INTRODUCTION

The oncolytic virotherapy field has significantly expanded in the last decade, and recently, several novel viral vectors have reached late phase clinical evaluation. Among the novel oncolytic viruses under development, the Edmonston vaccine strain of measles virus (MV-Edm) is a promising one, whose safety and efficacy are under development, the Edmonston vaccine strain of measles virus. However, the majority of published recombinant MV vectors are targeted against receptors that are present in human, but not host (rodent) tissues. This limits the ability to fully characterize issues such as safety, selectivity and antitumor efficacy of targeted oncolytic MVs in syngeneic models with an intact immune system. In order to obtain preclinical data more predictive of the potential virus–tumor–host interactions that may occur in humans, it is critical to develop viral agents directed at targets that are expressed in human and murine tumors and tissues in a similar manner.

The urokinase receptor (uPAR) is a clinically relevant target for novel biological therapies. We have previously rescued oncolytic measles viruses fully retargeted against human (MV-h-uPA) or murine (MV-m-uPA) uPAR. Here, we investigated the in vivo effects of systemic administration of MV-m-uPA in immunocompetent cancer models. MV-m-uPA induced in vitro cytoxicity and replicated in a receptor-dependent manner in murine mammary (4T1) and colon (MC-38 and CT-26) cancer cells. Intravenous administration of MV-m-uPA to 4T1 tumor-bearing mice was not associated with significant clinical or laboratory toxicity. Higher MV-N RNA copy numbers were detected in primary tumors, and viable viral particles were recovered from tumor-bearing tissues only. Non-tumor-bearing organs did not show histological signs of viral-induced toxicity. Serum anti-MV antibodies were detected at day 14 of treatment. Immunohistochemistry and immunofluorescence studies confirmed successful tumor targeting and demonstrated enhanced MV-m-uPA-induced tumor cell apoptosis in treated compared with control mice. Significant antitumor effects and prolonged survival were observed after systemic administration of MV-m-uPA in colon (CT-26) and mammary (4T1) cancer models. The above results show safety and feasibility of uPAR targeting by an oncolytic virus, and confirm significant antitumor effects in highly aggressive syngeneic immunocompetent cancer models.

The urokinase receptor (uPAR) is a glycosylphosphatidylinositol anchored cell surface receptor whose role in tumor progression and angiogenesis is well recognized. It is overexpressed in a variety of human and murine cancers, and its presence has been associated with metastatic potential and poor prognosis.

During normal conditions, tissue expression of uPAR is very limited, and restricted to during active tissue remodeling, injury and inflammation. Genetic and biological anti-uPAR strategies have shown to induce potent antitumor effects, and agents like anti-uPAR antibodies or uPAR targeted nanoparticles successfully target tumors and micrometastases in vivo, without affecting non-tumor-bearing organs.

MV-uPA is a novel oncolytic measles virus fully retargeted against uPAR. MV-uPA was engineered by displaying the aminoterminal fragment of either human (MV-h-uPA) or mouse (MV-m-uPA) urokinase in the C-terminus of a CD46 and SLAM ‘blind’ MV-H glycoprotein (H4AALS). The fully retargeted viruses were shown to bind to human or murine uPAR-expressing cells in a receptor and species-specific manner. In our previous report, we demonstrated antitumor effects of MV-h-uPA against human breast cancer xenografts. MV-m-uPA (which targets murine uPAR) offers the unique opportunity for in vivo characterization of the safety and antitumor effects of a fully retargeted oncolytic MV in syngeneic models of cancer, where the target is naturally expressed by tumors and tissues, similar to the human situation.

In this study, the safety, biodistribution, organ toxicity, targeting and antitumor effects of MV-m-uPA in syngeneic, immunocompetent cancer models were investigated. As uPAR is a highly relevant human and murine tumor target, results from our in vivo studies will be useful to predict safety and efficacy during preclinical and clinical development of uPAR targeted oncolytic viral therapies.
RESULTS

uPAR-dependent in vitro cytotoxicity and viral replication in murine cancer cells

To assess differences in MV-m-uPA-induced cytotoxicity in murine cancer cells with different levels of uPAR expression, receptor levels were determined in murine colon cancer (MC-38 and CT-26), murine mammary cancer (4T1) and melanoma (B16F10) cells. 4T1, MC-38 and CT-26 had increased uPAR expression compared with B16F10 cells, which had markedly less expression (Figure 1a and Supplementary Figure S2A, for quantitative analysis). This was correlated with successful infection, syncytia formation (Figures 1b and c, and Supplementary Figure S1), and significantly increased (P < 0.001 compared with controls) viral-induced cytotoxicity in uPAR-overexpressing cells (CT-26, MC-38 and 4T1), as opposed to B16F10 cells, where the levels of infection were markedly decreased (Figure 1d, and Supplementary Figure S2B). MV-m-uPA

