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

Eugene S. Bah[1], Rebecca A. Nace[2], Kah Whye Peng[2], Miguel Ángel Muñoz-Alía[2,*], Stephen J. Russell[2,3,*]

[1.]Medical Scientist Training Program, Mayo Clinic Alix School of Medicine

[2.]Department of Molecular Medicine, Mayo Clinic Rochester

[3.]Division of Hematology, Mayo Clinic Rochester, MN, USA

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 EGF receptor 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.

Keywords

*Co-Corresponding authors: Miguel Ángel Muñoz-Alía, Department of Molecular Medicine, Mayo Clinic, 200 First St SW, Rochester, MN 55905, USA. alia.miguel@mayo.edu Stephen J. Russell, Department of Molecular Medicine, Mayo Clinic, 200 First St SW, Rochester, MN 55905, USA. sjr@mayo.edu (S.J.R).

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] hematological[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 $10^{11}TCID_{50}$, 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 is targeted to antigens overexpressed on the tumor cell surface, and which have been further "stealthed" to evade neutralization by pre-existing 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].

MVs retargeted in this way were shown to escape neutralization by monoclonal antibodies 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]. Since 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 measles-immune cancer patients.

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's lymphoma Raji and Ramos cell lines, and Chinese hamster ovary (CHO) cells were purchased from American Type Tissue Culture collection (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] and CHO-NECTIN4 [25] CHO-αHIS[13], CHO-EGFR[14], CHO-CD38[26], CHO-dogSLAMtag[27] and Vero-αHIS[13] were cultured as previously described. The SKOV3ip.1-FLuc was authenticated using short tandem repeat DNA profiling, all other modified cell lines were not further authenticated. All cells were grown at 37 °C in a 5% CO_2 humidified incubator. All cell lines were tested 48 hours post 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 H from the 5804P wild type strain [25] or the previously generated SLAM Blind construct [26], were inserted between the PacI/SpeI restriction sites in the pCG vector or the PacI/SfiI restriction sites pTN vector bearing the respective scFvs[13] by ligation cloning to generate pCG CDV H, pTN CDV H -CD38, pTN CDV H_{RB}-CD38 and pTN CDV H_{RB}-EGFR expression plasmids(Fig.1A) The pCG CDV F plasmid was generated by excising out the MV F gene in pCG-MV F with HpaI and PacI and ligating an in-frame CDV F DNA coding sequence PCR amplified from a CDV field isolate. Recombinant viruses were generated by replacing MV F and H genes in the MV Moraten vaccine antigenome [22] (with an additional transcription unit after the P gene encoding for enhanced Green Fluorescence protein (eGFP) to facilitate visualization of virus during rescue) with the NarI/PacI CDV F gene fragment (from pCG-CDVF) and the PacI/SpeI CDVH$_{RB}$ EGFR or CDVH$_{RB}$-CD38 fragments (from pTN CDVH$_{RB}$ EGFR and pTN CDVH$_{RB}$-CD38, respectively), in compliance with the rule that recombinant MV genomes are hexametric. The sequences of all generated plasmids were verified by sanger sequencing. Viruses were rescued as described previously[22] and grown on Vero-αHis cells at 32 °C.

Approval to generate MV that evade neutralization was obtained from the Mayo Clinic Institutional Biosafety Committee (IBC) before initiating this project.

Measles immune human serum: The pooled human immune-measles AB serum used for all experiments was obtained from Valley Biomedical Inc. (Lot #C80553). This serum

has anti-MV IgG of 300 EU/mL and titer determined to be 256 by plaque reduction neutralization titer (PRN)[24]. Serum dilutions were made using Opti-MEM or cell culture medium.

Intercellular fusion assay: 1×10^4 cells seeded in each well of a 96 well tissue culture plate (Corning) were transfected the next day with 50ng each of pCG CDV F and the indicated engineered CDV H plasmid using FugeneHD (Promega) according to the manufacturer's instructions. 48 hours post transfection, cells were washed once with PBS, stained with 1% (weight/vol.) crystal violet solution and imaged with a light microscope at 100X magnification.

To assess sensitivity of intercellular fusion to measles immune human serum, 1×10^4 CHO-CD38 cells in each well of a 96 well plate were co-transfected the next day with 50ng each of either the pCG CDV F, pTN CDV H_{RB}-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 two-fold serial dilutions of heat inactivated pooled measles immune human AB serum. Photographs were taken 48 hours post transfection under a fluorescence microscope at 100X magnification.

Western Blot: To determine expression of engineered CDV H proteins, HEK293 cells (2×10^6/well in 6 well plate) were transfected with 2μg of the corresponding CDV H plasmids. 48 hours later, cells were washed with PBS, lysed with RIPA buffer for 30 mins at 4ºC, and centrifuged at 13000 RPM for 20 mins at 4ºC to clarify the lysate. 10 μL of lysate was mixed with an equal volume of 2X Laemmli buffer (Biorad), boiled at 95ºC for 5 mins, fractionated on a 7.5% Tris-HCl gel and transferred to a PVDF membrane. Immunoblotting to detect the CDV H was performed as previously described[22] using the following antibody dilutions: rabbit anti-CDV H cyt antibody (MC712, 1:5000)[21] kindly provided by Dr. Roberto Cattaneo, horse radish peroxidase (HRP)-conjugated goat anti-rabbit (Thermo Fisher Scientific; 1:10000), and HRP- conjugated mouse anti-B-actin (Sigma-Aldrich, A3854; 1:25000).

