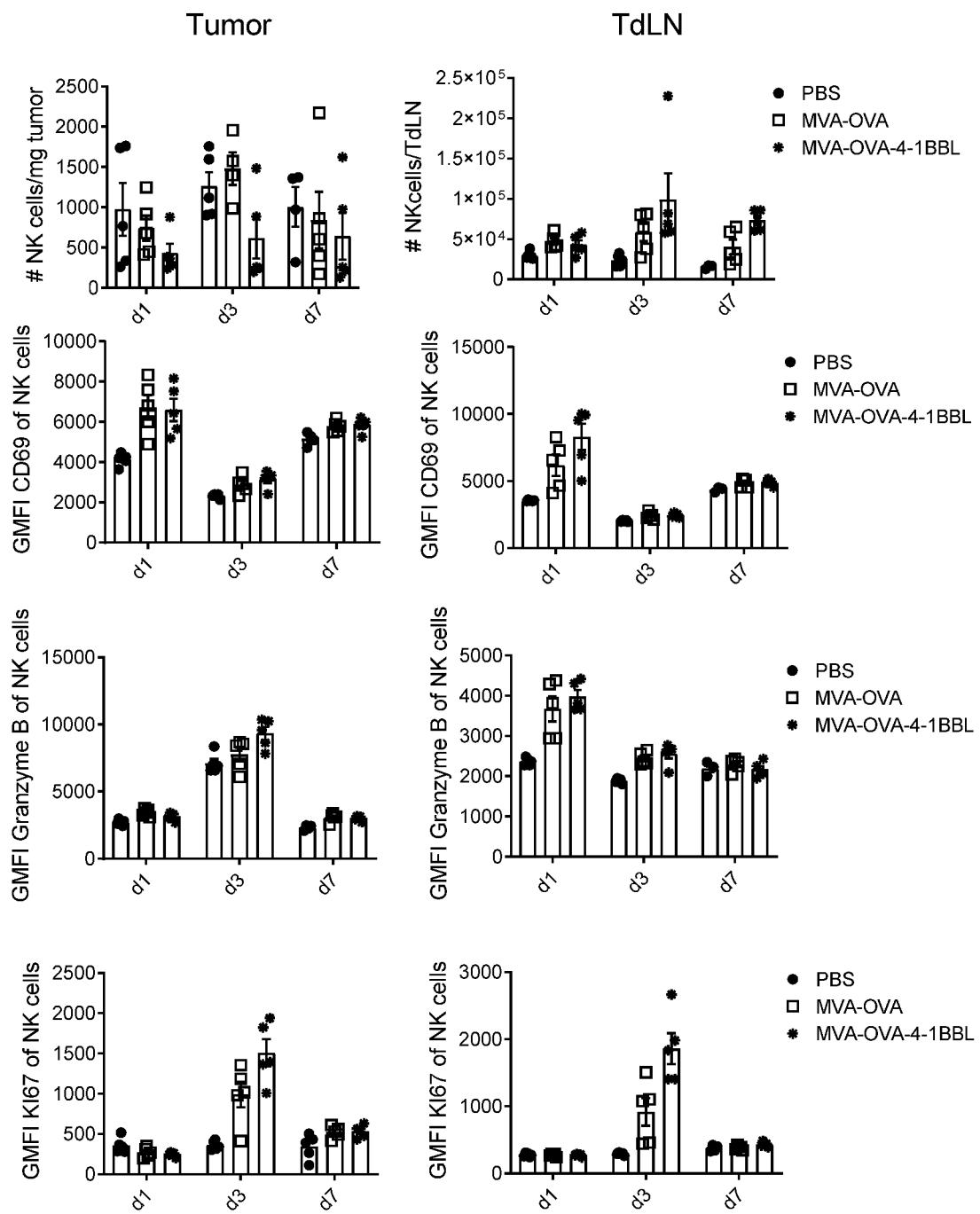


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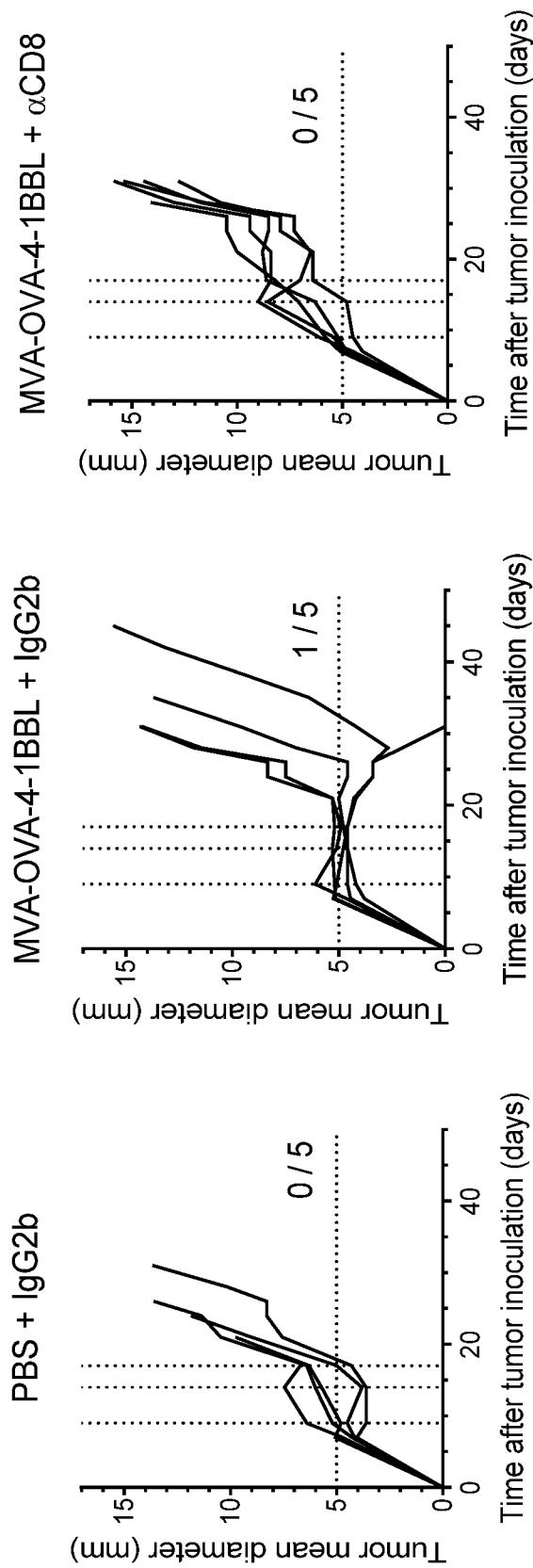
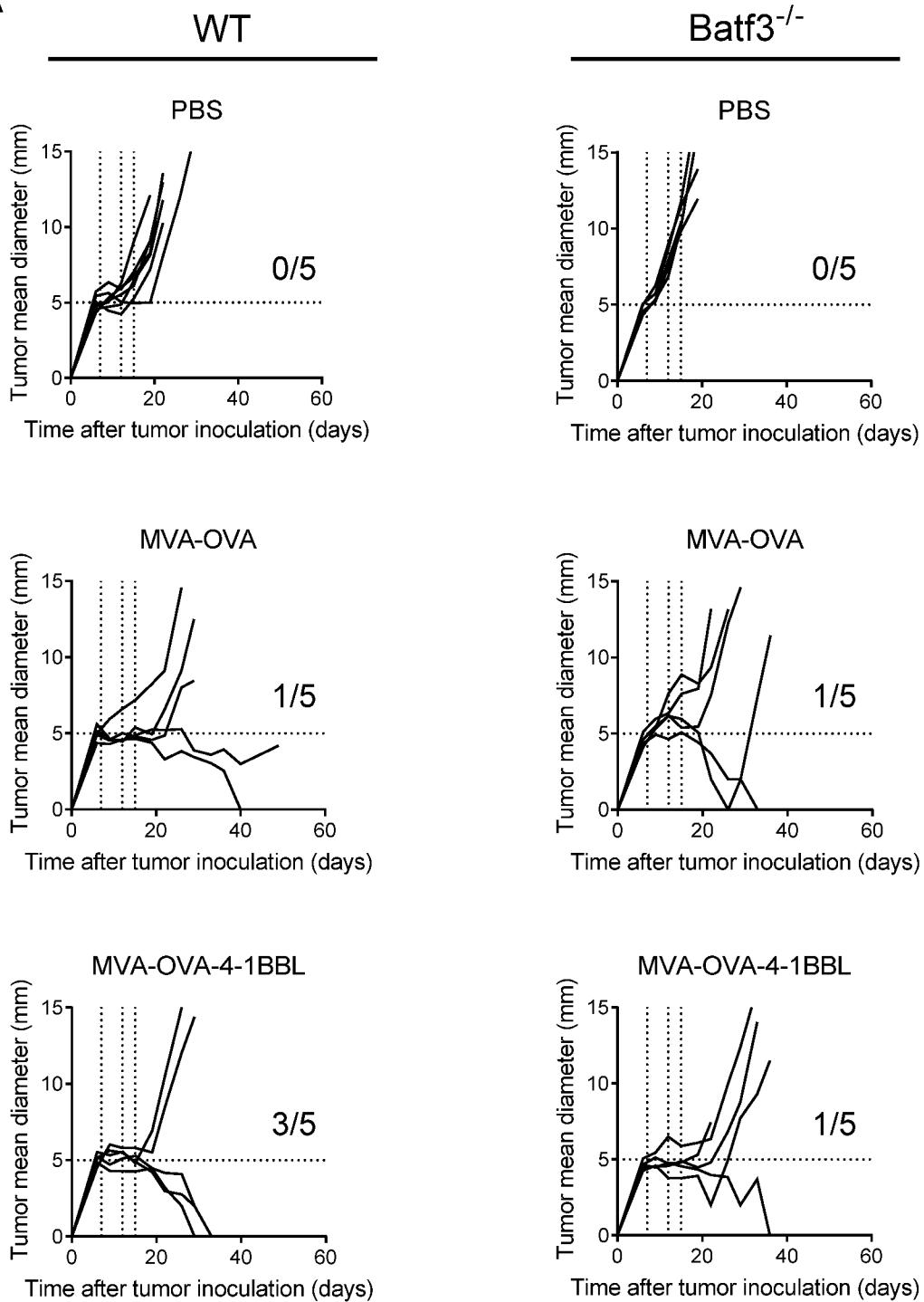