Figure 1. In vitro viral infection, cytotoxicity and replication by MV-m-uPA in murine cancer cells. (a) uPAR expression in mouse cancer cells MC-38, CT-26, 4T1 and B16F10 was assessed by flow cytometry (FCM), using murine anti-uPAR monoclonal antibodies (filled histograms) or isotype controls (open histograms). (b, c) Mouse cancer cells were infected with MV-m-uPA as indicated at an MOI = 1 and photographed 48 h after infection. Representative pictures of infected cells (b: light; c: fluorescence). Scale bar = 500 µm. Arrows indicate areas of virus-induced syncytia. (d) In vitro cytopathic effects of MV-m-uPA. Murine cancer cells were infected with MV-m-uPA at an MOI = 1 and viability was determined at different time points (48, 72 and 96 h) by Trypan blue exclusion and presented as percentage of controls. Bars represent averages ± s.d. of triplicate experiments, *P < 0.001. (e) MC-38, CT-26 and 4T1 cells were infected with MV-m-uPA (MOI = 3) and titers of virus were determined at different time points by the one-step growth curve.
successfully replicated in uPAR-overexpressing murine cancer cells (viral titers -TCID$_{50}$- at 48 and 72 h: MC-38 = 26,600/6300; CT-26 = 6309/199,000; 4T1: 3548/11,220). We observed that MV-m-uPA replicated at significantly higher levels in CT-26 cells ($P<0.001$) compared with 4T1 cells at 72 h (Figure 1e).

In vivo safety and biodistribution of MV-m-uPA after intravenous administration

The orthotopic 4T1 tumor model was established in immunocompetent female Balb/C mice. Tumor-bearing mice were treated with two doses of MV-m-uPA (1.5 x 10$^8$ TCID$_{50}$, total dose: 3 x 10$^8$ TCID$_{50}$) intravenously, and were killed at 2, 5 and 28 days after treatment. No significant toxicity or treatment-related deaths were observed throughout the study. No changes in feeding behavior or activity were observed, nor were signs of physical distress or neurotoxicity observed in treated mice.

Tumors and organs were harvested for viral biodistribution and toxicity studies. Total RNA was extracted from frozen specimens and quantitative reverse transcriptase-PCR for MV-N mRNA was performed. Significantly more viral RNA was detected in tumors compared with other organs at days 2 and 5 after treatment (Figure 2). There was a sizeable increase in viral copy numbers in tumor tissues at day 5 compared with day 2 ($P=0.0622$).

Viremia and antibody production

MV-m-uPA RNA was detected in the blood of treated animals at day 2, and levels significantly decreased at days 5, 14 and 28 after treatment (Figure 2h). At day 28, viral RNA was detected in 2/5 mice in the blood at low levels. Viral RNA was detected in the urine of treated animals at day 2, but significantly decreased at days 5 and 28 of treatment (Figure 2g). Serum from mice was obtained at days 7, 14 and 28 after treatment for determination of anti-MV antibody titer (Figure 2i).
serum anti-MV antibody. Although no anti-MV antibodies were detected at day 7, increasing titers were found in all mice (n = 3 per group), from days 14 to 28 (Figure 2i).

Histologic analysis of tumors and organs of mice treated with MV-m-uPA

Five days after virus treatment, tumors and major organs were removed from treated and control (untreated) tumor-bearing mice, for histological analysis (hematoxylin and eosin). As shown in Figure 3a, significant necrosis and inflammatory changes were observed in tumors from treated animals. Lung, kidney, brain, spleen and heart did not show histological signs of viral-induced toxicity. Of note, livers from some tumor-bearing mice treated with MV-m-uPA had few microscopic tumor foci, and areas of focal inflammation. To further investigate these findings, and rule out the possibility of viral-induced liver inflammation, we conducted additional experiments in MV-m-uPA-treated and -untreated tumor-bearing mice, as well as in tumor-free mice treated with virus. Mice were killed 5 days after treatment, and livers were analyzed. No signs of inflammation or other signs of organ toxicity were observed in the livers of tumor-free mice treated with MV-m-uPA (Figure 3b, center). In tumor bearing, untreated mice, obvious tumor foci (5/5 mice) and inflammatory changes were observed (Figure 3b, left). On the other hand, the prevalence of tumor foci was markedly decreased (2/5 mice) in tumor bearing, treated mice, and inflammation was not different compared with untreated mice (Figure 3b, right). No significant staining of viral (MV-N) protein was detected in the livers of treated mice (data not shown).

