Retargeted and Stealth-Modified Oncolytic Measles Viruses for Systemic Cancer Therapy in Measles Immune Patients

Eugene S. Bah, Rebecca A. Nace, Kah Whye Peng, Miguel Ángel Muñoz-Alía, and Stephen J. Russell

MOLECULAR CANCER THERAPEUTICS

ABSTRACT

Measles viruses (MV) are rapidly inactivated by anti-measles neutralizing antibodies, which has limited their clinical performance as oncolytic agents. Here, by substituting the H and F surface glycoproteins of MV with those from the homologous canine distemper virus (CDV) and engineering the CDV H attachment protein to target EGFR or CD38, we generated a fully retargeted MV capable of resisting neutralization by measles-immune human serum. The resultant recombinant MVs encoding retargeted CDV envelope glycoproteins had similar growth kinetics as the control MV, showed the expected engineered receptor specificities for cell entry, intercellular fusion, and target cell killing, and were blind to native CDV receptors. In contrast to the control MV, recombinant MVs incorporating CDV F and H glycoproteins retained full infectivity when exposed to high concentrations of pooled measles-immune human serum. Comparing viruses bearing MV or CDV glycoproteins in the SKOV3ip.1 model, only the virus bearing an EGFR-retargeted CDV envelope glycoprotein complex was capable of limiting tumor growth and extending the survival in measles immune mice. MV, “stealthed” and retargeted using engineered CDV surface glycoproteins, may be a promising platform to advance for systemic cancer therapy in measles immune patients.

Introduction

Oncolytic virotherapy using engineered measles viruses (MV) derived from the Edmonston vaccine lineage is a promising experimental approach to the treatment of cancer (1). MV can be engineered to selectively infect, replicate in, and destroy tumor cells with minimal toxicity to normal cells (1, 2). Clinical trials using engineered MVs to treat a variety of human cancers (3) including nervous system (4, 5), hematologic (6–8), gynecologic (9, 10), and urothelial malignancies (11), have shown promising results.

A major barrier to the systemic deployment of MV as an anticancer agent is the high prevalence of anti-measles antibodies in the general population (6, 8, 12). Also, while dose-limiting toxicities have not yet been encountered in MV clinical trials (12), even with systemic MV doses as high as 10 (11) TCID50, the concern remains that the systemic deployment of genetically armed MVs could be toxic to noncancerous cells expressing natural MV receptors, SLAMF1 and NECTIN4. We have therefore, been working to engineer oncolytic MV whose receptor attachment and entry specificity are targeted to antigens overexpressed on the tumor cell surface, and which have been further “stealthed” to evade neutralization by preexisting anti-measles antibodies.

MV has two surface glycoproteins, the receptor-binding attachment protein, H, and its fusogenic partner, F, that together mediate virus entry and intercellular fusion between infected and uninfected cells expressing the native virus receptors. By a poorly understood mechanism, H receptor binding triggers the depolymerization of the F trimer leading to membrane fusion. We previously developed a versatile system to redirect the specificity of MV attachment and entry by fusing specificity determining polypeptide ligands to the carboxy terminus of H and mutating key residues to disrupt its interactions with the native receptors (13–15).

MV retargeted in this way were shown to escape neutralization by mAbs targeting the receptor binding sites on H (16, 17), but remained highly susceptible to neutralization by polyclonal anti-measles neutralizing antibodies in measles-immune serum (16). Because all MV neutralizing antibodies target H or F (18, 19), we chose to substitute these surface glycoproteins with the corresponding proteins from a homologous morbillivirus.

Canine distemper virus (CDV) is a morbillivirus endemic in domestic dogs. CDV and MV surface glycoproteins are structurally similar, but serologically distinct. A proof-of-concept study was previously reported for the substitution of MV F and H proteins with homologous CDV glycoproteins to circumvent antibody-mediated neutralization of MV (20–22). However, the previously reported viruses were not specific for clinically relevant tumor-selective receptors (21) and were not blinded for native receptor use, so could still infect nontargeted human cells via the human NECTIN4 receptor (23).

To generate fully retargeted chimeric MVs capable of escaping neutralization by measles-immune human serum, we here substituted the MV F and H genes with homologous CDV F and CDV H genes mutated to destroy native receptor interactions and retargeted via C-terminally fused single-chain antibody fragments (scFv), specific for clinically relevant receptors, EGFR or CD38, that have been previously well-characterized for specificity in the context of retargeted MV (13). These retargeted viruses show the expected reprogramming of their receptor tropisms and are selectively oncolytic for receptor-positive tumors, even in mice passively immunized with measles-immune antiserum. Fully retargeted chimeric MVs that evade measles neutralizing antibodies may be suitable for clinical translation in patients with measles-immune cancer.

