RECOMBINANT VACCINIA VECTORS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 63/676,672, filed July 29, 2024, the disclosure of which is incorporated herein by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING XML

[0002] A Sequence Listing is provided herewith as a Sequence Listing XML, "BERK-528WO_SEQ_LIST" created on July 21, 2025, and having a size of 4,869 bytes. The contents of the Sequence Listing XML are incorporated by reference herein in their entirety.

INTRODUCTION

[0003] Cancer remains a leading cause of death in humans. Immunotherapeutic approaches, such as engineered CAR T-cells and immune checkpoint inhibitors, have proven clinically safe and effective to treat multiple tumor types. However, immunotherapies have shown low response rates in solid cancers that inactivate or exclude immune cells from the tumor microenvironment (TME). Genetically encoded immunostimulatory molecules delivered using oncolytic viral vectors (OVs), which are viruses whose tropism has been restricted to tumor cells, have shown promise to reprogram the immunological status of TMEs from "cold" to "hot" states leading to improved treatment potency and durability. For oncology, two OV platforms (one platform based on an oncolytic Herpesvirus and a second platform based on a replication-incompetent Adenovirus) have achieved full clinical approval from the U.S. Food and Drug Administration (FDA). Additional OVs have been tested in clinical trials worldwide, such as vectors based on Measles Virus, Vesicular Stomatitis Virus, Coxsackie Virus, Reovirus, and Vaccinia Virus (VACV).

[0004] VACV is a promising platform due to its safety record, demonstrated through its use as an FDA-approved vaccine to prevent orthopoxvirus infections and inclusion in > 112 oncology-focused clinical trials. Additionally, VACV has tunable specificity to cancer cells conferred by well-characterized endogenous gene deletions, can encode up to 40 kilobase pairs of heterologous DNA, replicates quickly in the cytoplasm eliminating risk of vector persistence

following treatment, and can facilitate therapeutic transgene delivery in hypoxic tumor environments. Despite these advantages, VACV has yet to meet the efficacy standards needed to achieve full clinical approval as an anti-cancer agent.

[0005] An additional advantage of VACV as a delivery system for anti-cancer molecules is its formation of extra-cellular enveloped virion (EEV), which is secreted from cells and contains a second outer envelope that is thought to enable improved delivery both within tumors and among distal tumor sites. However, < 1% of total vectors produced are in the EEV form for most natural VACV strains. One exception is the IHD-J strain of VACV, which encodes a variant (K151Q) of an EEV envelope protein known as A34R conferring increased EEV production. When incorporated into an oncolytic VACV backbone, a rationally designed point mutation at the 151st amino acid position in A34R (K1541E) has been shown to confer improved delivery efficiency and survival in mouse models.

[0006] There is a need in the art for compositions that improve gene delivery to cancer cells, such as viral vector variants that confer the increased formation of EEV.

SUMMARY

[0007] Certain aspects of the disclosure provides a recombinant vaccinia virus (rVACV) comprising a variant A34R protein. An rVACV disclosed herein comprises a variant A34R protein, which, as compared to a VACV comprising a wild-type A34R protein, exhibits increased production of rVACV, particularly, in an extra-cellular enveloped virion (EEV) form.

[0008] In certain embodiments, an rVACV comprises a variant A34R protein having a mutation, as compared to a wild-type A34R protein, in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position. In certain such embodiments, when the mutation is only in lysine in the 151st position, the mutation is not a substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E). Variant A34R proteins are also provided.

[0009] In some cases, the rVACV is modified to improve its tumor specificity. For example, an improved tumor specificity could be achieved by deletions in one or both of thymidine kinase (TK) and vaccinia growth factor (VGF) genes.

[0010] In certain cases, the rVACV is modified to further include a heterologous gene, for example, a heterologous gene that increases an anti-cancer property of the rVACV. Certain such heterologous genes include but are not limited to granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-2 variant (IL-2v), interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-23 (interleukin-23), interleukin-24 (IL-24), interleukin-36γ (IL-36γ), tumor necrosis factor (TNF), cGAS-STING antagonist, tumor-associated antigen (TAA), tumor neoantigen, interferon-α (IFN-α), β-galactosidase, and interferon-b (IFN-β).

[0011] Also provided herein are methods of delivering a heterologous gene to a cancer cell in a subject by administering to the subject an rVACV comprising the heterologous gene.

[0012] Further aspects of the disclosure provide a pharmaceutical composition comprising an rVACV disclosed herein. Also, provided herein are methods of treating a cancer in a subject by administering to the subject an rVACV or a pharmaceutical composition comprising an rVACV described herein.

[0013] Even further embodiments of the disclosure provide a nucleic acid comprising a nucleotide sequence that encodes a variant A34R protein described herein. In some cases, such nucleic acid is in a viral genome, plasmid, and/or host cell.

[0014] Additional embodiments of the disclosure provide a method of producing an rVACV by culturing a host cell comprising a nucleic acid encoding a variant A34R protein and purifying the rVACV. In some cases, the rVACV is in an EEV form.

[0015] Variants of vaccinia virus A34R protein are also provided. Certain such variant A34R proteins comprise, as compared to a wild-type A34R protein, a mutation in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position. In certain such embodiments, when the mutation is only in lysine in the 151st position, the mutation is not a substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E).

[0016] Variant nucleic acids encoding such variant A34R proteins are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIGS. 1A-1B provide schematics of EEV formation during VACV replication cycle. IMV = Intracellular Mature Virion. IEV = Intracellular Enveloped Virion. CEV = Cell-Associated Enveloped Virion. EEV = Extracellular Enveloped Virion. *Created using world-wide-website: Biorender.com.*

[0018] FIGS. 2A-2B. A. A schematic of the diversification and selection strategy used to identify the VACV A34R variants disclosed herein. A CytoEvolvR-based diversification strategy (as described in WO 2024/011173) was used to identify the VACV A34R protein variants Y144Y, V153T, K148E, R79L, Y73H, K151R, and K148E. A single substitution variant library and homologous recombination-based diversification strategy was used to identify the VACV A34R protein variants K65S, K65T, K65N, R81M, R88N, R88D, R91P, K151P, K165E, and K168D. B. The fold-enrichment (adjusted for the background Illumina error rate) of individual nucleic acid variants following CytoEvolvR-based diversification and three rounds of selection for EEV in oncolytic vaccinia vectors produced in human colorectal cancer (KM12) cells. The X axis represents the nucleotide location relative to the first nucleotide position of the start codon in the Vaccinia A34R gene.

[0019] FIGS. 3A-3C provide graphs showing increased EEV production conferred by engineered A34R variants incorporated into rVACV infected in BSC-1 cells. A. Ratios of EEV to total rVACV for several rVACV having different A34R variants. B. EEV titers quantified at 6, 12, 24, or 48 hours post infection. C. Cell counts quantified at 6, 12, 24, or 48 hours post infection.

[0020] FIGS. 4A-4B provide EEV titers (2A) and ratios of EEV to total rVACV (2B) produced in human colorectal cancer cells (KM12). The engineered variant comprising a K148E mutation in the A34R gene confers elevated EEV production at the 24 hours post-infection time point relative to the previously known K151E variant.

[0021] FIGS. 5A-5B describe comet assay of BSC-1 cells infected at low MOI with double deleted rVACV. A well of confluent, adherent cells that are permissive to rVACV were infected with rVACV at a low MOI and incubated at a 45-degree angle. rVACV variants that confer higher EEV production lead to longer "comet"-like plaques.

[0022] FIGS. 6A-6C provide cancer therapeutic testing of rVACV disclosed herein. A. Study schematic: MC38 cancer cells were administered to form a single tumor in mice. When the tumors became palpable, the indicated oncolytic vaccinia (OV) cargo was administered

intravenously with five repeat injections spaces 48 hours apart to mice. B. Probability of survival for each experimental condition. C. Tumor volume for each experimental condition.

[0023] FIGS. 7A-7C provide assessment of the delivery efficiency of rVACV disclosed herein. A. Study schematic: MC38 cancer cells were administered to form two separate tumors in mice. Subsequently, the indicated OV was administered intratumorally to the primary tumor. The secondary tumor was harvested 48 hours post-administration, and the OV in the secondary tumor was evaluated by infectious titer.