Virus rescue, tumor targeting and induction of apoptosis

To correlate viral RNA copy numbers with presence of viable virus in tissues, virus recovery assays were performed from tumors and selected tissues at days 5 and 28 after treatment. At day 5, no virus was rescued from lung, heart, spleen, brain samples, whereas virus was rescued in four of five tumor samples, and one of five liver samples in mice treated with MV-m-uPA (Figure 4a). At day 28, MV-m-uPA was rescued from two of five tumors samples, whereas none of the organs tested had viable virus (Figure 4b).

To further evaluate tumor targeting of the recombinant viruses after systemic administration, 4T1 tumor-bearing mice were treated with MV-m-uPA, and tumors were resected 72 h after the last injection, for immunohistochemistry determination of viral protein (MV-N) in treated tumors. Viral protein was detected in the tumors of treated animals, but not in the controls (Figure 4c).

Next, we evaluated the effects of systemic administration of MV-m-uPA on tumor cell apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL) and proliferation (Ki67). Tumors from mice treated with MV-m-uPA had higher frequency of TUNEL-positive areas compared with untreated controls (MV-m-uPA (9.79% ± 1.77) vs Ctrl (2.45% ± 0.9), P < 0.01; Figures 4c and d). No differences in tumor cell proliferation were observed between treated and control mice (data not shown).

![Figure 3. Histologic analysis of tumors and organs of mice treated with MV-m-uPA. (a) 4T1 tumor-bearing Balb/c mice (n = 5) were given two doses of 1.5 x 10^6 TCID50 of MV-m-uPA or PBS via tail vein. Mice were killed 5 days after virus treatment and primary tumors and major organs (lung, heart, liver, spleen and brain) were removed for histological analysis (hematoxylin and eosin). Arrows indicate the necrotic and inflammatory areas. White arrowheads (liver) represent tumor foci. Scale bar = 400 μm. (b) Effects of MV-m-uPA in the liver of tumor-bearing and tumor-free mice (n = 5 per group). Virus treatment and tissue processing was performed as in Materials and methods section. Representative pictures of livers in the three groups. Left: tumor-bearing mice treated with PBS (micrometastases detected in 5/5 mice). Center: tumor-free mice treated with virus. Right: tumor-bearing mice treated with virus (micrometastases detected in 2/5 mice, picture is shown from a mouse with positive micrometastases). Note micrometastatic foci (white arrowheads) and areas of inflammation (black arrows). Scale bar = 200 μm.](image-url)
Laboratory parameters

At day 5, blood samples were obtained for complete blood count and clinical chemistry (liver and renal function) analysis. Hematology studies showed that both treated and untreated tumor-bearing mice had elevated white blood cell counts, with the treated mice having higher white blood cell counts than the controls (Table 1). In addition, lymphocyte percentages were decreased in both treated and control groups, albeit the degree of relative lymphopenia was greater in virus-treated mice. Calculated absolute lymphocyte counts, however, were not markedly different between the two groups (5651 × 10^3/l ± 1 in treated mice vs 5348 × 10^3/l ± 1 in untreated mice). Otherwise, no clinically significant differences were observed in other hematological or chemistry values in treated vs control mice. To determine if the increase in white blood cell counts was directly related to the virus treatment, a separate group of tumor-free mice were treated with MV-m-uPA as described above, and complete blood counts were performed. No changes in the white blood cells or lymphocytes were observed in tumor-free mice treated with the virus (Supplementary Table 1).

In vivo antitumor effects

Next, the in vivo antitumor effects of systemic administration of MV-m-uPA were investigated. First, immunocompetent Balb/C mice bearing orthotopic 4T1 tumors (approximately 0.5 cm) were treated (n = 8 per group) with either vehicle, or escalating doses of MV-m-uPA, as follows: (A) 1 × 10^5 (total dose, divided in three individual doses of 3.33 × 10^4); (B) 1 × 10^6 (three doses of 3.33 × 10^5); (C) 1 × 10^7 (three doses of 3.33 × 10^6); (D) 5 × 10^7 (three doses of 1.66 × 10^7) TCID_{50}, as described in Materials and methods section. As shown in Figure 5a, treatment with MV-m-uPA (total) doses of 1 × 10^5 and 5 × 10^7 TCID_{50} was associated with significant delay in tumor growth (P < 0.0001) compared with control mice. This was associated with significant prolongation of survival in mice treated with 1 × 10^7 (P = 0.0001) or 5 × 10^7 (P = 0.0006) TCID_{50} of MV-m-uPA compared with controls (Figure 5b). Importantly, systemic administration of escalating doses of MV-m-uPA was not associated with acute and subacute toxicity in tumor-bearing mice during or after intravenous administration.

