**ORIGINAL ARTICLE**

Potent systemic therapy of multiple myeloma utilizing oncolytic vesicular stomatitis virus coding for interferon-β

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Multiple myeloma (MM) is an incurable malignancy of plasma secreting B cells disseminated in the bone marrow. Successful utilization of oncolytic virotherapy for myeloma treatment requires a systemically administered virus that selectively destroys disseminated myeloma cells in an immune-competent host. Vesicular stomatitis virus (VSV)-expressing interferon-β (IFNβ) is a promising new oncolytic agent that exploits tumor-associated defects in innate immune signaling pathways to destroy cancer cells specifically. We demonstrate here that a single, intravenous dose of VSV coding for IFNβ (VSV-IFNβ) specifically destroys subcutaneous and disseminated STG1 myeloma in an immune-competent myeloma model. VSV-IFN treatment significantly prolonged survival in mice bearing orthotopic myeloma. Viral murine IFNβ expression further delayed myeloma progression and significantly enhanced survival compared with VSV-expressing human IFNβ. Evaluation of VSV-IFNβ oncolytic activity in human myeloma cell lines and primary patient samples confirmed myeloma-specific oncolytic activity, but revealed variable susceptibility to VSV-IFNβ oncolysis. The results indicate that VSV-IFNβ is a potent, safe oncolytic agent that can be systemically administered to target and destroy disseminated myeloma effectively in immune-competent mice. IFNβ expression improves cancer specificity and enhances VSV therapeutic efficacy against disseminated myeloma. These data show VSV-IFNβ to be a promising vector for further development as a potential therapy for the treatment of MM.

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INTRODUCTION

Multiple myeloma (MM) is a disseminated malignancy of terminally differentiated plasma cells residing primarily in the bone marrow. The disease is associated with bone destruction, anemia and compromised immune function.¹ Current treatments for MM, including stem cell transplantation and high-dose chemotherapy, have significantly prolonged median survival up to 6 years, but still fall short as a cure. There is a clear need for the development and testing of novel treatment options that can be delivered intravenously to reach and destroy disseminated disease sites.²

Vesicular stomatitis virus (VSV) is a non-segmented, negative strand RNA virus of the Rhabdoviridae family that selectively kills malignant cells and has demonstrated oncolytic activity in various preclinical cancer models.³ VSV infections normally occur in livestock, causing only mild symptoms during naturally occurring human infections.⁴ Lethal infection of neural tissue in animal models prompted the use of viral engineering to attenuate VSV and minimize toxicity.⁵,⁶ Detection of viral infection in cells results in the activation of interferon (IFN) regulatory factors, and expression of Type I IFNs that can bind the z/β IFN receptor (IFNAR). z/β IFN receptor activation induces cellular innate immune responses by stimulating production of proteins that facilitate viral clearance by degrading viral intermediates, inhibiting translation, inducing apoptosis and activating release of pro-inflammatory cytokines.⁷,⁸ The Type I IFNs include several IFNα subtypes encoded by 14 pseudogenes, and IFNβ, which is encoded by a single gene.⁹ Although there have been differences described in receptor activation by IFNα and IFNβ,¹⁰ IFNβ has been described to be expressed early upon viral detection, inducing IFN regulatory factor-7 expression that activates further expression of the various IFNα genes.⁹ Of the five genes encoded by the VSV genome, the VSV matrix protein (M) facilitates evasion of innate immunity by blocking nucleocytoplasmic transport of mRNA preventing synthesis of IFN and other anti-viral or pro-inflammatory proteins.¹¹,¹² Aberrations leading to tumorigenesis also diminish innate immune response pathways, thereby rendering cancer cells susceptible to VSV replication and oncolysis.⁸ VSV attenuation can be achieved by deleting (or mutating) residue 51 of the M protein (VSV-MΔ51) to reverse viral suppression of IFN synthesis¹³ or by incorporation of the IFNα/β gene into the viral genome,¹⁴ inducing IFNα expression and activation of downstream anti-viral genes including IFNs, to promote viral clearance from non-cancerous tissues. Cancer cells that are weakly responsive to IFN remain permissive to viral propagation and oncolysis.