[0024] FIG. 8 provides further supporting data for the therapeutic relevance of EEV. Vaccinia was infected into BSC-1 cells. 24 hours post-infection, the EEV was isolated from the supernatant by centrifugation and incubating with an IMV-neutralizing antibody + varying concentrations of intravenous immunoglobulin (IVIg), which represents serum from ~10,000 human donors. In this study, the infectious titer of EEV was unaffected by IVIg, whereas IMV was neutralized by increasing concentrations of IVIg.

[0025] FIGS. 9A-9D show increased immune cell infiltration into the tumor cells. A. Quantification of $CD3^+$ cells, which represents the total T-cell count. B shows quantification of $CD8^+$ T Cells shows an increase in cytotoxic T-cell infiltration conferred by the rVACV encoding the K148E variant of A34R. C. Quantification of Granzyme B, which is released by effector $CD8^+$ T cells, shows elevated activity of effector T cells in the tumor conferred by the rVACV encoding the K148E variant of A34R. D. No difference in $CD4^+$ helper T cell infiltration. Mean ± SEM, One-way ANOVA, $^*P < 0.05$.

[0026] FIGS. 10A-10E show decreasing overall diversity and increasing enrichment in the VACV A34R variant population across four rounds of selection for EEV production. The A34R region was amplified from the VACV population after library incorporation but prior to selection (Round 0) and following one (Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells, then shotgun sequenced on Illumina next-generation sequencing technology. The reads were trimmed and aligned to the VACV A34R gene reference sequence. The frequency of each non-wild-type codon substitution was then quantified and ordered based on frequency. The number of overall VACV A34R variant sequences with greater than one read decreased in the population across each selection round, while leading variants increased in prevalence across each selection round.

[0027] FIG. 11 shows the frequency distribution of wild-type VACV A34R amino acid substitutions after library incorporation but prior to selection (Round 0) and following one

(Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells. The frequency of wild-type amino acids decreased at positions where non-wild-type variant enrichment was observed (e.g. amino acid position 65).

[0028] FIG. 12 shows the fold-enrichment of leading variants after library incorporation but prior to selection (Round 0) and following one (Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells. Consistent, positive enrichment was observed across each round of selection for VACV A34R protein variants K65S, K65T, K65N, R81M, R88N, R88D, R91P, K151P, K165E, and K168D. The enrichment profiles observed for these variants strongly indicate they confer functional improvements to the formation and function of VACV EEV.

[0029] FIGS. 13A-13K depict locations of selected amino acid variants on the predicted structure of the A34R protein.

DEFINITIONS

[0030] "VACV" is an abbreviation for vaccinia vector or vaccinia virus, and may be used to refer to the virion or virus itself or derivatives thereof. The term covers all subtypes and both naturally occurring and recombinant forms, except where required otherwise. The abbreviation "rVACV" refers to recombinant vaccinia virus or virion, also referred to as a recombinant VACV vector (or "rVACV vector"). The term "VACV" includes the intracellular mature virion (IMV), intracellular enveloped virion (IEV), cell-associated enveloped virion (CEV), and the extracellular enveloped virion (EEV).

[0031] An "rVACV" as used herein refers to a VACV comprising a polynucleotide sequence having a mutation as compared to a wild-type VACV sequence. In certain rVACV disclosed herein, the VACV has a mutation in the gene encoding the envelope protein A34R. An rVACV may also have a polynucleotide sequence not of the VACV origin (i.e., a polynucleotide heterologous to VACV), typically a sequence of interest for expression in a cell. The term rVACV vector encompasses both rVACV vector particles and rVACV vector plasmids.

[0032] A "VACV virus" or "VACV viral particle" or "rVACV vector particle" refers to a viral particle composed of at least one VACV capsid protein and an encapsidated polynucleotide rVACV. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other

than a wild-type VACV genome, such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "rVACV vector particle" or simply an "rVACV vector."

[0033] The terms "A34R," "A34R protein," and "A34R gene" refer to, as applicable in the context, A34R gene or protein of a VACV. A34R gene encodes a glycoprotein present in the outer membrane of EEV. A34R protein in a VACV virion is required for infectivity for VACV in the EEV form.

[0034] An "infectious" virus or viral particle is one that comprises a polynucleotide component capable of delivering into a cell for which the viral species is tropic. The term does not necessarily imply any replication capacity of the virus. As used herein, an "infectious" virus or viral particle is one that can access a target cell, can infect a target cell, and can express a heterologous nucleic acid in a target cell. Thus, "infectivity" refers to the ability of a viral particle to access a target cell, infect a target cell, and express a heterologous nucleic acid in a target cell. Infectivity can refer to *in vitro* infectivity or *in vivo* infectivity. Assays for counting infectious viral particles are described elsewhere in this disclosure and in the art. Viral infectivity can be expressed as the ratio of infectious viral particles to total viral particles. Total viral particles can be expressed as the number of viral genome copies. The ability of a viral particle to express a heterologous nucleic acid in a cell can be referred to as "transduction." The ability of a viral particle to express a heterologous nucleic acid in a cell can be assayed using a number of techniques, including assessment of a marker gene, such as a green fluorescent protein (GFP) assay (e.g., where the virus comprises a nucleotide sequence encoding GFP), where GFP is produced in a cell infected with the viral particle and is detected and/or measured; or the measurement of a produced protein, for example by an enzyme-linked immunosorbent assay (ELISA). Viral infectivity can be expressed as the ratio of infectious viral particles to total viral particles. Methods of determining the ratio of infectious viral particle to total viral particle are known in the art. See, e.g., Grainger et al. (2005) *Mol. Ther.* 11:S337 (describing a TCID50 infectious titer assay); and Zolotukhin et al. (1999) *Gene Ther.* 6:973.

[0035] A "replication-competent" virus (e.g. a replication-competent VACV) refers to a phenotypically wild-type virus that is infectious, and is also capable of being replicated in an infected cell (e.g. in the presence of a helper virus or helper virus functions).

[0036] The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may

be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0037] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST/. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wisconsin, USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See *Meth. Mol. Biol.* 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See *J. Mol. Biol.* 48: 443-453 (1970)

[0038] Of interest is the BestFit program using the local homology algorithm of Smith Waterman (Advances in Applied Mathematics 2: 482-489 (1981) to determine sequence identity. The gap generation penalty will generally range from 1 to 5, usually 2 to 4 and in many embodiments will be 3. The gap extension penalty will generally range from about 0.01 to 0.20 and in many instances will be 0.10. The program has default parameters determined by the sequences inputted to be compared. Preferably, the sequence identity is determined using the default parameters determined by the program. This program is available also from Genetics Computing Group (GCG) package, from Madison, Wisconsin, USA.

[0039] Another program of interest is the FastDB algorithm. FastDB is described in Current Methods in Sequence Comparison and Analysis, Macromolecule Sequencing and Synthesis,

Selected Methods and Applications, pp. 127-149, 1988, Alan R. Liss, Inc. Percent sequence identity is calculated by FastDB based upon the following parameters:

Mismatch Penalty: 1.00; Gap Penalty: 1.00; Gap Size Penalty: 0.33; and Joining Penalty: 30.0.

[0040] A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

[0041] The term "recombinant" as applied to a nucleic acid means that the nucleic acid is different, for example, in polynucleotide sequence, from a nucleic acid found in nature. Similarly, the term "recombinant" as applied to a protein means that the protein is different, for example, in amino acid sequence, from a protein found in nature.

[0042] A recombinant virus is a viral particle comprising a recombinant nucleic acid or a recombinant protein.

[0043] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.

[0044] An "expression vector" is a vector comprising a region that encodes a polypeptide of interest, and is used for effecting the expression of the protein in an intended target cell. An expression vector may also comprise control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components available in the art.

[0045] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally linked is a heterologous promoter. Thus, for example, an rVACV that includes a heterologous nucleic acid encoding a heterologous gene product is an rVACV that includes a nucleic acid not normally included in a naturally-occurring, wild-type VACV, and the encoded heterologous gene product is a gene

product not normally encoded by a naturally-occurring, wild-type VACV. As another example, a variant VACV protein that comprises one or more amino acid mutations as compared to the corresponding wild-type protein is a variant VACV protein.