Figure 20

A**Figure 21**

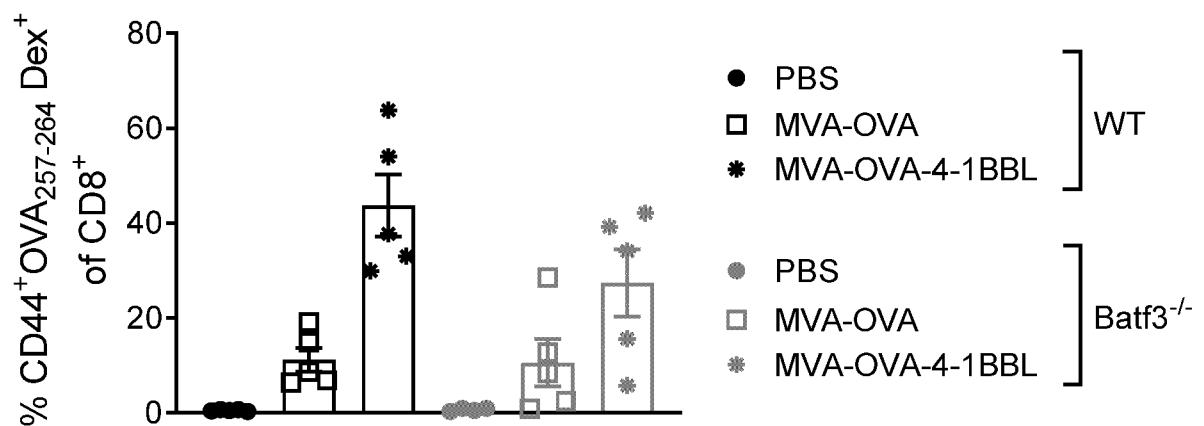
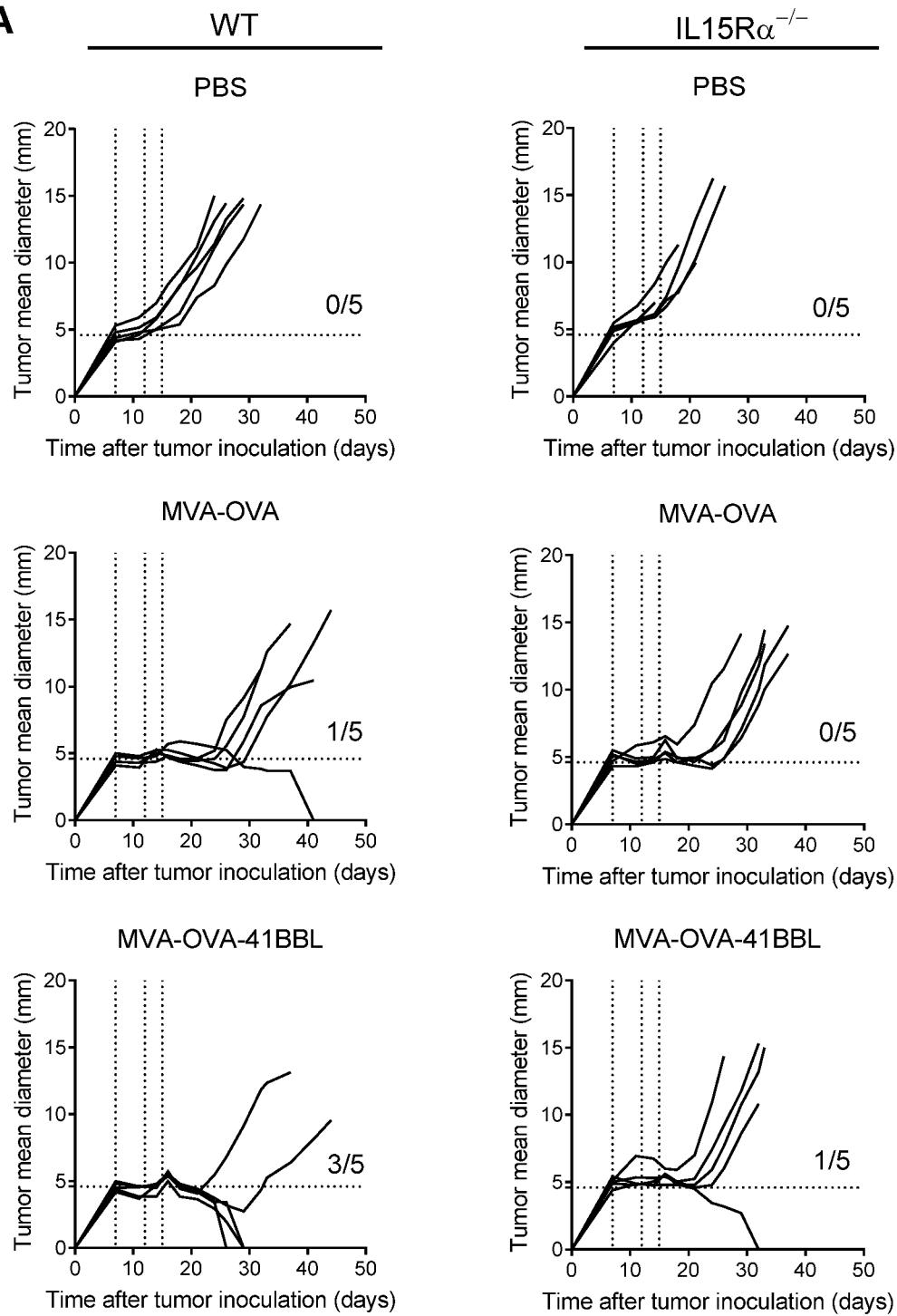
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Figure 21 (cont'd.)