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Materials and Methods

Cell culture

The African green monkey kidney cells (Vero), baby hamster kidney cells (BHK), human glioblastoma cell line (U87-MG), human embryonic kidney cells (HEK293), human Burkitt lymphoma Raji and Ramos cell lines, and Chinese hamster ovary (CHO) cells were purchased from ATCC and grown in the ATCC recommended media. ATCC authenticates cell lines using short tandem repeat DNA profiling. The modified cell lines: SKOV3ip.1-FLuc (24), CHO-NECTIN4 (25), CHO-EGFR (14), CHO-CD38 (26), CHO-dogSLAMtag (27), and Vero-γHIS (13) were cultured as described previously. The SKOV3ip.1-FLuc was authenticated using short tandem repeat DNA profiling, all other modified cell lines were not further authenticated. All cell lines were grown at 37°C in a 5% CO₂ humidified incubator. All cell lines were tested 48 hours after thawing for Mycoplasma contamination using a Universal Mycoplasma Detection Kit (ATCC; 30-1012K) and all cell lines routinely tested negative. Cells were not maintained in tissue culture for more than 20 passages.

Generation of expression plasmids and recombinant viruses.

CDV F from the 5804P wild-type strain (25) or the previously described field isolate. The modiﬁed cell lines were grown in the ATCC recom- mended media. ATCC authenticates cell lines using short tandem repeat DNA pro- filing. The modiﬁed cell lines: SKOV3ip.1-FLuc (24), CHO-NECTIN4 (25), CHO-EGFR (14), CHO-CD38 (26), CHO-dogSLAMtag (27), and Vero-γHIS (13) were cultured as described previously. The SKOV3ip.1-FLuc was authenticated using short tandem repeat DNA profiling, all other modified cell lines were not further authenticated. All cell lines were grown at 37°C in a 5% CO₂ humidified incubator. All cell lines were tested 48 hours after thawing for Mycoplasma contamination using a Universal Mycoplasma Detection Kit (ATCC; 30-1012K) and all cell lines routinely tested negative. Cells were not maintained in tissue culture for more than 20 passages.

To assess sensitivity of intercellular fusion to measles-immune human serum, 1 × 10⁶ CHO-CD38 cells in each well of a 96-well plate were cotransfected the next day with 50 ng each of either the pCG CDV F, pTN CDV Hαβ-CD38, and pCDNA3.1-eGFP, or the pCG-MV F, pTN MV HaalsCD38, and pCDNA3.1 GFP using SuperFect (Qiagen) according to the manufacturer’s instructions. Two hours after transfection, the transfection medium was replaced with culture medium containing 2-fold serial dilutions of heat-inactivated pooled measles-immune human antibody serum. Photographs were taken 48 hours after transfection under a fluorescence microscope at 100× magnification.

Western blot analysis.

To determine expression of engineered CDV H proteins, HEK293 cells (2 × 10⁶/well in 6-well plate) were transfected with 2 µg of the corresponding CDV H plasmids. Forty-eight hours later, cells were washed with PBS, lysed with RIPA buffer for 30 minutes at 4°C, and centrifuged at 13,000 rpm for 20 minutes at 4°C to clarify the lysate. Lysate (10 µL) was mixed with an equal volume of 2 × Laemmli Buffer (Bio-Rad), boiled at 95°C for 5 minutes, fractionated on a 7.5% Tris-HCl gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting to detect the CDV H was performed as described previously (22) using the following antibody dilutions: rabbit anti-CDV H cyt antibody (MC712, 1:5,000; ref. 21) kindly provided by Dr. Roberto Cattaneo (Mayo Clinic, Rochester, MN), horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Thermo Fisher Scientific; 1:10,000), and HRP-conjugated mouse anti-B-actin (Sigma-Aldrich, A3854; 1:25,000).

For immunoblot analysis of chimeric viruses, 2 × 10⁵ TCID₅₀ of each virus stock was directly mixed with an equal volume of denaturing loading buffer, boiled at 95°C for 5 minutes, and fractionated on a 7.5% gel. Proteins were transferred to a PVDF membrane and immunoblot- ted in the standard fashion using primary antibodies: a rabbit antibody against MV N (N12) and rabbit antibodies directed against the cytoplasmic tails of CDV H (MC712; 1:2,000; ref. 21) or MV H (1:2,000; ref. 21; all kind gifts of Dr. Roberto Cattaneo); and an HRP-conjugated goat anti-rabbit (1:10,000) secondary antibody as described previously (22).

Virus growth kinetics.

Vero-γHIS cells (2 × 10⁵) in each well of a 6-well plate were infected with the respective viruses at an MOI = 0.03 for 2 hours in 1 mL Opti-MEM at 37°C. After which, the infection media were replaced with 2 mL/well of fresh growth media and the cells incubated at 37°C. Cells were harvested at the indicated times by scraping into 1 mL of growth media, subjected to three freeze–thaw cycles, centrifuged at 1,200 rpm for 10 minutes at 4°C, and viral titers in the supernatant determined on Vero-γHIS cells. The experiment was repeated three times.

Flow cytometry.

The surface expression of EGFR and CD38 on human tumor cell lines was performed as described previously (13) using an Alexa Fluor647–conjugated anti-human EGFR (matuzumab) mAb (R&D Systems; FAB10023R) and FITC-conjugated Mouse anti-Human CD38 (BD Biosciences; catalog no., 555459), respectively.

In vitro infection, virus neutralization, and cell killing.