[0046] The terms "genetic alteration" and "genetic modification" (and grammatical variants thereof), are used interchangeably herein to refer to a process wherein a genetic element (e.g., a polynucleotide) is introduced into a cell other than by mitosis or meiosis. The element may be heterologous to the cell, or it may be an additional copy or improved version of an element already present in the cell. Genetic alteration may be achieved, for example, by transfecting a cell with a recombinant plasmid or other polynucleotide through any process known in the art, such as electroporation, calcium phosphate precipitation, or contacting with a polynucleotide-liposome complex. Genetic alteration may also be achieved, for example, by transduction or infection with a DNA or RNA virus or viral vector. Generally, the genetic element is introduced into a chromosome or mini-chromosome in the cell; but any alteration that changes the phenotype and/or genotype of the cell and its progeny is included in this term.

[0047] A cell is said to be "stably" altered, transduced, genetically modified, or transformed with a genetic sequence if the sequence is available to perform its function during extended culture of the cell *in vitro*. Generally, such a cell is "heritably" altered (genetically modified) in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

[0048] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component. Polypeptides such as cytokines, inflammatory agents, anti-inflammatory agents, and the like, when discussed in the context of delivering a gene product to a mammalian subject, and compositions therefor, refer to the respective intact polypeptide, or any fragment or genetically engineered derivative thereof, which retains the desired biochemical function of the intact polypeptide. Similarly, references to nucleic acids encoding cytokines, inflammatory agents, anti-inflammatory agents, and the like, for use in delivery of a gene product to a mammalian subject (which may be referred to as "transgenes" to be delivered to a recipient cell), include polynucleotides encoding the intact polypeptide or any fragment or genetically engineered derivative possessing the desired biochemical function.

[0049] An "isolated" plasmid, nucleic acid, vector, virus, virion, host cell, or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are increasingly more isolated. An isolated plasmid, nucleic acid, vector, virus, host cell, or other substance is in some embodiments purified, e.g., from about 80% to about 90% pure, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least about 99%, or more, pure.

[0050] As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes inhibiting the disease, i.e., arresting its development; and relieving the disease, i.e., causing regression of the disease.

[0051] The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses, camels, etc.); mammalian farm animals (e.g., sheep, goats, cows, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.). In some cases, the individual is a human.

[0052] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0053] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention,

subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0054] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0055] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an rVACV virion" includes a plurality of such virions and reference to "the variant protein" includes reference to one or more variant proteins and equivalents thereof known to those skilled in the art, and so forth. The claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[0056] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0057] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0058] Vaccinia virus is an enveloped DNA virus, which replicates entirely in the cytoplasm. Vaccinia virus genome is about 200 kb, which is larger than a typical virus. FIGS. 1A-1B provide a schematic of EEV formation during the VACV replication cycle. The replication cycle includes the following steps: 1) first uncoating, 2) early gene expression, 3) second uncoating, 4) DNA replication, 5) intermediate gene expression, 6) late gene expression, 7) virion assembly and egress. Increasing the ratio of VACV vectors produced in the EEV form improves cancer treatment outcomes.

[0059] The A34R gene of VACV encodes a glycoprotein in the outer membrane of EEV. A34R protein is involved in cell-to-cell transmission of vaccinia virus.

[0060] The rVACV described herein comprise a variant A34R gene or protein. These rVACV could be used deliver genetically encoded therapeutic cargoes for cancer treatments. Also, these vectors could be used synergistically to boost efficacy of existing immunotherapeutic treatment modalities in immunologically cold tumors.

[0061] Thus, in some aspects, the present disclosure provides rVACV with altered envelope protein, A34R. The rVACV exhibits enhanced production of the virus in the EEV form. In some cases, the rVACV comprises a heterologous nucleic acid. Also provided herein are methods of delivering a gene product to a cancer cell in a subject.

[0062] An example of a wild-type or consensus A34R protein sequence is given below:

[0063] MKSLNRQTVSRFKKLSVPVAIMMILSTIISGIGTFLHYKEELMPSACANGWIQYD KHCYLDTNIKMSTDNAVYQCRKLRARLPRPDTRHLRVLFSIFYKDYWVSLKKTNDKW LDINNDKDIDISKLTNFKQLNSTTDAEACYIYKSGKLVKTVCKSTQSVLCVKKFYK (SEQ ID NO: 1).

[0064] An example of a wild-type gene encoding an A34R protein is given below:

[0065] ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTCGGTGC CGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACATTTCTGC ATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATACAATACGAT AAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATAATGCGGTTTATCAG TGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATACTAGACATCTGAGAGTATT

GTTTAGTATTTTTTATAAAGATTATTGGGTAAGTTTAAAAAAGACCAATGATAAATG
GTTAGATATTAATAATGATAAAGATATAGATATTAGTAAATTAACAAATTTTAAAC
AACTAAACAGTACGACGGATGCTGAAGCGTGTTATATATACAAGTCTGGAAAACTG
GTTAAAACAGTATGTAAAAGTACTCAATCTGTACTATGTGTTAAAAAATTCTACAA
GTGA (SEQ ID NO: 2).

[0066] In some cases, the rVACV comprises a variant A34R, wherein, as compared to a vaccinia virion comprising a wild-type A34R protein, for example, an A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises a mutation in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0067] As compared to a VACV comprising a wild-type A34R protein, the rVACV disclosed herein exhibit increased production of rVACV, for example, in the EEV form.

[0068] In some cases, as compared to a wild-type A34R protein, for example, an A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

[0069] In some cases, the rVACV comprises a variant A34R having only one mutation in one of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0070] In some cases, rVACV comprises a variant A34R having mutations in exactly two of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the

73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0071] In some cases, rVACV comprises a variant A34R having mutations in exactly three of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0072] In some cases, rVACV comprises a variant A34R having mutations in exactly four of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0073] In some cases, rVACV comprises a variant A34R having mutations in exactly five of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0074] In some cases, rVACV comprises a variant A34R having mutations in exactly six of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0075] In some cases, rVACV comprises a variant A34R having mutations in exactly seven of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0076] In some cases, rVACV comprises a variant A34R having mutations in exactly eight of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th

position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0077] In some cases, rVACV comprises a variant A34R having mutations in exactly nine of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0078] In some cases, rVACV comprises a variant A34R having mutations in exactly ten of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0079] In some cases, rVACV comprises a variant A34R having mutations in exactly eleven of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0080] In some cases, rVACV comprises a variant A34R having mutations in all of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0081] In some cases, rVACV comprises a variant A34R having mutations in at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, or at least eleven of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[0082] When the mutation is only in lysine in the 151st position, the mutation is not the substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E).

[0083] In a specific embodiment, an rVACV comprises a variant A34R having a mutation K148E. In a further specific embodiment, an rVACV comprises a variant A34R having the mutation K148E and the mutation K151E.

[0084] In a specific embodiment, an rVACV comprises a variant A34R having the mutation K148E.

[0085] In certain embodiments, an rVACV comprises a variant A34R having the mutation K151R.

[0086] In certain embodiments, an rVACV comprises a variant A34R having the mutation K151P.

[0087] In certain embodiments, an rVACV comprises a variant A34R having the mutation Y73H.

[0088] In certain embodiments, an rVACV comprises a variant A34R having the mutation R79L.

[0089] In certain embodiments, an rVACV comprises a variant A34R having the mutation V153T.

[0090] In certain embodiments, an rVACV comprises a variant A34R having the mutation T8I.

[0091] In a specific embodiment, an rVACV comprises a variant A34R having the mutation K65S.

[0092] In a further specific embodiment, an rVACV comprises a variant A34R having the mutation K65T.

[0093] In certain embodiments, an rVACV comprises a variant A34R having the mutation K65N.

[0094] In further embodiments, an rVACV comprises a variant A34R having the mutation R81M.

[0095] In even further embodiments, an rVACV comprises a variant A34R having the mutation R88N.

[0096] In a further specific embodiment, an rVACV comprises a variant A34R having the mutation R88D.

[0097] In certain embodiments, an rVACV comprises a variant A34R having the mutation R91P.

[0098] In further embodiments, an rVACV comprises a variant A34R having the mutation K151P.

[0099] In certain embodiments, an rVACV comprises a variant A34R having the mutation K165E.

[00100] In further embodiments, an rVACV comprises a variant A34R having the mutation K168D.