A**Figure 22**

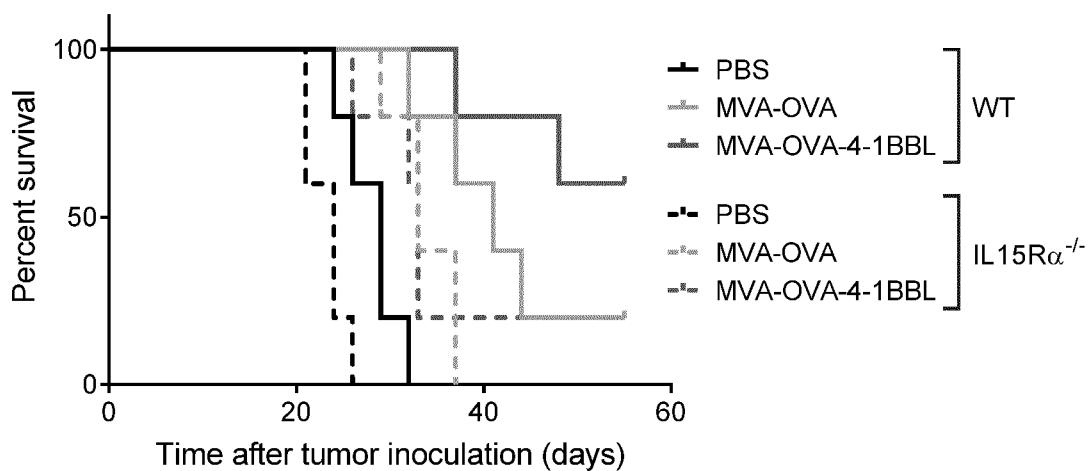
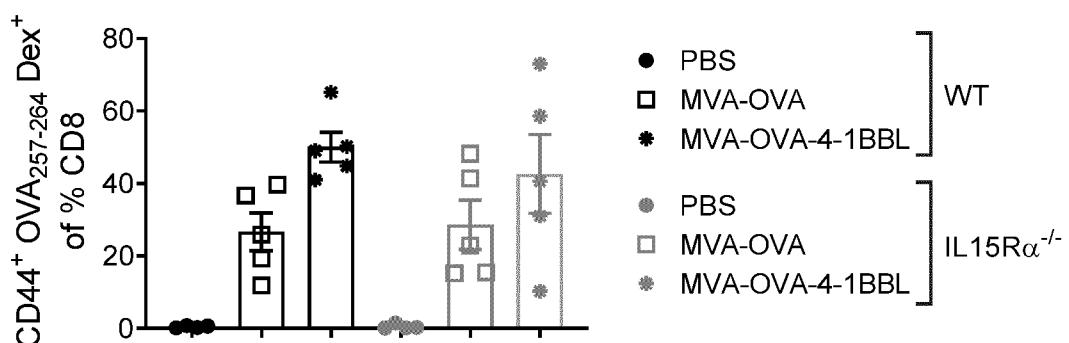
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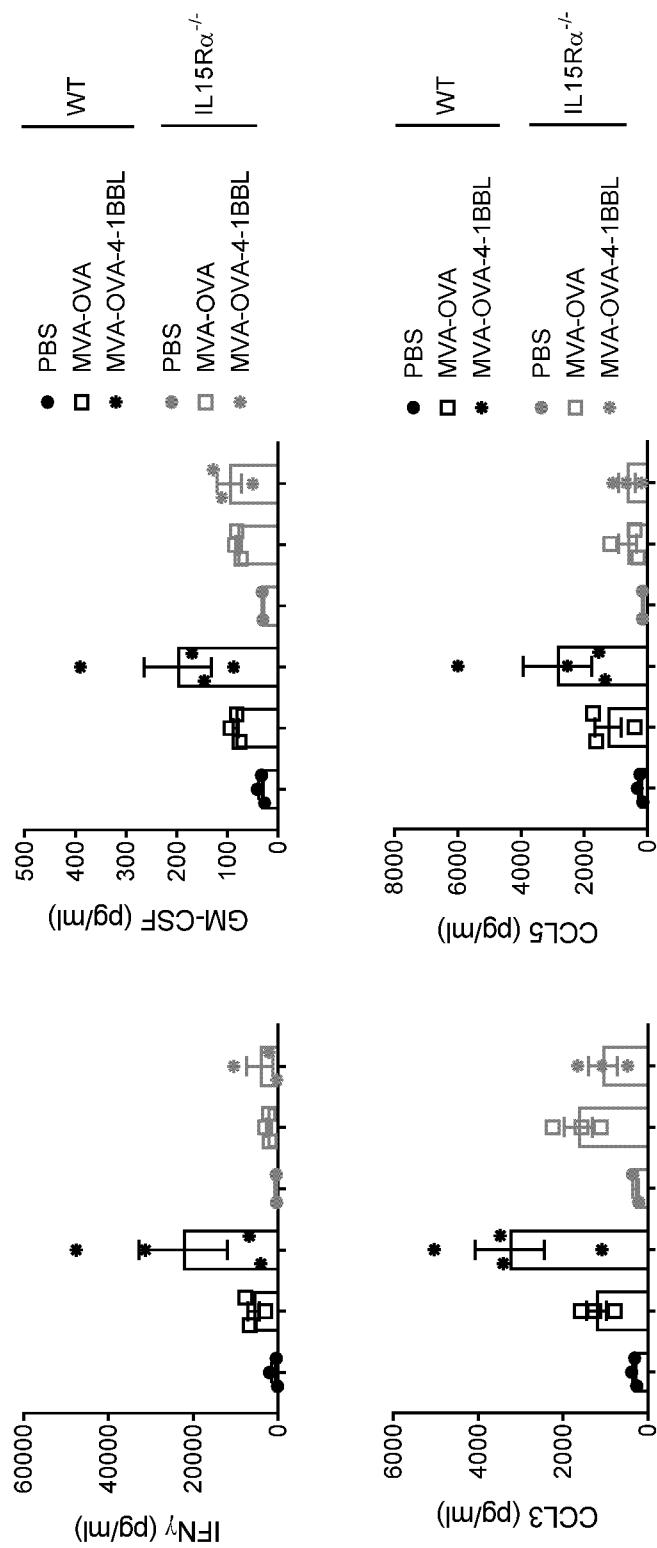
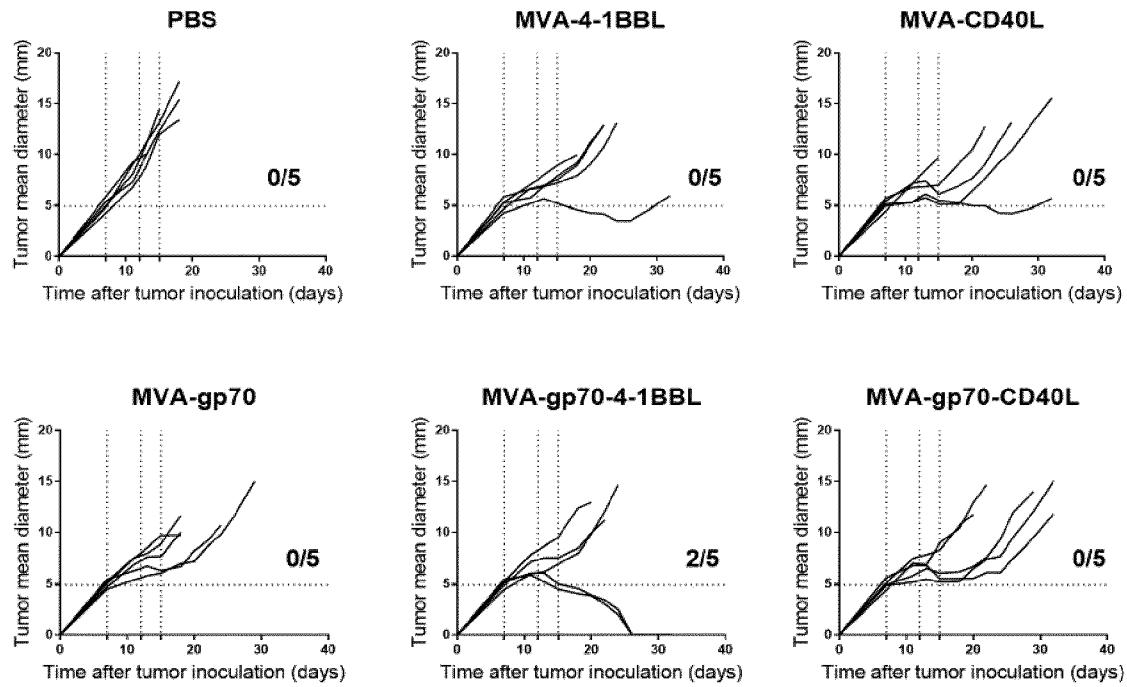