Adherent cells (10⁴) or 10⁵ suspension cells were infected in one well of a 96-well plate at an MOI of 0.5 (titers determined on Vero-γHIS cells) in 50 µL Opti-MEM for 3 hours at 37°C before the addition of complete growth medium. Forty-eight hours after infection, cells...
were examined and photographed under a fluorescence microscope at 100× magnification.

For the neutralization assay, 30 infectious virus units of each virus were incubated with serial dilutions of heat-inactivated pooled anti-body human serum (Valley Biomedical, Inc. Lot #C80553) in Opti-MEM at 37°C in quadruplicates for an hour. The mix was then used to infect a 90% confluent monolayer of Vero-aHis cells in a 96-well plate for 2 hours at 37°C. After the infection, fresh culture medium was added, and the cells were cultured at 37°C. Seventy-two hours later, wells were observed for the appearance of viral cytopathic effect (CPE). The reciprocal of the dilution at which there was no viral CPE in all four wells for each virus was reported as the neutralization titer. The experiment was repeated two times.

For in vitro cytotoxicity, 1 × 10^6 SKOV3ip.1-Fluc cells in each well of 96-well plate were infected with the respective viruses in quadruplicates in Opti-MEM media for 3 hours at an MOI of 10 and cultured at 37°C in culture medium. Seventy-two hours later, cytotoxicity was determined by MTT Assay (Promega) according to the manufacturer’s instructions.

In vivo experiments.

All animal experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and performed in accordance with IACUC-established guidelines. For all experiments, we used female 6- to 8-week-old athymic nude mice (Taconic).

To assess specificity of infection in vivo, 5 × 10^6 cells in 200 μL PBS were implanted intraperitoneally, followed 10 days later by six intraperitoneal virus treatments of 2 × 10^6 TCID50 (200 μL) MVCDVenv-EGFR or MVCDVenv-CD38 (n = 4/group) or control treatment (200 μL of Vero-aHis cells lysate; n = 3) every other day. To compare...
efficacy of MV<sup>EGRF</sup> with MV<sup>CDVenvEGRF</sup> 5 × 10<sup>6</sup> cells in 200 µL PBS were implanted intraperitoneally, followed 10 days later by a single treatment of 200 µL control (Vero-αHis cells lysate) or 2 × 10<sup>5</sup> TCID<sub>50</sub> of MV<sup>EGRF</sup> or MV<sup>CDVenvEGRF</sup> (n = 5/group). For the immune evasion studies, 2.5 × 10<sup>6</sup> SKOV3ip.1-Fluc cells were implanted into the peritoneal cavity of athymic nude mice (n = 10/group), followed 6 days later by passive immunization of animals in the serum group as described previously (24). For these experiments, we used 200 µL of pooled human measles-immune serum (Valley Biomedical, Inc; Lot #CB0553). Three hours following passive immunization, animals were treated with a single intraperitoneal injection of 200 µL control treatment (Vero-αHis cells lysate) or 2 × 10<sup>5</sup> TCID<sub>50</sub> of MV<sup>EGRF</sup> or MV<sup>CDVenvEGRF</sup>. Tumor burden was monitored by weekly luminescence Imaging (IVIS Xenogen; Perkins) and total body luminescence was quantified from these images using the Live Imaging Software (IVIS Xenogen; Perkins) according to the manufacturer’s protocol. Animals were euthanized when they developed ascites. For the subcutaneous model, 5 × 10<sup>6</sup> SKOV3ip.1-Fluc cells were implanted in 100 µL PBS were implanted subcutaneously in the right flank and treated with six intratumoral injections of 100 µL control treatment (Vero-αHis cells lysate; n = 3) or 1 × 10<sup>5</sup> TCID<sub>50</sub> of MV<sup>EGRF</sup> or MV<sup>CDVenvEGRF</sup> (n = 4/group) every other day. Tumor volume was monitored by caliper measurements and animals were sacrificed if they met euthanasia criteria. Tumor volume was calculated by using the formula: 0.5 × length × width × width.

**Statistical analysis**

Statistical analyses and calculations were performed with the GraphPad Prism Software (GraphPad). We used the Kaplan–Meier method to graph the survival of treated animals, and the log-rank test to compare the differences in the survival curves. Differences in subcutaneous tumor volumes and total body luminescence were assessed by two-way ANOVA with Turkey multiple comparison test to correct for multiple comparisons. Unpaired Student t test was used to compare differences between two groups. A P<0.05 was considered to be statistically significant for all analyses.

**Results**

**Retargeting CDV H to EGFR and CD38**

Because the vaccine strains of CDV have evolved to use other uncharacterized receptor(s) in addition to the known CDV receptors (28), we focused our engineering efforts on a wild-type CDV H. The CDV H coding sequence of the wild-type strain (5804P; ref. 29) or the previously engineered receptor blind variant (CDV H #8; ref. 30) were cloned as shown in Fig. 1A into an expression vector with or without C-terminally fused scFvs targeting EGRF or CD38 to generate expression plasmids for CDV H, CDV H-CD38, CDV H<sub>Ha</sub>-CD38, and CDV H<sub>Ha</sub>-EGRF. These plasmids were transiently transfected into human embryonic kidney (HEK293) cells and the lysates were assessed for protein expression by Western blot analysis using an antibody directed against the cytoplasmic tail of CDV H protein (21). The immunoblot in Fig. 1B shows that all proteins were equally expressed and the scFv-tagged proteins (CDV H-CD38, CDV H<sub>Ha</sub>-CD38, and CDV H<sub>Ha</sub>-EGRF) had the expected approximately 25 kDa increase in molecular weight compared with the unmodified CDV H protein.