[00101] In some cases, in addition to the amino acid mutations in the A34R described above, an rVACV also comprises a silent mutation in the codon encoding the 144th amino acid position of the variant A34R. Particularly, in a wild-type gene encoding a wild-type A34R protein, the gene has the sequence shown below in SEQ ID NO: 2 above. The bolded, italicized, and underlined TAC codon encodes for a tyrosine residue (shown below). In a variant A34R gene, the TAC codon is mutated to TAT codon, which also encodes for tyrosine. Thus, in some cases, in addition to the amino acid mutations in the A34R described above, an rVACV also comprises a gene encoding A34R protein where the codon encoding the tyrosine at position1 44 is mutated to produce a silent mutation so that the mutated codon also encodes for a tyrosine.

[00102] ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTC GGTGCCGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACAT TTCTGCATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATACAA TACGATAAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATAATGCGGTT TATCAGTGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATACTAGACATCTGAG AGTATTGTTTAGTATTTTTTATAAAGATTATTGGGTAAGTTTAAAAAAGACCAATGA TAAATGGTTAGATATTAATAATGATAAAGATATAGATATTAGTAAATTAACAAATT TTAAACAACTAAACAGTACGACGGATGCTGAAGCGTGTTATATA***TAC***AAGTCTGGA AAACTGGTTAAAACAGTATGTAAAAGTACTCAATCTGTACTATGTGTTAAAAAATT CTACAAGTGA (SEQ ID NO: 2).

[00103] In a certain embodiment, the disclosure provides an rVACV that comprises an A34R gene having a silent mutation in the codon encoding the 144th amino acid of the A34R protein. The sequences of a wild-type and mutated A34R genes are discussed below. Accordingly, in some cases, an rVACV comprises a gene encoding A34R where the codon encoding the tyrosine at position 144 is mutated to produce a silent mutation so that the mutated codon also encodes for a tyrosine. For example, the codon "TAC" is mutated to the codon "TAT."

[00104] In some cases, an rVACV disclosed herein exhibits enhanced rVACV production, for example, in the EEV form.

[00105] In some cases, an rVACV disclosed herein produces higher titers, for example, 10- to 100-fold higher, such as 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, or 100-fold higher titer as compared to VACV comprising a wild-type A34R protein. Examples of such increases in the titers of rVACV disclosed herein is provided in FIG. 3B and FIG. 4A.

[00106] In some cases, an rVACV produce higher ratio of EEV form to total rVACV produced in the cells. For example, an rVACV disclosed herein produces 10- to 150-fold higher, such as 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 110-fold, 120-fold, 130-fold, 140-fold, or 150-fold higher ratio of EEV form to total rVACV produced in the cells. Examples of such increases in the ratios of EEV to total VACV produced in rVACV disclosed herein is provided in FIG. 3A and FIG. 4B.

CANCER TROPISM OF rVACV

[00107] As discussed elsewhere in this disclosure, in some cases, the rVACV disclosed herein could be used for treating a cancer in a subject. To that end, the rVACV act as oncolytic viruses. Oncolytic viruses can selectively target and kill cancer cells while not affecting or minimally affecting the normal cells. Such ability of oncolytic viruses to selectively infect and replicate within tumor cells while not affecting or minimally affecting the normal cells is called "cancer tropism" or "oncotropism."

[00108] Certain modifications are known to render oncolytic viruses better suited for survival and/or multiplication in cancer or tumor environment, thus, improving cancer tropism of such viruses. Certain approaches to improve cancer tropism of oncolytic viruses include: 1) modifying oncolytic viruses to target cancer cells either in the entry stage or during post-entry replication steps; 2) utilizing IFN-sensitive viruses that cannot replicate in normal cells but survive and multiply in cancer cells with defective IFN pathways; and 3) deleting one or more immune-evasion genes from the viruses.

[00109] Certain modifications to improve cancer tropism in oncolytic viruses include a deletion in the virus' vaccinia growth factor (VGF) and thymidine kinase (TK) genes. An rVACV with these deletions is referenced herein as a double deleted rVACV.

[00110] Also, mutation of lysine at position 151 to glutamic acid (K151E) of A34R protein increased cancer tropism by inducing virus spread and the formation of EEV.

[00111] Moreover, deleting B18R and expressing IFN-β can produce a virus that is unable to infect normal cells but replicates within cancer cells and exhibits enhanced tumor killing.

[00112] Additional modifications to improve cancer tropism in oncolytic viruses, including VACV, are known in the art and such embodiments are within the purview of the disclosure. Certain such modifications are described by Enow *et al.* (2023), *Viruses*, 15(11): 2262.

[00113] Accordingly, certain embodiments of rVACV disclosed herein comprise one or more genetic modifications that improve cancer tropism of rVACV. In certain such example, rVACV comprises a deletion in the virus' VGF and/or TK genes. In another such example, rVACV comprises a deletion in the virus' VGF and TK genes.

ONCOLYTIC PROPERTY OF rVACV

[00114] The term "oncolytic property" refers to the speed and efficiency of an oncolytic virus to kill cancer cells. Oncolytic viruses with higher oncolytic property would kill target cancer cells faster and with higher efficiency.

[00115] Several genetic modifications are known to increase oncolytic property of an oncolytic virus. As an example, such modifications include incorporating into the genome of the oncolytic virus one or more heterologous nucleic acids that encode agents that enhance or restore antitumor immunity. Certain such agents include immune checkpoint inhibitors, chimeric antigen receptors (CARs), and antigen-specific T-cell receptors (TCRs). Additional options include granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-2 variant (IL-2v), interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-23 (interleukin-23), interleukin-24 (IL-24), interleukin-36γ (IL-36γ), tumor necrosis factor (TNF), cGAS-STING antagonist, tumor-associated antigen (TAA), tumor neoantigen, interferon-α (IFN-α), β-galactosidase, and interferon-b (IFN-β).

[00116] In further embodiments, an rVACV disclosed herein is modified to increase its oncolytic property. This can be done by incorporating into the rVACV genome a heterologous nucleic acid that encodes an agent that enhances or restores antitumor immunity. Heterologous

nucleic acids that encode one or more of the agents discussed in the preceding paragraph could be used to increase the oncolytic properties of rVACV disclosed herein.

[00117] Additional examples of heterologous nucleic acids that encode agents to increase oncolytic properties of oncolytic viruses are well-known in the art and use of such heterologous nucleic acids in the rVACV disclosed herein is within the purview of the disclosure.

[00118] In a specific example, the disclosure provides an rVACV comprising a heterologous gene encoding IL-2, for example, IL-2v transgene expressed off the poxvirus synthetic early-late promoter. IL-2v differs from IL-2 by three specific mutations that block binding with the CD25 receptor (*i.e.* IL-2v does not bind CD25, whereas IL-2 binds CD25).

[00119] In certain such example, an rVACV further comprises a deletion in one or both of TK and VGF.

[00120] In some cases, an rVACV of the present disclosure comprises a heterologous nucleotide sequence encoding both a heterologous nucleic acid gene product and a heterologous polypeptide gene product. Where the gene product is an RNA, in some cases, the RNA gene product encodes a polypeptide. Where the gene product is an RNA, in some cases, the RNA gene product does not encode a polypeptide. In some cases, an rVACV of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding a single heterologous gene product. In some cases, an rVACV of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding two heterologous gene products. Where the single heterologous nucleic acid encodes two heterologous gene products, in some cases, nucleotide sequences encoding the two heterologous gene products are operably linked to the same promoter. Where the single heterologous nucleic acid encodes two heterologous gene products, in some cases, nucleotide sequences encoding the two heterologous gene products are operably linked to two different promoters. In some cases, an rVACV of the present disclosure comprises a single heterologous nucleic acid comprising a nucleotide sequence encoding three heterologous gene products. Where the single heterologous nucleic acid encodes three heterologous gene products, in some cases, nucleotide sequences encoding the three heterologous gene products are operably linked to the same promoter. Where the single heterologous nucleic acid encodes three heterologous gene products, in some cases, nucleotide sequences encoding the three heterologous gene products are operably linked to two or three different promoters. In some cases, an rVACV of

the present disclosure comprises two heterologous nucleic acids, each comprising a nucleotide sequence encoding a heterologous gene product.

Recombinant Regulatory Sequences

[00121] In some cases, a nucleotide sequence encoding a gene product of interest is operably linked to a transcriptional control element. For example, in some cases, a nucleotide sequence encoding a gene product of interest is operably linked to a constitutive promoter. In other cases, a nucleotide sequence encoding a gene product of interest is operably linked to an inducible promoter. In some instances, a nucleotide sequence encoding a gene product of interest is operably linked to a tissue-specific or cell type-specific regulatory element. For example, in some instances, a nucleotide sequence encoding a gene product of interest is operably linked to a cancer cell-specific promoter or a promoter that is overactive in a cancer cell as compared to a normal cell. Various such specific promoters are well-known in the art and use of such promoters in the rVACV disclosed herein is within the purview of the disclosure.

