An Immunocompetent Murine Model for Oncolysis with an Armed and Targeted Measles Virus

Guy Ungerechts[1], Christoph Springfeld[1]*, Marie E Frenzke[1], Johanna Lampe[1], William B Parker[2], Eric J Sorscher[3] and Roberto Cattaneo[1]

[1]Molecular Medicine Program and Virology and Gene Therapy Track, Mayo Clinic College of Medicine, Rochester, Minnesota, USA; [2]Southern Research Institute, Birmingham, Alabama, USA; [3]University of Alabama at Birmingham, Birmingham, Alabama, USA

An immunocompetent model is required to test therapeutic regimens for clinical trials with the oncolytic measles virus (MV). Toward developing this model, a retargeted MV that enters murine colon adenocarcinoma cells forming tumors in syngeneic C57BL/6 mice was generated. Since MV infection tends to be less efficient in murine than in human cells, the targeted virus was also armed with the prodrug convertase, purine nucleoside phosphorylase (PNP), and named MV-PNP-antiCEA. We have shown before that in cultured cells, infection with this virus activated the prodrug, 6-methylpurine-2′-deoxyriboside (MeP-dR), causing extensive cytotoxicity. When injected intratumorally (IT), MV-PNP-antiCEA inhibited subcutaneous tumor growth marginally, but subsequent administration of the prodrug enhanced the oncolytic effect. Systemic delivery of MV-PNP-antiCEA alone had no substantial oncolytic effects, but in combination with the prodrug it was therapeutic, revealing synergistic effects between virus and prodrug. Immunosuppression with cyclophosphamide (CPA) retarded the appearance of MV neutralizing antibodies and enhanced oncolytic efficacy: survival was 100%, with 9 out of 10 animals going into complete remission. This immunocompetent murine model facilitates the testing of therapeutic regimens for clinical trials.

Received 13 April 2007; accepted 25 July 2007; published online 21 August 2007. doi:10.1038/sj.mt.6300291

INTRODUCTION

Oncolytic agents are being developed based on adenoviruses, herpes simplex virus 1, poxviruses, reoviruses, vesicular stomatitis virus and measles virus (MV).[1–3] This biological anti-cancer therapy concept has progressed from mere pre-clinical experiments to phase III clinical trials in humans,[4] at present the first oncolytic therapy regimen is operational.[5] The efficacy of new oncolytic vectors can be tested in human cancer cell xenografts implanted in immunodeficient mice. In particular, pre-clinical MV efficacy trials have been based on immunodeficient mouse–human tumor xenograft models.[6–9] However, this approach does not allow for analysis of the effects of the immune system.

The therapeutic efficacy of oncolysis can be reduced by premature viral clearance. Frequent intratumoral (IT) administrations of a reovirus are necessary in immunocompetent mice to achieve the same levels of tumor regression as in a severe combined immunodeficient mouse model.[10] Immunosuppressive drugs like cyclophosphamide (CPA) and cyclosporine A, or T-cell depletion using antibodies against CD4 or CD8, enhance the oncolytic efficacy of many viruses including reovirus,[11] herpes simplex virus 1[12] and adenovirus;[13] early viral clearance interferes with adenovirus efficacy in clinical trials.[14–16] To study these effects, immunocompetent models permissive of oncolytic adenovirus vectors have been established.[17,18]

Conversely, the immune system might increase the efficacy of tumor killing: the induction of tumor-specific cytotoxic T lymphocytes, or antibodies, or cytokines such as the tumor necrosis factor can enhance the therapeutic outcome.[19] These observations promoted the development of virus-based vaccines that induce specific immune responses for clinical trials in cancer therapy.[20] In addition, immunostimulatory cytokines expressed from recombinant oncolytic viruses enhance systemic immune responses that complement viral cytotoxicity.[21–24]

The development of an immunocompetent model for MV oncolysis is complicated by the lack of a receptor on mouse cells.[25] However, MV can be retargeted to a designated receptor by displaying ligands on the viral attachment protein, the hemagglutinin (H).[26,27] Surface proteins that have been successfully targeted by recombinant MV displaying appropriate ligands include the human carcinoembryonic antigen (CEA).[27] This protein is expressed in MC38cea cells, a murine colon adenocarcinoma cell line that is tumorigenic in syngeneic C57BL/6 mice.[28] MC38cea cells can be infected with MV-antiCEA, but viral replication is suboptimal, resulting in very low titers.[27] This is because post-entry events contribute to MV tropism being restricted to primate cells.[29–31] Thus, the oncolytic efficacy of MV may be low in mouse models.

To enhance oncolytic efficacy, viruses have been armed with genes for prodrug converting enzymes.[32] For this study we armed

**Present address: Department of Gastroenterology, University Hospital Heidelberg, Infectious Diseases and Intoxications, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.*

***Correspondence:** Roberto Cattaneo, Mayo Clinic Rochester, Molecular Medicine Program, Guggenheim 1838, 200 First Street SW, Rochester, Minnesota, 55902, USA. E-mail: cattaneo.roberto@mayo.edu*

© The American Society of Gene Therapy

the MV with *Escherichia coli* purine nucleoside phosphorylase (PNP), a prodrug convertase. PNP converts 6-methylpurine-2′-deoxyriboside (MeP-dR) to 6-methylpurine (MeP). This highly diffusible substance is metabolized to toxic adenosine triphosphate analogs, which inhibit DNA, RNA, and/or protein synthesis immediately.[33] In this study we show that a CEA-targeted and convertase-armed MV inhibits the growth of syngeneic MC38cea tumors in C57BL/6 mice after IT, or after intravenous (IV) injection of the virus with subsequent prodrug administration. We also show that oncolytic efficacy can be enhanced by a single administration of an immunosuppressive drug before treatment.

RESULTS

Generation and characterization of armed and targeted MV

To enter mouse cells forming tumors in syngeneic animals, we generated a recombinant MV whose attachment protein hemagglutinin (H) is fused to a single-chain antibody, thus mediating entry through the human protein CEA expressed on these cells (**Figure 1a**). In addition, to enhance oncolysis, the *E. coli* PNP gene was inserted in the viral genome (**Figure 1a**). The recombinant MV that displays the human CEA single chain antibody, maintains entry through the human receptors, but this fact is not relevant in the syngeneic mouse model used here.