For immunoblot analysis of chimeric viruses, 2×10^5 $TCID_{50}$ of each virus stock was directly mixed with an equal volume of denaturing loading buffer, boiled at 95ºC for 5 mins and fractionated on a 7.5% gel. Proteins were transferred to a PVDF membrane and immunoblotted in the standard fashion using a rabbit antibody against MV N (N12), rabbit antibodies directed against the cytoplasmic tails of CDV H (MC712; 1:2000)[21] or MV H (1:2000)[21], (all kind gifts of Dr. Roberto Cattaneo) primary antibodies; and HRP-conjugated goat anti-rabbit (1:10000) secondary antibody as previously described[22]

Virus growth kinetics: 2×10^5 Vero-αHis cells in each well of a 6 well plate were infected with the respective viruses at an MOI of 0.03 for 2 hours in 1 mL optiMEM at 37ºC. After which, the infection media was replaced with 2 ml / well of fresh growth media and the cells incubated at 37ºC. Cells were harvested at the indicated times by scraping into 1mL of growth media, subjected to 3 freeze-thaw cycles, centrifuged at 1200RPM for 10

mins at 4ºC and viral titers in the supernatant determined on Vero-αHis. The experiment was repeated 3 times.

Flow cytometry: The surface expression of EGFR and CD38 on human tumor cell lines was performed as previously described[13] using an Alexa Fluor647-conjugated anti-human EGFR (matuzumab) Mab (R&D systems; FAB10023R) and FITC-conjugated Mouse anti-Human CD38 (BD Biosciences; Cat: 555459), respectively.

In vitro infection, virus neutralization, and cell killing: 10^4 adherent cells or 10^5 suspension cells were infected in one well of a 96 well plate at an MOI of 0.5 (titers determined on Vero-αHis cells) in 50 μL Opti-MEM for 3 hours at 37°C before the addition of complete growth medium. 48 hours after infection, cells were examined and photographed under a fluorescence microscope at 100X magnification.

For the neutralization assay, 30 infectious virus units of each virus was incubated with serial dilutions of heat inactivated pooled AB human serum (Valley Biomedical, Inc. Lot # C80553) in Opti-MEM at 37°C in quadruplicates for hour. The mix was then used to infect a 90% confluent monolayer of Vero-αHis cells in a 96 well plate for 2 hours at 37°C. After the infection, fresh culture medium was added, and the cells cultured at 37°C. 72 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 4 wells for each virus is reported as the neutralization titer. The experiment was repeated 2 times.

For in vitro cytotoxicity, 1×10^4 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. 72 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 6–8 week old athymic nude mice (Taconic).

To assess specificity of infection *in vivo* 5×10^6 cells in 200μL PBS were implanted i.p. followed 10 days later by 6 i.p. virus treatments of 2×10^6 TCID$_{50}$ (200 μL) MV$^{CDVenv-EGFR}$ or MV$^{CDVenv-CD38}$ (n=4/group) or control treatment (200 μL of Vero-αHis cells lysate; n=3) every other day. To compare efficacy of MV^{EGFR} to MV^{CDVenvEGFR} 5×10^6 cells in 200μL PBS were implanted i.p. followed 10 days later by a single treatment of 200 μL control (Vero-αHis cells lysate) or 2×10^6 TCID$_{50}$ of MV^{EGFR} or MV^{CDVenvEGFR} (n=5/group). For the immune evasion studies, 2.5×10^6 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 previously described[24]. For these experiments we used 200 μL of pooled human measles immune serum (Valley Biomedical, Inc; Lot # C80553). Three hours following passive immunization, animals were treated with a single intraperitoneal injection of 200 μL control treatment (Vero-αHis cells lysate) or 2×10^6 TCID$_{50}$ of MV^{EGFR} or MV^{CDVenvEGFR}. 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, 5×10^6 SKOV3ip.1-Fluc cells in 100 μL PBS were implanted subcutaneously in the right flank and treated with 6 intratumoral injections of 100 μL control treatment (Vero-αHis cells lysate; n=3) or 1×10^6 TCID$_{50}$ of MV^{EGFR} or MV^{CDVenvEGFR} (n=4/group) every other day. Tumor volume was monitored by caliper measurements and animals sacrificed if they met euthanasia criteria. Tumor volume was calculated by using the formula $0.5 \times$ length $\times$ width $\times$ width.

Statistical analysis

Statistical analyses and calculations were performed with the GraphPad Prism software (Graphpad Software, San Diego, CA). We used the Kaplan-Maier 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's multiple comparison test to correct for multiple comparisons. Unpaired Student's t test was used to compare differences between two groups. A $p < 0.05$ was considered to be statistically significant for all analyses.

Results:

1. Retargeting CDV H to EGFR and CD38

Since the vaccine strains of CDV have evolved to use other uncharacterized receptor(s) in addition to the known CDV receptors[30] we focused our engineering efforts on a wild type CDV H. The CDV H coding sequence of the wild type strain (5804P)[28] or the previously engineered receptor blind variant (CDV H $_{RB}$)[29] were cloned as shown in Fig. 1A into an expression vector with or without C-terminally fused scFvs targeting EGFR or CD38 to generate expression plasmids for CDV H, CDV H-CD38, CDV H$_{RB}$-CD38 and CDV H$_{RB}$-EGFR. These plasmids were transiently transfected into human embryonic kidney (HEK293) cells and the lysates assessed for protein expression by western blot 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$_{RB}$-CD38 and CDV H$_{RB}$-EGFR) had the expected ~25kDa increase in molecular weight compared to the unmodified CDV H protein.