Pharmaceutical Compositions

[00122] The present disclosure provides a pharmaceutical composition comprising: a) a subject rVACV, as described above; and b) a pharmaceutically acceptable carrier, diluent, excipient, or buffer. In some embodiments, the pharmaceutically acceptable carrier, diluent, excipient, or buffer is suitable for use in a human.

[00123] Such excipients, carriers, diluents, and buffers include any pharmaceutical agent that can be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, &

Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

THERAPEUTIC METHODS

[00124] Certain aspects of the present disclosure provide a method of treating a cancer. Certain such methods comprise administering a therapeutically effective amount of an rVACV to a subject having a cancer. The rVACV can be administered systemically, such as via intravenous, intraperitoneal, or subcutaneous routes, or into a tumor.

[00125] The oncolytic properties of the rVACV provide the anti-cancer effects in a subject. In specific embodiments, the treated cancer is a melanoma cancer, ovarian cancer, colorectal cancer, cervical cancer, hepatocellular carcinoma, pancreatic cancer, breast cancer, or head and neck cancer, brain cancer, esophageal cancer, skin cancer, lung cancer, thymic cancer, uterine cancer, bladder cancer, testicular cancer. A "therapeutically effective amount" will fall in a relatively broad range that can be determined through experimentation and/or clinical trials. For example, for *in vivo* injection, a therapeutically effective dose may be in the order of from about 10^4 to about 10^{12} of the rVACV vectors. Specific effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[00126] For example, one of ordinary skill in the art could readily determine an effective amount of rVACV by testing for an effect on one or more parameters, for example, oncolytic efficacy, subject's tolerance to the treatment, stage and severity of cancer, spread of cancer, etc.

[00127] In some cases, administering an effective amount of an rVACV of the present disclosure results in a decrease in the rate of cancer progression, e.g. a 2-fold, 3-fold, 4-fold, 5-fold, or 10-fold decrease in the rate of cancer progression. In some cases, administering an effective amount of an rVACV of the present disclosure results in reduction of tumor load in a subject, e.g., reduction of tumor load by between 10% to 100%, such as 20%, 40%, 60%, 80%, or 100%.

[00128] As mentioned above, in some cases, rVACVs may also include one or more heterologous nucleic acids. Accordingly, certain aspects of the disclosure provide a method of delivering a gene product to a cancer cell in an individual, the method comprising administering to a subject rVACV virion disclosed herein.

[00129] The gene product can be a suitable gene product, for example, as described elsewhere in this disclosure. Delivering a gene product to cancer cells in a subject can provide improved oncolytic properties of the rVACV.

[00130] In some embodiments, more than one administration (e.g., two, three, four or more administrations) may be employed to achieve the desired level of gene expression. In some cases, the more than one administration is administered at various intervals, e.g., daily, weekly, twice monthly, monthly, every 3 months, every 6 months, yearly, etc. In some cases, multiple administrations are administered over a period of time from 1 month to 2 months, from 2 months to 4 months, from 4 months to 8 months, from 8 months to 12 months, from 1 year to 2 years, from 2 years to 5 years, or more than 5 years.

[00131] In some cases, administering rVACV to a subject to treat a cancer comprises administering to the subject a carrier cell comprising rVACV. A carrier cell comprising rVACV can be administered systemically, such as via intravenous, subcutaneous, or intraperitoneal routes. In some cases, the carrier cells are autologous carrier cells, i.e., cells obtained from the subject so that administering such cells back into the subject would not induce immune reaction against the administered cells.

[00132] In some cases, specific cells are isolated from a subject, infected with the rVACV, and optionally multiplied *in vivo* before administering those cells back into the subject. In specific embodiments, the carrier cells are macrophages, B cells, or T cells.

[00133] In some cases, the carrier cells and/or the rVACV can be genetically modified to express on the cells' surface molecules that allow binding of the carrier cells to the molecules on the cancer cells. Such binding facilitates delivery of the rVACV to the target cancer cells. Various cell surface molecules that are specific for cancer cells or are highly enriched as compared to normal cells are well known in the art and use of such molecules to facilitate the interaction of carrier cells with the target cancer cells is within the purview of the disclosure.

NUCLEIC ACIDS

[00134] The present disclosure provides an isolated nucleic acid comprising a nucleotide sequence that encodes a variant A34R protein as compared to a wild-type A34R protein, for example, an A34R protein having the sequence of SEQ ID NO: 1.

[00135] In some cases, the nucleic acid encodes a variant A34R, wherein, as compared to a wild-type A34R protein, for example, an A34R protein having the sequence of SEQ ID NO: 1,

the variant A34R protein comprises a mutation in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00136] In some cases, as compared to a wild-type A34R protein, for example, an A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

[00137] In some cases, the nucleic acid encodes a variant A34R having only one mutation in one of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00138] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly two of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00139] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly three of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in

the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00140] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly four of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00141] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly five of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00142] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly six of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00143] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly seven of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00144] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly eight of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00145] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly nine of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00146] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly ten of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00147] In some cases, the nucleic acid encodes a variant A34R having mutations in exactly eleven of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00148] In some cases, the nucleic acid encodes a variant A34R having mutations in all twelve of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00149] In some cases, the nucleic acid encodes a variant A34R having mutations in at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or all twelve of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00150] When the mutation is only in lysine in the 151st position, the mutation is not a substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E).

[00151] In a specific embodiment, the nucleic acid encodes a variant A34R having a mutation K148E. In a further specific embodiment, a nucleic acid encodes a variant A34R having the mutation K148E and the mutation K151E.

[00152] In a specific embodiment, the nucleic acid encodes a variant A34R having the mutation K148E.

[00153] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation K151R.

[00154] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation K151P.

[00155] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation Y73H.

[00156] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation R79L.

[00157] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation V153T.

[00158] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation T8I.

[00159] In a specific embodiment, the nucleic acid encodes a variant A34R having the mutation K65S.

[00160] In a further specific embodiment, the nucleic acid encodes a variant A34R having the mutation K65T.

[00161] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation K65N.

[00162] In further embodiments the nucleic acid encodes a variant A34R having the mutation R81M.

[00163] In even further embodiments, the nucleic acid encodes a variant A34R having the mutation R88N.

[00164] In a further specific embodiment, the nucleic acid encodes a variant A34R having the mutation R88D.

[00165] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation R91P.

[00166] In further embodiments, the nucleic acid encodes a variant A34R having the mutation K151P.

[00167] In certain embodiments, the nucleic acid encodes a variant A34R having the mutation K165E.

[00168] In further embodiments, the nucleic acid encodes a variant A34R having the mutation K168D.

[00169] In some cases, a nucleic acid comprises mutations, in addition to mutations that cause the amino acid mutations in the A34R described above, a silent mutation in the codon encoding the 144th amino acid position of the variant A34R. Particularly, in a wild-type gene encoding a wild-type A34R protein, the gene has the sequence shown below in SEQ ID NO: 2. The bolded and underlined TAC codon encodes for a tyrosine residue. In a variant nucleic acid, the TAC codon is mutated to TAT codon, which also encodes for tyrosine. Thus, in some cases, in a nucleic acid, in addition to causing the amino acid mutations in the A34R described above, the codon encoding the 144th tyrosine is mutated to produce a silent mutation so that the mutated codon also encodes for a tyrosine.

[00170] ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTC
GGTGCCGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACAT
TTCTGCATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATACAA
TACGATAAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATAATGCGGTT
TATCAGTGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATACTAGACATCTGAG
AGTATTGTTTAGTATTTTTTATAAAGATTATTGGGTAAGTTTAAAAAAGACCAATGA
TAAATGGTTAGATATTAATAATGATAAAGATATAGATATTAGTAAATTAACAAATT
TTAAACAACTAAACAGTACGACGGATGCTGAAGCGTGTTATATA***TAC***AAGTCTGGA
AAACTGGTTAAAACAGTATGTAAAAGTACTCAATCTGTACTATGTGTTAAAAAATT
CTACAAGTGA (SEQ ID NO: 2).

