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Engineering Oncolytic Measles Virus to Circumvent the Intracellular Innate Immune Response

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Abstract

The innate antiviral responses of tumor cells are often impaired but may still be sufficient to impede the intratumoral spread of an oncolytic virus. Here, we establish that the oncolytic measles virus (MV-eGFP) induces interferon (IFN) production in human myeloma and ovarian cancer cells. In addition, MV gene expression and virus progeny production were inhibited by IFN treatment of these tumor cells. The P gene of wild-type measles virus encodes P/V/C proteins known to antagonize IFN induction and/or response. We therefore engineered MV-eGFP for IFN evasion and more efficient intratumoral spread by arming it with the P gene from wild-type IC-B strain MV, thus generating MV-eGFP-Pwt. The chimeric virus exhibited reduced IFN sensitivity and diminished capacity to induce IFN in BJAB lymphoma, ARH-77 myeloma cells, and activated peripheral blood mononuclear cells. Interestingly, unlike the wild-type MV, MV-eGFP-Pwt was unable to shut down IFN induction completely. In immunocompromised mice bearing human myeloma xenografts, intravenously administered MV-eGFP-Pwt showed significantly enhanced oncolytic potency compared to MV-eGFP. These results indicate that oncolytic viruses are subject to control by the innate immune defenses of human tumor cells and may therefore be more effective if their natural ability to combat innate immunity is maintained.

INTRODUCTION

Live attenuated Moraten measles vaccine and recombinant measles virus (MV) derivatives generated from the infectious clone Edmonston tag (Edmtag) have potent antineoplastic activity *in vivo*.[1–6] The virus selectively induces potent cytopathic effects (CPEs) of intercellular fusion in tumor cells and minimal damage in normal cells.[2,3] The tumor selectivity of MV is in part due to tumor cells expressing high levels of the viral receptor, CD46, resulting in enhanced CPE and preferential killing of these cells.[7,8] Three phase I trials evaluating MV in patients with recurrent ovarian cancer, multiple myeloma, or glioblastoma are in progress. However, the success of virotherapy and eradication of tumor cells depends on a complex interplay between viral replication, tumor cell growth, and the host innate and adaptive immune responses. Mathematical modeling suggested that the ideal oncolytic virus should have rapid but tumor-selective intratumoral spread and possess immune avoidance strategies.[9] The interferon (IFN) system, in particular type-I IFN (IFN-α and IFN-β), is a powerful antiviral innate defense, which involves detection of viral infection and production of IFN-β (and a small subset of IFN-α) by the infected cell, often mediated through activation of the cellular transcription factors, nuclear factor-κB (NFκB), ATF-2/c-Jun, and IRF-3.[10] IFN released from infected cells binds to IFN-α/β receptors and

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results in activation of the Jak/signal transducer and activator of transcription (STAT) signaling cascade and in transcriptional induction of IFN-stimulated genes. Autocrine and paracrine amplification of the IFN-α/β response pathway results in synthesis and activation of antiviral proteins that shut down protein synthesis and prevent viral replication.[11]

To combat the cellular innate immune response, many viruses encode antagonist molecules that block one or more steps of the IFN response pathway.[11] The P/V/C proteins of paramyxoviruses have been identified as IFN antagonist molecules.[10] The measles phosphoprotein (P) is a basic component of the viral RNA polymerase whereas the C and V proteins are nonstructural accessory proteins encoded within the P gene.[12] It was recently reported that these proteins, in particular P and V, contribute to MV immune evasion by inhibiting IFN-induced STAT nuclear translocation or by suppressing STAT1 and STAT2 phosphorylation.[13–16] Studies have reported differences in IFN induction and antagonizing properties of the wild-type versus attenuated MV strains. As such, wild-type MV isolates induce significantly lower production of IFN by phytohemagglutinin-stimulated peripheral blood lymphocytes than attenuated MV strains and the V protein of the wild-type MV IC-B strain, but not the attenuated Edmtag strain, blocks IFN-α signaling.[17,18]

In contrast to normal cells, tumor cells are generally thought to have defects in their IFN response pathways and are unable to mount effective antiviral responses. Thus, IFN-inducing oncolytic viruses or viruses defective in evading the innate IFN immune response have been developed to take advantage of this to achieve selective virus replication in tumor cells.[19–23] However, as we demonstrate in this study, IFN induction in tumor cells can potentially compromise viral gene expression and limit potency of the oncolytic virus. We determined that oncolytic MVs derived from the MV-Edmtag infectious molecular clone induced significantly higher levels of IFN in normal and tumor cells than wild-type MV. In addition, MV gene expression was compromised in IFN-treated tumor cells. We thus sought to develop a recombinant MV capable of circumventing the innate immune response. A chimeric MV based on the Edmtag platform, but armed with a wild-type P gene, was generated and evaluated *in vitro* and *in vivo* for antineoplastic activity. We demonstrated that the chimeric virus induces significantly less IFN in tumor cells and has enhanced oncolytic potency against human multiple myeloma xenografts in mice compared to the parental virus.

RESULTS

Tumor cells produce IFN upon infection by attenuated MV

An enhanced GFP reporter gene was inserted upstream of the nucleocapsid gene in the full-length infectious cDNA clone of attenuated Edmonston MV (MV-Edmtag) to generate MV-eGFP.[24] Induction of IFN production by tumor cells after infection by attenuated MV-eGFP, nonattenuated MV-VR24 (purchased from American Type Culture Collection ATCC), and vesicular stomatitis virus expressing GFP was quantified using IFN-specific enzyme-linked immunosorbent assay (ELISA) kits. As shown in Figure 1a, MV-eGFP induced significant amounts of IFN-β in a panel of low-passage ovarian carcinoma cells derived from Mayo Clinic patients (OV17, OV167, OV202, OV207). No detectable levels of IFN-α were found. MV-eGFP induced IFN-α, but not detectable levels of IFN-β, in infected human ARH-77 cells and in primary myeloma cells isolated from bone marrow aspirates of myeloma patients (Figure 1b).

Attenuated MV expression is inhibited in IFN-treated tumor cells

As tumor cells produce IFN upon MV-eGFP infection, we next evaluated the effect of IFN on MV gene expression. The MV-CEA virus used in the experiments was constructed on the

MV-Edmtag platform where the human carcinoembryonic antigen (CEA) gene was inserted upstream of the nucleocapsid gene.[6] Virally encoded CEA released into the culture media by infected cells allows precise quantitation of viral gene expression. As shown in Figure 2a, levels of CEA in the conditioned media of MV-infected ovarian cancinoma cells were reduced 2–50-fold in the presence of IFN. Similarly, an inhibition of viral CEA gene expression (2–12-fold) was observed in IFN-treated myeloma cells (Figure 2b). MV virus progeny production was also inhibited (1–2 logs lower) in IFN-pretreated ovarian carcinoma and myeloma cells (Figure 2c).

