MODIFIED EXTRACELLULAR ENVELOPED VIRUS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No 62/916,035 filed Oct 16, 2019, which is incorporated by reference herein in its entirety.

INCORPORATION BY REFERENCE

[0002] All publications, patents, patent applications, and NCBI accession numbers mentionedin this specification are herein incorporated by reference to the same extent as if each individualpublication, patent, or patent application was specifically and individually indicated to be incorporated by reference, and as if set forth in their entireties. In the event of a conflict betweena term as used herein and the term as defined in the incorporated reference, the definition of this disclosure controls.

SUMMARY

[0002a] In one aspect, provided herein is an oncolytic virus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations that are in amino acid residues that are positively charged at pH 5, and wherein the A34R protein is mutated relative to a sequence within a wild-type A34R protein (SEQ ID No. 4).

[0002b] In another aspect, provided herein is use of the oncolytic virus as described herein, in the manufacture of a medicament for the treatment of a cancer.

[0002c] In another aspect, provided herein is an oncolytic virus as described herein for use in therapy.

[0003] One ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations, wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4). In some ~~embodiment~~examples, the mutation in the position corresponding to position Lys119 is Lys119Glu. In some ~~embodiment~~examples, the mutation in the position corresponding to position Lys151 is Lys151Glu. In some ~~embodiment~~examples, the at least two mutations in positions corresponding to positions Lys119 and Lys151 of the wild-type vaccinia virus A43R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.

[0004] One ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non- naturally occurring mutations that are in amino acid residues that are positively charged at pH 5, within the wild-type A34R protein (SEQ ID No. 4).

[0005] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non- naturally occurring mutations that are not at position 110 of the wild-type A34R protein (SEQ ID No. 4).

[0006] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-

naturally occurring mutations that are not at aspartic acid residues within the wild-type A34R protein (SEQ ID No. 4).

[0007] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non- naturally occurring mutations that are independently in alanine, arginine, asparagine, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine residues within the wild-type A34R protein (SEQ ID No. 4).

[0008] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising a non-naturally occurring mutation at a lysine residue that is at a position other than position Lys151 of the wild-type A34R protein (SEQ ID No. 4).

[0009] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising a non-naturally occurring mutation at position Lys119 of the wild-type A34R protein (SEQ ID No. 4).

[0010] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are in amino acid residues that are positively charged at pH 5, within the wild-type A34R protein (SEQ ID No. 4), wherein the modifiedoncolytic poxvirus generates an increased number of comet tail type plaques in a viralplaque forming assay, compared to an otherwise identical oncolytic virus that does not comprise the at least two non-naturally occurring mutations.

[0011] Another ~~embodiment~~example provides a modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non- naturally occurring mutations, wherein if either of the non-naturally occurring mutations are at position 110 within the wild-type A34R protein (SEQ ID No. 4), the amino acid encoded is not an asparagine residue.

[0012] Another ~~embodiment~~example provides a modified oncolytic poxvirus that demonstrates an increased resistance to neutralizing antibodies compared to a wild-type strain of the oncolytic poxvirus, wherein the increased resistance is measured by number of plaques produced by the modified oncolytic poxvirus or the wild-type strain in a viral plaque assay, following treatment with an anti-L1 NR-45114 antibody or an anti-VIG antibody, and wherein the modified oncolytic poxvirus produces at least about 55,000 plaque forming units/mL.

[0013] Another ~~embodiment~~example provides a modified oncolytic poxvirus that produces at least about 55,000 plaque forming units/mL, in a viral plaque assay, following treatment with a neutralizing antibody.

[0014] In some ~~embodiment~~examples, the neutralizing antibody is an anti-L1 NR-45114 antibody or an anti-VIG antibody. In some ~~embodiment~~examples, the A34R protein or the fragment thereof further comprises a mutation at position Lys151 of the wild-type A34R protein (SEQ ID No. 4). In some ~~embodiment~~examples, the amino acid residues that are positively charged at pH5 are lysine residues. In some ~~embodiment~~examples, the nucleic acid comprises a nucleotide sequence that is at least about 80% homologous to the coding sequence within the viral gene VACWR157, or a fragment thereof. In some ~~embodiment~~examples, the nucleic acid comprises a nucleotide sequence that is at least about 80% homologous to the nucleotide sequence set forth as SEQ ID No. 3. In some ~~embodiment~~examples, at least one of the two non- naturally occurring mutations is at position Lys119 of the wild-type A34R protein (SEQ ID No. 4). In some ~~embodiment~~examples, the non-naturally occurring mutation is at position Lys119 of the wild-type A34R protein (SEQ ID No. 4). In some ~~embodiment~~examples, the mutation at position Lys119 of the wild-type A34R protein (SEQ ID No. 4) is Lys119Glu. In some ~~embodiment~~examples, at least one of the two non-naturally occurring mutations is at position Lys151 of the wild-type A34R protein (SEQ ID No. 4). In some ~~embodiment~~examples, the mutation at position Lys151 of the wild-type A34R protein (SEQ ID No. 4) is Lys151Glu.