Figure 23

A**B****Figure 24**

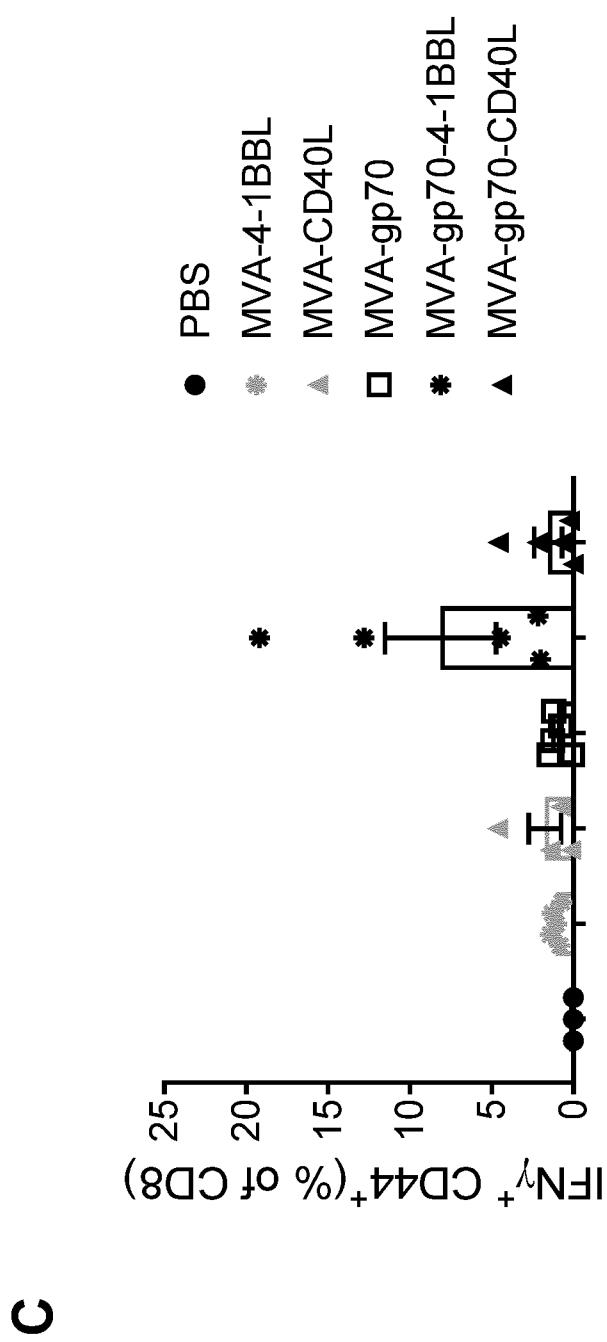


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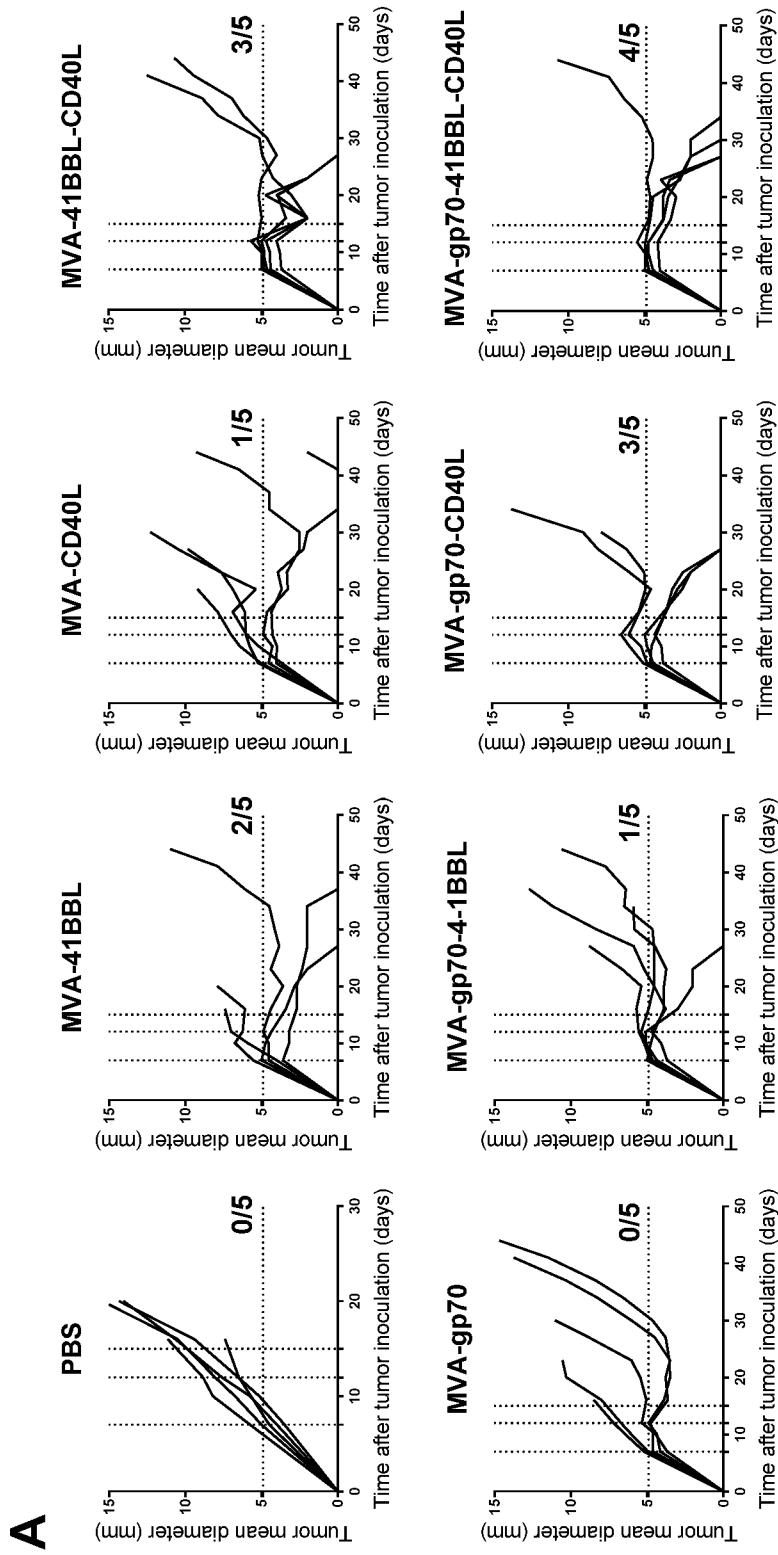


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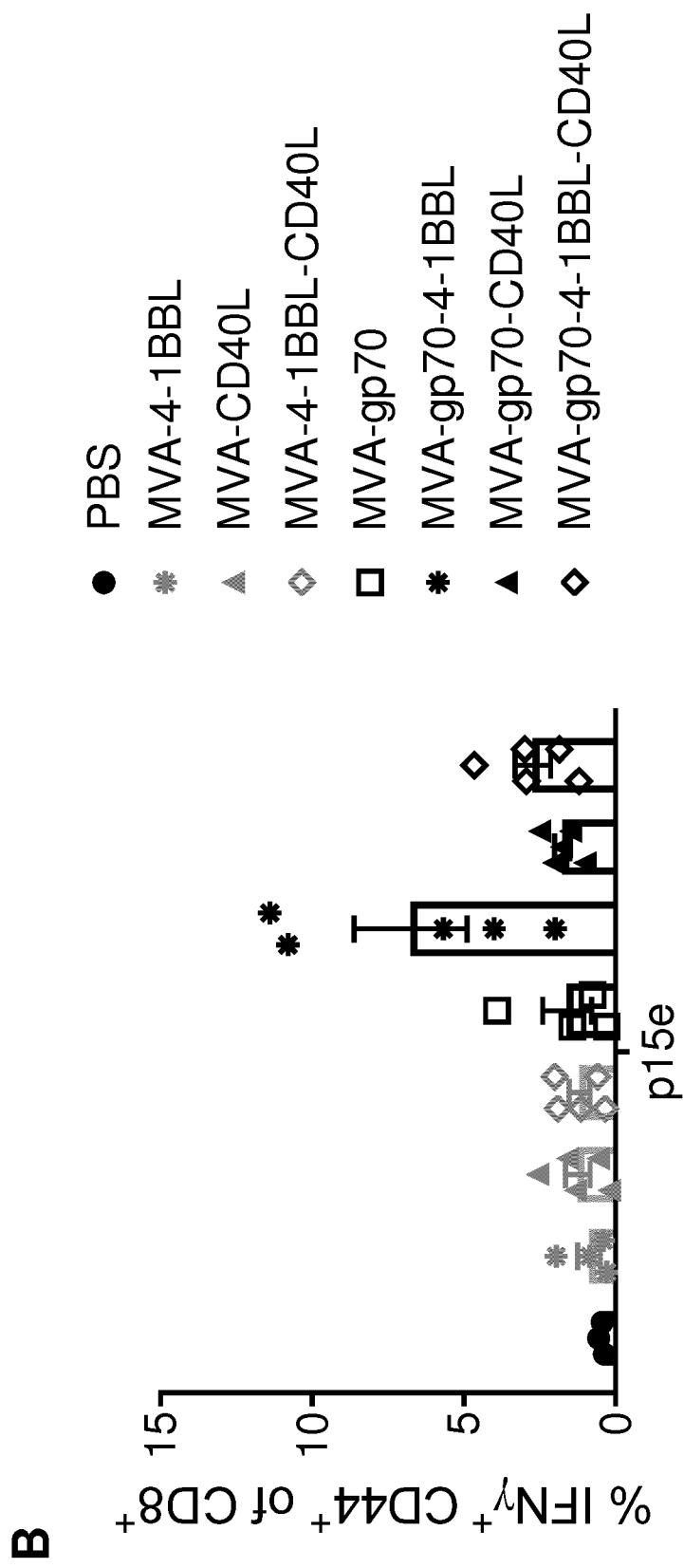


