

CANINE DISTEMPER VIRUS HEMAGGLUTININ AND FUSION POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application Serial No. 62/913,111, filed October 9, 2019. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

SEQUENCE LISTING

10 The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 25, 2020, is named 07039-1936WO1_SL.txt and is 1,096,580 bytes in size.

1. Technical Field

15 This document relates to canine distemper virus (CDV) hemagglutinin (H) and fusion (F) polypeptides. For example, this document relates to CDV H polypeptides, CDV F polypeptides, recombinant viruses (e.g., vesicular stomatitis viruses (VSVs)) containing CDV H polypeptides and/or CDV F polypeptides, nucleic acid molecules encoding a CDV H polypeptide and/or CDV F polypeptide, methods for making
20 recombinant viruses (e.g., VSVs) containing CDV H polypeptides and/or CDV F polypeptides, and methods for using recombinant viruses (e.g., VSVs) containing CDV H polypeptides and/or CDV F polypeptides to treat cancer or infectious diseases.

2. Background Information

25 Viruses such as VSVs, measles viruses (MeVs), and adenoviruses can be used as oncolytic viruses to treat cancer. Vesicular stomatitis virus (VSV) is a member of the Rhabdoviridae family. The VSV genome is a single molecule of negative-sense RNA that encodes five major polypeptides: a nucleocapsid (N) polypeptide, a phosphoprotein (P) polypeptide, a matrix (M) polypeptide, a glycoprotein (G) polypeptide, and a viral
30 polymerase (L) polypeptide.

SUMMARY

 This document provides methods and materials related to CDV H and/or CDV F polypeptides. For example, this document provides CDV H polypeptides, CDV F polypeptides, recombinant viruses (e.g., vesicular stomatitis viruses (VSVs)) containing
35 CDV H polypeptides and/or CDV F polypeptides, nucleic acid molecules encoding a

A fluorescence focus reduction neutralization assay was performed with MeV and CDV antisera. Different dilutions of the antiserum were pre-incubated for 1 hour at 37°C with a fixed amount of virus. The mix was then used to infect Vero-HIS cells, and the amount of virus in control wells without antibodies was set to 100%. All neutralization curves represent the mean and SD of four replicate curves run on the same 96-well plate. (F) Receptor-selectivity of recombinant viruses. CHO cells expressing the relevant receptors were infected with the recombinant viruses at the indicated MOI. GFP auto-fluorescence was recorded 2 days later.

Figure 4. Oncolytic activity of CD46-targeted envelope chimeric MeV. CDB17 SCID mice were implanted subcutaneously with the human myeloma cell line U266.B1. When tumor volume reached 500 mm³, mice were randomized and left untreated (PBS control) or treated once with 1 x 10⁵ TCID₅₀ particles administered intravenously. Tumor volumes (B) and survival (C) was recorded afterwards.

Figure 5. Schematic of exemplary recombinant VSVs according to some embodiments. VSV-hIFN β -NIS: VSV Indiana was engineered to express human interferon beta (hIFN β) at the M/G intergenic region, and human sodium iodide symporter (NIS) at the G/L intergenic region and rescued as described elsewhere (Naik *et al.*, *Leukemia*, 26:1870-78 (2012)). VSV expressing CDV-F_{22458/16} (with the CDV F polypeptide signal peptide replaced with that of MeV F polypeptide) and CDV-H₅₈₀₄ were generated using the pVSV-smart platform. Point mutations in the CDV-H₅₈₀₄ polypeptide, Y539A and R529A, were introduced by site-directed mutagenesis to ablate natural tropisms to canine Nectin-4 and SLAMF1 polypeptides, respectively. Targeted viruses were generated by displaying EGFR or CD38-targeted scFvs at the C-terminus of CDV-H₅₈₀₄ with an IGES linker peptide and an H6 polyhistidine tag (SEQ ID NO: 21). Retargeted VSV-CDV F/H constructs were rescued on Vero-anti-H6, allowing infection, virus amplification, and fusion of target cells. The titers for each recombinant virus are indicated.

Figure 6. Monolayers of the indicated CHO cells (wild-type or stably overexpressing the indicated receptors) were mock infected or infected (MOI = 0.2) with VSV-CDV-FH-GFP or VSV-CDV-F/H_{aa}- α EGFR-GFP. Fluorescent micrographs were taken under 100X magnification at the indicated times. GFP expression (green) correlates with virus infection and spread within the monolayers.