[0015] One ~~embodiment~~example provides a modified oncolytic poxvirus that expresses an A34R protein comprising mutations Lys119Glu and Lys151Glu

[0016] In some ~~embodiment~~examples, positions 305-307 of SEQ ID No. 3 comprises nucleotides GAA or GAG. In some ~~embodiment~~examples, positions 451-453 of SEQ ID No. 3 comprises nucleotides GAA or GAG. In some ~~embodiment~~examples, the modified oncolytic poxvirus produces a greater amount of an extracellular enveloped virus form than an intracellular mature virus form, as compared to an otherwise identical oncolytic virus that does not comprise the at least two non-naturally occurring mutations. In some ~~embodiment~~examples, the modified oncolytic poxvirus produces a greater amount of an extracellular enveloped virus form than an intracellular mature virus form, as compared to an otherwise identical oncolytic virus that does not comprise the non-naturally occurring mutation. In some ~~embodiment~~examples, the modified oncolytic poxvirus further comprises an exogenous nucleic acid that codes for at least one of: a therapeutic protein or a diagnostic protein. In some ~~embodiment~~examples, the exogenous nucleic acid can code for at least one of: a chemokine

receptor, a membrane associated protein, a microbial protein that is capable of degrading hyaluronan, a microbial protein, SOCS3, PH-20, HMGB1, PIAS3, IL15, IL15-Rα, LIGHT, ITAC, fractalkine, CCL5, N1L, an immune checkpoint modulator, a metabolic modulating protein, or any combinations thereof, such as a fusion protein comprising any combination of the above (such as a metabolic modulating protein and a cytokine). In some ~~embodiment~~examples, the exogenous nucleic acid that codes for a chemokine receptor, wherein the chemokine receptor comprises at least one of CXCR4 and CCR2. In some ~~embodiment~~examples, the exogenous nucleic acid that codes for the membrane associated protein. In some ~~embodiment~~examples, the membrane associated protein comprises a membraned associated hyaluronidase. In some ~~embodiment~~examples, the membrane associated hyaluronidase comprises PH-20. In some ~~embodiment~~examples, the PH-20 is GPI-anchored. In some ~~embodiment~~examples, the exogenous nucleic acid that codes for the microbial protein that is capable of degrading hyaluronan, wherein the microbial protein comprises a secreted hyaluronidase. In some ~~embodiment~~examples, the secreted hyaluronidase comprises at least one of HysA, lin, sko, and rv, or any combinations thereof. In some ~~embodiment~~examples, the exogenous nucleic acid that codes for the microbial protein. In some ~~embodiment~~examples, the microbial protein comprises HysA. In some ~~embodiment~~examples, the modified oncolytic poxvirus further comprises a modification in the genome of the virus, wherein the modification comprises a mutation or a deletion of the B5R gene. In some ~~embodiment~~examples, the modification in the genome of the virus, wherein the modification comprises a mutation or a deletion in a SCR region of the B5R gene, wherein said SCR region comprises SCR1, SCR3, SCR4, or any combinations thereof, and wherein the SCR region does not comprise SCR2. In some ~~embodiment~~examples, the modified oncolytic poxvirus further comprises a mutation or a deletion of a viral gene selected from a group consisting of: Thymidine kinase (TK), B8R, B18R, B15R, K7R, C6L, K4L, F8L, F9L, F10L, F17R, E1L, E4L, E6R, E8R, E10R, E11L, O2L, I1L, I2L, I3L, I5L, I7L, I8R, G1L, G3L, G4L, G5.5R, G7L, G9R, L1R, L3L, L4R, L5R, J1R, J4R, J6R, H1L, H2R, H3L, H4L, H5R, H6R, D1R, D2L, D3R, D6R, D7R, D8L, D11L, D12L, D13L, A2.5L, A3L, A4L, A5R, A6L, A7L, A9L, A10L, A13L, A14L, A15L, A16L, A17L, A18R, A21L, A24R, A25L, A26L, A27L, A28L, A29L, A30L, A31R, A34R, A42R, A45R, A46R, A52R, and any combinations thereof. In some ~~embodiment~~examples, the modified oncolytic poxvirus comprises the mutation or deletion of viral gene A52R. In some ~~embodiment~~examples, the modifiedoncolytic poxvirus comprises (i) the exogenous nucleic acid that codes for a chemokine receptor, wherein the chemokine receptor comprises at least one of CXCR4 and CCR2;

(ii) the exogenous nucleic acid that codes for PIAS3; (iii) a mutation or deletion of the thymidine kinase gene; (iv) the mutation or deletion of the A52R gene. In some ~~embodiment~~examples, the virus is suitable for systemic delivery. In some ~~embodiment~~examples, the virusis capable of immune evasion. In some ~~embodiment~~examples, the systemic delivery comprises oral administration, parenteral administration, intranasal administration, sublingual administration, rectal administration, transdermal administration, or any combinations thereof. In some ~~embodiment~~examples, the parenteral administration comprises an intravenous injection. In some ~~embodiment~~examples, the virus is suitable for intratumoral delivery. In some ~~embodiment~~examples, the poxvirus is a vaccinia virus.