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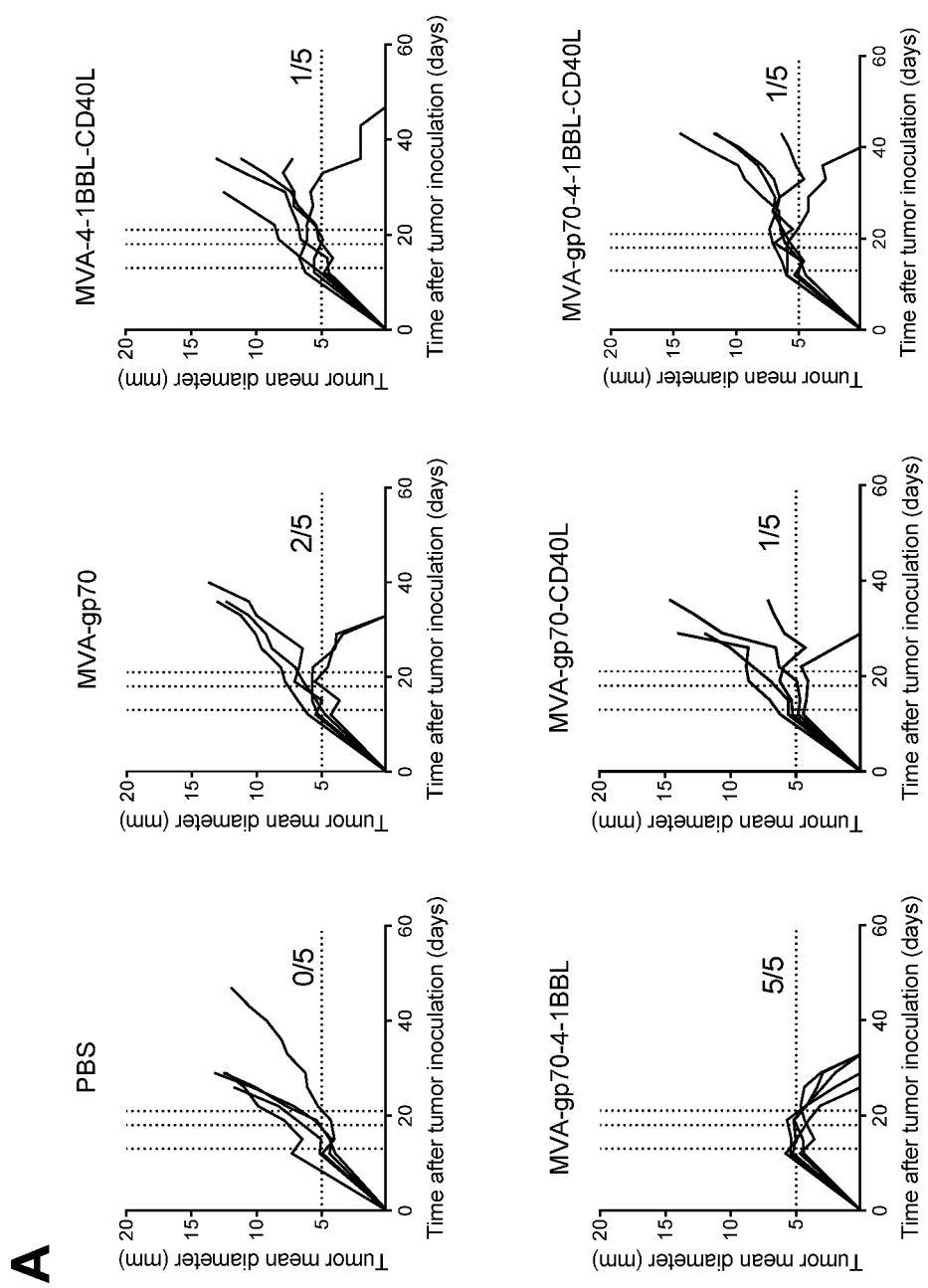


Figure 26

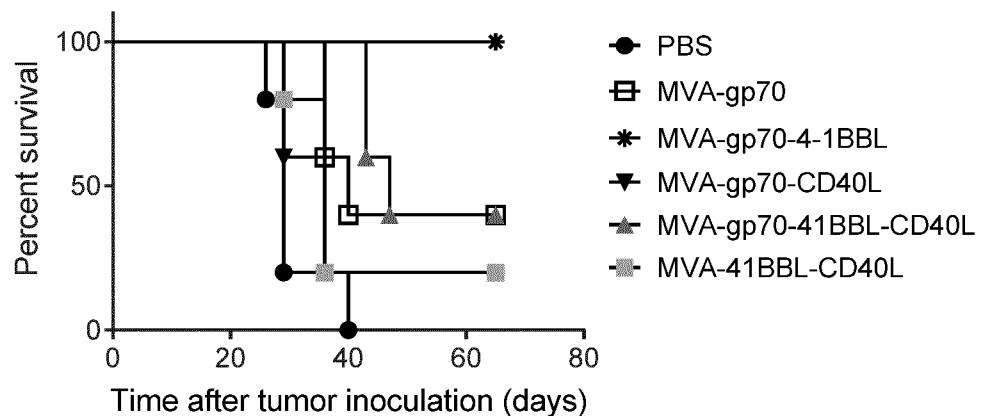