[00171] In a certain embodiment, the disclosure provides a nucleic acid that encodes a A34R protein, for example, a wild-type A34R protein having the sequence of SEQ ID NO: 1, but has a silent mutation in the codon encoding the 144th amino acid of the A34R protein. The sequences of a wild-type and mutated A34R genes are discussed below. Accordingly, in some cases, the disclosure provides a nucleic acid encoding A34R where the codon encoding the 144th

tyrosine is mutated to produce a silent mutation so that the mutated codon also encodes for a tyrosine. For example, the codon "TAC" is mutated to the codon "TAT" thereby producing the sequence provided below:

[00172] ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTC GGTGCCGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACAT TTCTGCATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATACAA TACGATAAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATAATGCGGTT TATCAGTGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATACTAGACATCTGAG AGTATTGTTTAGTATTTTTTATAAAGATTATTGGGTAAGTTTAAAAAAGACCAATGA TAAATGGTTAGATATTAATAATGATAAAGATATAGATATTAGTAAATTAACAAATT TTAAACAACTAAACAGTACGACGGATGCTGAAGCGTGTTATATA***TAT***AAGTCTGGA AAACTGGTTAAAACAGTATGTAAAAGTACTCAATCTGTACTATGTGTTAAAAAATT CTACAAGTGA (SEQ ID NO: 3).

HOST CELLS

[00173] In a further aspect, the present disclosure provides host cells, e.g., isolated (genetically modified) host cells, comprising a variant nucleic acid disclosed herein. The variant nucleic acid can be a part of an rVACV genome.

[00174] A subject host cell can be an isolated cell, e.g., a cell in *in vitro* culture or in an organism, i.e., *in vivo*.

[00175] An rVACV disclosed herein can be used to generate rVACV in the virion form. Thus, the present disclosure provides an rVACV that, when introduced into a suitable cell, can provide for production of rVACV.

[00176] A subject host cell is useful for producing a subject rVACV, as described below. Where a subject host cell is used to produce a subject rVACV, it is referred to as a "packaging cell." In some embodiments, a subject host cell is stably genetically modified with a subject nucleic acid. In other embodiments, a subject host cell is transiently genetically modified with a subject nucleic acid.

[00177] An rVACV genome comprising a variant nucleic acid can be introduced stably or transiently into a host cell, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, liposome-mediated transfection, and the like.

A stable transformation generally further includes a selectable marker, e.g., any of several well-known selectable markers such as neomycin resistance, and the like.

[00178] A subject host cell is generated by introducing a variant nucleic acid, for example, a VACV genome comprising a variant nucleic acid, into any of a variety of cells, e.g., mammalian cells, including, e.g., murine cells, and primate cells (e.g., human cells). Suitable mammalian cells include, but are not limited to, primary cells and cell lines, where suitable cell lines include, but are not limited to HeLa, Vero, Vero B4, HeLa S3, BS-C-1, HuTK$^-$ 143B, CEF, BHK-21, HEK293Ad, HEK293 parental, HEK293T, RK13, U2OS, or CHO cells.

[00179] Further embodiments of the disclosure provide methods of producing rVACV by culturing cells comprising rVACV and purifying rVACV produced in the cells.

Examples of Non-Limiting Aspects of the Disclosure

[00180] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure numbered 1-32 are provided below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

[00181] Aspect 1. A recombinant vaccinia virion (rVACV) comprising a variant A34R protein, wherein, as compared to a vaccinia virion comprising a wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises a mutation in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position,

wherein, as compared to a vaccinia virion comprising the wild-type A34R protein, the rVACV exhibits increased production of extra-cellular enveloped virion (EEV), and wherein

when the mutation is only in lysine in the 151st position, the mutation is not a substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E).

[00182] Aspect 2. The rVACV of Aspect 1, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosinc in 73rd position to histidinc (Y73H), arginine in 79th position to lysinc (R79L), valinc in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

[00183] Aspect 3. The rVACV of Aspect 2, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

[00184] Aspect 4. The rVACV of Aspect 2, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

[00185] Aspect 5. The rVACV of Aspect 2, comprising the amino acid mutation K148E.

[00186] Aspect 6. The rVACV of Aspect 5, further comprising the amino acid mutation K151E.

[00187] Aspect 7. The rVACV of any one of the preceding Aspects, wherein the gene encoding the variant A34R comprises a silent mutation in the codon encoding the 144th amino acid of the variant A34R.

[00188] Aspect 8. A recombinant vaccinia virion (rVACV), wherein the gene encoding the A34R protein comprises a silent mutation in the codon encoding the 144th amino acid of the A34R protein.

[00189] Aspect 9. The rVACV of any one the preceding Aspects, further comprising deletions in one or both of thymidine kinase (TK) and vaccinia growth factor (VGF) genes.

[00190] Aspect 10. The rVACV of any one of the preceding Aspects, wherein the increased production of EEV comprises an increase ratio of EEV production to total rVACV production.

[00191] Aspect 11. The rVACV of any one of the preceding Aspects, wherein the increased production of EEV comprises increased production of rVACV.

[00192] Aspect 12. The rVACV of any one of the preceding Aspects, further comprising a heterologous gene.

[00193] Aspect 13. The rVACV of Aspect 12, wherein the heterologous gene increases the oncolytic property of the rVACV.

[00194] Aspect 14. The rVACV of Aspect 12 or 13, wherein the heterologous gene is selected from: granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-12 (IL-12), interleukin-2, interleukin-2 variant, interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-23 (interleukin-23), interleukin-24 (IL-24), interleukin-36γ (IL-36γ), tumor necrosis factor (TNF), interferon-α (IFN-α), β-galactosidase, and interferon-b (IFN-β).

[00195] Aspect 15. A pharmaceutical composition comprising:

[00196] a) a recombinant vaccinia virion (rVACV) of any one of Aspects 1-13; and

[00197] b) a pharmaceutically acceptable excipient.

[00198] Aspect 16. A method of delivering a heterologous gene to a cancer cell in a subject, the method comprising administering to the subject an rVACV of any one of Aspects 12-14 or a composition comprising the rVACV of any one of Aspects 12-14 and a pharmaceutically acceptable excipient.

[00199] Aspect 17. A method of treating a cancer in a subject, the method comprising administering to the subject an effective amount of an rVACV of any one of Aspects 1-14 or the composition of Aspect 15.

[00200] Aspect 18. An isolated nucleic acid comprising a nucleotide sequence that encodes a variant A34R protein, wherein, as compared to a wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises mutations in one or more of the following amino acids: lysine in 148th position, lysine in the 151st position, tyrosine in 73rd position, arginine in 79th position, valine in 153rd position, threonine in 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00201] Aspect 19. The isolated nucleic acid of Aspect 18, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), and threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

[00202] Aspect 20. The isolated nucleic acid of Aspect 19, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

[00203] Aspect 21. The isolated nucleic acid of Aspect 19, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

[00204] Aspect 22. The isolated nucleic acid of Aspect 18, wherein the variant A34R protein comprises the amino acid mutation K148E.

[00205] Aspect 23. The isolated nucleic acid of Aspect 22, wherein the variant A34R protein further comprises the amino acid mutation K151E.

[00206] Aspect 24. A genetically modified host cell comprising the nucleic acid of any one of Aspects 18 to 23.

[00207] Aspect 25. A method of producing a recombinant vaccinia virion (rVACV) the method comprising culturing the host cell of Aspect 24 and purifying the rVACV.

[00208] Aspect 26. The method of producing the rVACV of Aspect 25, wherein the rVACV is in an extra-cellular enveloped virion (EEV) form.

[00209] Aspect 27. A variant vaccinia virus A34R protein, comprising, as compared to a wild-type A34R protein having the sequence of SEQ ID NO: 1, mutations in one or more of the following amino acids: lysine in 148th position, lysine in the 151st position, tyrosine in 73rd position, arginine in 79th position, valine in 153rd position, threonine in 8th position, lysine in the

65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

[00210] Aspect 28. The variant vaccinia virus A34R protein of Aspect 27, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

[00211] Aspect 29. The variant vaccinia virus A34R protein of Aspect 28, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

[00212] Aspect 30. The variant vaccinia virus A34R protein of Aspect 28, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

[00213] Aspect 31. The variant vaccinia virus A34R protein of Aspect 27, wherein the variant A34R protein comprises the amino acid mutation K148E.

