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Authors
Lal, Sangeet
Raffel, Corey

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Using Cystine Knot Proteins as a Novel Approach to Retarget Oncolytic Measles Virus

Sangeet Lal1 and Corey Raffel1

1Department of Neurological Surgery, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA 94158, USA

INTRODUCTION

Standard therapy for malignant brain tumors has failed to achieve meaningful prolongation of survival. For example, combined radiation therapy and chemotherapy with temozolomide, the current standard of care for glioblastoma multiforme (GBM), has been shown to prolong overall survival only 2.5 months over irradiation alone, and most patients still progress to death caused by the tumor.1 While the success of treating the most common pediatric malignant brain tumor, medulloblastoma, is better, with 5-year survival rates of 70% for localized tumors, CSF-disseminated medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT) lead to open phase 1 trials for the treatment of recurrent intracerebral GBM and recurrent intraparenchymal and/or CSF-disseminated medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT).8,9

The use of replication-competent oncolytic viruses (OVs) as anticancer agents has promise as a tumor-specific therapy. In our laboratory, we have been investigating a vaccine strain of measles virus (MV) as a potential therapy for pediatric brain tumors. We have demonstrated efficacy in the treatment of intraparenchymal and cerebrospinal fluid (CSF)-disseminated murine xenograft models of medulloblastoma and in the treatment of intraparenchymal atypical teratoid/rhabdoid tumor.4–6 Similarly, MV has proven efficacious in the treatment of a murine xenograft model of intracerebral GBM.7 Animal safety studies have demonstrated no evidence of toxicity from injection of MV into the brain or CSF, leading to open phase 1 trials for the treatment of recurrent intracerebral GBM and recurrent intraparenchymal and/or CSF-disseminated medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT).8,9

One of the advantages of MV is the ease with which the virus can be retargeted to cell surface antigens of choice. The production of such viruses has proven laborious, as the receptor-binding motif used is often a single-chain antibody. An alternative receptor-binding motif may be cystine knot proteins (CKPs). These small peptides of 20–50 amino acids containing two or three disulfide bonds bind to a specific target with single-digit nanomolar to picomolar binding coefficients.10 In this paper, we manufacture a retargeted MV that uses a CKP that binds αvβ3, αvβ5, and α5β1 integrins with single-digit nanomolar affinity to retarget MV to the integrins (MV-CKPint). MV-CKPint infected, replicated in, and killed human glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma (DIPG), and melanoma cancer cells in vitro, all of which express the target integrins. MV-CKPint activity was competitively blocked by echistatin, an integrin binding peptide. When the CKP was cleaved from the viral H protein at an included protease site, virus activity was abrogated. When delivered intravenously (i.v.), the retargeted virus reached a subcutaneous glioblastoma tumor bed and produced cytopathic effects similar to that shown by intratumoral injection of the virus. Because these target integrins are overexpressed by tumor vascular endothelium, MV-CKPint may allow for effective therapy with i.v. injection. These results indicate for the first time that CKPs can be used to retarget MV for a receptor of choice. In addition, MV-CKPint provides proof of principle for the use of a CKP of interest to retarget any enveloped virus for both oncolytic and gene therapy purposes.

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Modified measles virus (MV) has effective oncolytic activity preclinically and is currently being investigated in clinical trials for various types of cancer. We investigated the use of cystine knot proteins (CKPs) to direct MV activity. CKPs are short polypeptides that bind their targets with high affinity. We used a CKP that binds αvβ3, αvβ5, and α5β1 integrins with single-digit nanomolar affinity to retarget MV to the integrins (MV-CKPint). MV-CKPint infected, replicated in, and killed human glioblastoma, medulloblastoma, diffuse intrinsic pontine glioma (DIPG), and melanoma cancer cells in vitro, all of which express the target integrins. MV-CKPint activity was competitively blocked by echistatin, an integrin binding peptide. When the CKP was cleaved from the viral H protein at an included protease site, virus activity was abrogated. When delivered intravenously (i.v.), the retargeted virus reached a subcutaneous glioblastoma tumor bed and produced cytopathic effects similar to that shown by intratumoral injection of the virus. Because these target integrins are overexpressed by tumor vascular endothelium, MV-CKPint may allow for effective therapy with i.v. injection. These results indicate for the first time that CKPs can be used to retarget MV for a receptor of choice. In addition, MV-CKPint provides proof of principle for the use of a CKP of interest to retarget any enveloped virus for both oncolytic and gene therapy purposes.