Figure 7. Monolayers of the indicated CHO cells (wild-type or stably expressing receptors EGFR or CD38) were infected (MOI = 0.1) with VSV-CDVFH_{aa}-αEGFR or VSV-CDVFH_{aa}-αCD38. After 42 hours, cell monolayers were fixed with paraformaldehyde and stained with crystal violet. Photos were taken under 40X magnification.

Figure 8. Therapeutic effects of chimeric VSV-CDVFH_{aa}-αEGFR against xenografted human ovarian cancer in the peritoneum. Female 5-6 week-old athymic nude mice (Envigo, Indianapolis, IN) were implanted intraperitoneally with 2x10⁶ SKOV3ip.1-Fluc cells (200 μL/mouse) (day -7). 7 days post-implantation (day 0), tumor-bearing mice were randomized based on firefly luciferase signals using IVIS spectrum (Perkin Elmer, Hopkinton, MA). Mice were identified by microchip and ear notch. Following randomization, mice received a single dose of 1x10⁷ TCID₅₀ of virus or saline control (250 μL/mouse) via intraperitoneal injection. Mice were euthanized if they developed ascites, subcutaneous injection site tumors that were >10% of body weight, or if they lost >20% of body weight. All surviving mice were euthanized at the end of the experiment (day 92 after virus treatment). Kaplan-Meier survival curves were plotted and compared by log-rank sum test. Clinical observations and weight were recorded three times per week until end of study or euthanasia of the mouse.

Figure 9 is a nucleic acid sequence (SEQ ID NO:1) of a CDV H open reading frame encoding a CDV H polypeptide (SEQ ID NO:2).

Figure 10 is a nucleic acid sequence (SEQ ID NO:3) of a CDV F open reading frame encoding a CDV F polypeptide (SEQ ID NO:4).

Figure 11. Retargeting wild-type CDV envelope to EGFR and CD38. (A) Schematic representation of cloning strategy to generate retargeted wild-type CDV H polypeptides (top). Standard one-letter amino acid abbreviations are used to denote the changes introduced to ablate native receptor (SLAMF1 and Nectin-4) usage (bottom). The amino acid numbering is with respect to SEQ ID NO:5. The single-chain antibody fragments are displayed as a C-terminal extension of the H glycoprotein using a Factor Xa (Fxa) cleavage site (an IEGR amino acid sequence). There is an optional six-histidine tag (SEQ ID NO: 21) in all of the constructs to facilitate virus rescue on Vero-His cells. (B) Co-transfection experiments demonstrating targeted fusion competence of receptor blind H polypeptide

targeted to CD38. CHO-CD38 cells in 12 well plates were co-transfected with either CMV driven CDV F plasmid and CMV driven wild-type CDV H-CD38 or CMV driven receptor blind CDV H-CD38, and cells were fixed, stained and imaged 24 hours later. (C) Targeted cell fusion mediated by CDV H constructs was resistant to pooled measles immune human serum. CHO-CD38 cells were co-transfected with CMV driven H and F plasmids together with a CMV driven GFP plasmid for visualization and incubated with indicated dilutions. Cells were photographed 24 hours after transfection. (D) Chimeric measles viruses bearing targeted CDV H polypeptides retain specificity on a panel of CHO cells expressing the desired receptors or human tumor cell lines with the desired receptor. Cell lines were infected with the respective viruses at an MOI of 0.5, and photographed were taken 48 hours later. (E) Schematic design of experiment to test *in vivo* oncolytic efficacy and specificity of chimeric measles viruses with retargeted CDV envelope. Nude mice were implanted with 5×10^6 SKOV3ip-fluc cells either subcutaneously (SQ) or intraperitoneally (IP) followed by 6 doses of 1×10^6 TCID₅₀/mL intratumorally (IT) for subcutaneous tumors or 2×10^6 TCID₅₀/mL IP for IP tumors every other day beginning on day 10. (F) Individual tumor volumes of subcutaneous SKOV3ip tumors treated with respective viruses (top) and survival of animals with intraperitoneal tumors treated with respective viruses (bottom).

Figure 12 is an alignment of a representative number of CDV H polypeptides. The top sequence (designated AF378705.1_America1) is SEQ ID NO:5 and used for numbering purposes where indicated. Figure 12 discloses SEQ ID NOS 5, 31-109, 105, 110-145, and 227-228, respectively, in order of appearance.