[00214] Aspect 32. The variant vaccinia virus A34R protein of Aspect 27, wherein the variant A34R protein further comprises the amino acid mutation K151E.

EXAMPLES

[00215] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular

weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Generation of rVACV having enhanced production

[00216] A directed evolution approach was utilized, which involved CRISPR-guided, error prone DNA polymerases (as described in WO 2024011173) coupled with supernatant/antibody-based selections to uncover novel and non-obvious variants of A34R that confer increased EEV vector production.

[00217] Enriched rVACV having A34R variants were isolated from the selected library and individually tested. The effect of each A34R variant on EEV production was tested *in vitro*. To that end, the rVACV comprising specific variants were incorporated into a double-deleted VACV (Western Reserve Strain lacking thymidine kinase (TK) and vaccinia growth factor (VGF) function).

[00218] As shown in FIGS. 3A-3C, certain mutations in the A34R protein resulted in elevated EEV production relative to the total number of VACV vectors produced at 24 hours post-infection (HPI) in BSC-1 cells. These mutations include: K148E (OV-151), K151R (OV-171), Y73H (OV-154), R79L (OV-153), and V153T (OV-179).

[00219] Also, a nucleotide variant that resulted in a silent mutation at the 144th amino acid position of the A34R sequence while creating a T8I mutation in a previously unreported open reading frame (OV-172) exhibited elevated EEV production relative to the total number of VACV vectors produced at 24 hours post-infection (HPI) in BSC-1 cells (data not shown).

[00220] Additionally, one of the A34R variants identified in this screening was combined with an A34R variant previously known to confer increased EEV production, namely, K151E. Both the single K148E variant (OV-151) and double K148E/K151E variant (OV-180) conferred increased EEV/total VACV vector production at 24 HPI in BSC-1 cells (FIG. 3A), EEV titer at 24 HPI in BSC-1 (FIGS. 3B-3C) and KM12 cells (FIG. 4A), EEV/total VACV ratio at 24 HPI in KM12 cells (FIG. 4B), and comet length at 48 HPI in BSC-1 cells (FIGS. 5A-5B).

[00221] Notably, the phenotype of the novel A34R K148E variant was distinct from that of the known A34R K151E variant as shown through the increased kinetics of EEV formation for vectors harboring the A34R K148E mutation (FIGS. 3B, 4A, and 4B).

[00222] Kidney cells from African Green Monkey (BSC-1) were infected with double-deleted VACV variants harboring point mutations that result in the indicated amino acid change(s) in the A34R gene. For the data presented in FIG. 3A, vectors were harvested 24 HPI, and the EEV was isolated by centrifugation and incubation in antibodies that neutralize the intracellular virion forms. The infectious titer of isolated EEV in plaque forming units (PFU) was divided by the titer (in PFU) of total VACV.

[00223] The EEV/total VACV ratios for each individual replicate were then divided by the mean EEV/total VACV derived from five replicates OV-18 (harboring the wild-type A34R sequence). FIGS. 3B-3C show data from a separate trial, where isolated EEV titer was quantified at 6, 12, 24, or 48 HPI. N = $\geq$ 3. FIGS. 4A-4B show data resulting from infecting human colorectal cancer cells (KM12) infected with double-deleted VACV variants harboring point mutations that confer the indicated amino acid mutation(s) in the A34R gene. Vectors were harvested 24 HPI, at which point EEV was isolated by centrifugation and incubation in antibodies to neutralize intracellular virion forms. FIG. 4A depicts the infectious titer of isolated EEV in PFU and FIG. 4B shows the number of EEV PFU divided by the titer (PFU) of total VACV.

[00224] The EEV/total VACV ratios for each individual replicate were then divided by the mean EEV/total VACV for five OV-18 (harboring the wild-type A34R sequence) replicates. N = $\geq$3.

[00225] FIGS. 5A-5B show data resulting from infecting African Green Monkey (BSC-1) cells with double-deleted VACV variants harboring the wild-type A34R protein or point mutations that confer the indicated amino acid mutation(s) in the A34R protein. The plate was tilted at a 25-degree angle and incubated at 37°C, 5% CO_2 for 72 hours post-infection, at which point the "comet" length was quantified using ImageJ as a way to visualize the improved delivery effects conferred by elevated EEV production.

Example 2: Generation of rVACV having cancer therapeutic properties

[00226] rVACV were produced that contained mutations in the A34R protein described herein. In addition to the A34R protein mutations, the rVACV was also further modified to

contain a transgene encoding interleukin-2 variant (IL-2v) under the poxvirus synthetic early-late promoter. Thus oncolytic rVACV were produced that expressed IL-2v in combination with the wild-type A34R sequence (OV-18), or novel A34R K148E variant (OV-151). Each OV was then tested in C57BL/6 mice bearing MC38 tumors to model colorectal carcinoma. OV-151 (A34R gene K148E mutation) selected variant slowed growth of the tumor and conferred increased survival relative to OV-18 (wild-type A34R) or the "vehicle" control treated with a mock injection.

[00227] FIGS. 6A-6C show that intravenous injection of the selected variant (OV-151 containing the K148E mutation in the A34R gene) conferred increased survival and decreased tumor burden relative to treatment with wild-type (OV-18) vector or vehicle control (mock injection). C57Bl6 mice were subcutaneously injected with 5E5 mouse colorectal cancer (MC38) cells. On days 3, 5, 7, 9, and 11, 3E7 plaque-forming units (pfu) of the indicated OV variant encoding IL-2v was delivered intravenously into the MC38 tumor-bearing mice. The tumor size was measured over the course of 34 days, and each mouse was euthanized when its tumor volume reached 1500 mm^2. FIG. 6B shows the survival of mice treated with vector (mock injection), WT (OV-18 encoding wild-type A34R), or selected variant (OV-151 encoding the A34R gene containing a K148E mutation). FIG. 6B shows the average tumor volume through the first 20 days of the study.

[00228] FIGS. 9A-9D provide evidence for increased immune cell infiltration into the tumor cells. FIG. 9A shows quantification of $CD3^+$ cells, which represents the total T-cell count. FIG. 9B shows quantification of $CD8^+$ T Cells shows an increase in cytotoxic T-cell infiltration conferred by the rVACV encoding the K148E variant of A34R. FIG. 9C shows quantification of Granzyme B, which is released by effector $CD8^+$ T cells, shows elevated activity of effector T cells in the tumor conferred by the rVACV encoding the K148E variant of A34R. FIG. 9D shows that no difference was observed in $CD4^+$ helper T cell infiltration.

[00229] FIGS. 7A-7C depict a bi-tumoral study aimed at simulating inter-tumoral dissemination during oncolytic virotherapy treatment. In this study, 5E5 MC38 cells were injected into two distal locations in C57Bl6 mice. When the tumor volumes reached ~250 mm^2, the "primary" tumor was intratumorally administered 1.5E7 pfu of the indicated OV. Both the primary tumor that received intratumorally injected OV and the "secondary" tumor that did not receive intratumorally injected OV was harvested at 48 hours post-infection, and the amount of OV vector present in each tumor was quantified by infectious titer in BSC-1 cells (and depicted

in pfu). FIG. 7B shows that there were no statistically significant differences in OV titer present in the primary tumor conferred by the selected variant (OV-151 encoding the A34R gene containing the K148E mutation) vs the WT (OV-18 encoding wild-type A34R) or vector (mock injection). On the other hand, the selected variant conferred significantly elevated levels of OV vector in the secondary tumor that did not receive the initial intratumoral injection relative to WT or vector, indicating that the elevated levels of EEV produced by the selected variant (OV-151 encoding the A34R gene containing the K148E mutation) led to improved inter-tumoral delivery efficiency.

[00230] FIG. 8 provides evidence that the EEV form of VACV is inherently resistant to pre-existing neutralizing antibodies and the complement system. In this experiment, HeLa cells were infected with double-deleted VACV Western Reserve at MOI = 0.1. Following 48 hours post-infection, EEV was isolated by centrifuging the supernatant to remove cell debris and incubating with 1) a concentration of anti-L1 antibody that had previously been shown to neutralize 100% of IMV particles and 2) the indicated concentrations of human serum collected from ~10,000 donors known as Intravenous Immunoglobulin (IVIg). IMV was isolated by separating the infected cells from their supernatant, freeze-thawing the infected cells three times, removing the cell debris by centrifugation, and incubating the IMV with the indicated concentrations of IVIg. Following a one-hour incubation at 37°C with the antibody and/or IVIg, the EEV or IMV samples were serially diluted and infected onto fresh HeLa cells. While increasing concentrations of IVIg led to >90% of IMV particles being neutralized, no concentration of IVIg neutralized EEV to the same degree. This data suggest that elevated EEV may be an advantageous feature to promote improved therapeutic gene delivery, particularly, in patients whose immune systems were previously exposed to vaccinia-based vaccines or oncolytic virotherapies.