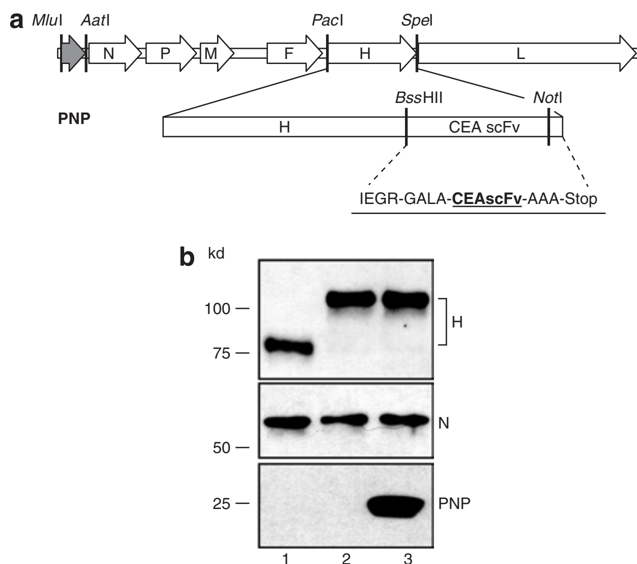

Figure 1 Construction and characterization of carcinoembryonic antigen (CEA)-targeted and/or purine nucleoside phosphorylase (PNP)-armed viruses. (**a**) Schematic drawing of a recombinant measles virus (MV) genome encoding a hybrid attachment protein (hemagglutinin, H) displaying an antiCEA single-chain antibody (scFv). The amino acid sequence of the linker preceding the scFv is indicated on the bottom line (one-letter code). An additional transcription unit coding for the *Escherichia coli* PNP gene was inserted upstream of the N gene. (**b**) Immunoblot analysis of proteins expressed by the unmodified MV (lane 1), MV-antiCEA (lane 2), and MV-PNP-antiCEA (lane 3). For detection of H and N, immunoblots of 5,000 infectious particles were performed. To confirm PNP expression, Vero cells were infected at multiplicity of infection 0.5, and cells were lysed for immunoblotting 36 hours post infection. Upper and center panel show expression of H and N viral proteins, respectively. Lower panel, PNP expression.

A targeted virus, MV-antiCEA,[27] and an armed and targeted virus, MV-PNP-antiCEA, were both generated. PNP expression was confirmed by immunoblotting: a band with an apparent molecular weight of 25 kd was detected in cells infected with MV-PNP-antiCEA (**Figure 1b**; lane 3). Reduced electrophoretic mobility of the hybrid H glycoprotein confirmed addition of the antiCEA scFv (**Figure 1b**; lanes 2 and 3). The N protein immunoblot analysis served as an internal control for viral protein incorporation (**Figure 1b**; lanes 1–3). Equivalent incorporation of the standard and the extended H proteins in viral particles was observed. The targeted and armed virus reached comparable titer levels, with equivalent kinetics, to the two other viruses (data not shown). Thus, targeting and arming were not seen to significantly compromise viral replication.

Targeted MV infect murine colon adenocarcinoma cells expressing human CEA

Mouse MC38cea cells expressing human CEA, the parental mouse cell line MC38, and simian Vero cells were inoculated at a multiplicity of infection (MOI) of 0.5 with three different MV and monitored after 36 hours for the appearance of syncytia, which would denote virus entry and replication (**Figure 2**). As expected, only MV-antiCEA and MV-PNP-antiCEA induced syncytia in MC38cea cells expressing the designated receptor human CEA (**Figure 2**; right column, middle and lower panels). The retargeted viruses did not fuse the parental cell line MC38 (**Figure 2**; middle column, middle and lower panels). All the MV showed similar cytopathic effects including widespread syncytia formation on Vero cells that express one of the MV receptors[34] (**Figure 2**; left

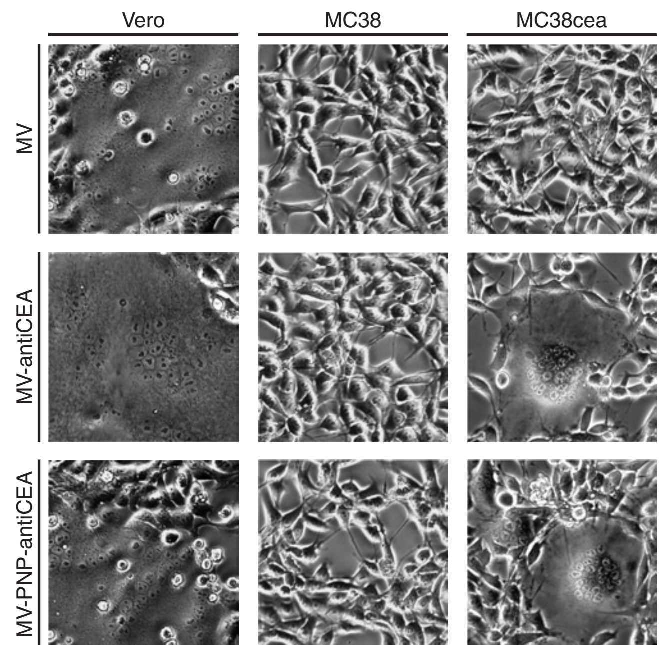

Figure 2 Selective infection of cells by recombinant viruses. Cells were infected with unmodified measles virus (MV) (upper row), MV-antiCEA (middle row), or MV-PNP-antiCEA (lower row) at multiplicity of infection 0.5 and photographed 36 hours later. Syncytia formation was documented on simian Vero cells (left column), murine MC38 (middle column), and MC38cea cells (right column). CEA, carcinoembryonic antigen; PNP, purine nucleoside phosphorylase.

column). MV infection resulted in complete Vero cell lysis after about 48 hours, whereas fusion and lysis of MC38cea cells was limited to small syncytia (data not shown). Thus, while targeting sustained MV entry in murine cells the infection was inefficient and cytotoxicity limited.