Figure 13 is an alignment of a representative number of CDV F polypeptides. The signal peptide sequence extends from amino acid position 1 to amino acid position 135. The top sequence (designated AF378705.1_America1) is SEQ ID NO:7 and used for numbering purposes where indicated. Figure 13 discloses SEQ ID NOS 7 and 146-226, respectively, in order of appearance.

Figure 14. CDV OL can infect cells lacking SLAMF1 and NECTIN4 receptors. (A) Assessment of infection of Vero cells by CDV isolates in comparison with the OL strain. Cells were infected at an MOI of 0.1 (determined on Vero-dogSLAMF1 cells) and stained with Hema-Quick 48 hours later for visualization. (B) A panel of CHO cells expressing different relevant receptors was infected with an eGFP reporter MeV

cells (ATCC, Cat.# CCL-86) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning Inc., Cat. #10-040-CV, Corning, NY, United States) as described elsewhere.

5 *Plasmids and Construction of full-genome rMeV*

To generate CDV 22458/16 expression plasmids, total RNA was extracted from CDV 22458/16 isolate-infected Vero/dog SLAMF1 cells (passage 1) using RNeasy Mini Kit (Qiagen, Hilden, Germany). Both CDV-H and CDV-F genes were reverse transcribed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Cat.# 11752050) and amplified by PCR with the following primers:

CDVH7050(+):AGAAACTTAGGGCTCAGGTAGTCC (SEQ ID NO: 22)

CDVH8949(-): TCGTCTGTAAGGGATTTCTCACC (SEQ ID NO: 23)

CDVF4857(+): AGGACATAGCAAGCCAACAGG (SEQ ID NO: 24)

CDVH7050(-): GGACTACCTGAGCCCTAAGTTTTCT (SEQ ID NO: 25)

PCR products were sequenced directly by Sanger (Genewiz, Plainfield NJ, USA) and cloned into pJET1.2 vector (Thermo Fisher). CDV H open reading frame (Figure 8) was next PCR amplified with a forward primer (5'-CCG GTA GTT AAT TAA AAC TTA GGG TGC AAG ATC ATC GAT AAT GCT CTC CTA CCA AGA TAA GGT G-3' (SEQ ID NO: 26)) and a reverse primer (5'-CTA TTT CAC ACT AGT GGG TAT GCC TGA TGT CTG GGT GAC ATC ATG TGA TTG GTT CAC TAG CAG CCT CAA GGT TTT GAA CGG TTA CAG GAG-3' (SEQ ID NO: 27)) and cloned into the PacI and SpeI (New England Biolabs, Ipswich MA, USA) restricted pCG vector (Cathomen *et al.*, *J. Virol.*, 72(2):1224-34 (1998)) using the InFusion HD kit (Takara, Shinagawa, Tokyo, Japan). The primers provided the PacI and SpeI restriction sites (underlined, respectively) as well as the MeV-H untranslated region of MeV-H (italic). Similarly, a CDV F open reading frame (Figure 9; amino acid residues 136-662 of SEQ ID NO:4) was cloned into HpaI/SpeI restricted pCG-CDV-F plasmid (von Messling *et al.*, *J. Virol.*, 75(14):6418-27 (2001)). The resulting plasmid pCG-CDV F 22458/16 possesses the MeV-F untranslated region and the MeV-F signal peptide.

Expression plasmids for CDV H/F Onderstepoort vaccine and 5804 isolate (von Messling *et al.*, *J. Virol.*, 75(14):6418-27 (2001)), as well as the MeV Nse are described

Rhabdoviruses, Measles Virus incorporating the targeted CDV H/F complexes could infect and fuse cells in a CD38 or EGFR-specific manner (Figure 11D). This infection-specificity also was observed with an array of tumor cell lines. Skov3pi and U87 cells (EGFR-positive) were infected by EGFR-targeted viruses, but not CD38-targeted viruses. On the contrary, Raji and Ramos cell lines (CD38 positive) were infected exclusively by the CD38-targeted viruses. As a control, cells expressing the 6xHIS pseudo receptor ("6xHIS" disclosed as SEQ ID NO: 21) were infected by all retargeted viruses. These results demonstrate that CDV F/H complexes can be used to avoid anti-measles immunity and drive cell-targeted entry and syncytia formation.

The *in vivo* oncolytic activity of CD38 and EGFR-targeted MeV were also investigated. SKOV3ip.1 tumors were implanted either subcutaneously or intraperitoneally into athymic nude mice, followed by virus treatment using the same route (Figure 11E). Although CD38-targeted viruses showed some therapeutic potency, the potency exhibited by the EGFR-targeted viruses was superior (Figure 11F).