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The use of replication-competent oncolytic viruses (OVs) as anticancer agents has promise as a tumor-specific therapy. In our laboratory, we have been investigating a vaccine strain of measles virus (MV) as a potential therapy for pediatric brain tumors. We have demonstrated efficacy in the treatment of intraparenchymal and cerebrospinal fluid (CSF)-disseminated murine xenograft models of medulloblastoma and in the treatment of intraparenchymal atypical teratoid/rhabdoid tumor.4–6 Similarly, MV has proven efficacious in the treatment of a murine xenograft model of intracerebral GBM.7 Animal safety studies have demonstrated no evidence of toxicity from injection of MV into the brain or CSF, leading to open phase 1 trials for the treatment of recurrent intracerebral GBM and recurrent intraparenchymal and/or CSF-disseminated medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT).8,9

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retarget MV and, indeed, suggest that CKPs can be used to retarget any enveloped virus.

RESULTS

The Integrin-Binding CKP Motif Is Expressed at the C Terminus of the H Protein

MV normally binds to its receptors via the H protein. The H protein encoded by the viral genome used to manufacture MV-CKPint has two mutations (Y481A and R533A) that eliminate binding to CD46 and SLAM, the normal MV receptors. The integrin-binding CKP was expressed at the carboxyl end of the H protein, resulting in its exposure on the surface of the virus (Figure 1A). The functionality of the MV-CKPint was verified by infecting anti-His (α-His)-Vero cells. These modified cells express membrane-bound single-chain antibody for hexahistidine and hence facilitate viral entry through binding of the 6xHis tag at the C terminus of the MV-CKPint virus (Figure 1B). We also tested this virus on the D283-medulloblastoma cell line (Figure 1B). The MV-CKPint virus successfully infected αHis-Vero and D283-med cells, and large syncytia fluorescing with the GFP were formed in both cell lines. In D283-med cells, GFP fluorescence is clearly visible in the perinuclear space, which is typical for MV. This finding indicates that adding the integrin-binding CKP to the H protein is functional and allows for infection, and replication of the virus.

MV-CKPint Infected Cells of Different Tumor Types

To investigate CKP-mediated cellular infection, we analyzed tumor cell lines of different origin, glioblastoma, diffuse intrinsic pontine glioma (DIPG), medulloblastoma, and melanoma for expression of target integrins. Flow cytometry confirmed that all tested cell lines expressed αvβ3, αvβ5, and α5β1 integrins on the membrane (Figure 2, right panel). The percentages of cells positive for each integrin dimers are shown in parentheses. The expression of the GFP protein in the MV-CKPint virus was used as a marker of infection. After infection with the virus, cell lines of all tested tumor types showed multiple green fluorescent syncytia (Figure 2, left panel). Syncytia formation became visible as early as 48 hr post-infection. The formation of syncytia indicates that MV-CKPint infects and grows in cells expressing target integrins.

MV-CKPint Replicates in and Kills Cells of Different Tumor Types

The replication competency of MV-CKPint was measured in a time course by flow cytometry. The intensity of GFP fluorescence in untreated control cells was used as a baseline. As a second approach, the titer of cell-associated virus was quantified daily after infection of U87 and MDAMB-435 cells with 0.2 MOI of the virus. As shown in Figure 4B, the titer of infectious virus particles in these cells.
increased gradually over the period of 4 days post-infection, demonstrating replication of the virus in these cell types. The replication efficiency of the retargeted MV-CKPint virus was compared with that of the parental Edmonston virus strain (MV-GFP). Human U87 glioblastoma cells were infected in parallel with both viruses at an MOI of 0.1, and the titer of cell-associated virus was quantified daily on Vero cells for 4 days post-infection. After 24 hr of infection, the titer of MV-GFP was higher with formation of many fluorescent syncytia, whereas no syncytia were observed and the titer of infectious particles was significantly lower for MV-CKPint virus (Figure 4C). Nevertheless, the titer of the infectious MV-CKPint virus particles increased over time and rose slightly higher than that of MV-GFP at 72 and 96 hr time points. This suggests that the initial replication and intracellular assembly of the retargeted MV-CKPint virus in U87 glioblastoma cells is slower than the parental MV, but the virus efficiently replicates and the titer of infectious virus increases over time.