Bystander effect of activated prodrug enhances MV cytotoxicity

To assess bystander cell killing by the toxified prodrug MeP, Vero cells were infected with MV-PNP-antiCEA at 0.1 MOI. MeP-dR (100 μmol/l) was added to the media 36 hours post infection for 12 hours. After heat-inactivation, different dilutions (1:100, 1:10, or 1:2) of conditioned media were transferred to a new sub-confluent Vero cell monolayer and living cells were counted after 72 hours (**Figure 3a**). Toxicity increased in accordance with the concentration of conditioned media (**Figure 3a**; hatched columns). No significant impact on viability was observed after incubating cells with supernatants of mock-infected, MV-infected cells, or with cells previously incubated with media containing 100 μmol/l MeP-dR (**Figure 3a**; black, gray, or white columns, respectively). Thus, MeP is efficiently secreted and even 1:100 media dilutions are found to be toxic.

We then tested whether arming MV with PNP enhanced the lysis of murine cells. MC38cea cells were infected with MV-PNP antiCEA at MOI of 0.01, 0.1, or 1 (**Figure 3b**). The prodrug MeP-dR (left panel), or no drug (right panel) were added to the medium 24 hours post infection, and cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide cell proliferation assay. The prodrug strongly enhanced the moderate cytotoxic effect of MV-PNP-antiCEA. MeP-dR (up to a concentration of 100 μmol/l) without concomitant virus infection had no significant impact on cell growth (cell viability >95% after 120 hours). Therefore, arming MV with PNP allows this human virus to be highly cytotoxic even for mouse cells after prodrug administration.

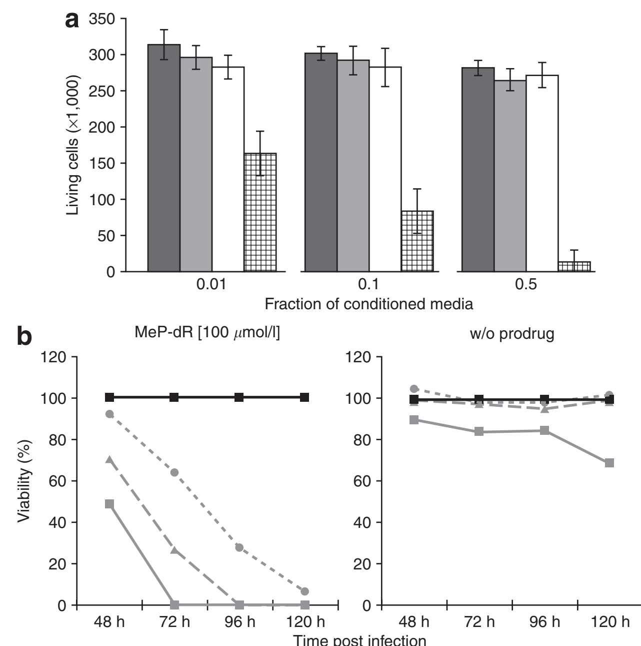

Figure 3 Cytotoxic efficacy of the activated prodrug. (**a**) Measurement of cell toxicity caused by conditioned media. Cells were mock-treated, infected with MV-PNP only, treated with 6-methylpurine-2′-deoxyriboside (MeP-dR) only, or infected with MV-PNP-antiCEA and MeP-dR was added 36 hours post infection for 12 hours. Different dilutions (0.01, 0.1, and 0.5) of conditioned and heat-inactivated media (mock-treated, dark gray column; MV-PNP infected, light gray column; MeP-dR-treated, white column; MV-PNP infected and MeP-dR treated, hatched column) were added to a new Vero cell monolayer. Numbers of living cells were determined after 72 hours. Columns, mean of three independent experiments; bars, SE. (**b**) Viability of mouse cells after infection, with or without prodrug administration. MC38cea cells were infected in quadruplicate with MV-PNP-antiCEA. Left panel, gray squares, triangles, and dots represent the mean of experiments at multiplicity of infection 1, 0.1, and 0.01, respectively, with 100 μmol/l MeP-dR (prodrug added *ab initio*). Right panel, gray symbols represent experiments without prodrug; black squares: mock-treated cells used to define 100% viability. CEA, carcinoembryonic antigen; MV, measles virus; PNP, purine nucleoside phosphorylase.

Low-level prodrug convertase expression in tumors sustains oncolysis

We then tried to assess or characterize tumor targeting, transgene expression (PNP activity) and the oncolytic efficacy of armed and retargeted MV in subcutaneous MC38cea tumors grown in syngeneic C57BL/6 mice. In an experiment designed to quantify viral transcription and PNP expression at the expected peak of replication, mice received either four IT or four IV injections of MV-PNP-antiCEA on days 6, 7, 8, and 9 post MC38cea cells implantation. On day 11, tumors were harvested and halved, RNA extracted from one half, and the number of viral N messenger RNA (mRNA) molecules were measured by quantitative analysis using the reverse transcription polymerase chain reaction method. On average, 1.7×10^6 MV N mRNA copies per 10^5 cells, or about 15–20 copies of N mRNA per average cell were detected after IT injection, and about 10 times lower levels were detected after IV administration of MV-PNP-antiCEA (**Figure 4a**). The efficiency of PNP expression was assessed on protein extracts

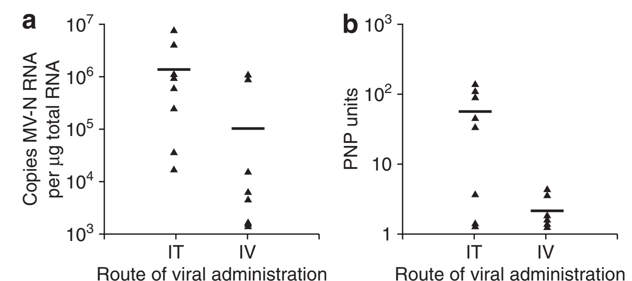

Figure 4 Tumor targeting and transgene expression of MV-PNP-antiCEA *in vivo*. One million MC38cea cells were implanted in the flank of C57BL/6 mice. Viral treatment was started when tumors reached a volume of 20–40 μl. Mice were injected either intratumorally or intravenously on four consecutive days with a total dose of 8×10^6 plaque forming units of MV-PNP-antiCEA. Measurements were made 2 days after the last viral application. Each experimental group consisted of eight mice. (**a**) Copies of MV N messenger RNA (per microgram total cellular RNA or approximately 10^5 cells) measured by quantitative reverse transcription polymerase chain reaction. (**b**) PNP activity in tumor tissue detected by an enzymatic assay is expressed as purine nucleoside phosphorylase (PNP) units (1 unit represents 1 nmol MeP-dR-converted/mg tumor/hour). CEA, carcinoembryonic antigen; IT, intratumorally; IV, intravenous; MV, measles virus.