Next, to assess receptor specificity of the engineered CDV H proteins, we co-transfected 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-EGFR, or CHO-CD38 cell lines (Fig. 1C). By 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 to the CDV H$_{RB}$-CD38 or CDV H$_{RB}$-EGFR 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 co-transfected 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.

2. Generation and *in vitro* characterization of chimeric MV incorporating retargeted CDV envelope glycoproteins (MV^{CDVenv})

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 cistron inserted upstream of the P gene)[22] with the respective CDV F and CDV H$_{RB}$-EGFR or CDV H$_{RB}$-CD38 protein-encoding genes. The resulting viruses were named MV$^{CDVenv-EGFR}$ and MV$^{CDVenv-CD38}$ respectively. As control, we also generated MV^{EGFR}, 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 EGFR (Haals-EGFR) (Fig.2A). All three viruses were rescued and propagated using the six-histidine tag rescue (STAR) system[13] and the incorporation of chimeric envelopes confirmed by immunoblot analysis (Fig. 2B). The retargeted viruses all exhibited slower growth kinetics compared to unmodified MV-Moraten, but compared to one another had similar growth kinetics on the Vero-αHis cell line (Fig. 2C) and yielded stocks with similar maximum titers of $3 \times 10^8 TCID_{50}$/mL for MV^{EGFR}, $2 \times 10^8 TCID_{50}$/mL, MV$^{CDVenv-EGFR}$, and $6 \times 10^7 TCID_{50}$/mL for MV$^{CDVenv-CD38}$. *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 measles viruses with the retargeted CDV envelope can be generated and propagated with the STAR pseudo-receptor system and that these viruses are resistant to neutralization *in vitro* by measles immune serum.

3. 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 post 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^{EGFR} and MV$^{CDVenv-EGFR}$ specifically infected CHO-EGFR cells with no discernable infection in CHO-CD38 cells, while MV$^{CDVenv-CD38}$ 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's lymphoma lines are positive for CD38 and negative for EGFR as confirmed by flow cytometric staining (Fig. 3B). As expected, MV^{EGFR} and $MV^{CDVenv-EGFR}$ infected only the EGFR positive tumor cell lines while $MV^{CDVenv-CD38}$ 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 post 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.

4. *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 non-invasive monitoring of tumor burden by bioluminescence (IVIS Xenogen) 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 6 intraperitoneal treatments of 2×10^6 $TCID_{50}$ of $MV^{CDVenv-EGFR}$, $MV^{CDVenv-CD38}$ 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 $MV^{CDVenv-EGFR}$ virus was able to control tumor growth, as demonstrated by a marked reduction in the tumor luminescence signal in this treatment group compared to the control treatment groups. Control of tumor growth was associated with significant prolongation in survival of animals in the $MV^{CDVenv-EGFR}$ treatment group compared to the $MV^{CDVenv-CD38}$ 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 $MV^{CDVenv-EGFR}$ translates to specific oncolytic efficacy *in vivo*.

5. Comparing the oncolytic activity of MV^{EGFR} versus $MV^{CDVenv-EGFR}$

To directly compare the oncolytic activities of the $MV^{CDVenv-EGFR}$ and MV^{EGFR} viruses, we used both subcutaneous and intraperitoneal SKOV3ip.1-Fluc tumor models. In the subcutaneous model we assessed the effect of 6 intratumoral treatments of 1×10^6 $TCID_{50}$ 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 $MV^{CDVenv-EGFR}$ and MV^{EGFR} were equally effective in this model, significantly impacting tumor volumes compared to control (p=0.0019 for MV^{EGFR} and p<0.0001 compared to control treatment). Tumor volumes (mean ±SD) were 27.5 ± 9.1 mm^3, 23.4 ± 11.8 mm^3 and 20.9 ± 2.5 mm^3 before virus injection and 1346.7 ± 527.6 mm^3, 67.3 ± 76.6 mm^3 and 9.8 ± 19.3 mm^3, 30 days after the first virus treatment for control, MV^{EGFR}, and $MV^{CDVenv-EGFR}$, 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 TCID$_{50}$) 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 to control treated animals on day 14 (p = 0.0473 for MV^{EGFR} and p = 0.0324 for MV$^{CDVenv-EGFR}$ compared to 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 MV^{EGFR} and 53 days for MV$^{CDVenv-EGFR}$ 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 MV$^{CDVenv-EGFR}$ is equipotent compared to the previously reported MV^{EGFR} in an EGFR-overexpressing tumor model.