[0017] One ~~embodiment~~example provides a process for engineering an oncolytic poxvirus comprising: (i) obtaining an oncolytic poxvirus DNA backbone vector, the oncolytic poxvirus DNA backbone vector comprising one or more modifications according to any one of the preceding claims; (ii) further modifying the oncolytic virus DNA vector to produce an engineered DNA vector; (iii) transfecting mammalian cells with the engineered DNA vector; (iv) culturing the mammalian cells under conditions suitable for viral replication; and (v) harvesting the viral particles.

[0018] In some ~~embodiment~~examples, the mammalian cells comprise HeLa cells, 293 cells, A549 cells, or Vero cells.

[0019] One ~~embodiment~~example provides a kit, comprising: the oncolytic poxvirus, a container; and instructions for administering said oncolytic virus to a subject to treat a disorder associated with pathological angiogenesis.

[0020] One ~~embodiment~~examples provides a method of treating a tumor, the method comprising administering to a subject a therapeutically effective amount of the oncolytic poxvirus according.

[0021] One ~~embodiment~~examples provides a method of treating a tumor, the method comprising administering to a subject a composition comprising patient-derived leukocyte cells infected with a modified oncolytic poxvirus that expresses an A34R protein comprising mutations at positions 119 and 151 of the wild-type A34 protein (SEQ ID No. 4), wherein the modified oncolytic poxvirus produces a population of viral particles in a tumor microenvironment. In some ~~embodiment~~examples, the patient-derived leukocyte cells comprise macrophages. In some ~~embodiment~~examples, the patient-derived leukocyte cells comprise tumor- targeted T cells. In some ~~embodiment~~examples, at least about 10% to at least about 90% of the population of viral particles are EEV particles, as measured in a viral plaque assay. In some ~~embodiment~~examples, the method further comprises harvesting the EEV particles from the

tumor microenvironment and intravenously administering the EEV particles to the subject. In some ~~embodiment~~examples, the modified oncolytic poxvirus is a modified oncolytic vaccinia virus.

[0022] One ~~embodiment~~example provides a process comprising infecting a culture of host cells with a population of modified oncolytic poxvirus that comprises at least about 10% to at least about 90% EEV particles, wherein the modified oncolytic poxvirus expresses an A34R protein comprising mutations at positions 119 and 151 of the wild-type A34 protein (SEQ ID No. 4). In some ~~embodiment~~examples, the modified oncolytic poxvirus is a modified oncolytic vaccinia virus.

[0023] One ~~embodiment~~example provides a method of treating a cancer, the method comprising administering to a patient a modified oncolytic virus comprising a nucleic acid that codesfor an A34R protein or a fragment thereof comprising at least two mutations, wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4). In some ~~embodiment~~examples, the at least two mutations n positions corresponding to positions Lys119 and Lys151 of a wild- type vaccinia virus A43R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.

[0024] One ~~embodiment~~example provides a method of treating a tumor, the method comprising administering to a patient a modified oncolytic virus comprising a nucleic acid that codesfor an A34R protein or a fragment thereof comprising at least two mutations, wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4). In some ~~embodiment~~examples, the at least two mutations n positions corresponding to positions Lys119 and Lys151 of a wild- type vaccinia virus A43R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.

[0025] In some ~~embodiment~~examples, the administering is via an intratumoral injection, anintravenous injection, or a combination thereof. In some ~~embodiment~~examples, the administering is via an intratumoral injection, an intravenous injection, or a combination thereof.

[0026] In some ~~embodiment~~examples, the method further comprises administering a furthertherapy, in combination with the oncolytic poxvirus, wherein the further therapycomprises at least one of: a chemotherapy, a radiation therapy, an oncolytic viral therapy with an additional virus, treatment with an immunomodulatory protein, a CAR T cellular therapy, an anti-cancer agent, an immunomodulatory agent, or any combinations thereof.

[0027] In some ~~embodiment~~examples, the further therapy comprises the immunomodulatory agent selected from the group consisting of: an anti-CD33 antibody or an antigen binding fragment thereof, an anti-CD11b antibody or an antigen binding fragment thereof, a COX2 inhibitor, a cytokine, a chemokine, an anti-CTLA4 antibody or an antigen binding fragment thereof, an anti-PD-1 antibody or an antigen binding fragment thereof, an anti- PD-L1 antibody or an antigen binding fragment thereof, and a TLR agonist.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] The novel features of the disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of this disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of this disclosure are utilized, and the accompanying drawings of which.

[0029] **FIG. 1** shows an exemplary assembly scheme to generate DNA for making arecombinant virus library.