© The American Society of Gene Therapy

obtained from the other tumor half: a mean of 59 PNP units was monitored after IT injection of MV-PNP-antiCEA, and about 20–25 times lower levels after systemic administration (**Figure 4b**). These low activity levels sustain oncolysis (see below).

Prodrug administration after IT injection extends mouse survival

Next, we assessed the effect of an IT injection of MV-PNP-antiCEA on tumor growth, with or without subsequent intraperitoneal administration of MeP-dR. Mice received the same treatment as above; control groups were mock-infected or treated only with prodrug, respectively, and tumor diameters measured every 3 days. The oncolytic effect of MV-PNP-antiCEA alone was not statistically significant (**Figure 5a**; dots; $P = 0.2381$ by the two sample t-test). However, prodrug administration in combination with virus infection was seen to produce an oncolytic effect (**Figure 5a**; triangles; $P = 0.0015$ by the two sample t-test, compared to mock treatment). MeP-dR alone showed no effect (**Figure 5a**; diamonds). Targeting the human CEA was necessary too to produce the oncolytic effect: MV that did not display an antiCEA single chain antibody was not effective in treating established subcutaneous MC38cea tumors after IT injection, or even when the infection preceded cell implantation (data not shown).

The inhibition of tumor growth resulted in survival benefits, as illustrated using a Kaplan–Meier estimate with a 1,500 µl tumor volume defined endpoint. Infection with MV-PNP-antiCEA combined with prodrug administration resulted in significant increase of survival compared to mock therapy or administration of MeP-dR alone (**Figure 5b**; $P = 0.0005$ and 0.002 by the two sample t-test). In this experimental approach, survival was significantly extended in treatment with the PNP vector combined with MeP-dR; this was also the case when compared to treatment with the virus alone (two sample t-test P-value = 0.0183). Thus, prodrug administration after IT virus infection significantly retarded tumor growth and extended mouse survival.

Systemic administration of MV-PNP-antiCEA and prodrug are synergistic

Since local administration of viral vectors is unsuitable for treatment of most cancer types, we tested the efficacy of the armed and CEA-targeted MV after its systemic administration via tail vein. **Figure 5c** shows that treatment with prodrug only (diamonds) or virus alone (dots) did not have a statistically significant effect compared to mock treatment (squares; $P > 0.5$ from two-sample t-test). However, the combination of MV-PNP-antiCEA and MeP-dR (triangles) did have a better therapeutic effect than mock, prodrug, or virus treatment alone did ($P = 0.0016/0.0288/0.009$ by two-sample t-test). Importantly, there is seen a tendency for synergism between MV-PNP-antiCEA and MeP-dR (P-value = 0.1477; interaction test from two factor analysis of variance model).

Again, retardation of tumor growth resulted in extended survival (**Figure 5d**; triangles). Prolongation of survival was significant only after combining the prodrug and virus as compared to the mock treatment (squares), MeP-dR treatment alone (diamonds), or virus treatment alone (dots; log-rank test P-value <0.001 for all events). Statistical analysis revealed significant synergistic effects between the virus and drug (P-value = 0.0163; the interaction test used a two factor analysis of variance model). Thus, this combination, of infection with an armed virus, along with prodrug administration, is seen to have synergistic effects.

Infection elicits a strong immune response that is delayed by CPA application

The next step of this study was to assess the strength of the humoral immune response against MV-PNP-antiCEA by measuring the neutralizing antibody titers 14 and 28 days after inoculation. To assess the impact of immune-suppression on antibody production

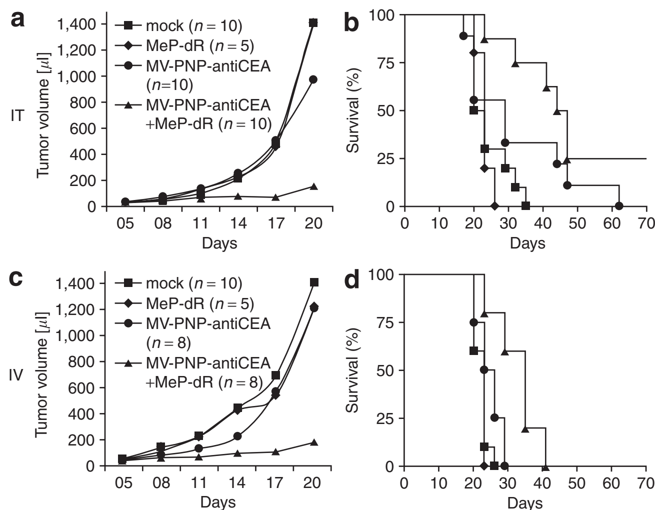

Figure 5 Oncolytic effects of the MV-PNP-antiCEA/prodrug system in immunocompetent C57BL/6 mice after intratumoral (IT) or intravenous (IV) administration. (**a** and **c**) Tumor volume measurements starting on day 5 after subcutaneous implantation. The administration of the virus injection was either IT (**a** and **b**) or IV (**c** and **d**). MeP-dR was administered intraperitoneally (60 mg/kg/dose) 24 hours post infection for three consecutive days. Kaplan–Meier survival curves after (**b**) IT or (**d**) IV injections of MV-PNP-antiCEA in the presence or absence of prodrug, compared to mock treatment. The defined endpoint was 1,500 µl tumor volume. CEA, carcinoembryonic antigen; MV, measles viruses; PNP, purine nucleoside phosphorylase.