6. *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 MV^{EGFR} and MV$^{CDVenv-EGFR}$, athymic mice with established intraperitoneal SKOV3ip.1-Fluc tumor xenografts were passively immunized with measles immune human AB serum (60 EU per mouse) and treated 3 hours later with a single intraperitoneal virus dose of 2×10^6 TCID$_{50}$ (Fig. 6A). Tumor growth was monitored by serial bioluminescence imaging as shown in Fig. 6B with the corresponding quantification of total body luminescence graphed in Fig. 6C. As expected, both MV^{EGFR} and MV$^{CDVenv-EGFR}$ reduced tumor burden(P<0.0001) and prolonged the survival (P<0.0001) of treated animals compared to control treatment in the absence of measles neutralizing antibodies. However, as anticipated, the efficacy of the MV^{EGFR} 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 to naïve mice treated with MV^{EGFR}. In contrast, the oncolytic activity of MV$^{CDVenv-EGFR}$ 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 to nonimmunized control mice treated with MV$^{CDVenv-EGFR}$. Together, these results demonstrate that MV$^{CDVenv-EGFR}$ 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. MV$^{CDVenv-EGFR}$ 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 measles virus 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 US 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 have therefore 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 to 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 the current 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). The current 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 monoclonal antibodies—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 (5804) because of its narrower range of receptor use compared to the H proteins from CDV vaccine strains[30]. While the mutations (D526A, I527S, S528A, R529A, Y547A, T548A) we introduced into the CDV H protein were initially reported to ablate only SLAM-dependent fusion[29] 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], due to 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 was 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 to the ascites concentrations present in ovarian cancer patients[24]. Clinical testing of the viruses described in this manuscript may therefore be warranted.

It is worth noting that despite the high doses of virus used in our ovarian cancer studies, neither MV[CDVenv-EGFR] or MV[EGFR] therapy was curative. Treated animals eventually succumbed to progressive intraperitoneal disease and were typically euthanized due to ascites formation. Examination of explanted peritoneal tumors from virus treated animals under fluorescent 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 the current study focused exclusively on the SKOV3ip.1 ovarian cancer model, we do plan to investigate the systemic administration of MV[CDVenv-EGFR] in other EGFR-overexpressing tumors such as lung