Conversely, CD38-targeted viruses exhibited no anti-tumor potency after intraperitoneal injection, whereas EGFR-targeted viruses resulted in complete tumor regression as observed by 100% percentage survival. Of note, no differences were observed between MeVs retargeted with either the MeV H/F complexes or CDV H/F. These results demonstrate that CDV H/F complexes can enhance the anti-tumoricidal properties of oncolytic measles virus without the problem of being neutralized by measles-induced neutralizing antibodies.

Example 2 – Further Analysis of CD46-specific Oncolytic Measles Viruses Resistant to Neutralization by Measles-Immune Human Serum

This example repeats some of the information and results from Example 1 in addition to providing additional results.

Heterologous combinations of wild-type CDV glycoproteins result in enhanced cell membrane fusion

The MeV coat was replaced with an alternate viral coat that would enable the virus to evade neutralization by anti-measles antibodies. To do this, wild-type CDV was

in the presence of immune serum exhibited high bioluminescence activity that continued to increase over time (Figure 27B), indicating that MeV could not exert oncolysis in the presence of pre-existent immunity. On the contrary, Kaplan-Meier survival curves indicated that, in the absence of immune serum, both oncolytic virus (OV) treatments significantly prolonged mouse survival, with a median survival times of 37 days for the MeV-treated mice and 53 days for the Stealth-treated mice versus a median survival time of 18 days for the control mice (Figure 27C). However, prolonged survival following MeV treatment was completely abrogated when mice received measles-immune serum, whereas MeV-Stealth treatment was still able to significantly prolong survival (median survival time of 28 days) with no statistical difference over treatment in the absence of immune serum.

These results demonstrate that CD46 targeting drives oncolysis in both a xenograft myeloma model and an orthotropic model of ovarian cancer, and that exchange of the MeV coat by the homologous CDV-H/F fusion apparatus shields MeV from MeV-immune human serum.

Methods and materials

Cell lines

Baby hamster kidney cells (BHK, Cat. # CCL-10, ATCC, Manassas, VA, USA), Human kidney epithelial cells (HEK293T) obtained from Dr. François-Loïc Cosset (Université de Lyon), and the human ovarian cancer cell line SKOV3ip.1-Fluc (Mader *et al.*, *Clin. Cancer Res.*, 15(23):7246-55 (2009)) were maintained in Dulbecco's modified Eagle's medium (DMEM; Cat. # SH30022.01, GE Healthcare Life, Pittsburg, PA, USA) supplemented with 5% fetal bovine serum (FBS; Cat. #10437-028; Thermo Fisher Scientific, Waltham, MA, USA). Vero African green monkey kidney cells (Vero, ATCC, Cat. # CCL-81) and their derivatives (expressing human NECTIN-4 (Noyce *et al.*, *Virology*, 436(1):210-20 (2013)), human SLAMF1 (Ono *et al.*, *J. Virol.*, 75(9):4399-401 (2001)) or a membrane-anchored single-chain variable fragment (scFv) specific for a hexahistidine peptide (6× HIS-tag (SEQ ID NO: 21)) (Nakamura *et al.*, *Nat. Biotechnol.*, 23(2):209-14 (2005)) were cultured in DMEM (Cat. # SH30022.01, GE Healthcare Life Sciences) as described elsewhere (Munoz-Alia *et al.*, *Viruses*, 11(8) (2019)). Vero cells constitutively

expressing the canine SLAMF1 molecule (Vero-dogSLAMF1) were generated by transduction and puromycin selection of a second-generation lentiviral vector (kindly provided by Dr. Lukkana Suksanpaisan [Imanis Life Science, Rochester, MN, USA]) encoding, under the control of the spleen focus-forming virus promoter, a codon-
 5 optimized SLAMF1 molecule from *Canis lupus familiaris* (GenBank NP_001003084.1) with an N-terminal FLAG-tag sequence (DYKDDDD (SEQ ID NO: 28)). Cells were maintained in DMEM supplemented with 5% FBS. The Chinese hamster ovary (CHO) cell line, CHO-CD46 cells, CHO-hSLAMF1 cells, CHO-dogSLAMF1 cells, CHO-NECTIN4 cells, CHO- α HIS cells, CHO-CD38 cells and the human myeloma cell line
 10 U266.B1 (kindly provided to us by Dr. David Dingli [Mayo Clinic, Rochester, MN]) were grown in RPMI 1640 medium supplemented with 10% FBS. Cells were incubated at 37 °C in 5% CO₂ with saturating humidity.