To further validate MV-m-uPA as a therapeutic viral vector in other immunocompetent models, we assessed the antitumor effects of MV-m-uPA in the CT-26 murine colon cancer model. CT-26 cells were implanted into the right flank of female Balb/c mice. When the tumors reached 0.5 cm, mice were treated with either vehicle, or escalating doses of MV-m-uPA, as follows: (A) 1 × 10^5 (total dose, divided in three individual doses of 3.33 × 10^4); (B) 1 × 10^6 (three doses of 3.33 × 10^5); (C) 1 × 10^7 (three doses of 3.33 × 10^6); (D) 5 × 10^7 (three doses of 1.66 × 10^7) TCID_{50}. Intravenous administration of MV-m-uPA at doses of 1 × 10^5 TCID_{50} and above resulted in significant (P < 0.0001) inhibition of tumor progression (Figure 5c), and significant prolongation of survival (1 × 10^5 vs ctrl: P = 0.0256, 1 × 10^6 vs ctrl: P = 0.0089, 5 × 10^6 vs ctrl; P = 0.0297; Figure 5d). No acute of subacute toxicity was observed in animals treated with escalating doses of MV-m-uPA compared with controls.

DISCUSSION

An important requisite for the development of a retargeted oncolytic virus from bench to bedside is the characterization of its safety, biodistribution and feasibility of systemic administration in syngeneic, immunocompetent models of cancer that resemble human malignancies. Preclinical development efforts have been made with measles viruses targeted or retargeted against CD38,7 CD20,31 CD133,32 EGFRvIII,6 IL-13,3 carcinoembionc antigen,33 prostate stem cell antigen,34 among others; however,
characterization of preclinical safety or virus–host interactions was limited by the use of either immunodeficient xenograft models, models where the target was not expressed by host tissues or immunocompetent models where targets were artificially expressed in murine cancer cells. Ideally, the retargeted oncolytic virus should be directed against targets that are biologically relevant and naturally expressed in murine cancer cells in a similar way as in humans. Under these conditions, data on safety, tissue distribution and tumor targeting may be more predictive of the human situation.

The uPAR is a clinically validated and biologically important target that is overexpressed in many human as well as murine cancers. Non-oncolytic viral strategies that target the uPAR have been shown to be tumor specific and to be associated with antitumor effects in vitro and in vivo. We have previously shown that human uPAR targeted viruses (MV-h-uPA) induce significant antitumor effects in human breast cancer xenograft model in vivo, in immunodeficient mice. The effects of this viral vector in syngeneic, immunocompetent cancer models have not been previously described.

In this study, we focused on characterizing the virus–tumor–host interactions in syngeneic cancer models using the murine uPAR retargeted MV (MV-m-uPA). We demonstrated uPAR-dependent in vitro cytotoxicity induced by MV-m-uPA, as well as successful in vivo viral replication in murine cancer cells that overexpress uPAR (4T1, CT-26, MC-38), whereas no efficacy was observed in murine melanoma cell lines (B16F10), which express low levels of uPAR.

Systemic administration of MV-m-uPA in immunocompetent mice bearing syngeneic mammary tumors was found to be safe and feasible. This was demonstrated both in the biodistribution studies (4T1 model), as well as the dose escalation studies in the efficacy studies (4T1 and CT-26 models). MV-m-uPA preferentially accumulates in tumor tissues after intravenous administration compared with other organs. The observation that viral RNA copy numbers increased in tumor tissues from days 2 to 5, while they decreased in other organs, as well as viable virus recovery in tumor tissues only strongly suggests tumor-selective viral replication. Tumor targeting by MV-m-uPA was also demonstrated by immunohistochemistry analysis of MV-N in treated, but not in control tumors (Figure 4c).