Next, to assess receptor specificity of the engineered CDV H proteins, we cotransfected a panel of CHO cells expressing the relevant receptors with each of the CDV H expression plasmids in combination with a CDV F expression plasmid. The readout for these experiments was syncytia formation resulting from intercellular fusion. As expected, we observed syncytia formation with the wild-type CDV H protein only in CHO cells expressing native CDV receptors canine SLAM or NECTIN4, and not in the parental CHO, CHO-EGRF, or CHO-CD38 cell lines (Fig. 1C). In contrast, the six mutations introduced into the CDV H receptor binding sites selectively eliminated native receptor–dependent fusion without impairing retargeted fusion via displayed ligands (compare the CDV H-CD38 row with the CDV H<sub>Ha</sub>-CD38 or CDV H<sub>Ha</sub>-EGRF rows). After confirming full retargeting of CDV H, we next evaluated the impact of measles neutralizing antibodies on the intercellular fusion mediated by the engineered CDV envelope. As shown in Fig. 1E, intercellular fusion mediated by the retargeted CDV H-CD38 was resistant to neutralization by pooled measles-immune human serum even with serum dilutions as low as 1:10. In contrast, intercellular fusion mediated by MV H-CD38 cotransfected with the MV F protein in CHO-CD38 cells was strongly inhibited even at high serum dilutions.

Taken together, these data demonstrate that wild-type CDV H can be fully retargeted and that intercellular fusion mediated by retargeted CDV H in concert with CDV F is resistant to neutralization by antibodies present in measles-immune human serum.

**Generation and in vitro characterization of chimeric MV incorporating retargeted CDV envelope glycoproteins (MV<sup>CDVenv</sup>)**

To generate fully retargeted chimeric MV bearing retargeted CDV envelopes, we substituted the MV F and H genes into the MV Moraten vaccine (vac2) genome (with an eGFP cisron inserted upstream of the P gene; ref. 22) with the respective CDV F and CDV H<sup>EGRF</sup> or CDV H<sub>Ha</sub>-EGRF or CDV H<sub>Ha</sub>-CD38 protein-encoding genes. The resulting viruses were named MV<sup>CDVenv-EGRF</sup> and MV<sup>CDVenv-CD38</sup>, respectively. As a control, we also generated MV<sup>EGRF</sup>, in which the MV H gene was replaced by a previously engineered sequence encoding a native, receptor blind MV H protein displaying a C-terminal scFv against EGRF (Haals-EGRF; Fig. 2A). All three viruses were rescued and propagated using the six-histidine tag rescue (STAR) system (13) and the incorporation of chimeric envelopes was confirmed by immunoblot analysis (Fig. 2B). All the retargeted viruses exhibited slower growth kinetics compared with unmodified MV-Moraten, but compared with one another had similar growth kinetics on the Vero-αHis cell line (Fig. 2C) and yielded stocks with similar maximum titers of 3 × 10<sup>8</sup> TCID<sub>50</sub>/mL for MV<sup>EGRF</sup>, 2 × 10<sup>7</sup> TCID<sub>50</sub>/mL for MV<sup>CDVenv-EGRF</sup>, and 6 × 10<sup>7</sup> TCID<sub>50</sub>/mL for MV<sup>CDVenv-CD38</sup>. In **in vitro** neutralization assays showed that the viruses bearing the retargeted CDV envelopes maintained their infectivity even in the presence of a high concentration (<1:10 dilution) of pooled measles-immune human serum. In contrast, the virus bearing the retargeted MV envelope was efficiently neutralized at serum dilutions as high as 1:320 (Fig. 2D). Collectively, these data demonstrate that chimeric MVs with the retargeted CDV envelope can be generated and propagated with the STAR pseudoreceptor system and that these viruses are resistant to neutralization in **in vitro** by measles-immune serum.

**Receptor specificity of chimeric MV bearing retargeted CDV envelopes**

To assess the receptor specificity of the chimeric-retargeted viruses, we infected the panel of CHO cells expressing the relevant receptors and monitored for GFP-expressing syncytia 48 hours after virus exposure as readout for infection. As shown in Fig. 3A, all chimeric viruses exhibited the expected receptor specificities. None
infected the parental CHO cells, whereas all viruses infected the CHO-αHis cell line via the interaction between their displayed His tag and the αHIs pseudoreceptor. MV<sup>EGFR</sup> and MV<sup>CDVenv-EGFR</sup> specifically infected CHO-EGFR cells with no discernable infection in CHO-CD38 cells, while MV<sup>CDVenv-CD38</sup> specifically infected the CHO-CD38 cells without infecting the CHO-EGFR cells. None of the viruses infected CHO cells expressing native CDV receptors, SLAM or NECTIN4.