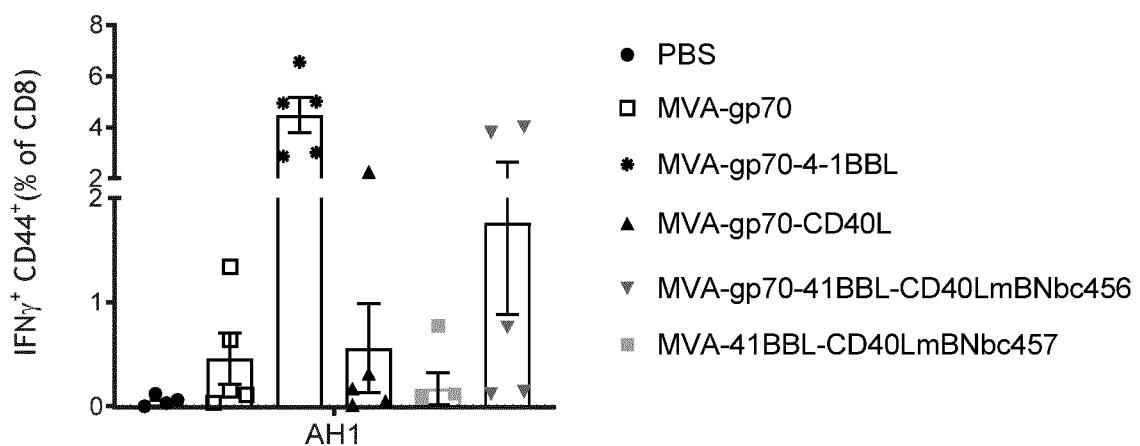
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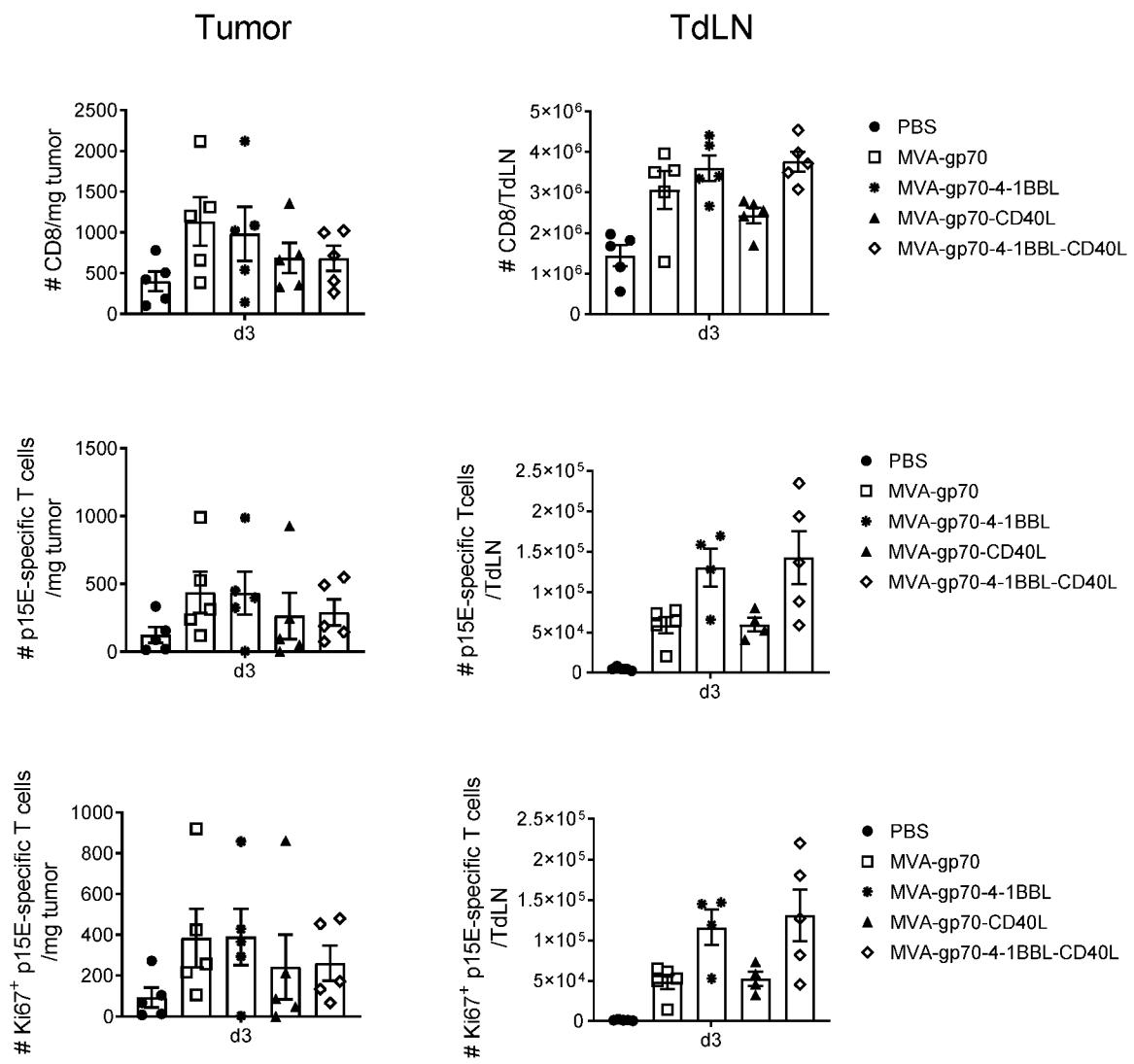