These results show that the MV-CKPint virus efficiently replicates in and kills tumor cells in vitro.

**Infection of MV-CKPint Is Dependent on the Integrin Binding of the CKP**

If the integrin-binding motif of CKP is responsible for cell binding and entry of the retargeted virus, the presence of a soluble antagonist of target integrins should inhibit the interaction of virus with cells, thereby blocking infection. To verify the requirement for integrin binding, we assessed infection and cell killing by MV-CKPint in the presence of echistatin, a potent competitive binder of αvβ3 and α5β1 integrins.12 Human glioblastoma and melanoma cells were infected with MV-CKPint virus at an MOI of 1 in the absence and presence of 1 μM soluble echistatin. As shown in Figure 5, echistatin significantly decreased killing of both cell lines by the MV-CKPint virus as a result of inhibition of cellular infection. However, inhibition
was not complete. Because echistatin and CKP in the virus compete to bind the same target integrins, a partial infection of cells with the virus may not be completely eliminated. In addition, echistatin has been shown to strongly bind and antagonize \( \alpha v \beta 3 \) and \( \alpha v \beta 1 \) integrins,\(^{13,14} \) but its interaction with \( \alpha v \beta 5 \) integrin, which is also a target of the CKP used in the MV-CKPint virus, is not well-known. This suggests that the retargeted virus utilizes CKP-mediated binding with target integrins for cellular entry.

To confirm integrin-binding-mediated cellular entry of the virus via CKP, we removed the CKP motif from the viral H protein using the activated factor X (FXa) protease cleavage site present right before the CKP sequence in the viral H protein (Figure 1A). Pre-incubation of virus with FXa protease markedly abrogated cell killing in dose-dependent manner (Figure 6). Proteolytic removal of the CKP moiety resulted in a more than 4-fold decrease in virus infectivity, thereby cell killing in U87 and MDAMB-435 cells, and a more than 2-fold decrease in Vero cells at 200 \( \mu \)g/mL FXa. Inhibition of viral infection was clearly demonstrated but was incomplete. This could be because CKP moiety was not completely removed by FXa even at 200 \( \mu \)g/mL. These results confirm that the integrin-binding CKP motif is required for cell binding and entry of the MV-CKPint virus.

**MV-CKPint Reaches Tumor Sites and Forms Syncytia after Intravenous Delivery**

We have established that MV-CKPint infects, replicates in, and kills U87-MG glioblastoma tumor cells in vitro. To investigate intravenous (i.v.) delivery of this modified virus, we injected mice bearing subcutaneous U87-MG glioblastoma tumors with a single dose of MV-CKPint (0.5 \( \times \) 10\(^6 \) TCID\(_{50} \) [50% tissue culture infective dose]/100 \( \mu \)L) or an equal volume of PBS (control) via the tail vein. Additional control mice were injected directly into the tumor with the same dose of MV-CKPint. Eight days post-injection, tumors were harvested and analyzed for the presence of MV by immunohistochemistry. In tumors from mice receiving i.v. injection of MV-CKPint, multiple scattered small syncytia were noticed; two representative regions of infection (foci 1 and 2) are shown in Figure 7. The syncytia stained positive for MV nucleoprotein, indicating for the presence of MV. For intratumoral injection, a large but localized region of MV nucleoprotein-positive syncytia was observed at the injection site. Additional sections from same tumors used for immunohistochemistry were also examined by immunofluorescence staining to confirm the presence of MV (Figure 7). This indicates that MV-CKPint virus successfully reached the tumor bed after i.v. injection.
In this paper, we have described a novel approach to manufacture a retargeted MV. We used a CKP that specifically and tightly binds to the relatively tumor-specific integrins αvβ3, αvβ5, and α5β1 to direct virus binding to tumor cells.11 We used a well-described "double-blind" virus system that abrogates binding by the virus H protein to its normal receptors to assure that the CKP was, in fact, responsible for binding of the virus to the cell. We show that cell killing by this retargeted MV-CKPint virus was comparable to that of the parental CD46-targeting Edmonston strain of MV on five different cell lines. Also, the retargeted virus efficiently replicated in U87 cells, suggesting that conjugation with a CKP does not have an adverse effect on the virus. We have demonstrated that echistatin, a competitive binder of the integrins, abrogates infection by the virus and that proteolytic cleavage of the CKP motif from the viral H protein also prevents infection and killing of cells. Thus, we have shown in two different ways that MV-CKPint uses the specific binding of the CKP for cell binding and entry.