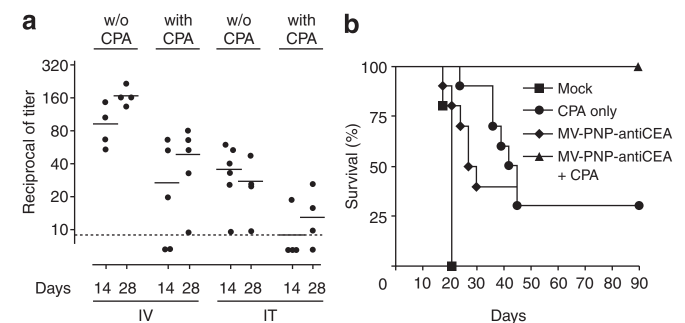

Figure 6 Influence of immune-suppression on measles virus (MV) neutralization and efficacy of oncolysis. (**a**) Neutralizing antibodies in the serum of mice treated with MV-PNP-antiCEA with or without cyclophosphamide (CPA) pre-treatment. Each dot represents one mouse; the mean of the group is indicated by a horizontal bar. The dotted line indicates the detection threshold. (**b**) Kaplan–Meier survival curves documenting the effects of immune-suppression on intratumoral (IT) treatment efficacy. The defined endpoint was 1,500 µl tumor volume. Each group consisted of 10 mice. CEA, carcinoembryonic antigen; IV, intravenous; PNP, purine nucleoside phosphorylase.

we administered CPA (**Figure 6a**). C57BL/6 mice (n = 5 in the IV group and n = 7 in the IT group) received either four IV or four IT injections of MV-PNP-antiCEA with or without CPA pre-administration (120–150 mg/kg intraperitoneal; 24 hours before viral treatment). As expected, after IV administration of MV-PNP-antiCEA without CPA immunosuppression, a robust immune response was elicited with a mean value of antibody titers around 1:95 on day 14 and 1:170 on day 28. This immune response was significantly suppressed to an average of around 1:30 on day 14 (P = 0.0007 by two-sample t-test) and 1:50 at day 28 (P = 0.0297) when CPA was pre-administered. The mean value of antibody titers was lower after IT administration of the virus (on average around 1:40 after 14 days), and very low (just above the detection level) when the IT-inoculated mice were immunosuppressed (P = 0.0161). Antibody titers after 28 days were found to be around 1:30 in non-immunosuppressed and around 1:15 in CPA treated mice (at this point of time the mice needed to be scarified due to the tumor burden; therefore there is no statistical significance for these values). The sera of naïve mice did not contain neutralizing antibodies against MV. Thus, we can conclude that CPA application delayed the humoral immune response.

Enhancement of oncolytic efficacy by immunosuppression

Next, we assessed the effect of immunosuppression on MV oncolysis after systemic as well as local inoculation was done with MV-PNP-antiCEA. C57BL/6 mice received the same treatment as above; control groups were mock-infected or treated only with CPA (120–150 mg/kg), respectively, and tumor diameters were measured every 3 days.

A combination of virotherapy and immunosuppression led to significantly retarded tumor growth as compared to mock-infection (data not shown). Strikingly, combination therapy led to a 100% survival rate, 90 days post IT treatment (**Figure 6b**). In 9 of the 10 mice killed after day 90 the tumor mass had regressed completely. In contrast, MV-PNP-antiCEA administered alone, or CPA alone only led to 30% remaining tumor-free 90 days post treatment. Pre-administration of CPA slowed tumor growth marginally after IV virotherapy, but did not extend the time of mouse survival (data not shown). We also noted that a single 120–150 mg/kg CPA dose had a therapeutic effect; in other murine models for viral oncolysis however, this dose is considered sufficient for immune-suppression but not for effective chemotherapy (in mice >400 mg/kg is required for effective chemotherapy).[11,13] Thus, immunosuppression in the context of MV treatment enhances oncolytic efficacy.

DISCUSSION

Oncolytic MV is emerging as effective in the field of experimental cancer therapeutics,[2,35] but so far an immunocompetent model possessing antitumoral efficacy has been lacking. In this study we have developed a colon adenocarcinoma tumor model in fully immunocompetent mice. These syngeneic tumors express human CEA, a target for recombinant MV displaying an antiCEA single-chain antibody on their attachment protein. We have shown here that arming of this virus with a prodrug convertase, and immunosuppression, amplifies its oncolytic effect. The mechanistic insights about MV oncolysis gained from this model will be used to plan second-generation clinical trials.

We observed that a low-level prodrug convertase expression sufficed to interfere with tumor progression. Only about two copies of N mRNA per average tumor cell were detected, whereas in cultured cells infected at high multiplicity, up to 3×10^4 copies of N mRNA per cell are produced.[36] Remarkably, at early points of time (until day 20 post tumor implantation), IT and IV administration elicited anti-tumor responses of similar strength; this was so even though an average PNP activity of 60 units was documented after IT, and only 2–3 units after IV administration. Thus, the 20–30 times lower amount of PNP expressed in the tumor after IV inoculation was as effective in inhibiting tumor growth as the higher amount expressed after IT inoculation. Similarly, an IV-injected CD20-targeted and PNP-armed MV, induced strong oncolytic effects in a lymphoma xenograft model, while producing PNP at comparable levels as the CEA-targeted and PNP-armed virus characterized here (data not shown). In both systems, a more homogenous virus distribution in the tumor after IV inoculation may account for this enhanced effect. It is to be noted that a homogenous mixture of 0.1% PNP expressing cells and 99.9% cells not expressing PNP in a tumor elicited substantial anti-tumor effects.[37] PNP activity in the range of 30 PNP units elicited anti-tumor effects in a subcutaneous glioma model in which a HSV-PNP vector was administered IT.[37,38] Taken together, these observations suggest that infection of less than 1 in 1,000 cells, if homogenously distributed in the tumor, sustains PNP expression levels sufficient to support oncolysis.

Immunosuppression with CPA enhanced the oncolytic effect of MV-PNP-antiCEA. Combination therapy with CPA led to 100% mouse survival 90 days post treatment and in 9 out of 10 mice to a complete regression of the tumor mass. CPA immunosuppression enhances the oncolytic efficacy of other viruses including reovirus, herpes simplex virus 1, and adenovirus.[11–13] At the time of approaching clinical trials, we are in the position of optimizing the window of immunosuppression elicited with CPA. It is worth noting as well, that the recombinant MV currently used in clinical trials of oncolysis has defects in two immune response evasion proteins;[31] studies in immunodeficient mice indicate that MV that expresses functional immune evasion proteins, is more effective than currently used vectors.[39] The mouse model presented here will allow us to compare the oncolytic efficacy of MV that retains a partial or complete immune response evasion protein function, in a fully immunocompetent host.