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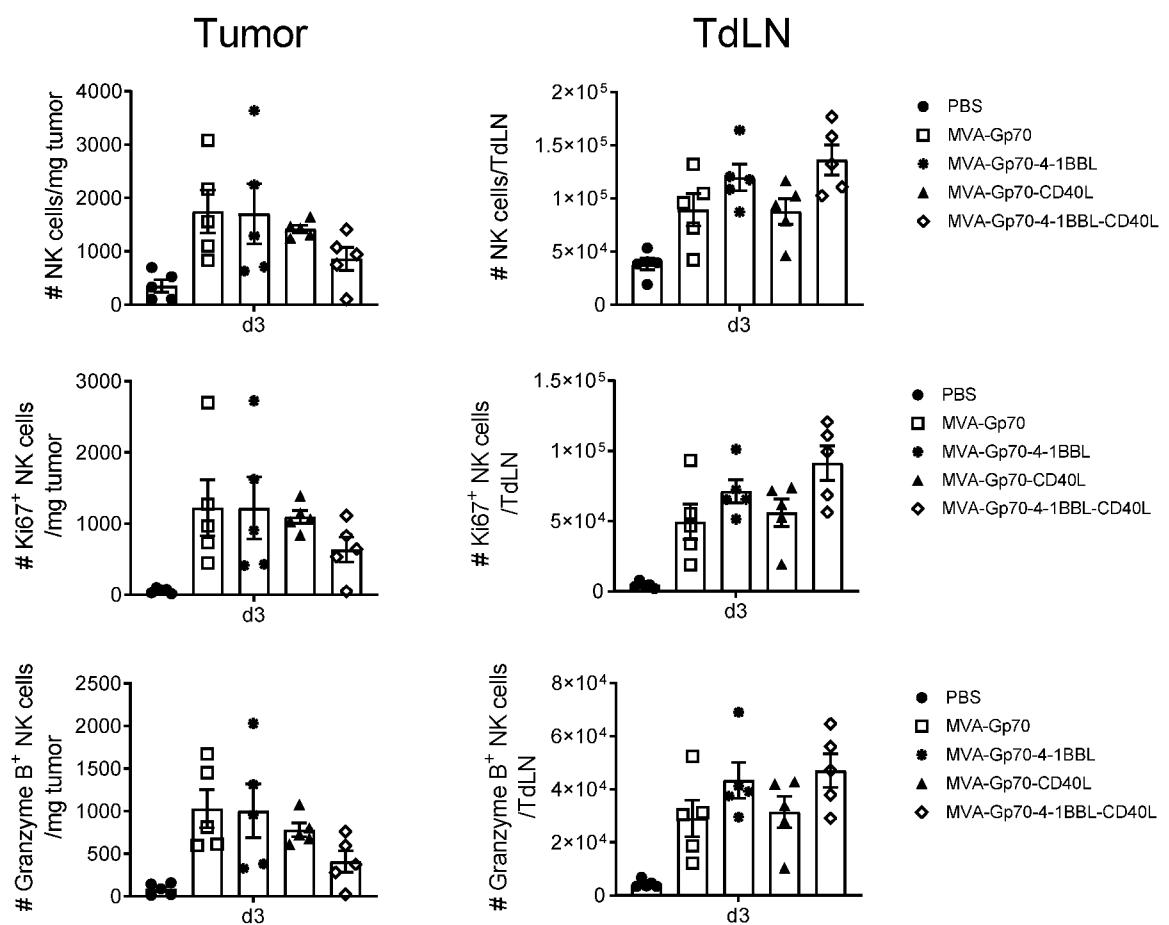