Wild-type V protein inhibits IFN-α/β induction by attenuated MV

Using a luciferase reporter assay, Ohno *et al.*[18] determined that attenuated MV, unlike wild-type MV, is defective in its ability to antagonize IFN signaling owing to mutations in its P/V proteins. To extend this observation, we sought to determine the effects of P, V, and C proteins on IFN induction and biosynthesis in MV-eGFP-infected Epstein-Barr virus (EBV) negative human Burkitt's lymphoma cell like (BJAB). Nine stable BJAB cell lines, each expressing P, V, or C proteins from each of the three MV strains MVwt323 (wild-type MV IC-B strain), MV-VR24 (nonattenuated MV), and MV-eGFP (attenuated MV-Edmtag) were generated by lentiviral transduction. Correct expression of P, V, and C proteins in the BJAB cells was verified by immunoblotting (Figure 3a). As shown in Figure 3b, expression of wild-type Vor VR24 V proteins significantly reduced IFN-β production ($P < 0.01$) by 2.9- and 2.1-fold, respectively, compared to infected parental BJAB cells. IFN-α production was also inhibited significantly ($P < 0.01$), 5.6- and 2-fold by wild-type V and VR24 V, respectively, compared to the infected parental cells (Figure 3b). In contrast, the expression of V protein from attenuated MV-eGFP or P and C proteins from all three viruses did not substantially affect IFN production (Figure 3b). The suppression of IFN induction and response was shown to be at the transcriptional level by semiquantitative RT-PCR analysis of MV-induced IFN-α, IFN-β, and MxA mRNA synthesis in BJAB cells expressing the respective P, V, or C proteins (Figure 3c). Levels of IFN-β and IFN-α13/1 mRNA transcripts in cells stably expressing V wild-type or V V24 proteins were inhibited compared to infected parental BJAB cells. MxA mRNA levels were suppressed only in cells stably expressing V wild-type protein (Figure 3c).

Generation and characterization of MV-eGFP-Pwt

The P gene from p(+)MV-eGFP (Figure 4a) was replaced with the wild-type P gene from p(+)MV323, which encodes the full-genome of the wild-type MV IC-B strain.[25] The chimeric virus with a wild-type P gene (MV-eGFP-Pwt) was rescued and efficiently propagated on Vero cells. This recombinant virus is essentially the same as MV-eGFP except that it expresses the P, V, and C proteins of wild-type MV. BJAB and ARH-77 cells express CD46 and signaling lymphocytic activation molecule (SLAM) and are permissive to infection by attenuated MVs (CD46-tropic) and the wild-type virus MVwt323, which uses only SLAM for entry.[26] The CPE induced by MV-eGFP-Pwt was similar to that of MV-eGFP and much more pronounced than that of MVwt323 (Figure 4b). The ability of MV-eGFP-Pwt to elicit IFN production was determined (Figure 4c–e). As shown in Figure 4c, MV-eGFP-Pwt induced considerably lower amounts (37- and 12.8-fold lower) of IFN-α and IFN-β than MV-eGFP in BJAB cells, at levels comparable to MV-VR24. In contrast, MVwt323 did not induce detectable levels of IFN. MV-eGFP-Pwt also induced less IFN (3–8.7-fold less IFN-α than MV-eGFP) in ARH-77 cells and phytohemagglutinin-activated peripheral blood mononuclear cells harvested from three different healthy donors (Figure 4d and e).

The growth kinetics of the chimeric virus was slightly different from that of MV-eGFP, although the maximal cell-associated virus titers were similar (around 10^7 PFU/ml) by 72 h

in the absence of IFN. However, the MV-eGFP-Pwt virus reached its maximal titer faster, at a rate comparable to that of the wild-type MVwt323 virus. The wild-type MVwt323 strain gave similar titers in the presence and absence of IFN at 72 h post infection and less than 1 log difference in the titers at 48 h, whereas the titer of MV-eGFP was 2–3 logs lower in the presence of IFN compared to the absence of IFN. The chimeric virus MV-eGFP-Pwt displayed intermediate sensitivity to the action of IFN with approximately 1 log difference between the titers in the presence and absence of IFN at 48 and 72 h (Figure 5).

MV-eGFP-Pwt killed myeloma cells more efficiently than MV-eGFP and is less sensitive to IFN-mediated restriction of viral infection

Viabilities of virally infected ARH-77 and KAS6/1 cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) cell proliferation assay. At 72 h after infection, cell death ranged from 0.5–50% (multiplicity of infection, MOI 0.01) to 53–80% (MOI 10.0). Cell killing by MV-eGFP or MV-eGFP-Pwt viruses was comparable in ARH-77 cells, but the chimeric virus demonstrated superior killing efficiency at the lower MOI in KAS6/1 myeloma cells ($P < 0.05$) and in other tested myeloma cell lines, JJN-3, RPMI-8226, and MM1 (Figure 6a, and data not shown). In myeloma cells, both viruses displayed a similar dose-dependent pattern of IFN-mediated restriction of viral infection. The percentage of infected ARH-77 cells ranged from 64 to 32% with IFN pretreatment (compared to 100% infection in untreated cells) for MV-eGFP-Pwt versus 39–21% for MV-eGFP. Similarly, the percentage of infected KAS6/1 cells ranged from 79 to 36% for the chimeric virus versus 50–30% for MV-eGFP (Figure 6b). MV-eGFP-Pwt appears to be more efficient ($P < 0.05$) in antagonizing the antiviral effects of IFN, particularly at the lower concentrations (Figure 6b).

Intravenous administration of MV-eGFP-Pwt caused faster regression of human myeloma xenografts compared to MV-eGFP

MV-eGFP-Pwt and MV-eGFP were given intravenously to CB.17. SCID mice bearing established (0.3–0.5 cm in diameter) subcutaneous human ARH-77 tumor xenografts. As shown in Figure 7a, intravenous administration (two doses of 4×10^6 50% tissue culture infectious dose ($TCID_{50}$)) of MV-eGFP-Pwt or MV-eGFP caused complete regression of ARH-77 tumor xenografts. However, the kinetics of tumor regression was different, with MV-eGFP-Pwt being the faster oncolyic virus, rendering complete eradication of all tumors 16 days earlier than MV-eGFP. The tumor volumes between the MV-eGFP-Pwt- and MV-eGFP-treated groups were significantly different at the point of complete tumor regression in the MV-eGFP-Pwt-treated group ($P < 0.05$). Thus, the chimeric virus exhibits enhanced potency and accelerated tumor regression in this ARH-77 myeloma model. In the KAS 6/1 model of myeloma, systemic virotherapy (four doses at 10^7 $TCID_{50}$/dose) caused significant inhibition of tumor growth compared to control animals ($P < 0.05$) (Figure 7b). Although the difference in the oncolytic potency between the two viruses was not as strong in this model compared to ARH-77, there was a trend for enhanced efficacy of the chimeric virus compared to MV-eGFP. Tumors harvested at necropsy and examined by confocal microscopy revealed patchy distribution of MV infection throughout the tumors and large syncytia 50 days after the last virus dose (Figure 7c).

To address the safety issue of a chimeric virus expressing Pwt, we injected 8-week-old CD46 transgenic mice intracerebrally under sterotactic guidance (5×10^4 $TCID_{50}$) with MV-eGFP or MV-eGFP-Pwt. No encephalitis, neurological deficits, behavioral changes, or impaired health were noted with either of the viruses for the observation period of 3 weeks. The biodistribution of viral gene expression (GFP) of both viruses was also compared after intraperitoneal injection (5×10^6 $TCID_{50}$) in $ifnar^{ko}$-CD46Ge transgenic mice. The pattern

of GFP expression was similar for both viruses, involving mainly the greater omentum, spleen, and parietal diaphragm as described previously.[27] No additional organs were infected by the MV-eGFP-Pwt virus.

DISCUSSION

Replication-competent oncolytic viruses represent a rapidly expanding modality for cancer therapy. Live attenuated MV has potent and tumor-selective oncolytic activity against a variety of human tumors.[2–5] However, some tumor types respond poorly to therapy, repeated doses of virus are often necessary for tumor control and complete eradication is not always attained.[6] We attribute these differences in tumor susceptibility to factors in the tumor microenvironment that restrict viral replication and efficient spread.[28] These factors include stroma architecture and intratumoral innate immune mechanisms that could potentially limit viral spread *in vivo*. To combat the innate immune response, we have engineered a chimeric MV that expresses immune avoidance proteins from the wild-type MV to evade the IFN system. We further demonstrated that this virus has enhanced oncolytic potency for systemic therapy of multiple myeloma xenografts *in vivo*.