In the treatment of solid tumors, MV has required intratumoral injection for efficacy.5 Indeed, both the GBM and the medulloblastoma phase 1 trials for solid tumor recurrence use direct injection of the tumor resection bed for virus delivery. Because the αvβ3, αvβ5, and α5β1 integrins targeted by the CKP used here have been shown to be expressed by tumor vascular endothelium, the use of this CKP may allow for effective therapy with i.v. injection of virus.15,16 To examine this possibility, we injected animals bearing flank tumors with a single dose of 5 × 106 TCID50 of MV-CKPint i.v. and harvested tumors for analysis at 8 days after injection. A localized large area of syncytia was observed in intratumoral-treated tumors compared to multiple small foci of infection with intravascular virus delivery. The successful delivery of MV-CKPint in xenograft tumors upon i.v. injection raises the possibility that the virus is infecting and replicating in tumor vascular endothelium, and the resultant virus is then spreading to the tumor cells. Further experimentation to compare the efficacy of treatment of solid tumors both in the flank and in the brain will be needed to determine the method of viral spread and overall efficacy of i.v. therapy.
An oncolytic adenovirus (Ad5) and MV have been engineered to express integrin-binding RGD motif on its glycoproteins. The modified virus demonstrated localization in vascular endothelial cells and slightly improved antitumor activity.

One of the advantages of MV in this regard is a relative receptor threshold effect. The more surface receptor present on a cell, the more efficacious the MV infection. Indeed, there is a threshold below which productive infection does not occur. This effect is important with regard to integrins. While the αvβ3, αvβ5, and α5β1 integrins have been described as tumor specific, low levels of expression on normal cell types such as dendritic cells, T lymphocytes, vascular smooth muscle cells, and bone have been reported. However, this may have no effect of MV toxicity, as the receptor expression of these normal cells may be too low to support productive infection. This is analogous to the situation with the CD46 receptor used by current oncolytic MVs. This protein, an inhibitor of complement-mediated lysis, is expressed at below MV threshold levels by many normal cell types, but is highly overexpressed, for obvious reasons, by most tumor cell types.

CKPs have been used to detect and monitor tumors of various types. In fact, the CKP used here has been labeled with radio-iodine and shown to specifically label medulloblastoma cells in a murine model. Similarly, chlorotoxin, a CKP isolated from scorpion venom, has been conjugated to a fluorescent marker to label tumors in vivo. This conjugate, called Tumor Paint, may allow for pre-resection labeling of tumor and intra-operative detection of small amounts of residual tumor, leading to a more complete tumor removal.

Indeed, the simplicity of CKP structure lends itself to the creation of CKP libraries. Such libraries could then be screened to identify a CKP that binds with high affinity to a target of choice. The selected CKP could then easily be incorporated into MV to direct infection by the virus.

One issue with the use of CKP for virus targeting is the requirement for correct peptide folding and correct formation of the disulfide bonds in the CPK for binding activity. It appears that passage of the viral protein containing the CKP through an infected cell’s endoplasmic reticulum provides the proper environment to assure proper folding and bond formation of the CKP. Passage of the viral protein through the endoplasmic reticulum (ER) occurs with most enveloped viruses. Currently, many enveloped viruses, including herpes simplex virus, Newcastle disease virus, and vesicular stomatitis virus, are used both for oncolysis and as gene therapy vectors. The results presented here provide proof of principle for the use of a CKP of interest to retarget any enveloped virus for both oncolytic and gene therapy purposes.

MATERIALS AND METHODS

Cell Culture

The human embryonic kidney cells (HEK293T), green monkey kidney cells (Vero), human glioblastoma (U87MG), diffuse intrinsic pontine glioma (DIPG-SF8628), melanoma (MDAMB-435), and medulloblastoma (DAOY, D283-med) cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine and maintained at 37 °C in a humidified incubator set at 5% CO₂. The authentication of all human cell lines was confirmed by short tandem repeat (STR) DNA profiling technique (DNA sequencing facility, UC Berkeley, Berkeley, CA, USA).