[0030] **FIG. 2** shows a comparison of viral plaque comet tails formed by different vacciniavirus strains.

[0031] **FIGs. 3A-3B** show results of neutralization assay carried out using different vacciniavirus strains (**FIG. 3A** shows results following treatment with anti-L1 NR-45114 antibody and **FIG. 3B** shows results following treatment with anti-L1R and VIG antibodies).

[0032] **FIG. 4** shows results cell viability (upper panel: MC38 cells; lower panel: HCT116cells) following infection with different vaccinia virus strains.

[0033] **FIG. 5** shows results of a viral replication assay in cancer cells (upper panel: HCT116cells; lower panel: MC38 cells), for different vaccinia virus strains.

DETAILED DESCRIPTION

[0034] While preferred embodiments of this disclosure have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from this disclosure. It should be understood that various alternatives to the embodiments of this disclosure described herein may be employedin practicing the disclosure. It is intended that the following claims define the scope of the

infection, and mutations in the EEV production-enhanced A34R were characterized by Sanger sequencing.

[0145] It was observed that all isolated clones shared two missense mutations: K119E and K151E, and one silent mutation at F129 (SEQ ID No. 2). The mutant protein was designated as WO34 (SEQ ID No. 2), and one corresponding clone was assigned UID WO0064R.002. The GFP reporters in this initial cohort of clones were not flanked by loxP sites and cannot beremoved. A codon-scrambled ORF encoding WO34 was synthesized and used to clone a transfer vector (pWR157-KE.R) that contained the loxP-flanked GFP. New recombinant viruses that encode WO34 were selected from fluorescent plaques and then used to infect cre recombinase-expressing cells. Reporter-lacking clones were selected after cre treatment.

Table 1: A34R gene and protein sequences

SEQ ID NO: 1	A34R	>NC_006998.1:143912-144418 Vaccinia virus, complete genome ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTCGGTGC CGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACATT TCTGCATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATA CAATACGATAAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATA ATGCGGTTTATCAGTGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATAC TAGACATCTGAGAGTATTGTTTAGTATTTTTTATAAAGATTATTGGGTAAGT TTAAAAAAGACCAATGATAAATGGTTAGATATTAATAATGATAAAGATATAG ATATTAGTAAATTAACAAATTTTAAACAACTAAACAGTACGACGGATGCTGA AGCGTGTTATATATACAAGTCTGGAAAACTGGTTAAAACAGTATGTAAAAGT ACTCAATCTGTACTATGTGTTAAAAAATTCTACAAGTGA
SEQ ID NO: 2	A34R modified	ATGAAATCGCTTAATAGACAAACTGTAAGTAGGTTTAAGAAGTTGTCGGTGC CGGCCGCTATAATGATGATACTCTCAACCATTATTAGTGGCATAGGAACATT TCTGCATTACAAAGAAGAACTGATGCCTAGTGCTTGCGCCAATGGATGGATA CAATACGATAAACATTGTTATTTAGATACTAACATTAAAATGTCTACAGATA ATGCGGTTTATCAGTGTCGTAAATTACGAGCCAGATTGCCTAGACCGGATAC TAGACATCTGAGAGTATTGTTTAGTATTTTTTATAAAGATTATTGGGTAAGT TTAAAAAAGACCAATGATAAATGGTTAGATATTAATAATGATGAGGATATAG ATATTAGTAAATTAACAAATTTCAAACAACTAAACAGTACGACGGATGCTGA AGCGTGTTATATATACAAGTCTGGAAAACTGGTTGAAACAGTATGTAAAAGT ACTCAATCTGTACTATGTGTTAAAAAATTCTACAAGTGA
SEQ ID NO: 3	A34R mutated (nucleic acid sequences) (nucleic acid differences with wild type A34R coding sequence are bold, and mutated codons underlined	atgaaatcgcttaatagacaaactgtaagtaggtttaagaagttgtcggtgc cggccgctataatgatgatactctcaaccattattagtggcataggaacatt tctgcattacaaagaagaactgatgcctagtgcttgcgccaatggatggata caatacgataaacattgttatttagatactaacattaaaatgtctacagata atgcggtttatcagtgtcgtaaattacgagccagattgcctagaccggatac tagacatctgagagtattgtttagtatttttttataaagattattgggtaagt ttaaaaaagaccaatgataaatggttagatattaataatgat**GaG**gatatag atattagtaaattaacaaattt**C**aaacaactaaacagtacgacggatgctga agcgtgttatatatacaagtctggaaaactggtt**Gaa**acagtatgtaaaagt actcaatctgtactatgtgttaaaaaattctacaagtga