In this study, we established that recombinant MV-eGFP, based on the attenuated Edmtag platform, is a more potent IFN inducer than nonattenuated or wild-type MVs in low-passage Mayo patient-derived ovarian cancer cell lines, BJAB, ARH-77, and primary myeloma cells freshly isolated from patients. Contrary to the general assumption that tumor cells are defective in the IFN response, the inhibitory effects of IFN on viral gene expression were clearly demonstrated in most of the tested cancer cells using a sensitive assay to monitor expression of a virally (MV-CEA) encoded CEA soluble marker peptide. Deregulation of IFN signaling and cancer-specific defects in innate antiviral responses have been reported for tumor cells and exploited for selective replication of oncolytic viruses.[19–23] From this study, it is apparent that attenuated MV gene expression in tumor cells is inhibited by IFN. Infection of normal cells, which have intact innate immune responses, within the tumor could also potentially restrict viral spread. Thus, effective immune evasion may be beneficial to increase anti-tumor potency *in vivo*.

For negative-strand RNA paramyxoviruses, the host evasion activities have been ascribed mostly to the viral P/V/C proteins. We therefore replaced the P gene of attenuated MV-eGFP with that of wild-type MV, thereby generating a recombinant virus expressing wild-type P, V, and C proteins. The IFN sensitivity of this chimeric virus was reduced compared to the parental virus, most significantly in the Vero-hSLAM cells and to a lesser degree in the tested myeloma cells. Ohno *et al.*[18] reported that wild-type MV strains are more resistant to the effects of IFN than attenuated strains in Vero-hSLAM cells. The chimeric MV-eGFP-Pwt virus has intermediate IFN sensitivity compared to either of the parental viruses. However, it is strategically far more advantageous for a virus not to induce IFN than to have to counteract cellular IFN antiviral response. Wild-type MV strains exhibit IFN-suppressive phenotype compared to laboratory attenuated strains that have IFN-inducing phenotype.[17] The engineered virus MV-eGFP-Pwt exhibited markedly diminished capacity to induce IFN than the parental virus, an ability that should favor replication and spread of this virus *in vivo*. According to published literature, proteins that counteract the innate cellular immune response are encoded mainly in the P gene.[10,11,29] An important observation with the chimeric virus, expressing all the wild-type P, V, and C proteins, is its inability to completely abrogate IFN production as the wild-type virus can. Incomplete shutdown may have been due to higher fusogenicity with rapid assimilation of uninfected cells into the syncytium, overwhelming the ability of the V protein to suppress IFN release. Also, another candidate protein that might play a role in the innate immune response is the MV

nucleocapsid protein, which activates IRF-3.[30] Clearly, other factors that predicted IFN evasion and viral pathogenicity remain to be elucidated in future studies.

Paramyxovirus V proteins have no cellular homologs, but exhibit a diverse range of host evasion activities, including prevention of apoptosis and inhibition of double-stranded RNA signaling,[31,32] cell-cycle alterations,[33] inhibition of activities of IFN-responsive STAT proteins,[13,15,34–36] and prevention of IFN biosynthesis.[31,32,37,38] Although elucidated for other paramyxoviruses, the mechanism of IFN induction silencing is still not clear for morbilliviruses. This study clearly identifies the MV wild-type V protein as a protein that can actively counteract both IFN-β and IFN-α induction, re-arming the virus with the ability to circumvent the innate immune response and spread efficiently in the host.

Different mechanisms could account for the enhanced *in vivo* antitumor activity of the chimeric virus, such as faster replication kinetics, enhanced cell killing, or evasion of antiviral response mechanisms, independent of IFN synthesis.[11,39] ARH-77 cells can produce and respond to IFN. In these cells, the chimeric virus induced comparable *in vitro* cell killing but was more effective at tumor elimination *in vivo*. It is tempting to postulate that superior anti-tumor efficacy of MV-eGFP-Pwt is due to its decreased sensitivity to IFN and induction of less IFN in infected ARH-77 cells compared to MV-eGFP. However, there might be a concern that re-arming attenuated MV with a wild-type P gene to permit more effective immune evasion will generate a more toxic agent and compromise patient safety. The likelihood that attenuated MV carrying a wild-type P gene might be pathogenic and become transmissible is very low as virus pathogenicity is complex and does not depend solely on P/V/C proteins. In addition, measles is a self-limiting disease and there is a high prevalence of MV antibody seropositivity either due to prior natural infection or vaccination. We did not observe any increased neurotoxicity of the chimeric virus after intracerebral injection in transgenic mice expressing human CD46 and we noted a similar pattern of biodistribution of viral gene expression after intraperitoneal injection of MV-eGFP and MV-eGFP-Pwt. It is also important to emphasize that the chimeric virus is not able to completely inhibit IFN biosynthesis and can still induce detectable quantities of IFN that are comparable to other nonattenuated Edm MV (MV-VR-24). In addition, we have now developed a highly efficient method to fully retarget MV tropism so that virus infection and CPE via its native receptors (CD46 and SLAM) are ablated and are mediated only through the targeted receptor such as CD38 or a-folate receptor.[40,41]

Taken together, our results suggest that to achieve more effective tumor control and tumor eradication via direct oncolysis and spread, an attenuated replication-competent virus requires innate immune avoidance resources. We have demonstrated that a chimeric MV virus, armed with wild-type proteins to silence the innate immune response has superior oncolytic activity than the parental virus for systemic therapy of multiple myeloma. This chimeric virus warrants further development for cancer therapy.

MATERIALS AND METHODS

Cell culture

The multiple myeloma cell lines were kind gifts of John Lust (RPMI 8226), Diane Jelinek (KAS6/1), and Rafael Fonseca (MM1, JJN-3) from Mayo Clinic (Rochester, MN). BJAB, an EBV-negative human Burkitt's lymphoma cell line, was a kind gift from M Peter (University of Chicago). ARH-77 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Primary myeloma cells (CD138-enriched) were isolated from bone marrow aspirates of patients with established myeloma after institutional review board approval and informed patient consent as described previously.[8] All cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS), except for KAS6/1 and

primary myeloma cells which were grown in media supplemented with 1 ng/ml interleukin-6 (Sigma, St Louis, MO). The human ovarian carcinoma cells, OV17, OV167, OV202, and OV207 were low-passage primary lines established at the Mayo Clinic. The SKOV3ip.1 ovarian tumor cells were a kind gift of Ellen Vitetta (University of Texas Southwestern Medical Center). OVCAR-3 ovarian tumor cells were purchased from ATCC. The ovarian cancer cell lines were maintained in alpha-minimal essential medium (Irvine Scientific, Irvine, CA) supplemented with 20% FBS and 2 nM L-glutamine. Vero-hSLAM, expressing human SLAM was grown in Dulbecco's modified Eagle's medium with 10% FBS and 0.5 mg of G418/ml.[25] The rescue helper cell line 293-3-46 was grown in Dulbecco's modified Eagle's medium with 10% FBS and 1.2 mg of G418/ml. Peripheral blood mononuclear cells were obtained from healthy volunteers, maintained in RPMI 1640 with 10% FBS and were activated with 2.5 μg/ml phytohemagglutinin (ICN Pharmaceuticals, Costa Mesa, CA) before infection. All cells were maintained at 371C in 5% CO_2.