cancers[35], and glioblastoma[36]. Furthermore, MV[CDVenv-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 manuscript is to improve the virus manufacturing yield. While we were eventually able to generate viral stocks with titers as high as 10^8 TCID$_{50}$/mL of the fully retargeted chimeric viruses for our *in vivo* animal experiments, retargeted MVs generally grow to much lower titers compared to 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 vaccination was introduced. Since there are over 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[46], 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 anti-tumor 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 non-human primates[49], the viruses described in this manuscript 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.

Acknowledgements

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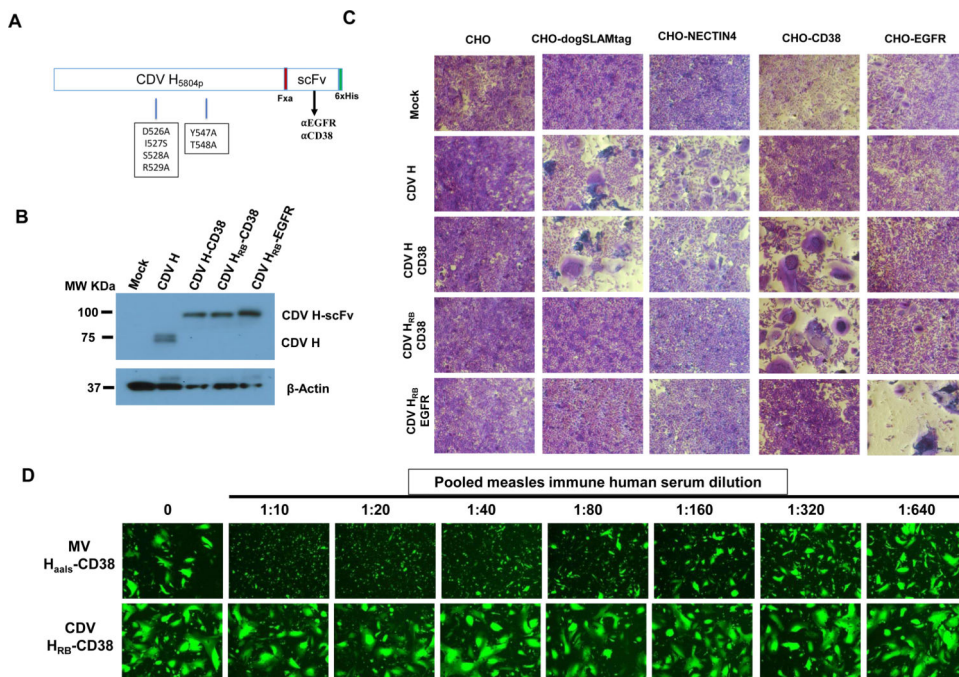
Figure 1: Retargeting the wild type CDV Hemagglutinin (H) protein to EGFR and CD38.
A) Schematic representation of retargeted CDV H protein constructs (not drawn to scale). Standard one-letter amino acid abbreviations are used to denote the mutations that ablate the use of the native receptors (dogSLAM and NECTIN4) thereby generating receptor blind CDV H (CDV H_{RB}). The single-chain antibody fragment (scFv) and a six-histidine tag (6xHis) is displayed as a C-terminal extension of H with a flexible linker containing the Factor Xa, Fxa, (IEGR) cleavage site. (B) Immunoblot analysis of HEK293 cell lysates after transient transfection of engineered CDV H expression plasmids (CDV H, CDV H-CD38, CDV H_{RB}-CD38, CDV H_{RB}EGFR) using an antibody directed against the cytoplasmic tail of CDV H. C) Co-transfection experiments evaluating receptor-specific intercellular fusion mediated by engineered CDV H and CDV F glycoproteins. CHO Cells expressing the desired receptors were co-transfected with a CDV F expression plasmid and the respective engineered CDV H expression plasmids, stained with crystal violet 48 hours post transfection and imaged (100X magnification). (D) Impact of pooled measles immune human serum on Intercellular fusion mediated by retargeted CDV envelope. CHO-CD38 cells were either co-transfected with the CDVH$_{RB}$-CD38 and CDV F or the homologous MV Haals-CD38 and MV F expression plasmids, and a GFP expression plasmid for visualization of fusion, and incubated with the indicated dilutions (made with culture medium) of pooled human serum (PRN = 256; anti-MV IgG titer = 300 EU/mL). Photographs were taken at 48 hours post transfection under a fluorescence microscope. 100X magnification
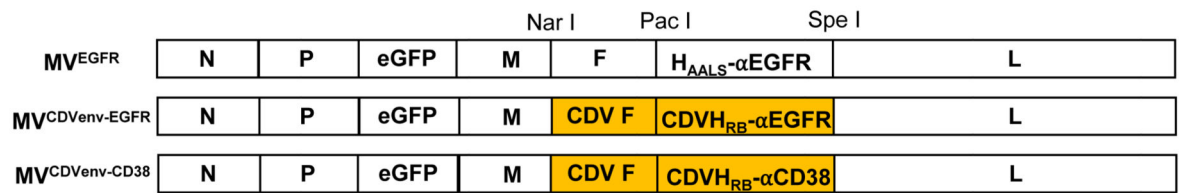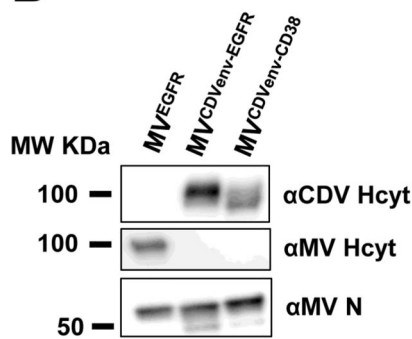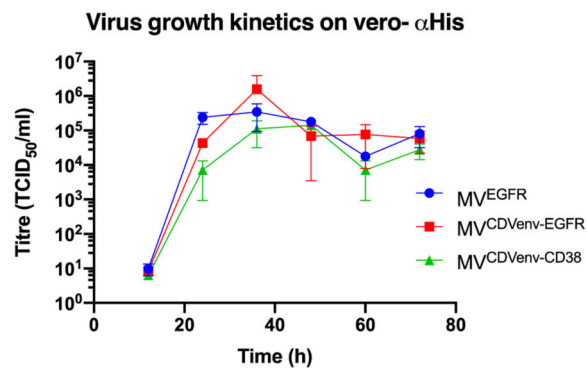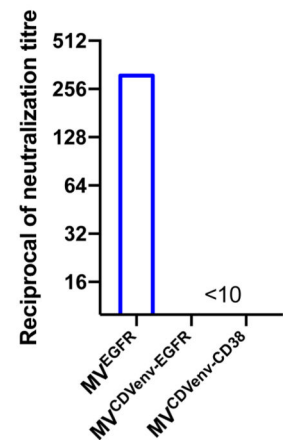
Figure 2: Generation and *in vitro* characterization of chimeric MeV bearing the CDV F and CDV H retargeted to EGFR and CD38 glycoproteins.