Entry specificity was further tested in a panel of four human tumor cell lines known to express either EGFR or CD38: the SKOV3ip.1 ovarian cancer and U87-MG brain tumor cell lines are positive for EGFR and negative for CD38, whereas the Raji and Ramos Burkitt lymphoma cell lines are positive for CD38 and negative for EGFR, as confirmed by flow cytometric staining (Fig. 3B). As expected, MV<sup>EGFR</sup> and MV<sup>CDVenv-EGFR</sup> infected only the EGFR-positive tumor cell lines, while MV<sup>CDVenv-CD38</sup> infected only the CD38-positive tumor cell lines (Fig. 3C). Receptor-dependent killing of SKOV3ip.1 tumor cells by the retargeted viruses was evaluated using an MTT assay as readout for cell viability 72 hours after infection. Consistent with the cell infectivity data, only the EGFR-retargeted viruses significantly diminished cell viability (Fig. 3D), indicating that EGFR-dependent entry and intercellular fusion translates to receptor-dependent cell killing.

**In vivo specificity and oncolytic activity of chimeric viruses in an EGFR-positive ovarian cancer tumor model**

To evaluate the in vivo specificity and oncolytic activity of the retargeted chimeric viruses, we utilized the EGFR-overexpressing SKOV3ip.1-Fluc orthotopic ovarian cancer xenograft tumor model (24, 31, 32). SKOV3ip.1-Fluc cells express luciferase, which allows for noninvasive monitoring of tumor burden by Bioluminescence Imaging (BLI). To assess for specificity of infection and oncolytic efficacy in vivo, SKOV3ip.1Fluc cells were implanted into the peritoneal cavity in athymic mice to develop a model of disseminated carcinomatosis peritonei, followed 10 days later by six intraperitoneal treatments of 2 × 10<sup>6</sup> TCID<sub>50</sub> of MV<sup>CDVenv-EGFR</sup>, MV<sup>CDVenv-CD38</sup>, or an equivalent volume of control cell lysate (from the producer Vero-αHis cell line), given every other day. Figure 4A shows that only the MV<sup>CDVenv-EGFR</sup> virus was able to control tumor growth, as demonstrated by a marked reduction in the tumor luminescence signal in this treatment group compared with the control treatment groups. Control of tumor growth was associated with significant prolongation in survival of animals in the MV<sup>CDVenv-EGFR</sup> treatment group compared with the MV<sup>CDVenv-CD38</sup> or control groups (median survival of 54 vs. 30 days; P = 0.01; Fig. 4B), indicating that the specificity of infection observed in vitro for the MV<sup>CDVenv-EGFR</sup> translates to specific oncolytic efficacy in vivo.

### Table A

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<th>Membrane</th>
<th>Recombinant</th>
<th>Pseudotype</th>
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### Figure 2

**Generation and in vitro characterization of chimeric MV bearing the CDV F and CDV H retargeted to EGFR and CD38 glycoproteins.**

A, Schematic representation of recombinant MV genome constructs. The MV F and MV H retargeted to EGFR genes in the MV vac2 antigenome were substituted with the homologous CDV F and either CDV<sub>HRB</sub>-EGFR or CDV<sub>HRB</sub>-CD38. There is an additional gene encoding for the eGFP inserted after the P gene to facilitate visualization of viruses during rescue, propagation, and in in vitro experiments. B, Western blot analysis of viral stocks (1 × 10<sup>7</sup> TCID<sub>50</sub>) using antibodies specific for MV N (αMV N), the cytoplasmic tail of CDV H (αCDV Hcyt), or the cytoplasmic tail of MV H (αMV Hcyt) demonstrated that engineered retargeted CDV H glycoproteins were incorporated into the chimeric viruses. C, Multi-step growth curves on Vero-αHis cells showed that the newly generated chimeric viruses had similar growth kinetics as the control MV<sup>EGFR</sup> virus. D, Neutralization assays using pooled measles-immune human serum showed that the retargeted chimeric viruses with the CDV envelope were less susceptible to neutralization. Viruses were exposed to 2-fold serial dilutions of pooled measles-immune serum prior to infecting Vero-αHis cells. The reciprocal of the lowest antibody dilution at which there was full viral CPE in two independent experiments was reported as the neutralization titer.

**Reciprocal of neutralization titre**

- MV<sup>EGFR</sup>: <10
- MV<sup>CDVenv-EGFR</sup>: 16
- MV<sup>CDVenv-CD38</sup>: 64
- MV<sup>CDVenv-EGFR</sup> treatment group compared with the MV<sup>CDVenv-CD38</sup> control group (median survival of 54 vs. 30 days; P = 0.01; Fig. 4B), indicating that the specificity of infection observed in vitro for the MV<sup>CDVenv-EGFR</sup> translates to specific oncolytic efficacy in vivo.