Preparation and Rescue of MV-CKPint

The cDNA of the integrin-specific CKP (EETI 2.5F) was commercially synthesized on Genscript (Piscataway, NJ, USA) using the amino acid sequence 5'-GCPRPRGDNPPLTCKQDSDCLAGVCGPNGFCG-3'. The sequence was flanked with 5'-SfiI and 3'-NotI restriction sites, and the enzyme-digested fragment was inserted in-frame at the C terminus of the MV H gene in the shuttle vector pTNH6aa. The H gene in this vector contains two point mutations resulting in the substitutions Y481A and R533A, which eliminates binding of the H protein to its normal receptors, CD46 and SLAM, respectively. After cloning the CKP, the entire H gene was isolated from pTNH6aa after digesting with PacI and SpeI restriction enzymes, and ligated into the corresponding sites in the p(+)MV-EGFP vector that codes for all six genes of MV. The viral plasmid was amplified by transforming One Shot Top10 competent cells (Life Technologies, Carlsbad, CA, USA) and purified using the plasmid maxi prep kit from QIAGEN (Valencia, CA, USA). The resulting MV-CKPint virus was prepared by transfecting HEK293T cells with the recombinant p(+)MV-EGFP-CKP vector along with packaging plasmids using the FuGENE HD transfection reagent (Promega, Madison, WI, USA). For robust preparation of MV-CKPint stocks, we used...
Vero-αHis cells. These cells are modified to express membrane-bound single-chain antibodies for anti-hexahistidine that allows cellular entry of this blind virus due to the 6xHis-tag attached to the H protein after the CKP sequence, as described before.\(^\text{37}\) Cells were scraped from plates in minimal volume of Opti-MEM media, lysed to release virus by two cycles of freeze-thaw in liquid nitrogen, and centrifuged at 10,000 \(\times g\) for 5 min to clarify virus stock from cellular debris. The aliquots of supernatant-containing virus were stored at \(-80^\circ\text{C}\), and the titer was determined as TCID\(_{50}\) by infecting αHis Vero cells in serial dilutions as described previously.\(^\text{38}\)

**In Vitro Cell Killing, Cytopathic Effect, and Virus Replication**

One hundred thousand tumor cells per well were seeded in 12-well plates. The next day, cells were infected with MV-CKPint at different MOIs in 0.5 mL of Opti-MEM media for 3 hr at \(37^\circ\text{C}\). Control wells were incubated in Opti-MEM only. The virus was removed and cells were maintained in the growth medium. After 96 hr, the number of live cells was determined by the trypan blue exclusion method and counted using Countess, the automated cell counting instrument (Thermo Fisher Scientific, Carlsbad, CA, USA). The percent survival from each treatment was calculated by dividing the number of viable cells in the infected well by the average of viable cells in control wells. The MV-CKPint virus expresses the EGFP, hence the infection of cells and formation of syncytia were confirmed by fluorescence microscopy. To examine the replication efficiency of the virus, we seeded \(2 \times 10^5\) U87 and MDAMB-435 cells per well in a six-well format plate and infected them with MV-CKPint virus at an MOI of 0.2 for 3 hr on rocker at \(37^\circ\text{C}\). To compare the replication of retargeted MV-CKPint virus with the parental Edmonston vaccine strain of MV (MV-GFP), we infected U87 cells (\(2 \times 10^5\) per well) in six-well plates in parallel with MV-CKPint virus and MV-GFP virus at an MOI of 0.1 for 3 hr on rocker at \(37^\circ\text{C}\). The incubator temperature was kept at \(37^\circ\text{C}\) for the first 48 hr and then decreased to 32°C until 96 hr post-infection, to avoid rupturing of syncytia to maximize collection of cell-associated virus. At 24, 48, 72, and 96 hr post-infection, supernatant was removed, and cells were scraped from the dish and lysed with two rounds of freeze-thaw in liquid nitrogen to harvest cell-associated virus particles. After centrifugation at 5,000 \(\times g\) for 5 min to remove cell debris, the supernatant was immediately used in the serial dilution assay on αHis Vero cells or Vero cells to quantify the titer of MV-CKP-int and MV-GFP virus, respectively.