Serum biochemistry studies did not show evidence of liver or renal toxicity (Table 1). The observation that white blood cell counts were elevated in treated and untreated tumor-bearing mice (Table 1), but not in treated non-tumor-bearing mice (Supplementary Table 1) suggests that changes in white blood cells may be secondary to tumor burden, and not directly due to the virus treatment. As expected in immunocompetent models, an antibody response was observed in treated mice at 14 days after systemic administration of MV-m-uPA.

Correlative histopathologic analysis of the organs where viral RNA was detected showed no signs of significant organ toxicity induced by the virus. The observation that the livers of some tumor-bearing mice treated with MV-m-uPA had higher viral RNA titers, viable virus (in 1/5 treated mice) and focal inflammatory changes can be explained by probable targeting by MV-m-uPA to liver micrometastases—which spontaneously develop in orthotopic 4T1 tumors—and not because of virus-induced hepatotoxicity. Lack of hepatotoxicity is supported by several lines of evidence, including lack of histological signs of liver injury in tumor-free mice.
treated with the virus (Figure 3b) and lack of abnormalities in serum chemistry (aspartate aminotransferase) in either treated tumor-free or tumor-bearing mice. MV-m-uPA targeting of liver micrometastases is supported by additional experiments showing that while all (5/5) tumor-bearing untreated mice had micrometastatic liver nodules, only 2/5 treated mice had metastatic foci. Studies to further characterize the abilities of MV-m-uPA to target and treat metastases in vivo are underway.

The biological significance of the findings was demonstrated by significant antitumor effects and prolonged survival in the very aggressive murine models of mammary carcinoma (4T1) and murine colon carcinoma (CT-26). TUNEL assays showed increased in vivo apoptosis in tumors treated with the retargeted virus compared with controls. This finding provides further in vivo validation of prior in vitro studies reporting that one mechanism of oncolytic measles virus cytotoxicity is induction of apoptosis. The in vitro and in vivo differences were more marked in the colon cancer than the mammary carcinoma model. The in vivo differences were correlated with the in vitro results (Figure 1), where CT-26 cells were found to be more sensitive for viral infection and permissive for viral replication than 4T1 cells, although uPAR expression was similar between the two cell lines. These findings may reflect differences in cellular innate antiviral responses, or in oncogenic pathways, which may render some cancer cells less sensitive to the cytotoxic effects of oncolytic viruses.

The in vivo antitumor effects (both antitumor response and survival) were more marked in the colon cancer than the mammary carcinoma model. The in vivo differences were correlated with the in vitro results (Figure 1), where CT-26 cells were found to be more sensitive for viral infection and permissive for viral replication than 4T1 cells, although uPAR expression was similar between the two cell lines. These findings may reflect differences in cellular innate antiviral responses, or in oncogenic pathways, which may render some cancer cells less sensitive to the cytotoxic effects of oncolytic viruses.

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4T1 cells (murine mammary carcinoma), MC-38 cells (murine colon carcinoma), CT-26 cells (murine colon cancer), B16F10 cells (murine melanoma) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin and streptomycin at 37 °C and 5% CO₂. Vero-r-siRNA cells55 were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C and 5% CO₂.

Flow cytometry
Mouse uPA receptor expression was detected in cancer cell lines by flow cytometry, using a phycocyanin-conjugated rat monoclonal anti-mouse uPAR (R&D Systems, Minneapolis, MN, USA), as previously described.30,41

Animal studies
Animal studies were approved by Institutional Animal Care and Use Committee of University of Miami.

Mouse biodistribution and organ toxicity studies
In all, 8- to 10-week-old female Balb/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were injected with 1 × 10⁵ 4T1 cells in 50 μl phosphate-buffered saline (PBS) into the fifth mammary fat pad. When tumors reached a diameter of 0.7 cm, mice (five mice per group per time point) were treated with MV-m-uPA at a dose of 1.5 × 10⁶ TCID₅₀ (in 100 μl PBS) via tail vein—every other day for three doses. At days 6 and 14 after treatment, mice were killed and blood was collected by retro-orbital puncture.

Additional studies were conducted to compare the effects of MV-m-uPA in the livers of tumor-bearing vs non-tumor-bearing mice. Three groups of mice (n = 5) were established: (a) tumor-free Balb/c mice treated with MV-m-uPA, 1.5 × 10⁶ TCID₅₀ (in 100 μl PBS for two doses), (b) 4T1 tumor-bearing Balb/c mice treated as above and (c) 4T1 tumor-bearing Balb/c mice treated with PBS. At days 5 after treatment, mice were killed and livers were removed and fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin for histological analysis by a veterinary pathologist. The presence of any lesions (inflammatory, necrotic, infectious, neoplastic, and so on) was assessed.