Viruses and infection assays

MV-eGFP and MVwt323 were propagated on Vero cells and Vero-hSLAM cells, respectively.[24,25] MV Edmonston VR-24, purchased from ATCC (Manassas, VA), MV-CEA, and vesicular stomatitis virus, expressing GFP (a kind gift from G Barber, University of Miami), were propagated on Vero cells. Viruses were titered on Vero or Vero-hSLAM cells and viral titers were expressed as $TCID_{50}$/ml. The infection assays of myeloma cells, lymphoma cells, and phytohemagglutinin-stimulated peripheral blood mononuclear cells were performed as described previously at MOI of 0.01, 0.1, 0.3, 0.5, 1, or 10.[2] Ovarian carcinoma cells were infected at MOI of 0.5 or 1.[3] At various time points, the CPEs were noted, cells were photographed and the percentage of MV-infected cells was estimated by flow cytometry (percentage GFP-positive cells). For MV-CEA, the amount of virally-encoded CEA marker peptide in the culture supernatants was analyzed using Bayer Centaur Immunoassay System. Cell viabilities of MV-infected myeloma cultures were evaluated with CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. For IFN sensitivity assays, cells were pretreated for 18–24 h before infection with different concentrations of recombinant human IFN-α (Cell Signaling Technology) or IFN-β (PBL Biomedical Laboratories). Forty-eight hours after infection, cells were photographed, MV infection and viral expression were estimated using flow cytometry or measurement of CEA levels. Virus growth curves were obtained by inoculating untreated or IFN-α pretreated Vero-hSLAM cells at an MOI of 0.01 $TCID_{50}$/cell. At the indicated time points, cells were scraped in Opti-MEM and subjected to two freeze–thaw cycles. Cell-associated viral titers were determined by $TCID_{50}$ titration on Vero-hSLAM cells.

Generation of chimeric MV-eGFP encoding the wild-type P gene

The *BsiWI*/*BsiWI*-digested fragment of p(+)MV-eGFP[24] was inserted into a shuttle vector pH6BS, which was modified from pTNH6,[42] to result in pSS8 (Figure 4a). The *XbaI*/*SalI*-digested fragment of p(+)MV323, a kind gift from K Takeuchi (University of Tsukuba),[25] was subcloned into pSS8 at the corresponding restriction sites. The *BsiWI*/*BsiWI*-digested fragment of the resulting plasmid was subcloned into the corresponding sites of p(+)MV-eGFP and the orientation was checked with *AatII* digestion. Recombinant virus MV-eGFP-Pwt was rescued from 293-3-46 helper cells as described previously.[43]

Generation of lentiviral vectors and cell lines stably expressing P, V, and C proteins

The vector pHR-SIN-CSGWdlNotI, expressing GFP,[44] was kindly provided by Y Ikeda (Mayo Clinic, Rochester, MN). The cDNAs encoding the P, V, and C proteins of the viruses were obtained by RT-PCR (SuperScriptTM One-step RT-PCR, Invitrogen, Carlsbad, CA)

from virus-infected cells using specific primers. The cDNAs were cloned into *BamHI–NotI* site of pHR-SIN-CSGWdlNotI to replace GFP and their sequences were verified by DNA sequencing. The V cDNAs were identified by the presence of a nontemplated nucleotide insertion at the RNA editing site. Vector stocks were produced by cotransfection of the vector plasmid with pCMVR8.91 and pMDG into 293 T cells using calcium phosphate co-precipitation method and used to transduce BJAB cells, using GFP-expressing lentiviral vector as control.

Flow cytometry and confocal microscopy

Cells were harvested, washed twice in ice-cold phosphate-buffered saline and fixed in 1% paraformaldehyde. Samples were run on a Becton-Dickinson FACScan Plus cytometer and analyzed using CellQuest software (Becton-Dickinson, San Jose, CA). Images of tumor sections were collected on an LSM510 laser scanning confocal microscope (Carl Zeiss, Inc. Oberkochen, Germany).

Western blotting and ELISA

Western blot was performed as described previously[40] using mouse anti-MV polyclonal antibody (for P and V proteins) and rabbit anti-C polyclonal antibody (for C protein), a generous gift from K. Takeuchi. ELISA specific for IFN-α was performed using a human IFN-α ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ) as per manufacturer's directions. IFN-β was measured using a human IFN-β ELISA kit (TFB; Tokyo, Japan or PBL Biomedical Laboratories) according to the manufacturer's instructions. IFN levels above 25 pg/ml or 25 IU/ml were considered as positive.

RT-PCR

Total cellular RNA was extracted using RNeasy mini kit (Qiagen) as per the manufacturer's instructions. Treatment with DNase I was performed to remove genomic DNA contamination. One-step RT-PCR kit (SuperScriptTM One-step RT-PCR; Invitrogen, Carlsbad, CA) was used for the reaction with primers specific for IFN-β,[45] IFN-α13/1,[46] MxA,[45] and GAPDH. The quantity of the template and the number of amplification cycles were optimized to ensure that reactions were stopped during the linear phase of product amplification, permitting semiquantitative comparisons of mRNA abundance between different RNA preparations.

In vivo studies

Animal studies were approved by Institutional Animal Care and Use Committee of the Mayo Foundation and were performed with 6-week-old female CB.17 ICR SCID mice. Twenty-four hours after total body irradiation (150 rad), mice were implanted subcutaneously in the right flank with 1×10^7 cells (ARH-77 or KAS6/1) in 100 μl phosphate-buffered saline. The animals were examined daily until the tumors were palpable, after which tumor size was measured twice a week in two dimensions using calipers. When the tumors reached a mean diameter of 0.3–0.5 cm, the mice were injected intravenously (i.v.) via the tail vein with MV-eGFP, MV-eGFP-Pwt, or saline. For the ARH-77 group (n = 8 for MV-eGFP-treated mice, n = 9 for MV-eGFP-Pwt-treated mice, n = 8 for controls), mice received two i.v. injections of 4×10^6 $TCID_{50}$ virus (total dose 8×10^6 $TCID_{50}$). For KAS6/1 group (n = 9 for MV-eGFP-treated mice, n = 10 for MV-eGFP-Pwt-treated mice, n = 9 for controls), mice received four i.v. injections of 10^7 $TCID_{50}$ virus (total dose 4×10^7 $TCID_{50}$). Animals were killed at the end of the experiment or when tumor burden reached 10% of body weight, or when tumors ulcerated. Tumor volume was calculated as $a^2 \times b \times 0.5$, where a is the width and b is the length of the tumor. Tumor volumes were expressed as mean ± SEM. Statistical analysis was performed comparing tumor volumes at each time

point using Student's *t*-test. $P < 0.05$ was considered to be statistically significant. For safety studies, six 8-week-old transgenic mice (3 mice/group) expressing human CD46 (CD46Ge) were injected intracerebrally with 5×10^4 $TCID_{50}$ of MV-eGFP or MV-eGFP-Pwt. The mice were monitored daily for 3 weeks for behavioral changes, neurologic deficits (using a noninvasive functional observational battery), and survival. For biodistribution experiments, six transgenic $Ifnar^{ko}$-CD46Ge mice (3 mice/group) were injected intraperitoneally with 5×10^6 $TCID_{50}$ of MV-eGFP or MV-eGFP-Pwt. Three days later, mice were dissected and the biodistribution of GFP expression was determined using a fluorescence microscope.