A) Schematic representation of recombinant MV genome constructs. The MV F and MV H retargeted to EGFR genes in the MV vac2 anti-genome were substituted with the homologous CDV F and either CDVH$_{RB}$-EGFR or CDVH$_{RB}$-CD38. There is an additional gene encoding for the enhanced green fluorescent protein (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 (1×10^5TCID$_{50}$) 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 virions. C) Multi-step growth curves on Vero-αHis cells showed that the newly generated chimeric viruses had similar growth kinetics as the control MV^{EGFR} virus. Vero-αHis cells were infected at an MOI of 0.03 and cell pellets were collected at the indicated time points and titrated on Vero-αHis cells after 3 three freeze thaw cycles. Error bars indicated SEM between 3 experiments. 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 two-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 is reported as the neutralization titer.
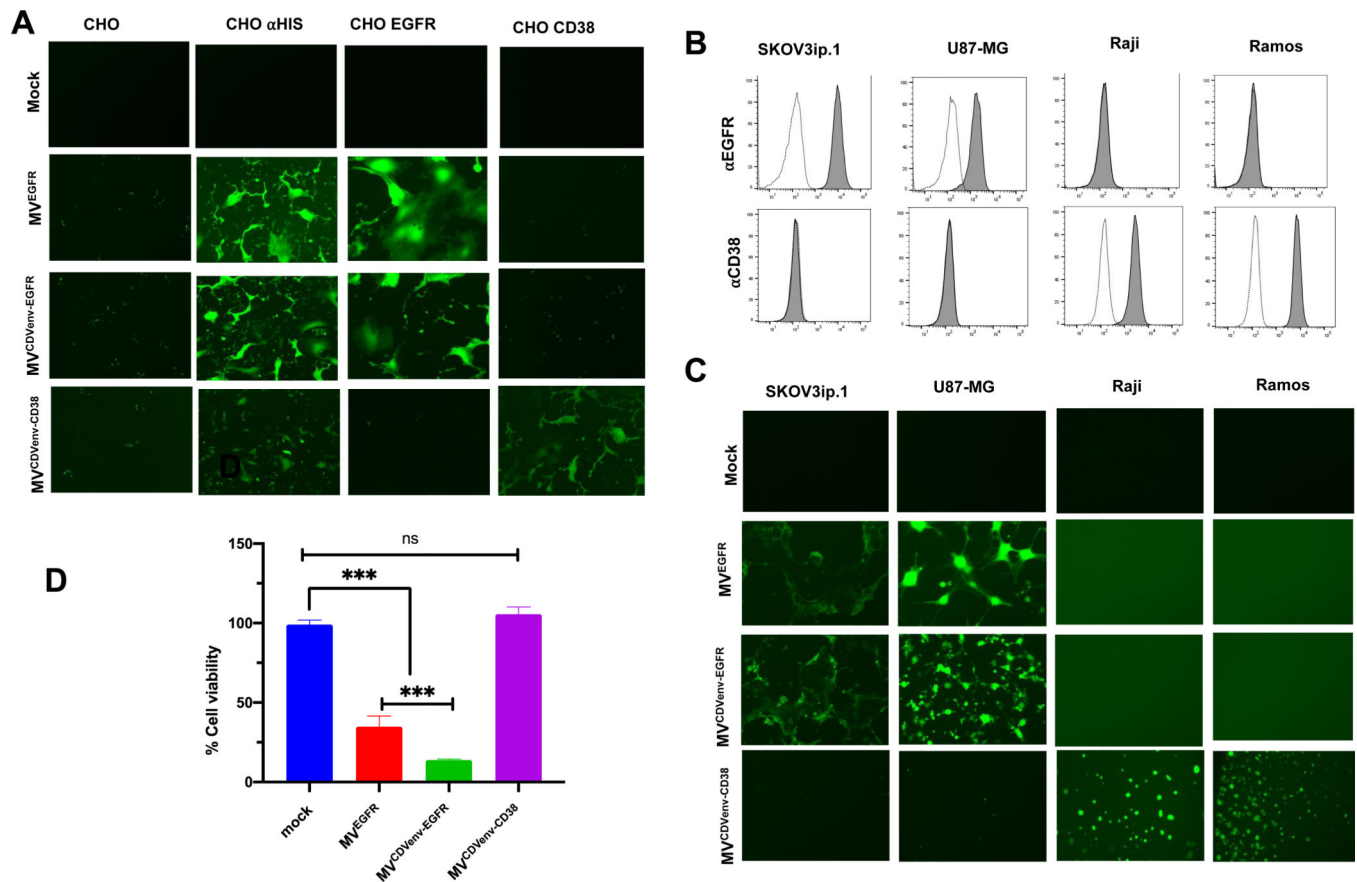
Figure 3. Receptor specificity of chimeric MV bearing the retargeted CDV envelope *in vitro*.
(A) Chimeric MV bearing CDV F and CDV H retargeted to EGFR or CD38 maintain receptor-specific infectivity on a panel of CHO cells with the desired receptors. Cells were infected with the respective viruses at an MOI of 0.5 and photographed 48 hours later using a fluorescence microscope; 100X magnification. (B) Flow cytometry analysis of selected human tumor cell lines that express either EGFR or CD38 stained (filled histograms) 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 maintain receptor-specific infectivity 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; 100X magnification. (D) *In vitro* cytotoxicity of retargeted chimeric viruses on the EGFR over-expressing SKoV3ip-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 determined 72 hours later by an MTT assay. Error bars indicate SEM between two experiments; ns = not significant, ***p<0.001
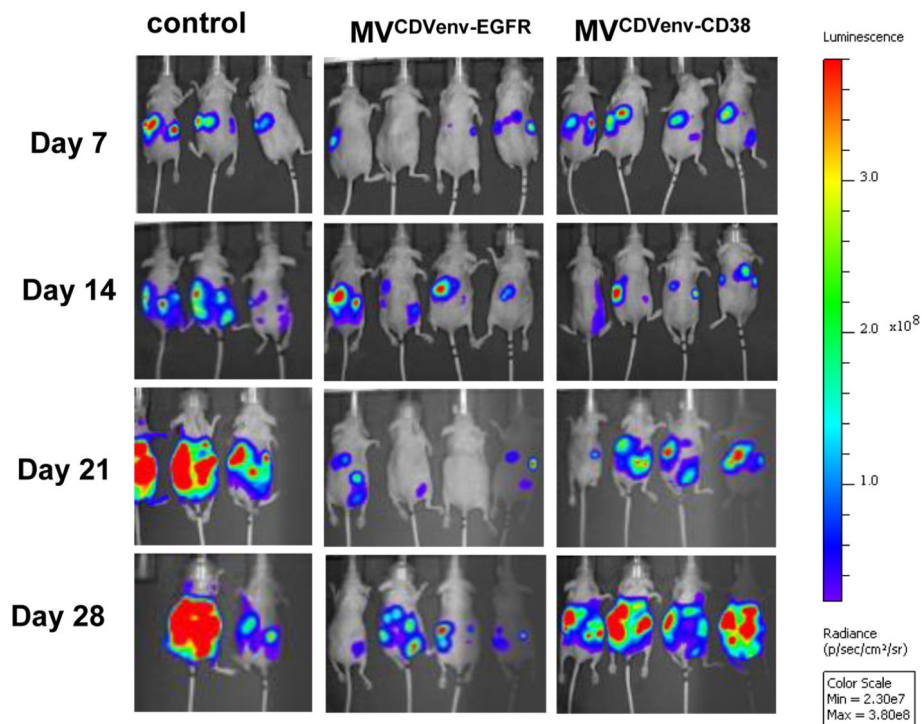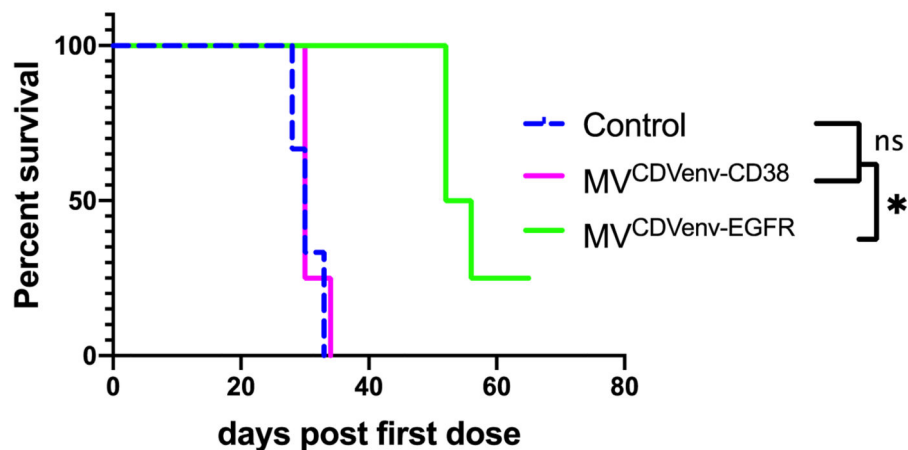
A