**Flow Cytometry**

The expression of \(\alpha\beta_\text{a2}, \alpha\beta_\text{v5},\) and \(\alpha_\text{b1}\) integrin dimers on cell surface was analyzed by fluorescence-activated cell sorting (FACS) analysis on live cells. Cells were removed from the dish with TrypLE (GIBCO, Grand Island, NY, USA), centrifuged in cold media, and the pellet was washed with cold flow cytometry (FC) buffer (PBS containing 0.1% sodium azide and 2 mM EDTA). Cells were incubated with phycoerythrin-conjugated primary antibodies for \(\alpha\beta_\text{a2}\) (EMD Millipore, Temecula, CA, USA), \(\alpha\beta_\text{v5}\) (R&D, Minneapolis, MN, USA), and the IgG-isotype control (eBioscience, San Diego, CA, USA). Unconjugated antibody was used for staining \(\alpha_\text{b1}\) integrin (EMD Millipore). All primary antibodies were incubated for 1 hr at 4°C. Alexa Fluor 594-conjugated secondary antibody for 30 min at 4°C was used for \(\alpha_\text{b1}\) integrin. After antibody incubations, cells were washed three times with FC buffer, and live cells were analyzed on the BD FACSCalibur using the Cell Quest software.

**Competitive Binding Assay and FXa Cleavage of MV-CKPint**

U87MG glioblastoma and MDAMB-435 melanoma cells were seeded at \(10^5\) cells per well in 12-well plates. Cells were washed with PBS and incubated with MV-CKPint at an MOI of 1 in the absence and presence of 1 \(\mu\text{M}\) echistatin in Opti-MEM medium for 3 hr on rocker at \(37^\circ\text{C}\). The supernatant containing unbound virus and echistatin was removed, and cells were grown in 10% FBS media. For the FXa proteolysis assay, prior to cellular infection, the number of MV-CKPint virus equivalent to an MOI of 1 was incubated with FXa protease (New England Biolabs, Ipswich, MA, USA) at final concentrations...
of 100 and 200 µg/mL in Opti-MEM media. The virus was incubated with FXa at room temperature for 1 hr and then for an additional 1 hr at 37°C in the water bath. The untreated and FXa-treated virus was added to cells for 3 hr at 37°C on a rocker in the 5% CO2 incubator. The unbound virus was removed, and cells were grown in 10% FBS media. At 96 hr post-infection, the number of live cells was counted with the Countess using the trypan blue method.

In Vivo Model and Immunohistochemistry

All animal procedures were approved and performed according to the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC) guidelines. Human U87 glioblastoma cells (1 × 10^6 in 100 µL) were injected subcutaneously in the right flank of 6-week-old NOD-SCID IL2Rγnull (NSG)-immunodeficient mice. At an approximate tumor diameter of 1 cm, mice were injected i.v. with 5 × 10^5 TCID50 of MV-CKPint virus diluted in 100 µL of PBS by the tail vein. Two mice were also injected with the virus by intratumoral injection and used as the positive control for immunohistochemistry analysis. After 8 days of virus injection, tumors were harvested and processed to detect measles virus nucleoprotein by immunohistochemistry (IHC) and immunofluorescence (red channel). Two representative foci of infection from i.v.-treated tumors (foci 1 and 2) and one for the IT injection are shown. Formation of syncytia and positive MV nucleoprotein reactivity in perinuclear cytoplasmic space (merge panel) was detected in both routes of delivery. Original magnification 400×. Scale bars, 20 µm.

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room temperature. After three washes with TBST, a coverslip was mounted with ProLong Gold antifade media containing DAPI (Molecular Probes, Thermo Fisher Scientific, OR, USA). The immunohistochemistry and immunofluorescence staining images were captured with the monochrome camera (400 × magnification) using the Zeiss Axiow A2 microscope. The merge channel images in Figure 7 were created using the ImageJ software from the NIH.

Statistical Analysis
Experiments were performed at least three times with three replicates per group of treatment. Statistical analysis was performed with Microsoft Office Excel using Student’s paired t test, and the level of significance was set at p < 0.05.

AUTHOR CONTRIBUTIONS
Overall Conception and Design: S.L. and C.R.; Experimental Methodology and Acquisition of Data: S.L.; Analysis and Interpretation of Data: S.L. and C.R.; Manuscript Writing and Revisions: S.L. and C.R.

CONFLICTS OF INTEREST
The authors do not have any conflict to disclose.

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Molecular Therapy: Oncolytics Vol. 7 December 2017 65
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