To circumvent neutralizing antibodies prevalent in the population through vaccination or natural disease, we envisage enclosing the MV replicative unit in the envelope of canine distemper virus (CDV) since it has marginal cross-reactivity with human sera. The CDV attachment protein can functionally substitute that of MV: recombinant MV together with CDV H is fully replication competent.[40] Moreover, we have shown before that both the envelope glycoproteins of MV and CDV are reciprocally interchangeable, and that the chimeric viruses have targeting competence (data not shown). We expect a MV with a CDV envelope to maintain some oncolytic efficacy in the presence of anti-MV immunity, and plan to test this hypothesis in C57BL/6 immunocompetent hosts. In conclusion, we have developed an

© The American Society of Gene Therapy

immunocompetent mouse model that allows us to design clinical protocols based on oncolytic MV that is either targeted, enclosed in foreign envelopes, or armed with prodrug convertases.

MATERIALS AND METHODS

Cell culture. Vero African green monkey kidney cells were purchased from American Type Culture Collection (Manassas, VA). MC38cea cells were kindly provided by Jeffrey Schlom.[28] Both cell lines were grown at 37 °C in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 10% fetal calf serum with penicillin/streptomycin in a humidified atmosphere of 5% CO_2.

Construction of recombinant MV. The *E. coli* PNP gene was amplified using the polymerase chain reaction method, from pSV-PNP[41] using primers providing the appropriate *Mlu*I and *Aat*II restriction sites. The *Mlu*I/*Aat*II-digested polymerase chain reaction product was cloned into the full-length complementary DNA p(+)MV[42] using the corresponding restriction sites, resulting in p(+)MV-PNP. The *Pac*I/*Spe*I-digested fragment from pCG-HXL[27] with CEA-retargeted H was exchanged for the corresponding fragment of p(+)MV and p(+)MV-PNP, respectively. The resulting full-length complementary DNAs were named p(+)MV-antiCEA and p(+)MV-PNP-antiCEA. Recombinant viruses were rescued as described previously.[42] To prepare virus stocks, Vero cells were infected at an MOI of 0.03 and incubated at 37 °C for 36 hours. Viruses were harvested by one freeze–thaw cycle from their cellular substrate and resuspended in Opti-MEM (Invitrogen, Carlsbad, CA). Titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) titration on Vero cells.

Immunoblot analysis. Viral samples (5,000 $TCID_{50}$) were mixed with an equal volume of sodium dodecyl sulfate loading buffer [130 mmol/l Tris (pH 6.8), 20% glycerol, 10% sodium dodecyl sulfate, 0.02% bromophenol blue, 100 mmol/l dithiothreitol]. These samples were denatured for 5 minutes at 95 °C, fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel, blotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), immunoblotted with anti-N protein antibody (Novus Biologicals, Littleton, CO) at 1:5,000 dilution, with anti-PNP antibody (a kind gift of Dr. Jeong S. Hong, University of Alabama) at 1:10,000 dilution and anti-H protein antibody[43] at 1:10,000 dilution according to BM Chemiluminescence Blotting Kit (Roche, Indianapolis, IN). After washing the primary antibody, the secondary horseradish peroxidase–conjugated goat anti-mouse IgG antibody (KPL, Gaithersburg, MD) was applied at 1:10,000 dilution for N protein detection. Horseradish peroxidase–conjugated anti-rabbit IgG (Calbiochem, San Diego, CA) at 1:5,000 dilution or 1:10,000 dilution, respectively, was applied for detection of H protein and PNP.

Virus infection. Each cell line (5×10^5 adherent cells/6-well plate) was incubated at an MOI of 0.5 in Opti-MEM for 3 hours at 37 °C. At the end of the incubation period, free viruses were removed and cells were maintained in the appropriate medium. At a time of 36 hours after infection, cells were photographed under phase contrast.

***Bystander killing measurement* in vitro.** Vero cells were seeded (5×10^5 cells/6-well plate), infected with MV-PNP at MOI 0.1 and incubated for 36 hours. MeP-dR was added to the media in a final concentration of 100 μmol/l for 12 hours. Supernatants were harvested and heat-inactivated at 60 °C for 30 minutes. Uninfected Vero cells (10^5 cells/12-well) were incubated for 72 hours with different dilutions of conditioned media. Living cells were counted after trypan blue staining. Experiments were done in triplicate.

Cell viability assay (MTT). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Cell Proliferation Kit I (MTT); Roche, Indianapolis, IN) assay was used to measure cell viability. Cells were grown in 96-well microtiter plates (5×10^4 cells/well) in the recommended culture medium in the either the presence or absence of the prodrug. Infections were at MOI of 0 (mock), 0.01, 0.1, or 1. Cell viability was measured by dye absorbency as determined by an optical density measurement of 595 nm on an automated enzyme-linked immunosorbent assay reader. The viability of cells, treated with or without prodrug, was calculated as the mean of quadruplicate optical density values, divided by the mean of quadruplicate optical density values of identically cultured cells in the absence of prodrug and virus (which served as control cells) and expressed as a percentage of the control cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed as recommended by the manufacturer.

Measurement of E. coli PNP activity in tumor allografts. Crude cell extracts were prepared as described previously[44] from subcutaneously implanted MC38cea tumors transduced with a total dose of 8×10^6 MV-PNP-antiCEA, obtained 5 days after first virus administration. The extracts were incubated with 100 μmol/l of MeP-dR, and product formation was measured by high performance liquid chromatographic analysis of the reaction mixture. Activity was expressed as PNP units; 1 unit represents 1 nmol MeP-dR-converted/mg tumor/hour.[45]

Assessment of tumor targeting. Tumors were established by inoculating MC38cea cells (10^6/100 μl per site) into the right flanks of 6–8 weeks C57BL/6 mice (Harlan, Indianapolis, IN). When tumors reached a volume of 20–40 μl, mice received either four IT or 4 IV injections of MV-PNP-antiCEA at 2×10^6 plaque forming units in 100 μl Opti-MEM (8×10^6 plaque forming units in total), on days 6, 7, 8, and 9 post implantation. On day 11 the mice were killed, and tumors were harvested and halved. One half was dissolved in RNAlater buffer. Levels of MV N mRNA in tumors harvested from each group ($n = 5$) were measured by quantitative reverse transcription polymerase chain reaction analysis, as described previously.[46] The other tumor half was used for PNP-activity measurements.