The potential of VSV as a novel myeloma therapy was assessed in the immune-competent STG1 myeloma model of MM. The STG1 murine myeloma cell line can be implanted in C57Bl/KaLwRij syngeneic mice to form rapidly growing subcutaneous tumors or injected intravenously to induce orthotopic myeloma that can be monitored by measuring IgG2b paraprotein secreted by myeloma cells.¹⁵ This allows novel MM therapies to be tested in a model that closely resembles disseminated myeloma in patients in the presence of intact anti-viral innate and adaptive immune responses.

Although VSV-MΔ51 has enhanced tumor specificity by inducing IFN production, viral replication is significantly compromised even in IFN-resistant cells.¹⁶ Having previously demonstrated that recombinant VSV-MΔ51 coding for the sodium iodide symporter has weak anti-myeloma activity,¹⁷ we hypothesized

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that VSV coding for IFN-β (VSV-IFNβ) would specifically destroy cancer cells in vivo while maintaining viral potency. IFNβ has historically been used as therapy against myeloma, albeit with weak and variable efficacy, and has been reported to have various antitumor properties, including direct pro-apoptotic activity, promotion of long-lasting antitumor immunity and inhibition of tumor blood vessel formation.\textsuperscript{18–20} IFNβ, however, has been utilized extensively in various oncolytic viral vectors, including adenovirus,\textsuperscript{21} vaccinia\textsuperscript{22} and measles viruses,\textsuperscript{23} demonstrating that viral vectors expressing IFNβ can be safely and successfully utilized for the treatment of cancer. VSV-expressing IFNβ has also previously shown antitumor efficacy in preclinical cancer models, including renal cell carcinoma\textsuperscript{13} and malignant pleural mesothelioma,\textsuperscript{24} whereas safety studies indicate that viral IFNβ expression successfully alleviates VSV neurotoxicity,\textsuperscript{25} making VSV-IFNβ a potentially potent and safe vector for systemic treatment of MM.

Here we report the potent oncolytic activity of intravenously administered VSV-IFNβ in an immune-competent model of MM. VSV is able to target specifically tumor sites in vivo inducing destruction of myeloma cells in subcutaneous tumors and within the bone marrow. Destruction of disseminated orthotopic myeloma resulted in transiently reduced and subsequently delayed disease burden and prolonged survival. Therapeutic efficacy was achieved in the presence of robust anti-viral antibody response with no detectable toxicity. We further demonstrate that VSV expressing murine IFNβ (mIFNβ) shows significantly enhanced therapy in mice bearing disseminated myeloma, making VSV-IFNβ a strong candidate as a potential new therapeutic vector for the treatment of myeloma.

**MATERIALS AND METHODS**

**Cells**

STG1 murine myeloma cell line, obtained from Dr Babatunde O. Oyajobi (University of Texas Health Sciences Center, San Antonio, TX) was grown in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 100 U ml\textsuperscript{−1} penicillin and 100 mg ml\textsuperscript{−1} streptomycin. These cells were not authenticated; however, confirmation of secretion of the specific IgG2b paraprotein (by IgG2b enzyme-lined immunosorbent assay (ELISA), see below) and tumor formation in the specific C57Bl/KaLwRij syngeneic mouse strain (and not in C57Bl mouse strain) provided evidence of correct myeloma cell identity. BHK-21 (baby hamster kidney 21) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and penicillin and streptomycin antibiotics. Human myeloma cell lines were obtained at Mayo Clinic courtesy of Dr Diane Jelinek (Kas 6/1, ALMC-1, JIN-3, KP-6 and ANBL-6, Rochester, MN) or Dr Rafael Fonseca (MM-1; Scottsdale, AZ). These cell lines were not authenticated. Human and murine bone marrow stromal cell lines, HS27A and SR4987, were obtained from American Type Culture Collection, HS27A, JNN-3 and MM-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, and penicillin and streptomycin antibiotics; KAS 6/1 and ANBL-6 cells were supplemented with 1 ng ml\textsuperscript{−1} interleukin-6. All cell lines were tested negative for mycoplasma contamination. Primary cells were obtained from bone marrow samples from patients with advanced MM at the Mayo Clinic. These cells were sorted in CD138\textsuperscript{−} myeloma cells and CD138\textsuperscript{+} normal bone marrow progenitor cells. Primary myeloma cells are grown in RPMI 1640 supplemented with 10% fetal bovine serum, and penicillin and streptomycin antibiotics and interleukin-6 (1 ng ml\textsuperscript{−1}).