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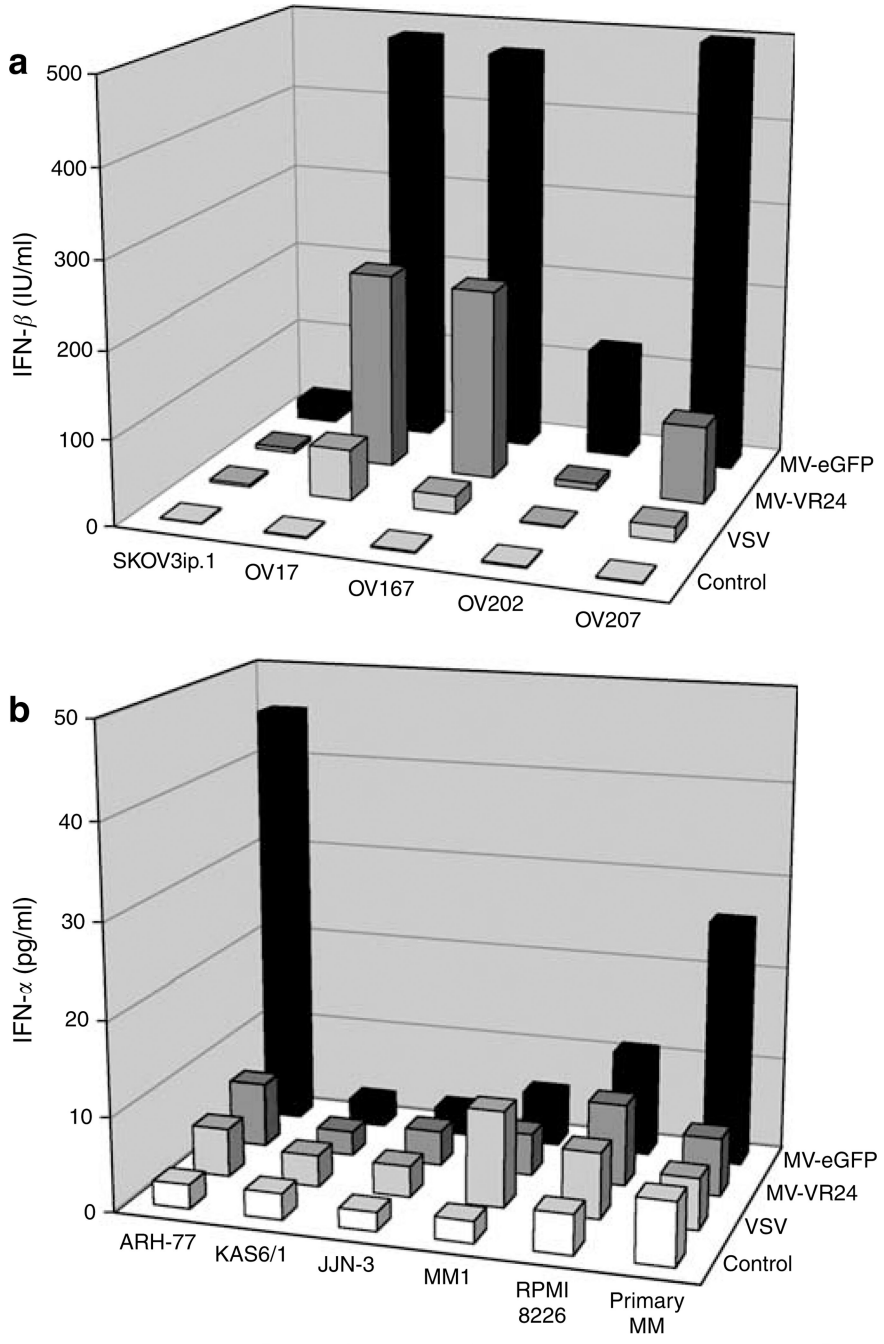

Figure 1. Attenuated MV (MV-eGFP) is a more potent IFN inducer than nonattenuated Edmonston MV-VR24

(**a**) Human ovarian cancer cells and (**b**) myeloma cells were mock infected (control) or infected with different viruses at MOI of 1.0. Conditioned media were collected 48 h after infection, placed on ice and IFN levels were measured using (**a**) IFN-β- or (**b**) IFN-α-specific ELISA. Data are representative of three separate experiments with similar results. OV17, OV167, OV202, and OV207 are low-passage cells derived from ovarian cancer patients and primary MM are $CD138^+$ malignant plasma cells isolated from the bone aspirate of a multiple myeloma patient.

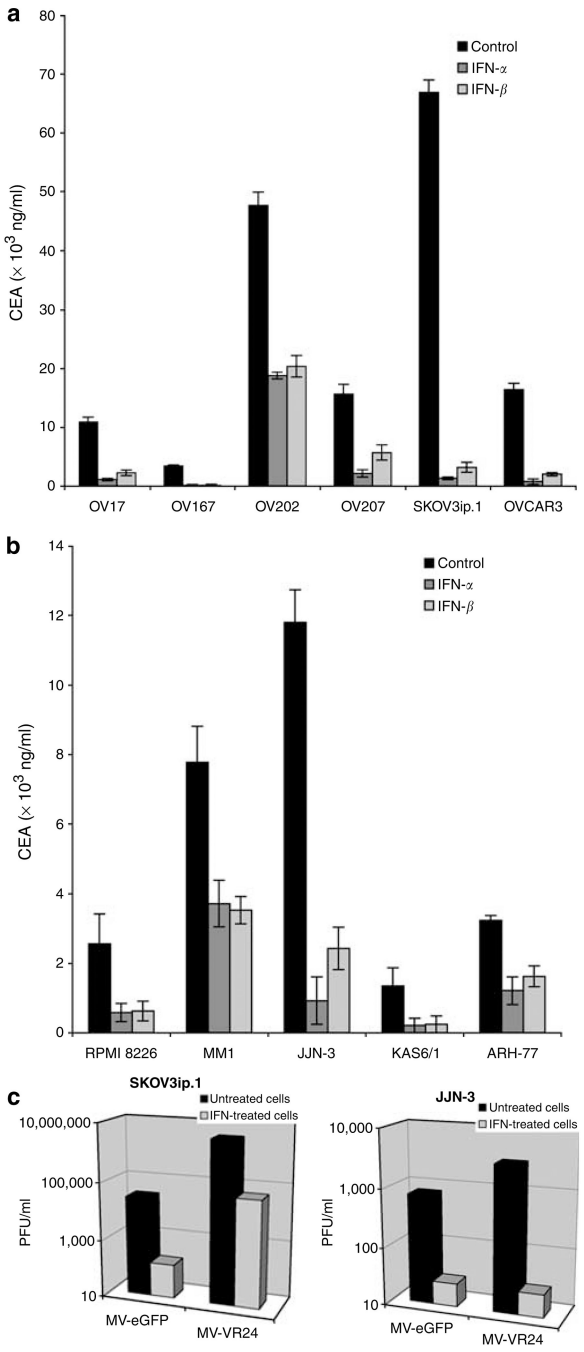

Figure 2. Attenuated MV gene expression in tumor cells is inhibited by IFN treatment

(**a**) Ovarian cancer cells or (**b**) myeloma cells were left untreated or pretreated with 500 IU/ml of IFN-α or IFN-β for 18–24 h, after which the cells were infected (MOI 1.0) with MV-CEA. Levels of virally encoded CEA in culture media were measured 48 h after infection. Error bars represent SD from three replicates. (**c**) Viral progeny production was inhibited in IFN-treated tumor cells. SKOV3ip.1 (ovarian carcinoma) or JJN-3 (myeloma) cells were treated with IFN as above and infected with MV-eGFP or MV-VR24 (MOI 0.5 for SKOV3ip.1 and MOI 1.0 for JJN-3). Virus progeny was released by freeze–thaw cycles 48 h later and titers determined on Vero cells.