![Image](https://example.com/image.png)
Comparing the oncolytic activity of MVEGFR versus MVCDVenv-EGFR

To directly compare the oncolytic activities of the MVCDVenv-EGFR and MVEGFR viruses, we used both subcutaneous and intraperitoneal SKOV3ip.1-Fluc tumor models. In the subcutaneous model, we assessed the effect of six intratumoral treatments of 1/2×10^6 TCID₅₀ of virus versus control cell lysate administered every other day starting 10 days after tumor implantation. The tumor volumes of subcutaneous tumors assessed by caliper measurements, shown in Fig. 5A, demonstrate that MVCDVenv-EGFR and MVEGFR were equally effective in this model, significantly impacting tumor volumes compared with control (P = 0.0019 for MVEGFR and P < 0.0001 compared with control) treatment. Tumor volumes (mean ± SD) were 27.5 ± 9.1 mm³, 23.4 ± 11.8 mm³, and 20.9 ± 2.5 mm³ before virus injection and 1,346.7 ± 527.6 mm³, 673.7 ± 76.6 mm³, and 9.8 ± 19.3 mm³, 30 days after the first virus treatment for control, MVEGFR, and MVCDVenv-EGFR, respectively. There was no statistical significance in tumor growth curves between the two virus treatments (P = 0.2234). Next, in the orthotopic SKO3ip.1-Fluc model, we compared the oncolytic activity of a single intraperitoneal virus treatment (2 × 10^6 TCID₅₀) versus control therapy 10 days after tumor cell implantation. Tumor burden was monitored weekly by luciferase imaging. As shown in Fig. 5B, the luciferase signal (i.e., tumor burden) increased more rapidly in the animals in the control group versus those in either virus treatment group. There was a statistically significant decrease in the whole-body luminescence of animals in the treatment groups compared with control-treated animals on day 14 (P = 0.0473 for MVEGFR and P = 0.0324 for MVCDVenv-EGFR compared with control), with no statistically significant difference in signal intensities between the two treatment groups on day 14 (P > 0.9999; Fig. 5C). This decrease in luminescence correlated with a statistically significant increase in median survival from 18 days for the control treatment to 45 days for the MVEGFR and 53 days for MVCDVenv-EGFR treatment groups (P = 0.0063 and P = 0.0015, respectively), with no difference in survival between the two treatment groups (P = 0.6217; Fig. 5D). Overall, these data show that MVCDVenv-EGFR is equipotent compared with the previously reported MVEGFR in an EGFR-overexpressing tumor model.

Figure 3.
Receptor specificity of chimeric MV bearing the retargeted CDV envelope in vitro. A, Chimeric MV bearing CDV F and CDV H retargeted to EGFR or CD38 maintains receptor-specific infectivity on a panel of CHO cell lines with the desired receptors. Cells were infected with the respective viruses at an MOI of 0.5 and photographed 48 hours later using a fluorescence microscope; magnification, 100×. B, Flow cytometry analysis of selected human tumor cell lines that express either EGFR or CD38 (filled histograms) stained with an antibody specific for EGFR (αEGFR) or CD38 (αCD38). Unstained controls are shown as unfilled histograms. C, Chimeric MV bearing CDV F and CDV H retargeted to EGFR or CD38 maintains receptor-specific infectivity in selected human tumor cell lines that express either EGFR or CD38. Cells were infected with the respective viruses at an MOI of 0.5 and photographed 48 hours later using a fluorescence microscope; magnification, 100×. D, In vitro cytotoxicity of retargeted chimeric viruses on the EGFR-overexpressing Sko3ip-Fluc tumor cell line showing that the specific infection by the viruses retargeted to EGFR leads to in vitro cytotoxicity. Cells were infected with respective viruses at an MOI of 10 and cytotoxicity was determined 72 hours later by an MTT assay. Error bars indicate SEM between two experiments; ns, not significant (***, P < 0.001).
In vivo oncolytic activity of chimeric viruses in measles immune mice bearing EGFR-positive SKOV3ip.1 tumors

To assess the impact of measles neutralizing antibodies on the oncolytic activity of MVEGFR and MVCDVenv-EGFR, athymic mice with established intraperitoneal SKOV3ip.1-Fluc tumor xenografts were passively immunized with measles-immune human antibody serum (60 EU/mouse) and treated 3 hours later with a single intraperitoneal virus dose of $2 \times 10^6$ TCID$_{50}$ (Fig. 6A). Tumor growth was monitored by serial BLI as shown in Fig. 6B with the corresponding quantification of total body luminescence graphed in Fig. 6C. As expected, both MVEGFR and MVCDVenv-EGFR reduced tumor burden ($P < 0.0001$) and prolonged the survival ($P < 0.0001$) of treated animals compared with control treatment in the absence of measles neutralizing antibodies. However, as anticipated, the efficacy of the MVEGFR was severely diminished in the presence of measles neutralizing antibodies, resulting in a statistically significant increase in the luminescence signal ($P < 0.0001$) and corresponding decrease in survival ($P < 0.0018$) for passively immunized mice compared with naive mice treated with MVEGFR. In contrast, the oncolytic activity of MVCDVenv-EGFR was maintained in the presence of neutralizing measles antibodies, with no statistically significant difference in tumor luminescence ($P = 0.7083$) or overall survival ($P = 0.3381$) for passively immunized mice compared with nonimmunized control mice treated with MVCDVenv-EGFR.

Together, these results demonstrate that MVCDVenv-EGFR retains full antitumor activity in vivo even in the presence of a high body fluid concentration of MV neutralizing antibodies.