**Viruses**

VSV coding for mIFNβ and human IFNβ (hIFNβ) were generated in the lab of Dr Glen N. Barber (University of Miami School of Medicine, Miami, FL) as described previously.\textsuperscript{14} The VSV-IFNβ gene contains the IFNβ gene at Xhol/Nhel restriction sites generated in between the G and L viral genes. VSV-GFP (VSV-expressing GFP) (Indiana strain) was also provided by Dr Glen N. Barber. VSV vectors were amplified using BHK-21 cells. BHK-21 cells plated in flasks were allowed to grow to ~80% confluency. Cells were infected at a multiplicity of infection (MOI) of 0.01 for 1 h in serum-free Dulbecco’s modified Eagle’s medium. Virus was then removed and cells incubated at 37 °C in 5% CO\textsubscript{2} incubator. Complete cytopathic effect was seen by ~48 h post infection. Culture medium was harvested, frozen in liquid nitrogen and thawed at 37 °C, subjected to low-speed centrifugation and filtered through a 0.2-μm filter. The supernatant was loaded on 10% (w/v) sucrose and centrifuged at 27 000 g for 2 h. Pelleted particles were resuspended in phosphate-buffered saline (PBS), aliquoted and stored at ~80 °C. Virus stock titers were measured by infection of BHK-21 cells plated in 96-well plates (7 x 10\textsuperscript{3} cells per well per 0.05 ml) with serial dilutions of virus stock. Tissue culture infective dose 50 (TCID\textsubscript{50}) values are determined by the Spearman and Karber equation.

**In vitro viral activity**

Viral replication was compared by infection of BHK-21 cells plated in 24-well plates (1 x 10\textsuperscript{5} cells per well per ml). These cells were incubated overnight, and mock infected or infected with VSV-GFP, VSV-mIFNβ and VSV-hIFNβ (MOI of 3.0, in Opti-MEM at 37 °C for 1 h). Cell supernatant was harvested at specified time points and viral titer was measured using the previously described method and calculated using the Spearman and Karber equation.

Viral growth characteristics were measured in myeloma suspension cells mock infected or infected with VSV viruses in Opti-MEM. Cell supernatant was harvested at specified time points to measure viral titer (using the titer measurement protocol described above) and secreted IFNβ by using an ELISA kit designed to detect mIFNβ specifically (PBL InterferonSource 42400-1). Cells were harvested to measure viability by flow cytometry using the LIVE/DEAD fixable dead cell stain kit (Invitrogen Molecular Probes, Eugene, OR, USA). Assays to measure viable cell proliferation were carried out on myeloma cells and measured using the MT3 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (ATTC 30–10100). Cells were mock infected or infected with VSV-GFP, VSV-mIFNβ or VSV-hIFNβ (MOI of 1.0 in Opti-MEM, 1 h at 37 °C). Virus was washed out and cells were seeded into 96-well plates (1 x 10\textsuperscript{5} cells per well per 100 μl) in their respective media. Plates were incubated for 72 h. MTT reagent (0.01 ml) was added to each well and incubated at 37 °C for 3 h. A measure of 0.1 mg/l detergent was added to cells to extract formazin and color intensity was measured using a microplate reader (570 nm). Experiments were performed in triplicate and results tabulated as a percentage of mock-infected cells.