SEQ ID NO: 4	Wild-typeA34R protein	>sp|P24761|A34_VACCW Protein A34 OS=Vaccinia virus (strain Western Reserve) OX=10254 GN=VACWR157 PE=1 SV=1 MKSLNRQTVSRFKKLSVPAAIMMILSTIISGIGTFLHYKEELMPSACANGWI QYDKHCYLDTNIKMSTDNAVYQCRKLRARLPRPDTRHLRVLFSIFYKDYWVS LKKTNDKWLDINNDKDIDISKLTNFKQLNSTTDAEACYIYKSGKLVKTVCKS TQSVLCVKKFYK
SEQ ID NO: 5	WO34 (A34 double mutant K119E K151E)	MKSLNRQTVSRFKKLSVPAAIMMILSTIISGIGTFLHYKEELMPSACANGWI QYDKHCYLDTNIKMSTDNAVYQCRKLRARLPRPDTRHLRVLFSIFYKDYWVS LKKTNDKWLDINNDEDIDISKLTNFKQLNSTTDAEACYIYKSGKLVETVCKS TQSVLCVKKFYK*

Viral plaque comet tails formed by EEV-enhanced vaccinia virus containing WO34

[0146] EEV-enhanced vaccinia viruses that were previously titered were diluted and placedover confluent BS-C-40 cells in 6-well plates. Plates were incubated and left undisturbed for 48 hours and then the cells were stained with crystal violet to view differences in comet tail formation caused by aberrant EEV production. Results are shown in **FIG. 2**. As is seen in **FIG. 2**, the WO34 containing vaccinia virus strain demonstrated enhanced comet-tail formation compared to the WI strain (A34R single mutation in WR strain) and WR strain (non-mutated A34R). The comet-tail formation of the WO34 containing vaccinia virus strainwas visibly increased.

Neutralization of the EEV-enhanced vaccinia virus containing WO34

[0147] In two separate experiments using different strains of vaccinia viruses (WO34 containing vaccinia virus, WI strain, IHD-J strain, and WR strain), HeLa cells were grown to confluency in 6-well plates and infected with 1 MOI (multiplicity of infection) of a vaccinia virus strain. After 24 hours, 1 mL of culture medium. was removed and centrifuged at 800g. About 500 µl of supernatant was then removed and used for viral plaque assay. During the serial dilution, samples were treated with anti-L1 NR-45114 antibody or VIG then incubated for 1 hour at 37 °C. Dilutions were then added to confluent 6 well plates of BS-C-40 cells for plaque assay. After 1.5 hours, media was replaced with CM10 with 3% CMC. After 48 hours,cells were stained with crystal violet to count viral plaques to determine the titer of virus in the HeLa cell supernatant and the blocking ability of neutralizing antibodies as shown in **FIGS.3A-3B** (FIG. 3A shows viral plaque titer followed by treatment with the anti-L1 NR-45114 antibody and FIG. 3B shows the same followed by treatment with the anti-L1R antibody and the VIG antibody).

Cell viability after infection with EEV-enhanced vaccinia virus containing WO34

[0148] In 96-well plates, HCT116 or MC38 cell lines were seeded and allowed to grow until90% confluent. Cells were then infected with 1 MOI of different vaccinia virus strains (WR

CLAIMS

1. An oncolytic virus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations that are in amino acid residues that are positively charged at pH 5, and wherein the A34R protein is mutated relative to a sequence within a wild-type A34R protein (SEQ ID No. 4).

~~**1.**~~**2.** ~~A modified~~The oncolytic ~~poxvirus~~ virus of claim 1, ~~comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations,~~ wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus ~~A43R~~ A34R protein (SEQ ID NO. 4).

~~**2.**~~**3.** The ~~modified~~ oncolytic ~~poxvirus~~virus of claim ~~1~~2, wherein the mutation in the position corresponding to position Lys119 is Lys119Glu.

~~**3.**~~**4.** The ~~modified~~ oncolytic virus of claim ~~1 or 2~~2 or 3, wherein the mutation in the position corresponding to position Lys151 is Lys151Glu.

~~**4.**~~**5.** The ~~modified~~ oncolytic virus of claim ~~1~~2, wherein the at least two mutations in positions corresponding to positions Lys119 and Lys151 of the wild-type vaccinia virus ~~A43R~~ A34R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.

~~**5.** A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are in amino acid residues that are positively charged at pH 5, within the wild-type A34R protein (SEQ ID No. 4).~~

6. The oncolytic virus of any one of claims 1-5, wherein the at least two mutations that are in amino acids residues that are positively charged at pH 5 are for amino acid residues that are negatively charged at pH 5.

~~**6.** A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are not at position 110 of the wild-type A34R protein (SEQ ID No. 4).~~

~~7. A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are not at aspartic acid residues within the wild-type A34R protein (SEQ ID No. 4).~~

~~8. A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are independently in alanine, arginine, asparagine, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine residues within the wild-type A34R protein (SEQ ID No. 4).~~

~~9. A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising a non-naturally occurring mutation at a lysine residue that is at a position other than position Lys151 of the wild-type A34R protein (SEQ ID No. 4).~~

~~10.~~7. ~~A modified~~The oncolytic ~~poxvirus~~ virus of claim 1, wherein one of the ~~comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising a non-naturally occurring~~at least two mutations is at position Lys119 of the wild-type A34R protein (SEQ ID No. 4).