B

Figure 4: *In vivo* specificity of chimeric MV in an EGFR positive human ovarian tumor xenograft model.

MV$^{CDVenv-EGFR}$ is specifically oncolytic in i.p. EGFR positive SKOV3i.p.1-Fluc tumors treated 10 days after tumor implantation with 6 doses of 2×10^6 TCID$_{50}$ of indicated virus treatment or control (n=3 control and n=4 for treatment group). (A) Serial bioluminescent imaging on the indicated days. (B) Overall survival of treated animals. *ns = not significant; * p<0.05*
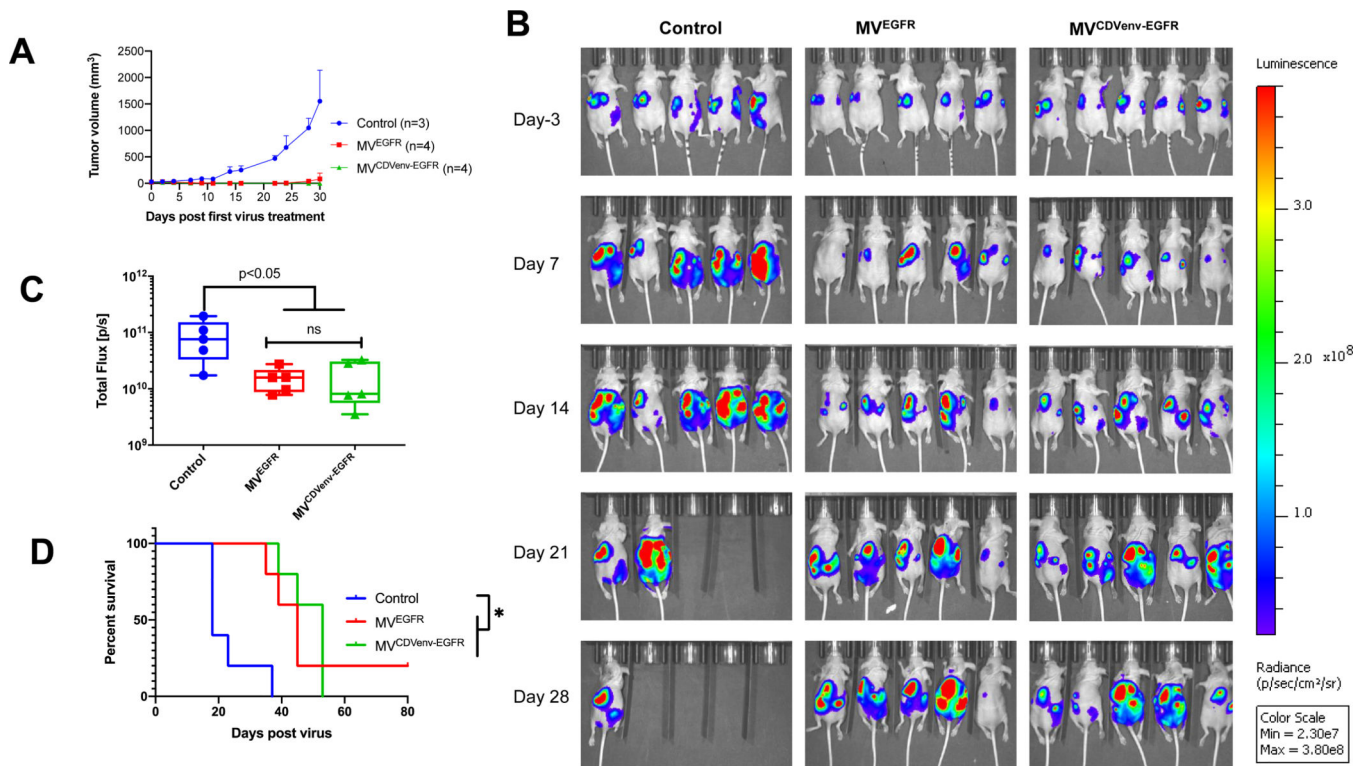
Figure 5: Comparison of the oncolytic efficacy of the EGFR retargeted viruses in the EGFR positive ovarian cancer tumor model.