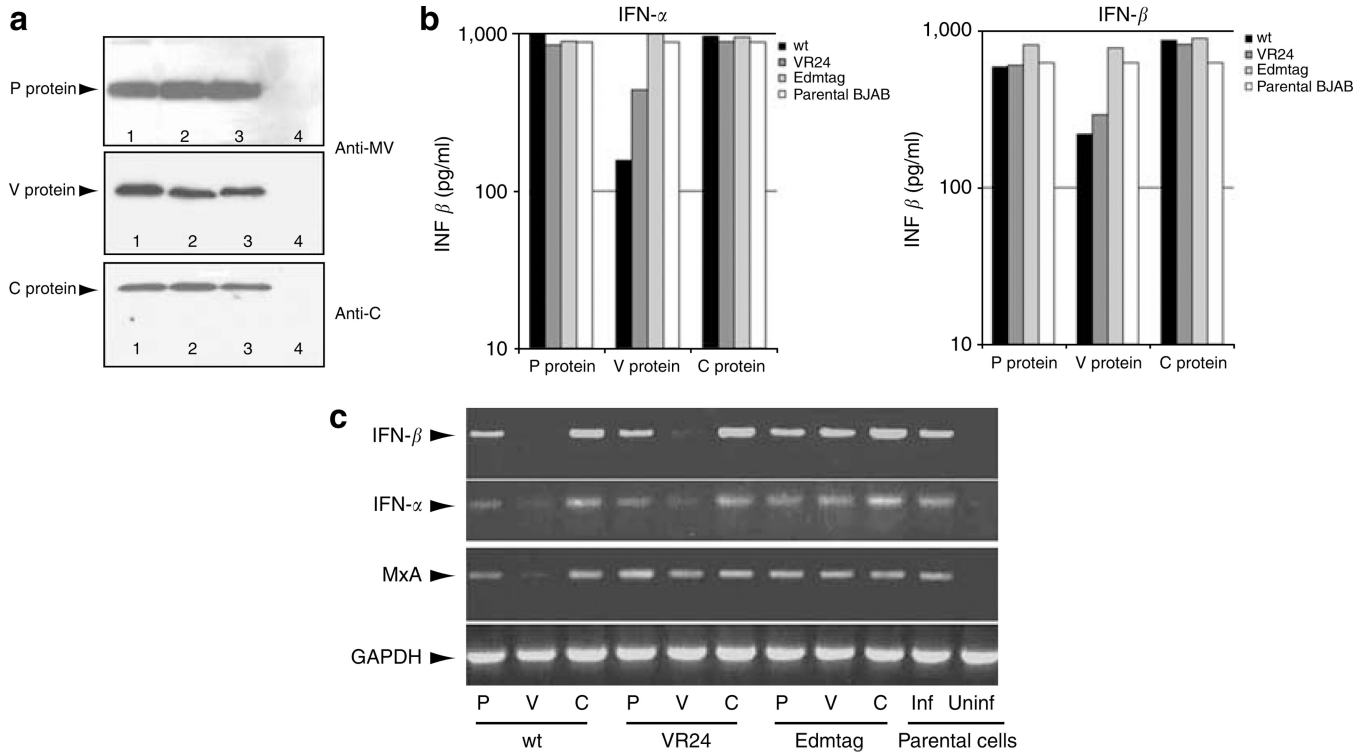

Figure 3. IFN induction and responses in BJAB cells individually expressing P, V, or C proteins from wild-type MV IC-B strain, nonattenuated Edm strain VR-24 and attenuated Edmtag strain MV-eGFP

(**a**) Confirmation of P, V, and C protein expression in BJAB cells. Lysates of BJAB cells expressing wild-type (1), VR-24 (2), attenuated MV-eGFP proteins (3), or control parental cells (4) were subjected to SDS-PAGE and immunoblotted using a mouse anti-MV polyclonal antibody, that detects P and V proteins, or a rabbit polyclonal antibody to measles C protein. (**b**) Wild-type V and VR24 V proteins suppress IFN-β and IFN-α production in BJAB cells. Parental or transduced BJAB cells, stably expressing P, V, or C proteins from the various viruses were infected with MV-eGFP at MOI of 1. Conditioned media were harvested 48 h after infection and IFN levels were measured by ELISA. Values were normalized for percentage of infected cells (GFP-positive cells from FACS analysis). Data are representative of three experiments with similar results. (**c**) Levels of IFN-β, IFN-α, and MxA mRNA transcripts were suppressed in BJAB cells expressing wild-type or VR24 V proteins. RT-PCR was performed on total cellular RNA from MV-eGFP-infected parental BJAB cells or cells expressing the respective P, V, or C proteins from the viruses.

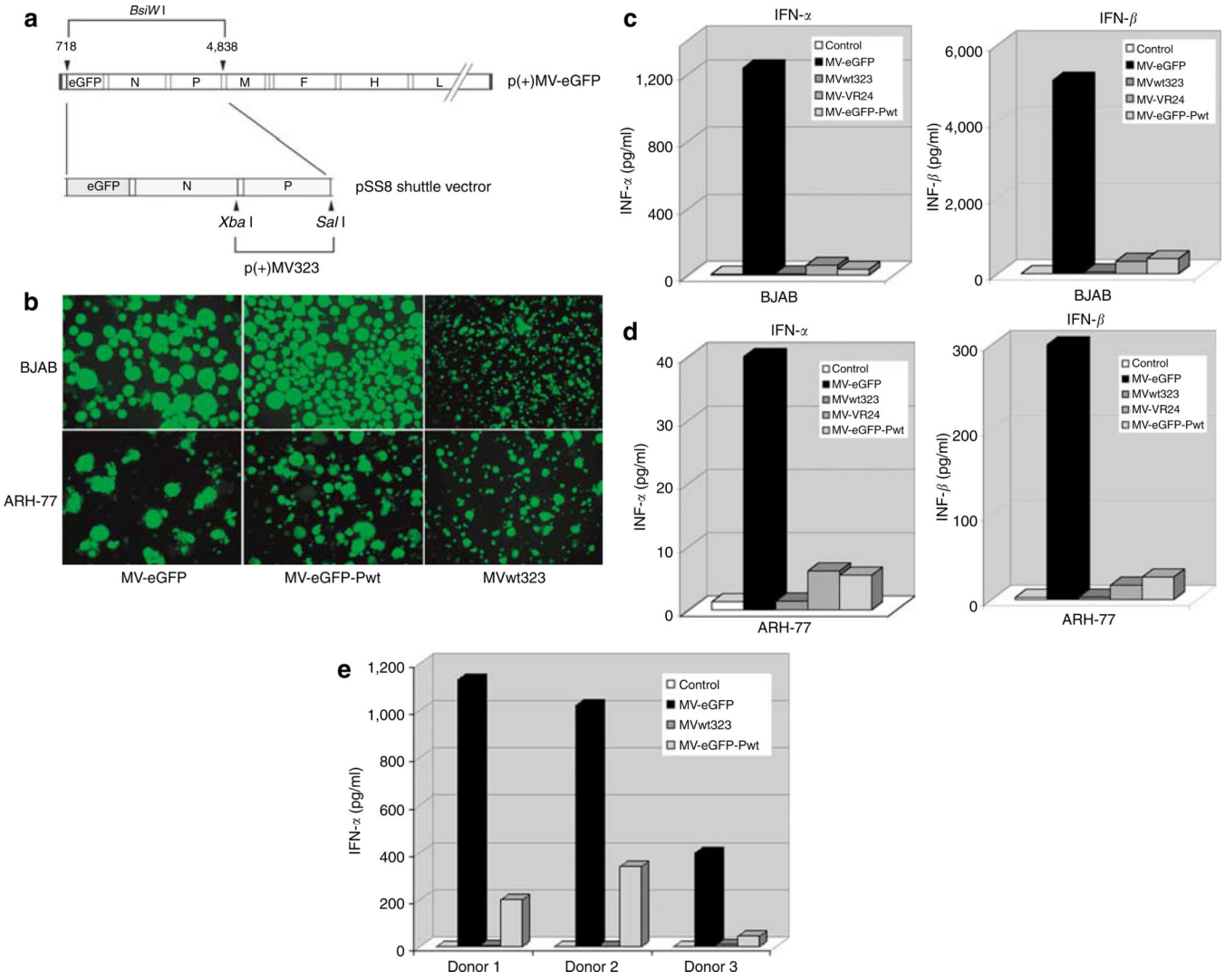

Figure 4. Generation of MV-eGFP-Pwt and its IFN-inducing properties in tumor and normal cells