~~11. A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations that are in amino acid residues that are positively charged at pH 5, within the wild-type A34R protein (SEQ ID No. 4), wherein the modified oncolytic poxvirus generates an increased number of comet tail type plaques in a viral plaque forming assay, compared to an otherwise identical oncolytic virus that does not comprise the at least two non-naturally occurring mutations.~~

~~12. A modified oncolytic poxvirus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two non-naturally occurring mutations, wherein if either of the non-naturally occurring mutations are at position 110 within the wild-type A34R protein (SEQ ID No. 4), the amino acid encoded is not an asparagine~~

~~residue.~~

~~**13.** A modified oncolytic poxvirus that demonstrates an increased resistance to neutralizing antibodies compared to a wild-type strain of the oncolytic poxvirus, wherein the increased resistance is measured by number of plaques produced by the modified oncolytic poxvirus or the wild-type strain in a viral plaque assay, following treatment with an anti-L1 NR-45114 antibody or an anti-VIG antibody, and wherein the modified oncolytic poxvirus produces at least about 55,000 plaque forming units/mL.~~

~~**14.** A modified oncolytic poxvirus that produces at least about 55,000 plaque forming units/mL, in a viral plaque assay, following treatment with a neutralizing antibody.~~

~~**15.** The modified oncolytic poxvirus of claim 10, wherein the neutralizing antibody is an anti-L1 NR-45114 antibody or an anti-VIG antibody.~~

~~**16.** The modified oncolytic poxvirus of claim 9 or 10, wherein the A34R protein or the fragment thereof further comprises a mutation at position Lys151 of the wild-type A34R protein (SEQ ID No. 4).~~

~~**17.** The modified oncolytic poxvirus of claim 1, wherein the amino acid residues that are positively charged at pH 5 are lysine residues.~~

~~**18.** The modified oncolytic poxvirus of any one of claims 5-12 and 16-17, wherein the nucleic acid comprises a nucleotide sequence that is at least about 80% homologous to the coding sequence within the viral gene VACWR157, or a fragment thereof.~~

~~**19.** The modified oncolytic poxvirus of any one of claims 5-12 and 16-17, wherein the nucleic acid comprises a nucleotide sequence that is at least about 80% homologous to the nucleotide sequence set forth as SEQ ID No. 3.~~

~~**20.** The modified oncolytic poxvirus of any one of claims 5-8, 11-12, and 17-19, wherein at least one of the two non-naturally occurring mutations is at position Lys119 of the wild- type A34R protein (SEQ ID No. 4).~~

~~21. The modified oncolytic poxvirus of any one of claims 9 and 18-19, wherein the non-naturally occurring mutation is at position Lys119 of the wild-type A34R protein (SEQ ID No. 4).~~

~~22. The modified oncolytic poxvirus of claim 10 or 21, wherein the mutation at positionLys119 of the wild-type A34R protein (SEQ ID No. 4) is Lys119Glu.~~

~~23. The modified oncolytic poxvirus of any one of claims 5-8, 11-12, 17-19, and 20-22, wherein at least one of the two non-naturally occurring mutations is at position Lys151 of thewild-type A34R protein (SEQ ID No. 4).~~

~~24. The modified oncolytic poxvirus of claim 23, wherein the mutation at positionLys151 of the wild-type A34R protein (SEQ ID No. 4) is Lys151Glu.~~

~~25. A modified oncolytic poxvirus that expresses an A34R protein comprising mutationsLys119Glu and Lys151Glu~~

~~26. The modified oncolytic poxvirus of any one of claims 19-23, wherein positions 305-307 of SEQ ID No. 3 comprises nucleotides GAA or GAG.~~

~~27. The modified oncolytic poxvirus of any one of claims 19-23 and 26, wherein positions 451-453 of SEQ ID No. 3 comprises nucleotides GAA or GAG.~~

~~28.~~**8.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of any one of claims ~~5~~1-~~8~~7~~, 11-12, and 17-27~~, wherein the ~~modified~~ oncolytic ~~poxvirus~~ virus produces a greater amount of an extracellular enveloped virus form than an intracellular mature virus form, as compared to an otherwise identical oncolytic virus that does not comprise the at least two ~~non-naturally occurring~~ mutations.

~~29. The modified oncolytic virus of any one of claims 9-10, 16, and 21-22, wherein the modified oncolytic poxvirus produces a greater amount of an extracellular enveloped virus form than an intracellular mature virus form, as compared to an otherwise identical oncolytic virus that does not comprise the non-naturally occurring mutation.~~

~~30.~~**9.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of any of one of claims 1-~~29~~8, further

comprising an exogenous nucleic acid that codes for at least one of: a therapeutic protein or a diagnostic protein.