Characterization of MV-m-uPA’s in vivo oncolytic effects in immunocompetent cancer murine models
Eight to 10-week-old female Balb/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were injected with 1 × 10⁷ 4T1 cells into the fifth mammary fat pad. CT-26 (2 × 10⁵) cells were inoculated into the right flank of Balb/c mice. When tumors reached 0.4–0.5 cm, mice (n = 5 per group) were treated with either PBS (control group) or escalating (total) doses of MV-m-uPA, given intravenously—every other day for two doses, or 100 μl PBS as control. At days 2, 5 and 28 after treatment, samples were collected for histological analysis by a veterinary pathologist. The presence of any lesions (inflammatory, necrotic, infectious, neoplastic, and so on) was assessed.

Viral RNA quantification
Total RNA was extracted from frozen specimens using the RNeasy tissue mini kit (Qiagen, Valencia, CA, USA) for heart, RNA from blood samples was isolated using the QiAamp RNA blood mini kit. RNA from urine was isolated using the QIAamp viral RNA minikit (Qiagen), following the manufacturer’s recommendations. Quantitative reverse transcriptase-PCR for MV-N (nucleoprotein) mRNA was performed as previously reported.42

Hematology and chemistry analysis
Whole blood and serum were harvested from mice at specific time points, and host toxicity was evaluated by assessment of changes in hematological and biochemical parameters using a Hemavet 1700 multispecies hematology analyzer (Drew Scientific, Dallas, TX, USA) and an Ortho Vitros 250 analyzer (Ortho-Clinical Diagnostics, Rochester, NY, USA), respectively.

Anti-MV antibody assay
Mice bearing 4T1 tumors were treated as described above, killed (n = 3 per time point) at 7, 14 and 28 days after treatment and serum was collected for anti-MV antibody studies. The MV-specific IgG titer was measured by an indirect immunofluorescence test using MV antigen substrate slides (Bion Enterprises, Des Plaines, IL, USA) according to the manufacturer’s instructions, and performed as described.33,43 The protocol was modified using goat anti-mouse IgG fluorescein isothiocyanate-conjugated antibodies (Invitrogen, Grand Island, NY, USA) for detection of murine antibodies.

Virus recovery
Tissues were weighed and homogenized in three volumes (v/v) of Opti-MEM utilizing mechanical crushing and a single freeze–thaw cycle. The supernatant was clarified by centrifugation and 10-fold serial dilutions of samples were prepared in Opti-MEM. Aliquots (50 μl) of each dilution were plated in 96-well plates containing Vero cells and TCID₅₀ titrations were performed. TCID₅₀ calculations were normalized per gram of tissue.

Immunohistochemistry studies
Tissue samples were collected and frozen, and cryostat sections were fixed in cold acetone for 10 min and endogenous peroxidase activity were quenched with 0.3% H₂O₂ for 10 min. The slides were washed in PBS and incubated with biotinylated mouse anti-MV-nucleoprotein antibody (Chemicon International, Temecula, CA, USA) for 30 min at 37 °C. After washing in PBS, the slides were developed with VECTASTAIN ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidine (DAB) horseradish peroxidase substrate (Vector Laboratories) according to the manufacturer’s instructions.

TUNEL assay
Apoptosis was detected with an in situ cell death detection kit (Roche Applied Science, Indianapolis, IN, USA), as previously described by us.42

Statistical analysis
The data in this study fell into three types: (1) continuous measures where only one observation was made on the experimental unit; (2) continuous measures where there were repeated observations, usually over time, where each experimental unit had multiple observations under different conditions; and, (3) time to survival after tumor transplant. The data in type 1 were analyzed using the analysis of variance and multiple linear and non-linear regression. Subgroup comparisons were made after the overall analyses using the Student’s t-test in single degree of freedom contrasts. Analyses were performed using SAS PROC GLM and SAS PROC REG statistical programs (SAS institute, Cary, NC, USA). The data from type 2 observations were analyzed using mixed model analysis of variances suitable for repeated measures. Single degree of freedom contrasts were performed between and within conditions, at specific times, and across time using the methods proposed by.46 The survival data were analyzed using Kaplan–Meir stratified analyses in SAS PROC LIFETEST and the Cox Proportional Hazards model in SAS PROC PHREG. Statistical significance was set at P = 0.05 with adjustments for multiple comparisons when appropriate.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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