(A) MV[EGFR] and MV[CDVenvEGFR] have similar oncolytic efficacy in a subcutaneous tumor model. Tumor volumes of subcutaneously implanted SKOV3i.p.1-Fluc cells treated with 6 intratumoral doses of 2×10^6 $TCID_{50}$ of the respective viruses or control (producer Vero-αHis lysate). (B-D) MV[EGFR] and MV[CDVenvEGFR] have similar oncolytic efficacy in an orthotopic, intra peritoneal model. (B) Serial bioluminescent imaging on the indicated days to monitor tumor burden of mice with i.p. SKOV3i.p.1-Fluc tumors treated with 2×10^6 $TCID_{50}$ of indicated virus treatment or control (n=5 per group) 10 days post tumor implantation. (C) Quantification of total body luminescence (tumor burden) on day 14 post treatment; error bars indicate SEM. (D) Overall survival of treated animals. ns= not significant; *p<0.05
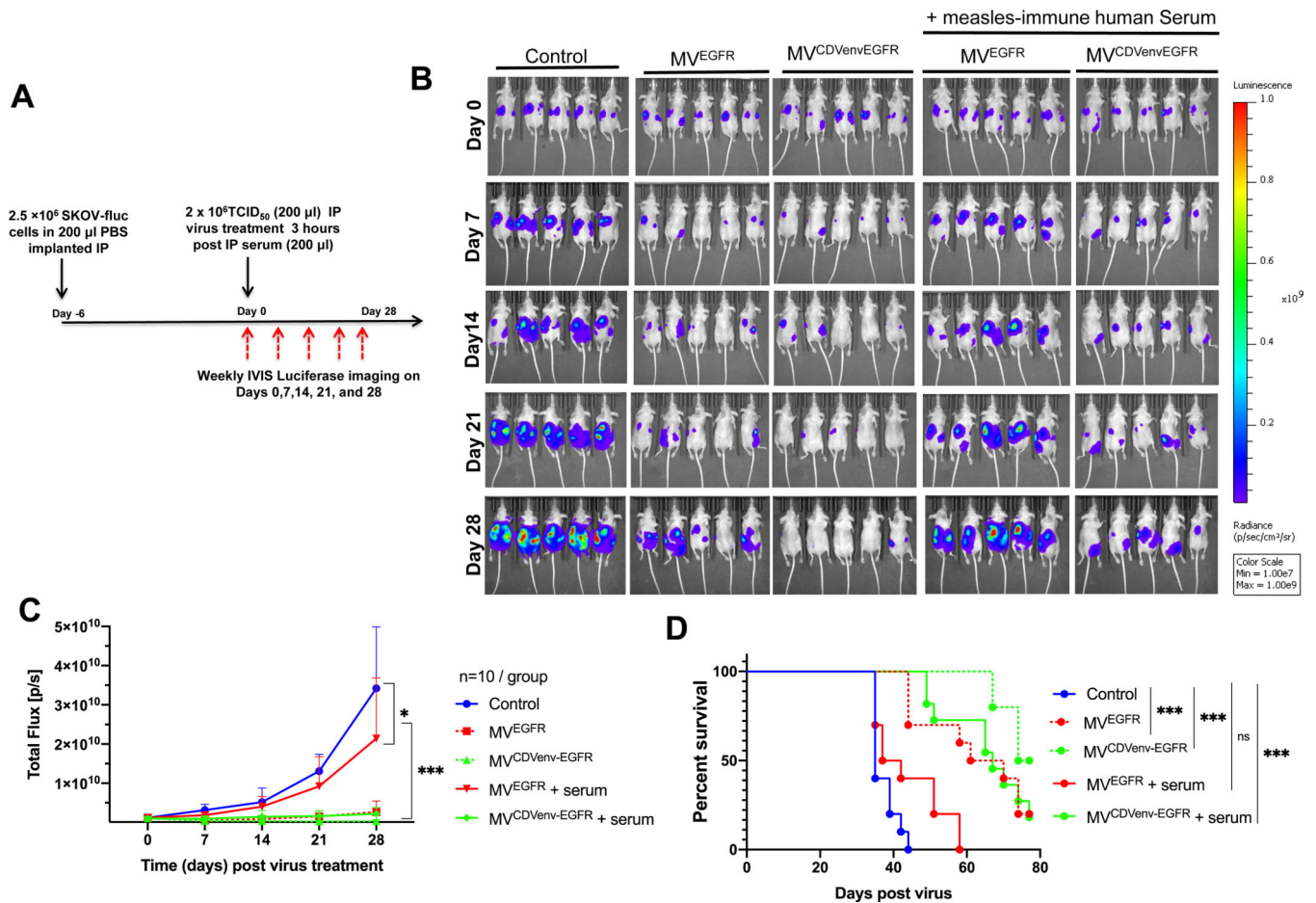
Figure 6: Impact of pre-existing measles neutralizing antibodies on the oncolytic activity of EGFR-retargeted viruses.

$MV^{CDVenvEGFR}$ maintained oncolytic efficacy in the presence of pre-existing anti-measles neutralizing antibodies. A) schematic of experimental design. Athymic mice with i.p. SKOV3ip.1Fluc tumors were treated with a single i.p. injection of 2×10^6 $TCID_{50}$ of MV^{EGFR}, $MV^{CDVenvEGFR}$ 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) quantification (C) of serial bioluminescent imaging and (D) overall survival of treated animals *p<0.05; *** P<0.0001.