**In vivo studies**

The 4- to 6-week-old female C57Bl/KaLwRij mice were obtained from Harlan (Horst, The Netherlands). Tumors were implanted in mice by a subcutaneous injection of 5 x 10\textsuperscript{6} cells in 100 μl PBS. Subcutaneous tumors were established by day 14 post STG1 implantation. Tumor burden was monitored by serial caliper measurements to measure tumor volume. Mice were randomized and treated with a single, intravenous dose of 100 μl PBS (control treatment), VSV-mIFNβ or VSV-hIFNβ at a dose of 1 x 10\textsuperscript{7} TCID\textsubscript{50} per mouse. Mice reached sacrifice criterion if tumor burden exceeded 10% of body weight, if mice were unable to access food or water or if tumors had indications of ulceration. Mice were culled at days 1 and 3 post VSV administration (n=2 per day). Tumor, brain and mouse serum was harvested. Serum was further harvested at days 5, 7, 10, 15 and 30 post VSV administration (two mice or surviving mice at each time point). Harvested tissues were processed and analyzed as described below. Orthotopic STG1 myeloma was established in syngeneic C57Bl/KaLwRij mice by intravenous administration of 5 x 10\textsuperscript{5} STG1 cells. Mice were administered with a single, intravenous dose of 100 μl PBS, or VSV-mIFNβ or VSV-hIFNβ (5 x 10\textsuperscript{7} TCID\textsubscript{50} per mouse) at day 21 post-injection. Myeloma burden was monitored by measurement of serum IgG2b using mouse IgG2b ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX, USA; E90-109). Mice were monitored for symptoms of myeloma, including weight loss, hindlimb paralysis or the growth of subscapular plasmacytomas and killed according to the previously described criterion.
Tissue analysis

Serum collected at days 1 and 3 was analyzed for mIFN and IFN using IFN and IFN ELISA kits (PBL InterferonSource, Piscataway, NJ, USA). Serum was also used to measure generation of anti-VSV neutralizing antibodies. Serum was pre-incubated for 30 min at 56°C to inactivate complement. Serial twofold dilutions of inactivated serum were incubated with 500 TCID50 VSV-GFP for 1 h at 37°C. The mixture of serum and virus was then plated onto Vero cells in 96-well plates. Anti-VSV antibody titer was quantified as the minimum dilution of serum that failed to protect Vero cells from VSV-induced complete cytopathic effect. Harvested organs, tumor and brain were segmented into two portions, one portion flash frozen and another portion stored in 10% formalin. Frozen tumor and brain sections were weighed, and macerated to form tissue suspension. Tissue suspension was subjected to two rounds of freeze--thaw (in liquid nitrogen) and centrifuged to collect tissue supernatant. Tissue supernatant was titered as previously described to measure viral recovery per mg of tissue. Tissues stored in formalin were embedded in paraffin, sectioned and subject to staining with hematoxylin and eosin. Tissue sections were subject to independent histopathological analysis by Dr Ronald Marler, Mayo Clinic (Scottsdale, AZ).