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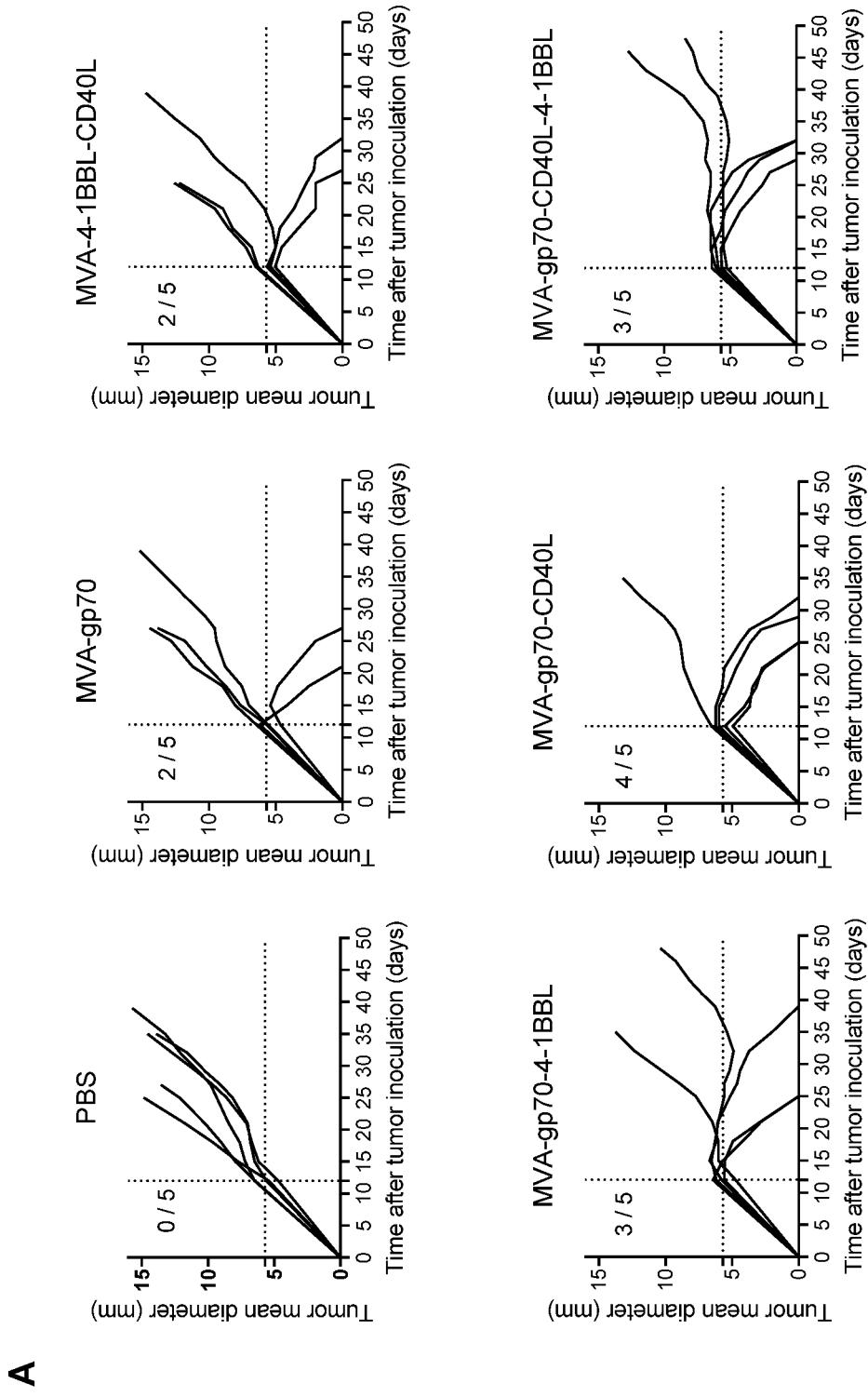


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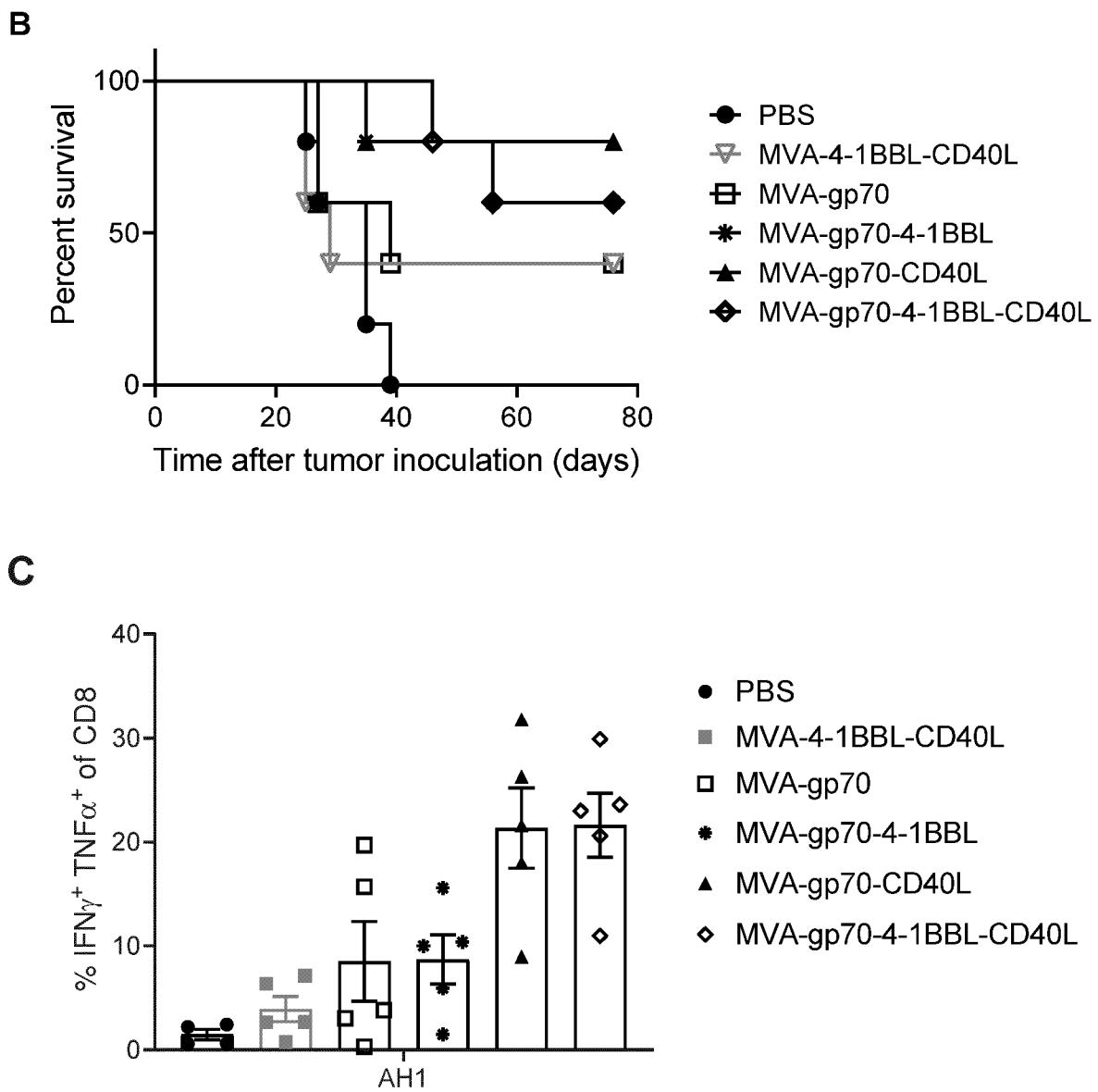


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