Assessement of oncolytic efficacy. Tumors were established and treated as described above; MeP-dR (60 mg/kg/dose) was injected intraperitoneally on days 10, 11, and 12 after implantation. The control animals (mock therapy groups) were injected with equal volumes of Opti-MEM containing no virus. Tumor diameters were measured every third day and the volume (product of $0.5 \times$ length $\times$ length $\times$ width) was calculated as mean ± SEM for each group. The animals were killed when the tumor burden reached a volume of 1,500 μl (approximately 10% of body weight).

Neutralizing antibody assay. For the neutralization assay, blood was collected from the tail vein. Serum was prepared after clotting at 37 °C for 1 hour and centrifugation at 10,000*g* for 10 minutes. Anti-MV antibodies were measured as described previously.[47]

Statistical analyses. Tumor volume data were analyzed by using the analysis of variance program suite. A two-sample *t*-test was used to make pairwise comparisons between the treatment groups. The survival data was analyzed using the Kaplan–Meier method and the log-rank test was used to test for significance between the groups. Only mice that reached a specific level (with a tumor burden >1,500 μl) were analyzed and two-sample *t*-test was used to make pairwise comparisons between the groups. P-values < 0.05 were considered statistically significant and the JMP program version 6 was used for making all these analyses.

ACKNOWLEDGMENTS

Mayo and Siebens Foundations and a grant (UN 254 1-1) from the Deutsche Forschungsgemeinschaft (German Research Foundation) helped support this study. We thank Steve Russell and Richard Vile for comments regarding this manuscript, Jeong Hong for providing the PNP-antibody, and Nathan Foster for his statistical analyses. Conflict-of-interest disclosure: W.B.P. and E.J.S. have a significant equity position in PNP Therapeutics.