Example 3: Selection of additional rVACV variants that confer positive fitness for elevated EEV production in cancer cells.

[00231] FIGS. 10A-10E show decreasing overall diversity and increasing enrichment in the VACV A34R variant population across four rounds of selection for EEV production. The A34R region was amplified from the VACV population after library incorporation but prior to selection (Round 0) and following one (Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells. The A34R regions

were then shotgun sequenced on Illumina next-generation sequencing technology. The reads were trimmed and aligned to the VACV A34R gene reference sequence. The frequency of each non-wild-type codon substitution was then quantified and ordered based on frequency. The number of overall VACV A34R variant sequences with greater than one read decreased in the population across each selection round, while leading variants increased in prevalence across each selection round.

[00232] FIG. 11 shows the frequency distribution of wild-type VACV A34R amino acid substitutions after library incorporation but prior to selection (Round 0) and following one (Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells. The frequency of wild-type amino acids decreased at positions where non-wild-type variant enrichment was observed (e.g. amino acid position 65).

[00233] FIG. 12 shows the fold-enrichment of leading variants after pre-synthesized single substitution variant library incorporation into double-deleted VACV but prior to selection (Round 0) and following one (Round 1), two (Round 2), three (Round 3), or four (Round 4) rounds of selection for EEV in KM12 colorectal cancer cells. Consistent, positive enrichment was observed across each round of selection for VACV A34R protein variants K65S, K65T, K65N, R81M, R88N, R88D, R91P, K151P, K165E, and K168D. In our experience with directed evolution screens of pre-synthesized single substitution variant libraries, the enrichment profiles observed for these variants strongly indicate they confer functional improvements to the formation and function of VACV EEV.

[00234] FIGS. 13A-13K depict the location of selected amino acid variants on the AlphaFold2-predicted structure of the A34R protein. Variants K65 (FIG. 13A), Y73 (FIG. 13B), R79 (FIG. 13C), R81 (FIG. 13D), R88 (FIG. 13E), R91 (FIG. 13F), K148 (FIG. 13G), K151 (FIG. 13H), V153 (FIG. 13I), K165 (FIG. 13J), and K168 (FIG. 13K) are highlighted in black.

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[00235] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

We claim:

1. A recombinant vaccinia virion (rVACV) comprising a variant A34R protein, wherein, as compared to a vaccinia virion comprising a wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises a mutation in one or more of the following amino acids: lysine in the 148th position, lysine in the 151st position, tyrosine in the 73rd position, arginine in the 79th position, valine in the 153rd position, threonine in the 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position,

wherein, as compared to a vaccinia virion comprising the wild-type A34R protein, the rVACV exhibits increased production of extra-cellular enveloped virion (EEV),

and wherein when the mutation is only in lysine in the 151st position, the mutation is not a substitution of the lysine in the 151st position with glutamine (K151Q) or with glutamic acid (K151E).

2. The rVACV of claim 1, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

3. The rVACV of claim 2, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

4. The rVACV of claim 2, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

5. The rVACV of claim 2, comprising the amino acid mutation K148E.

6. The rVACV of claim 5, further comprising the amino acid mutation K151E.

7. The rVACV of any one of the preceding claims, wherein the gene encoding the variant A34R comprises a silent mutation in the codon encoding the 144th amino acid of the variant A34R.

8. A recombinant vaccinia virion (rVACV), wherein the gene encoding the A34R protein comprises a silent mutation in the codon encoding the 144th amino acid of the A34R protein.

9. The rVACV of any one the preceding claims, further comprising deletions in one or both of thymidine kinase (TK) and vaccinia growth factor (VGF) genes.

10. The rVACV of any one of the preceding claims, wherein the increased production of EEV comprises an increased ratio of EEV production to total rVACV production.

11. The rVACV of any one of the preceding claims, wherein the increased production of EEV comprises increased production of rVACV.

12. The rVACV of any one of the preceding claims, further comprising a heterologous gene.

13. The rVACV of claim 12, wherein the heterologous gene increases the oncolytic property of the rVACV.

14. The rVACV of claim 12 or 13, wherein the heterologous gene is selected from: granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-12 (IL-12),

interleukin-2, interleukin-2 variant (IL-2v), interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-23 (interleukin-23), interleukin-24 (IL-24), interleukin-36γ (IL-36γ), tumor necrosis factor (TNF), interferon-α (IFN-α), β-galactosidase, and interferon-b (IFN-β).

15. A pharmaceutical composition comprising:
a) a recombinant vaccinia virion (rVACV) of any one of claims 1-13; and
b) a pharmaceutically acceptable excipient.

16. A method of delivering a heterologous gene to a cancer cell in a subject, the method comprising administering to the subject an rVACV of any one of claims 12-14 or a composition comprising the rVACV of any one of claims 12-14 and a pharmaceutically acceptable excipient.

17. A method of treating a cancer in a subject, the method comprising administering to the subject an effective amount of an rVACV of any one of claims 1-14 or the composition of claim 15.

18. An isolated nucleic acid comprising a nucleotide sequence that encodes a variant A34R protein, wherein, as compared to a wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises mutations in one or more of the following amino acids: lysine in 148th position, lysine in the 151st position, tyrosine in 73rd position, arginine in 79th position, valine in 153rd position, threonine in 8th position, lysine in the 65th position, arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

19. The isolated nucleic acid of claim 18, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in

the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

20. The isolated nucleic acid of claim 19, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

21. The isolated nucleic acid of claim 19, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

22. The isolated nucleic acid of claim 18, wherein the variant A34R protein comprises the amino acid mutation K148E.

23. The isolated nucleic acid of claim 22, wherein the variant A34R protein further comprises the amino acid mutation K151E.

24. A genetically modified host cell comprising the nucleic acid of any one of claims 18 to 23.

25. A method of producing a recombinant vaccinia virion (rVACV) the method comprising culturing the host cell of claim 24 and purifying the rVACV.

26. The method of producing the rVACV of claim 25, wherein the rVACV is in an extracellular enveloped virion (EEV) form.

27. A variant vaccinia virus A34R protein, comprising, as compared to a wild-type A34R protein having the sequence of SEQ ID NO: 1, mutations in one or more of the following amino acids: lysine in 148th position, lysine in the 151st position, tyrosine in 73rd position, arginine in 79th position, valine in 153rd position, threonine in 8th position, lysine in the 65th position,

arginine in the 81st position, arginine in the 88th position, arginine in the 91st position, lysine in the 165th position, and lysine in the 168th position.

28. The variant vaccinia virus A34R protein of claim 27, wherein, as compared to the wild-type A34R protein having the sequence of SEQ ID NO: 1, the variant A34R protein comprises one or more of the following amino acid mutations: lysine in 148th position to glutamate (K148E), lysine in the 151st position to arginine (K151R), lysine in the 151st position to proline (K151P), tyrosine in 73rd position to histidine (Y73H), arginine in 79th position to lysine (R79L), valine in 153rd position to threonine (V153T), threonine in 8th position to isoleucine (T8I), lysine in the 65th position to serine (K65S), lysine in the 65th position to threonine (K65T), lysine in the 65th position to asparagine (K65N), arginine in the 81st position to methionine (R81M), arginine in the 88th position to asparagine (R88N), arginine in the 88th position to aspartate (R88D), arginine in the 91st position to proline (R91P), lysine in the 165th position to glutamate (K165E), and lysine in the 168th position to aspartate (K168D).

29. The variant vaccinia virus A34R protein of claim 28, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamate (K151E).

30. The variant vaccinia virus A34R protein of claim 28, wherein when the variant A34R protein does not comprise the K151R mutation, the variant A34R comprises a mutation in lysine in 151st position to glutamine (K151Q).

31. The variant vaccinia virus A34R protein of claim 27, wherein the variant A34R protein comprises the amino acid mutation K148E.

32. The variant vaccinia virus A34R protein of claim 27, wherein the variant A34R protein further comprises the amino acid mutation K151E.