~~31.~~**10.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of claim ~~30~~9, wherein the exogenous nucleic acid can code for at least one of: a chemokine receptor, a membrane associated protein, a microbial protein that is capable of degrading hyaluronan, a microbial protein, SOCS3, PH-20, HMGB1, PIAS3, IL15, IL15-Rα, LIGHT, ITAC, fractalkine, CCL5, N1L, an immune checkpoint modulator, a metabolic modulating protein, or any combinations thereof.

~~32.~~**11.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of claim ~~31~~10, comprising the exogenous nucleic acid that codes for a chemokine receptor, wherein the chemokine receptor comprises at least one of CXCR4 and CCR2.

~~**33.** The modified oncolytic poxvirus of claim 31 or 32 comprising the exogenous nucleic acid that codes for the membrane associated protein.~~

~~**34.** The modified oncolytic poxvirus of claim 33, wherein the membrane associatedprotein comprises a membraned associated hyaluronidase.~~

~~**35.** The modified oncolytic poxvirus of claim 34, wherein the membrane associated hyaluronidase comprises PH-20.~~

~~**36.** The modified oncolytic poxvirus of claim 35, wherein the PH-20 is GPI-anchored.~~

~~**37.** The modified oncolytic virus of any one of claims 31 - 36, comprising the exogenousnucleic acid that codes for the microbial protein that is capable of degrading hyaluronan, wherein the microbial protein comprises a secreted hyaluronidase.~~

~~**38.** The modified oncolytic poxvirus of claim 37, wherein the secreted hyaluronidasecomprises at least one of HysA, lin, sko, and rv, or any combinations thereof.~~

~~**39.** The modified oncolytic poxvirus of any one of claims 31-38, comprising theexogenous nucleic acid that codes for the microbial protein.~~

~~**40.** The modified oncolytic poxvirus of claim 43, wherein the microbial protein comprises HysA.~~

~~**41.** The modified oncolytic poxvirus of any one of claims 1-40, further comprising a modification in the genome of the virus, wherein the modification comprises a mutation or a deletion of the B5R gene.~~

~~**42.** The modified oncolytic poxvirus of claim 41, comprising the modification in the genome of the virus, wherein the modification comprises a mutation or a deletion in a SCR region of the B5R gene, wherein said SCR region comprises SCR1, SCR3, SCR4, or any combinations thereof, and wherein the SCR region does not comprise SCR2.~~

~~**43.** The modified oncolytic poxvirus of any one of claims 1-42, further comprising a mutation or a deletion of a viral gene selected from a group consisting of: Thymidine kinase (TK), B8R, B18R, B15R, K7R, C6L, K4L, F8L, F9L, F10L, F17R, E1L, E4L, E6R, E8R, E10R, E11L, O2L, I1L, I2L, I3L, I5L, I7L, I8R, G1L, G3L, G4L, G5.5R, G7L, G9R, L1R, L3L, L4R, L5R, J1R, J4R, J6R, H1L, H2R, H3L, H4L, H5R, H6R, D1R, D2L, D3R, D6R, D7R, D8L, D11L, D12L, D13L, A2.5L, A3L, A4L, A5R, A6L, A7L, A9L, A10L, A13L, A14L, A15L, A16L, A17L, A18R, A21L, A24R, A25L, A26L, A27L, A28L, A29L, A30L, A31R, A34R, A42R, A45R, A46R, A52R, and any combinations thereof.~~

~~**44.**~~**12.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of any one of claims 1-11~~-43~~, further comprising the mutation or deletion of viral gene A52R.

~~**45.** The modified oncolytic poxvirus of any one of claims 31-44,comprising (i) the exogenous nucleic acid that codes for a chemokine receptor, wherein the chemokine receptor comprises at least one of CXCR4 and CCR2; (ii) the exogenous nucleic acid that codes for PIAS3; (iii) a mutation or deletion of the thymidine kinase gene; (iv) the mutation or deletionof the A52R gene.~~

~~**46.** The modified oncolytic poxvirus of any one of claims 1-45, wherein the virus issuitable for systemic delivery.~~

~~**47.** The modified oncolytic poxvirus of any one of claims 1-46, wherein the virus~~

~~is capable of immune evasion.~~

~~**48.** The modified oncolytic poxvirus of claim 46 or 47, wherein the systemic delivery comprises oral administration, parenteral administration, intranasal administration, sublingual administration, rectal administration, transdermal administration, or any combinations thereof.~~

~~**49.** The modified oncolytic poxvirus of claim 48, wherein the parenteral administrationcomprises an intravenous injection.~~

~~**50.** The modified oncolytic poxvirus of any one of claims 1-49, wherein the virus issuitable for intratumoral delivery.~~

13. The oncolytic virus of any one of claims 1-12, wherein the oncolytic virus is modified at a genome level.

14. The oncolytic virus of any one of claims 1-13, wherein the oncolytic virus is a pox virus.

~~**51.**~~**15.** The ~~modified~~ oncolytic ~~poxvirus~~ virus of ~~any one of claims 1-50~~claim 14, wherein the poxvirus is a vaccinia virus or a myxoma virus.