© The American Society of Gene Therapy

REFERENCES

1. Parato, KA, Senger, D, Forsyth, PA and Bell, JC (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* **5**: 965–976.
2. Liu, TC, Galanis, E and Kirn, D (2007). Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* **4**: 101–117.
3. Springfeld, C, von Messling, V, Frenzke, M, Ungerechts, G, Buchholz, CJ and Cattaneo, R (2006). Oncolytic efficacy and enhanced safety of measles virus activated by tumor-secreted matrix metalloproteinases. *Cancer Res* **66**: 7694–7700.
4. Liu, TC and Kirn, D (2007). Systemic efficacy with oncolytic virus therapeutics: clinical proof-of-concept and future directions. *Cancer Res* **67**: 429–432.
5. Garber, K (2006). China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst* **98**: 298–300.
6. Blechacz, B, Splinter, PL, Greiner, S, Myers, R, Peng, KW, Federspiel, MJ *et al.* (2006). Engineered measles virus as a novel oncolytic viral therapy system for hepatocellular carcinoma. *Hepatology* **44**: 1465–1477.
7. Grote, D, Russell, SJ, Cornu, TI, Cattaneo, R, Vile, R, Poland, GA *et al.* (2001). Live attenuated measles virus induces regression of human lymphoma xenografts in immunodeficient mice. *Blood* **97**: 3746–3754.
8. Peng, KW, Ahmann, GJ, Pham, L, Greipp, PR, Cattaneo, R and Russell, SJ (2001). Systemic therapy of myeloma xenografts by an attenuated measles virus. *Blood* **98**: 2002–2007.
9. Peng, KW, TenEyck, CJ, Galanis, E, Kalli, KR, Hartmann, LC and Russell, SJ (2002). Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res* **62**: 4656–4662.
10. Coffey, MC, Strong, JE, Forsyth, PA and Lee, PW (1998). Reovirus therapy of tumors with activated Ras pathway. *Science* **282**: 1332–1334.
11. Hirasawa, K, Nishikawa, SG, Norman, KL, Coffey, MC, Thompson, BG, Yoon, CS *et al.* (2003). Systemic reovirus therapy of metastatic cancer in immune-competent mice. *Cancer Res* **63**: 348–353.
12. Fulci, G, Breymann, L, Gianni, D, Kurozomi, K, Rhee, SS, Yu, J *et al.* (2006). Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. *Proc Natl Acad Sci USA* **103**: 12873–12878.
13. Di Paolo, NC, Tuve, S, Ni, S, Hellstrom, KE, Hellstrom, I and Lieber, A (2006). Effect of adenovirus-mediated heat shock protein expression and oncolysis in combination with low-dose cyclophosphamide treatment on antitumor immune responses. *Cancer Res* **66**: 960–969.
14. Nemunaitis, J, Ganly, I, Khuri, F, Arseneau, J, Kuhn, J, McCarty, T *et al.* (2000). Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55 kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res* **60**: 6359–6366.
15. Kirn, D (2001). Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene Ther* **8**: 89–98.
16. Vasey, PA, Shulman, LN, Campos, S, Davis, J, Gore, M, Johnston, S *et al.* (2002). Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol* **20**: 1562–1569.
17. Wang, Y, Hallden, G, Hill, R, Anand, A, Liu, TC, Francis, J *et al.* (2003). E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models. *Nat Biotechnol* **21**: 1328–1335.
18. Thomas, MA, Spencer, JF, La Regina, MC, Dhar, D, Tollefson, AE, Toth, K *et al.* (2006). Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Res* **66**: 1270–1276.
19. Melcher, A, Gough, M, Todryk, S and Vile, R (1999). Apoptosis or necrosis for tumor immunotherapy: what's in a name? *J Mol Med* **77**: 824–833.
20. Jaeger, E, Karbach, J, Gnjatic, S, Neumann, A, Bender, A, Valmori, D *et al.* (2006). Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci USA* **103**: 14453–14458.
21. Toda, M, Iizuka, Y, Kawase, T, Uyemura, K and Kawakami, Y (2002). Immuno-viral therapy of brain tumors by combination of viral therapy with cancer vaccination using a replication-conditional HSV. *Cancer Gene Ther* **9**: 356–364.
22. Bernt, KM, Ni, S, Tieu, AT and Lieber, A (2005). Assessment of a combined, adenovirus-mediated oncolytic and immunostimulatory tumor therapy. *Cancer Res* **65**: 4343–4352.
23. Fernandez, M, Porosnicu, M, Markovic, D and Barber, GN (2002). Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *J Virol* **76**: 895–904.
24. Grote, D, Cattaneo, R and Fielding, AK (2003). Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. *Cancer Res* **63**: 6463–6468.
25. Yanagi, Y, Takeda, M and Ohno, S (2006). Measles virus: cellular receptors, tropism and pathogenesis. *J Gen Virol* **87**: 2767–2779.
26. Schneider, U, Bullough, F, Vongpunsawad, S, Russell, SJ and Cattaneo, R (2000). Recombinant measles viruses efficiently entering cells through targeted receptors. *J Virol* **74**: 9928–9936.
27. Hammond, AL, Plemper, RK, Zhang, J, Schneider, U, Russell, SJ and Cattaneo, R (2001). Single-chain antibody displayed on a recombinant measles virus confers entry through the tumor-associated carcinoembryonic antigen. *J Virol* **75**: 2087–2096.
28. Robbins, PF, Kantor, JA, Salgaller, M, Hand, PH, Fernsten, PD and Schlom, J (1991). Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line. *Cancer Res* **51**: 3657–3662.
29. Avota, E, Avots, A, Niewiesk, S, Kane, LP, Bommhardt, U, ter Meulen, V *et al.* (2001). Disruption of Akt kinase activation is important for immunosuppression induced by measles virus. *Nat Med* **7**: 725–731.
30. Palosaari, H, Parisien, JP, Rodriguez, JJ, Ulane, CM and Horvath, CM (2003). STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. *J Virol* **77**: 7635–7644.
31. Devaux, P, von Messling, V, Songsungthong, W, Springfeld, C and Cattaneo, R (2007). Tyrosine 110 in the measles virus phosphoprotein is required to block STAT1 phosphorylation. *Virology* **360**: 72–83.
32. Hermiston, TW and Kuhn, I (2002). Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther* **9**: 1022–1035.
33. Parker, WB, Allan, PW, Shaddix, SC, Rose, LM, Speegle, HF, Gillespie, GY *et al.* (1998). Metabolism and metabolic actions of 6-methylpurine and 2-fluoroadenine in human cells. *Biochem Pharmacol* **55**: 1673–1681.
34. Naniche, D, Varior-Krishnan, G, Cervoni, F, Wild, TF, Rossi, B, Rabourdin-Combe, C *et al.* (1993). Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J Virol* **67**: 6025–6032.
35. Heinzerling, L, Kunzi, V, Oberholzer, PA, Kundig, T, Naim, H and Dummer, R (2005). Oncolytic measles virus in cutaneous T-cell lymphomas mounts antitumor immune responses *in vivo* and targets interferon-resistant tumor cells. *Blood* **106**: 2287–2294.
36. Cattaneo, R, Rebmann, G, Schmid, A, Baczko, K, ter Meulen, V and Billeter, MA (1987). Altered transcription of a defective measles virus genome derived from a diseased human brain. *EMBO J* **6**: 681–688.
37. Gadi, VK, Alexander, SD, Kudlow, JE, Allan, P, Parker, WB and Sorscher, EJ (2000). *In vivo* sensitization of ovarian tumors to chemotherapy by expression of *E. coli* purine nucleoside phosphorylase in a small fraction of cells. *Gene Ther* **7**: 1738–1743.
38. Bharara, S, Sorscher, EJ, Gillespie, GY, Lindsey, JR, Hong, JS, Curlee, KV *et al.* (2005). Antibiotic-mediated chemoprotection enhances adaptation of *E. coli* PNP for herpes simplex virus-based glioma therapy. *Hum Gene Ther* **16**: 339–347.
39. Haralambieva, I, Iankov, I, Hasegawa, K, Harvey, M, Russell, SJ and Peng, KW (2007). Engineering oncolytic measles virus to circumvent the intracellular innate immune response. *Mol Ther* **15**: 588–597.
40. von Messling, V, Zimmer, G, Herrler, G, Haas, L and Cattaneo, R (2001). The hemagglutinin of canine distemper virus determines tropism and cytopathogenicity. *J Virol* **75**: 6418–6427.
41. Sorscher, EJ, Peng, S, Bebok, Z, Allan, PW, Bennett, LL Jr. and Parker, WB (1994). Tumor cell bystander killing in colonic carcinoma utilizing the *Escherichia coli* DeoD gene to generate toxic purines. *Gene Ther* **1**: 233–238.
42. Radecke, F, Spielhofer, P, Schneider, H, Kaelin, K, Huber, M, Dotsch, C *et al.* (1995). Rescue of measles viruses from cloned DNA. *EMBO J* **14**: 5773–5784.
43. Cathomen, T, Naim, HY and Cattaneo, R (1998). Measles viruses with altered envelope protein cytoplasmic tails gain cell fusion competence. *J Virol* **72**: 1224–1234.
44. Parker, WB, Allan, PW, Hassan, AE, Secrist, JA III, Sorscher, EJ and Waud, WR (2003). Antitumor activity of 2-fluoro-2′-deoxyadenosine against tumors that express *Escherichia coli* purine nucleoside phosphorylase. *Cancer Gene Ther* **10**: 23–29.
45. Hughes, BW, King, SA, Allan, PW, Parker, WB and Sorscher, EJ (1998). Cell to cell contact is not required for bystander cell killing by *Escherichia coli* purine nucleoside phosphorylase. *J Biol Chem* **273**: 2322–2328.
46. Peng, KW, Frenzke, M, Myers, R, Soeffker, D, Harvey, M, Greiner, S *et al.* (2003). Biodistribution of oncolytic measles virus after intraperitoneal administration into Ifnar-CD46Ge transgenic mice. *Hum Gene Ther* **14**: 1565–1577.
47. Zhu, YD, Heath, J, Collins, J, Greene, T, Antipa, L, Rota, P *et al.* (1997). Experimental measles. II. Infection and immunity in the rhesus macaque. *Virology* **233**: 85–92.