Discussion

Here, we substituted the MV F and H genes with CDV F and CDV H retargeted to EGFR or CD38, and thereby generated fully retargeted oncolytic MVs capable of escaping neutralization by preexisting measles antibodies. MVCDVenv-EGFR was potently oncolytic in athymic mice bearing aggressive, intraperitoneally disseminated, EGFR-overexpressing, SKOV3ip.1 human ovarian cancer tumors, even after the mice had been passively immunized against MV using pooled human serum.

Ovarian cancer, the second most common cancer of the female genitourinary tract, is a lethal disease accounting for an estimated 13,980 number of deaths in the United States in 2019 (33). Intraperitoneal EGFR-targeted MV virotherapy is an interesting possibility for the treatment of this disease. Ovarian cancer often remains localized to the peritoneal cavity even in patients with relapsed and treatment refractory disease, and we, therefore, have been developing MV as a potential intraperitoneal therapy for this malignancy (9, 10, 24, 31). A recently completed phase I clinical trial of intraperitoneally
administered MV doubled the median overall survival of heavily pretreated, platinum-resistant ovarian cancer patients compared with historical controls (10), despite the fact that the enrolled patients had high titers of measles neutralizing antibodies in their blood and peritoneal fluid (10). Our preclinical studies have underscored the importance of circumventing antibody neutralization of MV to maximize the antitumor potency of the approach and, with this goal in mind, we are clinically evaluating the intraperitoneal administration of ex vivo–infected autologous mesenchymal stem cell carriers as an antibody-resistant virus delivery system (24).

In this study, we have pursued an alternative approach to circumvent antibody neutralization, using envelope glycoprotein exchange in conjunction with tropism engineering via surface display of single-chain antibodies (scFv). This study builds on previous reports of chimeric MV bearing CDV envelope glycoproteins (20–22). However, in contrast to previous studies, our current approach has clinical translational relevance because we have targeted the virus to EGFR, a clinically relevant ovarian cancer marker (34) also expressed at high levels in several other cancers (35, 36), and to CD38, a clinically relevant target in multiple myeloma (37). The scFvs we displayed in this study are derived from mAbs, EGFR (matuzumab) and CD38 (daratumumab), that have already been well-characterized for specificity and toxicity in humans, as well as in the context of retargeted MV (13). We elected here to substitute measles H with a wild-type CDV H (5804P) because of its narrower range of receptor use compared with the H proteins from CDV vaccine strains (28). While the mutations (D526A, I527S, S528A, R529A, Y547A, and T548A) we introduced into the CDV H protein were initially reported to ablate only SLAM-dependent fusion (38), we have confirmed here (Fig. 1C), as others have shown (38), that these mutations also ablate NECTIN4-dependent fusion making our vector completely “blind” to native pathogenicity-determining CDV receptors. Given the similarity of the CDV and MV envelopes, we anticipate that there will be little limitation to the diversity of tumor-associated surface antigens that can be targeted using these chimeric viruses (15).

MV does not replicate efficiently in murine cells (39), because of, as yet, undefined intracellular barriers to the viral replication cycle, independent of receptor expression. Thus, we did not test our viruses in syngeneic immunocompetent tumor models. Instead, we used the extensively characterized intraperitoneal SKOV3ip.1-Fluc tumor model in athymic mice, in which we could adoptively transfer measles-immune human serum. Therefore, we did not assess the potential contributions of the other compartments of the immune system to the antitumor efficacy of this new vector. However, data previously generated in the SKOV3ip.1 model were deemed sufficiently informative to justify initial clinical testing of MV in ovarian cancer, and the measles antibody titers achieved by passive immunization in our efficacy studies are comparable with the ascites concentrations present in patients with ovarian cancer (24). Clinical testing of the viruses described in this article may therefore be warranted.

Figure 5.
Comparison of the oncolytic efficacy of the EGFR-retargeted viruses in the EGFR-positive ovarian cancer tumor model. A, MVEGFR and MVCDVenvEGFR have similar oncolytic efficacy in a subcutaneous tumor model. Tumor volumes of subcutaneously implanted SKOV3ip.1-Fluc cells treated with six intratumoral doses of $2 \times 10^6$ TCID$_{50}$ of the respective viruses or control (producer Vero-cells lysate). B–D, MVEGFR and MVCDVenvEGFR have similar oncolytic efficacy in an orthotopic, intraperitoneal model. B, Serial BLI on the indicated days to monitor tumor burden of mice with intraperitoneal SKOV3ip.1-Fluc tumors treated with $2 \times 10^6$ TCID$_{50}$ of indicated virus treatment or control ($n = 5$ per group) 10 days after tumor implantation. C, Quantification of total body luminescence (tumor burden) on day 14 after treatment; error bars indicate SEM. D, Overall survival of treated animals. ns, not significant (*, $P < 0.05$).
It is worth noting that despite the high doses of virus used in our ovarian cancer studies, neither MVCDVenv-EGFR or MVEGFR therapy was curative. Treated animals eventually succumbed to progressive intraperitoneal disease and were typically euthanized because of ascites formation. Examination of explanted peritoneal tumors from virus-treated animals under fluorescence light showed strong expression of GFP indicating that these tumors were persistently infected with the virus. The presence of an intact T-cell immune response might facilitate clearance of these virus-infected cells, and this will be tested in future adoptive T-cell transfer experiments, which may also facilitate the evaluation of immunotherapy combination approaches such as MV virotherapy plus checkpoint inhibition to achieve more durable cures (40). While this study focused exclusively on the SKOV3ip.1 ovarian cancer model, we do plan to investigate the systemic administration of MVCDVenv-EGFR in other EGFR-overexpressing tumors such as lung cancers (35) and glioblastoma (36). Furthermore, MVCDVenv-CD38 will be evaluated in CD38-overexpressing models of multiple myeloma (26)