(**a**) Schematic representation of the full-length infectious molecular clone of attenuated MV-eGFP and the cloning strategy for generating MV-eGFP-Pwt, which expresses the wild-type P gene. The *Xba*I/*Sal*I fragment, containing the Pwt gene, was digested from a plasmid encoding the full-length infectious clone of wild-type MV IC-B strain, p(+)MV323. The fragment was subcloned into a shuttle vector pSS8 using the corresponding restriction sites and inserted as a *BsiW*I fragment into the full-length p(+)MV-eGFP plasmid to generate p(+)MV-eGFP-Pwt. (**b**) Photographs of BJAB and ARH-77 cells infected with MV-eGFP, MV-eGFP-Pwt or MVwt323 (MOI 1.0) taken at 48 h after infection. (**c**–**e**) MV-eGFP-Pwt induced less IFN in comparison with MV-eGFP, but did not completely silence IFN biosynthesis compared to MVwt323. IFN-α or IFN-β levels in (**c**) BJAB lymphoma cells, (**d**) ARH-77 cells, or (**e**) peripheral blood mononuclear cells from three different healthy donors' infected with MV viruses at an MOI of 1.0. Conditioned media were collected on ice 48 h later and IFN levels were measured by specific ELISA. IFN values were normalized for percentage of infected cells quantified by FACS analysis. Data are representative of three separate experiments with similar results.

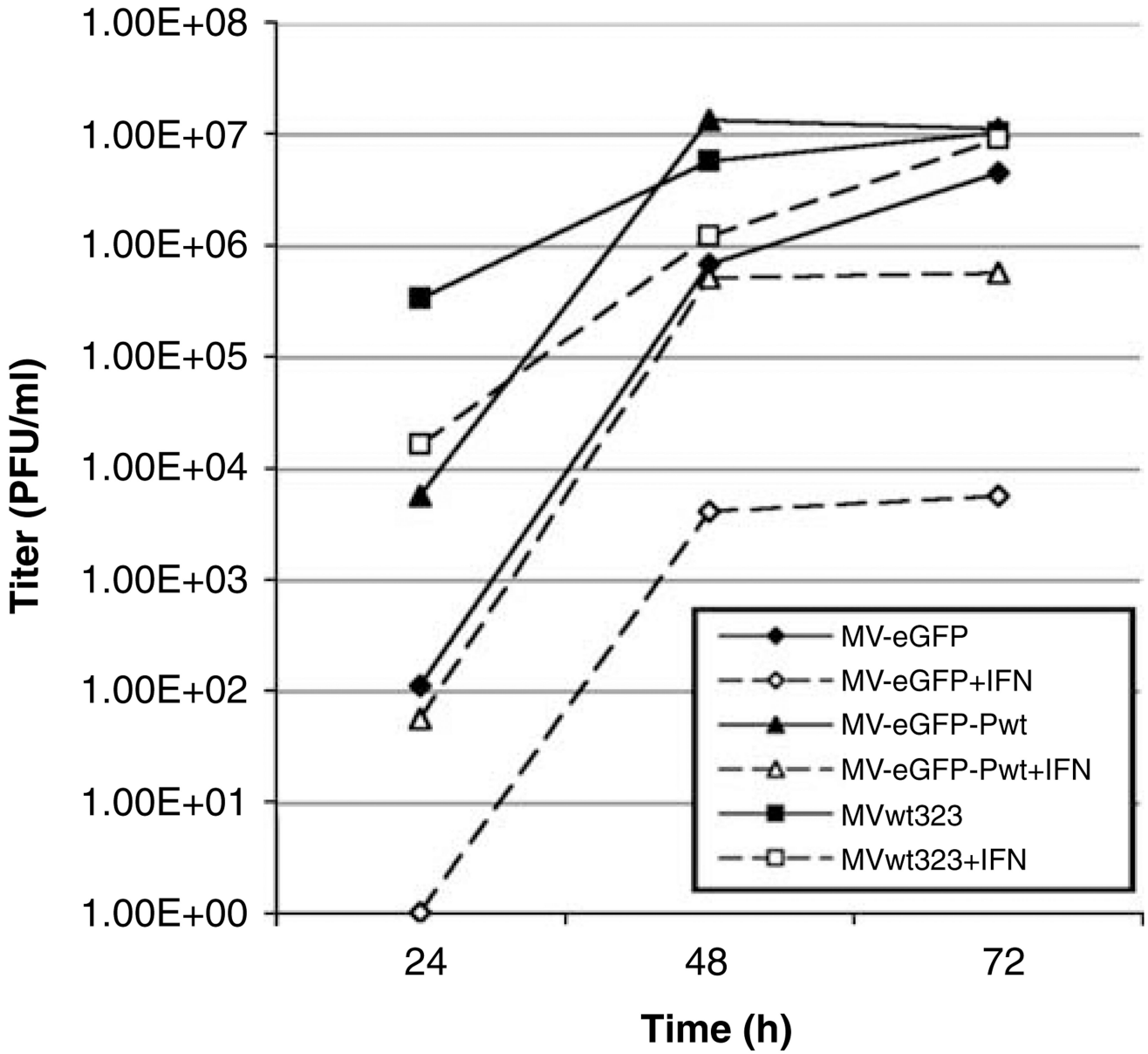

Figure 5. Comparison of growth kinetics of MV-eGFP-Pwt with the parental viruses

Vero cells stably expressing human SLAM (Vero-hSLAM) were left untreated or pretreated with 500 IU/ml IFN-α for 18–24 h. Cells were infected with MV-eGFP, MV-eGFP-Pwt, and MVwt323 at an MOI of 0.01 $TCID_{50}$/cell. At the indicated time points, the cells were scraped in Opti-MEM, subjected to two freeze-thaw cycles and cell-associated viral titers were determined by $TCID_{50}$ titration.