RESULTS

VSV-expressing IFNβ has enhanced cancer specificity without viral attenuation

Recombinant VSV coding for IFNβ (VSV-IFNβ) was generated by incorporation of the IFNβ gene cDNA into the previously constructed pVSV-XN2 plasmid containing the full-length positive strand VSV antigenome.14 PCR-generated IFNβ cDNA was inserted into a unique restriction site between the viral G and L genes coding for the viral surface glycoprotein and polymerase protein, respectively. Functional virus was recovered as described previously.25 Newly generated VSV-IFNβ vectors, containing transgenes coding for either mIFNβ (VSV-mIFNβ) or hIFNβ (VSV-hIFNβ), had similar growth characteristics to VSV-GFP in BHK cells, a cell line that is not responsive to either mIFNβ or hIFNβ (not shown). VSV oncolytic efficacy against myeloma was initially assessed in vitro, indicating that VSV-GFP, VSV-mIFNβ and VSV-hIFNβ viruses replicate rapidly in StG1 mouse myeloma cells following infection at an MOI of 3.0 (Figure 1a). VSV-mIFNβ induction of VSV-IFNβ (Figure 1b), which did not impede VSV oncolysis of StG1 cells compared with VSV-hIFNβ and VSV-GFP (Figure 1c). Evaluation of VSV-IFNβ specificity indicated that VSV-mIFNβ kills StG1 myeloma cells, but has diminished cytotoxicity in StG1 immortalized bone marrow stromal cells. VSV-GFP, however, kills both cell types effectively in vitro (Figure 1d). Overall these results demonstrate the ability of VSV-IFNβ to target and kill StG1 myeloma cells while exhibiting enhanced specificity in vitro with diminished potency in non-cancerous murine bone marrow stromal cells.

VSV-IFNβ has potent therapeutic efficacy against MM in immune-competent mice

Safety studies with VSV-IFNβ have previously demonstrated that viral expression of host-specific IFNβ diminished toxicity in preclinical cancer models.5 VSV-IFNβ oncolytic efficacy against MM was assessed in vivo in C57Bl/KaLwRij bearing syngeneic StG1 subcutaneous myeloma tumors. Mice were administered with a single, intravenous dose of 1 × 106 TCID50 VSV-mIFNβ, VSV-hIFNβ or 100 μl PBS via tail vein injection. Tumor responses were monitored and categorized into tumor progression, tumor regression or regression, followed by relapse as shown (Figure 2a). All mice treated with PBS had rapid tumor progression and were killed. In all, 100% of tumors regressed in response to VSV-mIFNβ treatment, whereas 80% of tumors regressed following VSV-hIFNβ treatment, with no associated symptoms of neurotoxicity. These tumor responses correlated with significantly prolonged survival of mice treated with VSV-mIFNβ (P = 0.0018) or VSV-hIFNβ (*P = 0.04) compared with PBS-treated mice using log-rank survival comparisons (data not shown). In vivo production of mIFNβ or hIFNβ was confirmed by the analysis of serum from treated mice at days 1 and 3 post VSV administration by ELISA (Figure 2b). A small proportion of mice with regressing tumors relapsed and mice were eventually killed owing to tumor burden (Figure 2a).

Adaptive immune responses against systemically administered virus in the form of neutralizing antibodies are a significant
VSV-mIFNβ specifically and rapidly destroys myeloma tumors. VSV-mIFNβ activity in vivo was further evaluated by the analysis of tumors and brains harvested from mice following intravenous virus treatment. Brain tissues were analyzed to monitor potential VSV toxicity, which proceeds by infection of neural tissues. Portions of tumors and brains from PBS- and VSV-mIFNβ-treated mice were sectioned and stained using hematoxylin and eosin. Histopathological analysis of harvested tumors showed extensive necrosis in tumors from VSV-mIFNβ-treated mice, with low to no necrosis in PBS-treated mice (Figures 3a and b). The degree of tumor necrosis was quantified, indicating increased tumor cell death at day 3 post VSV-mIFNβ treatment (Figure 3c). To confirm that tumor destruction was associated with viral oncolysis, supernatants from processed tumors were titrated showing viral load of 1 × 10^6 TCID₅₀ per mg of tissue in VSV-mIFNβ-treated mice and 1 × 10^3 TCID₅₀ per mg of tissue in VSV-hIFNβ-treated mice (Figure 2c). Although the differences are not significant, tumor-specific viral burden and degree of tissue necrosis increased from day 1 to day 3 post viral administration, suggesting that VSV is able to undergo effectively both viral replication and by 72 h induce cell death in >50% of tumor cells. There were no indications of necrosis or inflammation, or virus recovered from brains of VSV-mIFNβ-treated mice (Figure 3a and data not shown).