A critical parameter requiring further optimization for the effective clinical translation of the stealthed and retargeted MVs described in this article is to improve the virus manufacturing yield. While we were eventually able to generate viral stocks with titers as high as 10^8 TCID50/mL of the fully retargeted chimeric viruses for our in vivo animal experiments, retargeted MVs generally grow to much lower titers compared with MVs with native envelope glycoproteins, and are more challenging to propagate. Following their initial rescue, the viruses propagate very slowly, until they have been through several tissue culture passages. This could be related to the use of an artificial receptor (the αHis pseudoreceptor) on the cells that are used for virus amplification, or to altered kinetics of virus maturation in infected cells. Significant further optimization of virus yield may be achievable using alternative cell substrates (41), alternative receptors for virus propagation, or by virus engineering, perhaps through cytoplasmic tail truncations of the surface glycoproteins (42) or substitution of the M gene (20, 43). An alternative approach to bypass the MV manufacturing difficulty might be to incorporate the targeted CDV envelope glycoprotein complexes into other viral platforms such as adenovirus (14) or VSV (44, 45) that are easier to manufacture at high titer.

By design, we used the Edmonston lineage measles vaccine backbone with a remarkable safety record both as a vaccine and oncolytic agent in clinical trials to construct our viruses. The genome of this backbone has remained stable, with no reversions to pathogenicity reported to date, in the past 50+ years since routine childhood measles

Figure 6. Impact of preexisting measles neutralizing antibodies on the oncolytic activity of EGFR-retargeted viruses. MVCDVenvEGFR maintained oncolytic efficacy in the presence of preexisting anti-measles neutralizing antibodies. A, Schematic of experimental design. Athymic mice with intraperitoneal SKOV3ip.1 Fluc tumors were treated with a single intraperitoneal injection of 2 x 10^5 TCID50 of MVEGFR, MVCDVenvEGFR, or 200 μL control (producer Vero-αHis lysate), with or without 200 μL (60 EU) of pooled human measles-immune neutralizing serum added 3 hours before virus treatment (n = 10 mice/group). Representative (5 animals/group) images (B), quantitation of serial BLI (C), and overall survival of treated animals (D; *, P < 0.05; **, P < 0.0001; ns, not significant).
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vaccination was introduced. Because there are more than 40 different attenuating mutations dispersed all over the genome, of which mutations in the innate immune antagonizing P/V/C gene are the most important (45), we retained all of the core MV proteins, to ensure that our viruses would be safe and not cause disease in humans. Moreover, these core proteins harbor T-cell epitopes that could enhance the antitumor effect of MV by eliminating residual virus-infected tumor cells (47). While wild-type CDV can cause severe disease in dogs (48) and has been reported capable of pathogenicity in other species including nonhuman primates (49), the viruses described in this article do not use any of the natural virus receptors responsible for CDV pathogenicity (48) and are fully retargeted to receptors that are not compatible with normal virus biology (50). Moreover, most pet dogs are vaccinated against CDV. Of course, additional extensive formal toxicology studies in consultation with the FDA will be needed before any human clinical trials can be planned.

In summary, envelope glycoprotein exchange between MV and CDV allowed us to generate chimeric MVs retargeted to EGFR or CD38 that were resistant to neutralization by anti-measles antibodies in serum from measles immune human subjects. As such, these viruses may warrant clinical advancement for the treatment of measles immune patients with disseminated malignancies overexpressing EGFR or CD38. Furthermore, the platform might be suitable for generating additional “stealthed” and retargeted MV tailored for the treatment of a variety of human cancers.

Disclosure of Potential Conflicts of Interest

K.W. Peng reports being a cofounder and officer of Vyriad, outside the submitted work, as well as technology licensed to Vyriad. M.A. Muñoz-Alia reports lecture fees paid by a commercial entity (honoriaz). S.J. Russell reports being a cofounder and officer of Vyriad, and Mayo Clinic holds equity in Vyriad. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

E.S. Bah: Conceptualization, data curation, formal analysis, investigation, methodology, writing-original draft, writing-review and editing. R.A. Nace: Resources, investigation, methodology, writing-review and editing. K.W. Peng: Resources, writing-review and editing. M.A. Muñoz-Alia: Conceptualization, data curation, supervision, investigation, methodology, writing-review and editing. S.J. Russell: Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing-original draft, project administration, writing-review and editing.

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Reference


Retargeted and Stealth-Modified Oncolytic Measles Viruses for Systemic Cancer Therapy in Measles Immune Patients

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