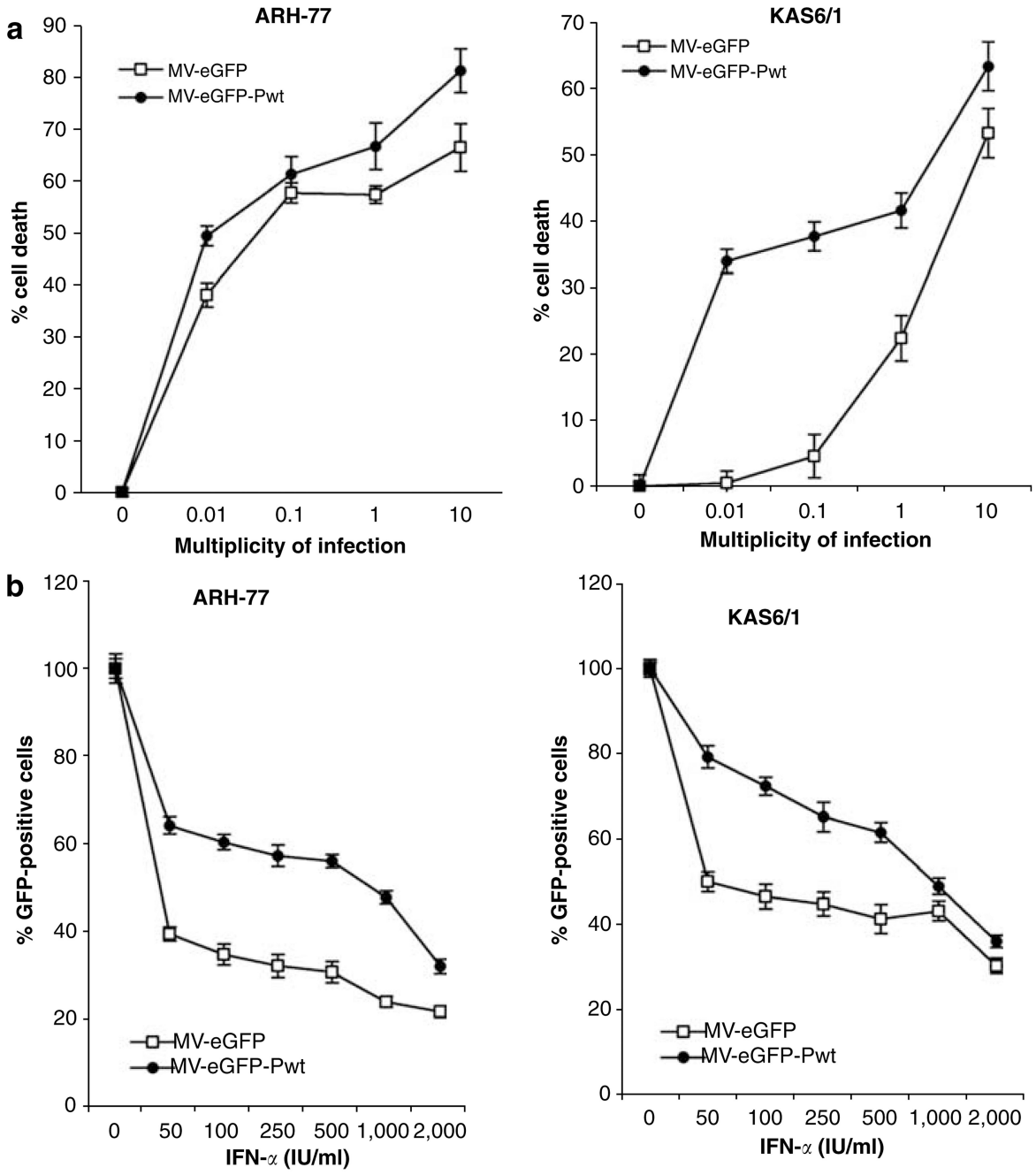

Figure 6. ***In vitro*** **analysis of the oncolytic potential of MV-eGFP-Pwt**

(**a**) Cell killing potential of oncolytic MV-eGFP and the chimeric virus, MV-eGFP-Pwt. ARH-77 and KAS6/1 myeloma cells were infected with either viruses at different MOIs (0.01, 0.1, 1, and 10), and the cell viability was determined using CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) 72 h later. The percentages of cell death in the infected cultures were calculated in comparison with the uninfected control. Error bars represent SD from three replicates. (**b**) MV-eGFP-Pwt is less sensitive to the antiviral effects of IFN, compared to MV-eGFP. ARH-77 and KAS6/1 cells were left untreated or were pretreated for 18–24 h with different concentrations of IFN-α (50–2,000

IU/ml), after which they were infected with the two viruses at MOI 1.0. MV infection was assessed using FACS analysis 48 h after infection. Data are presented as percentage of infected GFP-positive cells in comparison with the control (100% infected cells in the absence of INF). Error bars represent SD from three replicates.

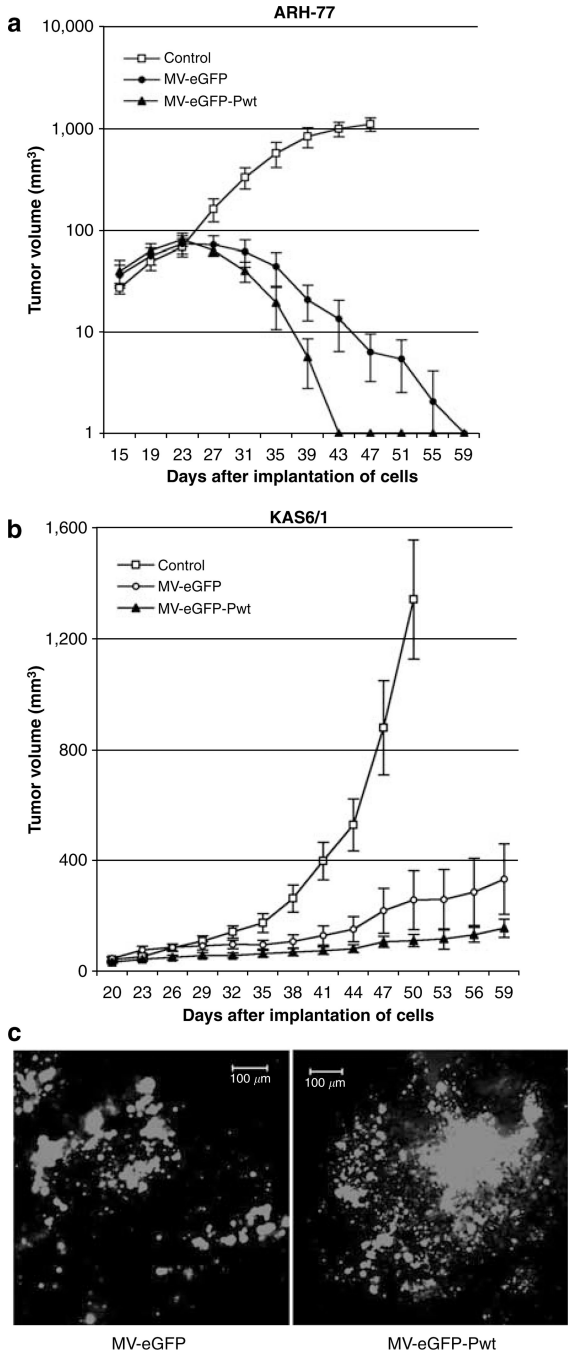

Figure 7. Therapeutic efficacy of MV-eGFP-Pwt for systemic therapy of human myeloma xenografts

(**a**) MV-eGFP-Pwt accelerated tumor regression of established subcutaneous ARH-77 xenografts in SCID mice, compared to MV-eGFP. Mice received two i.v. injections of viruses, each dose of 4×10^6 $TCID_{50}$. Numbers of mice per treatment groups are $n = 8$ (MV-eGFP), $n = 9$ (MV-eGFP-Pwt), and $n = 8$ (control saline). Error bars indicate SEM. (**b**) Systemic virotherapy of KAS6/1 myeloma xenografts caused significant inhibition of tumor growth with both administered viruses. Viruses were applied i.v. four times 1×10^7 $TCID_{50}$. Number of mice per treatment groups are $n = 9$ (MV-eGFP), $n = 10$ (MV-eGFP-Pwt), and n

= 9 (control saline). Error bars indicate SEM. (**c**) Confocal microscopy of MV-eGFP-treated and MV-eGFP-Pwt-treated KAS6/1 tumors revealed large syncytia of infected tumors.