Of note, histopathological analysis revealed minimal to negligible infiltration of immune cells at the site of tumor at days 1 and 3 post-treatment, suggesting that neither the presence of virus nor viral destruction of tumor cells stimulated early infiltration of inflammatory immune cells. Analysis of tumors at day 15 post PBS, VSV-mIFNβ or VSV-hIFNβ administration indicated slight mixed inflammatory infiltrate, including macrophages and lymphocytes in tumors treated with VSV-mIFNβ (Table 1). This suggests that mIFNβ expression may enhance late infiltration of immune cells at tumor site. These data collectively indicate that systemically administered VSV-mIFNβ localizes to the site of tumor to induce tumor destruction. There is no observed toxicity and no detectable virus in the brain demonstrating that VSV-mIFNβ can be safely administered intravenously at this dose level.

VSV-mIFNβ has potent therapeutic efficacy against disseminated myeloma. The potent efficacy of systemically administered VSV-mIFNβ against subcutaneous myeloma tumors prompted studies to evaluate the ability of VSV-mIFNβ to reach and destroy disseminated myeloma. Orthotopic myeloma was established in mice by intravenous implantation of STG1M cells. STG1M myeloma cells, like myeloma in patients, secrete monoclonal antibodies, specifically an IgG2b serum paraprotein. Myeloma burden was assessed by IgG2b ELISA as described previously.17 ELISA quantification of serum IgG2b showed a significant increase by day 21 post STG1M injection indicative of increasing myeloma burden (Figure 4a; ***P = 0.0019). Myeloma burden and survival were monitored following PBS, VSV-mIFNβ or VSV-hIFNβ treatment. PBS-treated mice had rapidly progressing myeloma and were killed owing to weight loss or hindlimb paralysis. A single, intravenous dose of 5 × 10^5 TCID₅₀ data indicate that systemically administered VSV-mIFNβ has potent and specific activity against myeloma tumors in vivo in immune-competent mice. At this dose level, VSV-mIFNβ demonstrates oncolytic efficacy with no associated toxicity, regardless of mIFNβ expression. The transient expression of detectable IFNβ and rapid generation of neutralizing antibodies against VSV-mIFNβ suggests that tumor regression occurs in response to a limited period of viral activity before antibody-mediated viral clearance, prompting further analysis of early tumor-specific viral activity.

Barrier to effective oncolytic virotherapy.26,27 VSV especially is a highly immunogenic virus.26 Neutralizing antibodies were detected in the serum by day 5 post-treatment, with no significant difference in antibody titer generated between VSV-mIFNβ- and VSV-hIFNβ-treated mice (Figure 2c). No anti-VSV neutralizing antibodies were detected in PBS-treated mice. Overall these
VSV-mIFNβ or VSV-hIFNβ delayed myeloma progression and significantly prolonged survival of myeloma bearing mice (Figures 4b and c). Analysis of spine and femur sections harvested from myeloma bearing mice at 48 h post-treatment showed foci of myeloma cells packed in the bone marrows of PBS-treated mice, whereas bone marrows of VSV-mIFNβ-treated mice contained regions of necrosis, indicating that VSV-mIFNβ could reach and destroy disseminated foci of myeloma within the bone marrow (Figure 5). There was no appreciable inflammatory infiltrate within the bone marrow of either PBS-, VSV-mIFNβ- or VSV-hIFNβ-treated mice at 48 h post-treatment. We further observed that systemic VSV-mIFNβ treatment significantly prolonged survival of mice bearing disseminated myeloma compared with VSV-hIFNβ-treated mice, with one mouse being completely cured of systemic disease (*P = 0.021). The basis of IFNβ-induced enhancement of oncolytic efficacy in mice with disseminated myeloma is as yet unknown, although the hypotheses include direct anti-myeloma activity, IFNβ effects on the bone marrow microenvironment and potentially IFNβ-induced antitumor immune responses.