Plasmids and construction of full-genome recombinant measles virus (MeV)

15 To generate canine distemper virus (CDV) SPA.Madrid/22458/16 expression plasmids, total RNA was extracted from CDV SPA.Madrid/22458/16 isolate–infected Vero/dog SLAMF1 cells (passage 1) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Both the CDV-hemagglutinin (H) and CDV-fusion (F) genes were reverse transcribed with SuperScript III Reverse Transcriptase (Cat. # 11752050, Thermo Fisher
 20 Scientific) and amplified by PCR with the following primers: CDVH7050(+): 5'-AGAAAACTTAGGGCTCAGGTAGTCC;-3' (SEQ ID NO: 22) CDVH8949(-): 5'-TCGTCTGTAAGGGATTTCTCACC-3' (SEQ ID NO: 23); CDVF4857(+): 5'-AGGACATAGCAAGCCAACAGG-3' (SEQ ID NO: 24) and CDVH7050(-): 5'-GGACTACCTGAGCCCTAAGTTTTCT-3' (SEQ ID NO: 25). PCR products were
 25 sequenced directly by Sanger sequencing (Genewiz, Plainfield NJ, USA) and cloned into the pJET1.2 vector (Thermo Fisher Scientific). Next, the CDV-H open reading frame was PCR amplified with a forward primer (5'-CCG GTA GTT AAT TAA AAC TTA GGG TGC AAG ATC ATC GAT AAT GCT CTC CTA CCA AGA TAA GGT G-3'(SEQ ID NO: 26)) and a reverse primer (5'-CTA TTT CAC ACT AGT GGG TAT GCC TGA TGT CTG GGT GAC ATC ATG TGA TTG GTT CAC TAG CAG CCT CAA GGT TTT
 30 GAA CGG TTA CAG GAG-3' (SEQ ID NO: 27)) and cloned into a PacI and SpeI-restricted (New England Biolabs, Ipswich, MA, USA) pCG

by using the unique MluI/AatII restriction sites. Rescue of rMeVs was carried out employing the START system (Nakamura *et al.*, *Nat. Biotechnol.*, 23(2):209-14 (2005)).

Expression of recombinant proteins

5 A plasmid encoding the CD46-Fc fusion protein was produced by fusing the CD46 ectodomain (residues 35-328) with the Fc domain of IgG1 (pfc1-hg1e3; InvivoGen, San Diego, CA, USA). The scFvs K1, K2, and A09 were designed with the VL and VH sequences separated by a GSSGGSSSG flexible linker (SEQ ID NO: 29), codon-optimized, synthesized and cloned into pUC57-Kan (GenScript). A fourth scFv
10 (N1E) was designed with the VH and VL sequences separated by an SSGGGGS linker (SEQ ID NO: 30), codon-optimized, synthesized by Creative Biolabs (Shirley, NY) and cloned into pCDNA3.1+ (Invitrogen). For the IgG constructs, scFvs were cloned into the unique AgeI and KpnI sites of pHL-FcHIS (Cat.# 99846, Addgene, Cambridge, MA, USA), harboring the coding sequence for a secretion signal and a C-terminal human Fc
15 region followed by a 6× HIS-tag (SEQ ID NO: 21). The recombinant proteins were expressed by transfecting Expi293F suspension cells (Thermo Fisher) in serum-free Expi293 expression medium (Thermo Fisher) in shaker flasks following the manufacturer's instructions. The culture supernatants containing the recombinant proteins were collected and passed through a Protein G chromatography cartridge (Cat.#
20 89926, ThermoFisher). Bound recombinant proteins were eluted with 0.1 M glycine (pH 2.0), followed by immediate neutralization with 1 M Tris (pH 8.0), and the isolated proteins were concentrated with an Amicon Ultra centrifugal concentrator (Millipore Sigma, Burlington, MA, USA). CD46 and NECTIN4 were released from the Fc region by incubation with HRV 3C Protease (Thermo Fisher) at a 1:200 ratio. A final
25 purification step was performed using a Superdex 75 10/300 gel filtration column (GE Healthcare) equilibrated in phosphate-buffered saline (PBS). Protein concentrations were calculated from the protein extinction coefficient as determined from the amino acid composition.

30 *Fusion assays*

Cells were transfected using Fugene HD (PROMEGA, Fitchburg WI, USA) or TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison WI, USA). For a