16. Use of the oncolytic virus of any one of claims 1-15, in the manufacture of a medicament for the treatment of a cancer.

~~**52.** A process for engineering an oncolytic poxvirus comprising: (i) obtaining an oncolytic poxvirus DNA backbone vector, the oncolytic poxvirus DNA backbone vector comprising one or more modifications according to any one of the preceding claims; (ii) further modifying the oncolytic virus DNA vector to produce an engineered DNA vector; (iii) transfecting mammalian cells with the engineered DNA vector; (iv) culturing the mammalian cells under conditions suitable for viral replication; and (v) harvesting the viral particles.~~

~~**53.** The process of claim 52, wherein the mammalian cells comprise HeLa cells, 293 cells, A549 cells, or Vero cells.~~

~~54. A kit, comprising: an oncolytic poxvirus according to any one of claims 1-53, a container; and instructions for administering said oncolytic virus to a subject to treat a disorder associated with pathological angiogenesis.~~

~~55.~~17. ~~A method of treating a tumor, the method~~The use of claim 16, wherein~~comprising administering to a subject~~ a therapeutically effective amount of an oncolytic ~~poxvirus~~ virus according to any one of claims 1-~~54~~15 is to be administered to a subject.

~~56. A method of treating a tumor, the method comprising administering to a subject a composition comprising patient-derived leukocyte cells infected with a modified oncolytic poxvirus that expresses an A34R protein comprising mutations at positions 119 and 151 of the wild-type A34 protein (SEQ ID No. 4), wherein the modified oncolytic poxvirus producesa population of viral particles in a tumor microenvironment.~~

~~57. The method of claim 56, wherein the patient-derived leukocyte cells comprise macrophages.~~

~~58. The method of claim 56, wherein the patient-derived leukocyte cells comprise tumor-targeted T cells.~~

~~59. The method of claim 56, wherein at least about 10% to at least about 90% of thepopulation of viral particles are EEV particles, as measured in a viral plaque assay.~~

~~60. The method of any one of claims 56-59, further comprising harvesting the EEVparticles from the tumor microenvironment and intravenously administering the EEV particles to the subject.~~

~~61. The method of any one of claims 56-60, wherein the modified oncolytic poxvirus is amodified oncolytic vaccinia virus.~~

~~62. A process comprising infecting a culture of host cells with a population of modified oncolytic poxvirus that comprises at least about 10% to at least about 90% EEV particles, wherein the modified oncolytic poxvirus expresses an A34R protein comprising mutations at positions 119 and 151 of the wild-type A34 protein (SEQ ID No. 4).~~

~~63. The process of claim 62, wherein the modified oncolytic poxvirus is a modifiedoncolytic vaccinia virus.~~

~~64. A method of treating a cancer, the method comprising administering to a patient a modified oncolytic virus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations, wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4).~~

~~65. The method of claim 64, wherein the at least two mutations n positions correspondingto positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.~~

~~66. A method of treating a tumor, the method comprising administering to a patient a modified oncolytic virus comprising a nucleic acid that codes for an A34R protein or a fragment thereof comprising at least two mutations, wherein the at least two mutations are in positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4).~~

~~67. The method of claim 66, wherein the at least two mutations n positions corresponding to positions Lys119 and Lys151 of a wild-type vaccinia virus A43R protein (SEQ ID NO. 4) are Lys119Glu and Lys151Glu, respectively.~~

~~68.~~18. The ~~method~~ use of ~~any one of claims 64-67~~claim 16 or 17, wherein the oncolytic virus is to be administered ~~administering is~~ via an intratumoral injection, an intravenous injection, or a combination thereof.

~~69. The method of any one of claims 55-61, wherein the administering is via an intratumoral injection, an intravenous injection, or a combination thereof.~~

~~70.~~19. The ~~method~~ use of any one of claims ~~68 or 69~~16-18, wherein said medicament is to be administered~~the method further comprises administering a further therapy,~~ in combination with ~~the~~ a further therapy~~oncolytic poxvirus~~, wherein the further therapy comprises at least one of: a chemotherapy, a radiation therapy, an oncolytic viral therapy

withan additional virus, treatment with an immunomodulatory protein, a CAR T cellular therapy, an anti-cancer agent, an immunomodulatory agent, or any combinations thereof.

~~71.~~**20.** The ~~method~~ use of claim ~~70~~19, wherein the further therapy comprises the immunomodulatory agent selected from the group consisting of: an anti-CD33 antibody or anantigen binding fragment thereof, an anti-CD11b antibody or an antigen binding fragment thereof, a COX2 inhibitor, a cytokine, a chemokine, an anti-CTLA4 antibody or an antigen binding fragment thereof, an anti-PD-1 antibody or an antigen binding fragment thereof, an anti-PD-L1 antibody or an antigen binding fragment thereof, and a TLR agonist.

21. An oncolytic virus of any one of claims 1-15 for use in therapy.