VSV coding for IFNβ has variable oncolytic activity in myeloma cells
Systemically administered VSV-IFNβ has demonstrated potent in vivo efficacy against both subcutaneous and disseminated myeloma, indicating that VSV-IFNβ is a promising oncolytic candidate for myeloma therapy. These studies, however, have been limited to the evaluation of viral oncolytic activity in 5TGM1 cells and in mice bearing 5TGM1 myeloma. The use of VSV-IFNβ as a potential anti-myeloma therapy will depend on the ability of VSV-IFNβ to exert oncolytic activity on various types of myeloma. A panel of human myeloma cell lines was infected with VSV-mIFNβ or VSV-hIFNβ and cell viability was measured 72 h later. These data show firstly that VSV-mIFNβ is potently cytopathic against all myeloma cell lines in the selected panel including HS27A, a human bone marrow stromal cell line (Figure 6a). HS27A cells, however, were relatively resistant to VSV-hIFNβ oncolysis,

| Treatment | Days post treatment | Inflammatory infiltrate |
|-----------|---------------------|------------------------|
| PBS       | 15                  | M18                    |
| VSV-mIFNβ | 15                  | M10                    |
| VSV-hIFNβ | 15                  | M37                    |

Abbreviations: H&E, hematoxylin and eosin; hIFNβ, human interferon-β; inf, infiltrate; i.v., intravenous; mIFNβ, murine IFNβ; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus; VSV-IFNβ, VSV coding for IFNβ. Inflammatory infiltrate in tumors processed for H&E 15 days post i.v. PBS, VSV-mIFNβ or VSV-hIFNβ administration. Inflammatory infiltrate are categorized as none, minimal or slight for mixed infiltrate including fibroblasts, macrophages and lymphocytes.

Figure 3. Tumor necrosis and virus recovery following systemic delivery of vesicular stomatitis virus coding for murine interferon-β (VSV-mIFNβ). Representative appearance of brain and tumor tissues harvested from mice at day 3 post intravenous (i.v.) treatment with (a) phosphate-buffered saline (PBS) or (b) 1 × 10⁶ tissue culture infectious dose 50 (TCID₅₀) VSV-mIFNβ. Tissues were harvested, paraffin-embedded and processed for hematoxylin and eosin (H&E). Images are shown at × 200 or × 400 magnification. (c) Quantification of tumor necrosis from H&E tumor sections from n = 2 mice culled at day 1 (unshaded) or day 3 (shaded) following i.v. treatment with PBS (white) or VSV-mIFNβ (red). Degree of necrosis was measured using four-point scale: (1) < 25% tissue necrosis; (2) 25–50% tissue necrosis; (3) 50–75% tissue necrosis; and (4) > 75% tissue necrosis. (d) Viral load in tumor harvested at day 1 (unshaded) and day 3 (shaded) post i.v. PBS or VSV-mIFNβ treatment. Tumor-specific viral load was measured by maceration of tumors and measurement of viral titer in the supernatant of tumor suspensions. Results are plotted as viral titer per mg of tissue.
indicating that viral expression of hIFNβ suppresses VSV-induced cell death in responsive human bone marrow stromal cells. hIFNβ production also diminished VSV oncolytic activity in four of the six cell lines in the myeloma panel, indicating that these cells were responsive to IFNβ production, albeit weakly when compared with non-cancerous bone marrow stromal cells (Figure 6a). These data indicate that oncolytic activity of VSV-IFNβ may be diminished in myeloma cells that retain responsiveness to IFN. VSV-IFNβ anti-myeloma activity was also assessed in primary myeloma cell lines.

Viral oncolytic activity and specificity are gauged by measuring the ability of VSV-hIFNβ to kill five primary patients samples sorted into CD138+ myeloma cells and CD138- non-myeloma cells. Cell death following infection was measured by staining cells with a dead cell marker and monitored using flow cytometry. In four out of five samples, >80% of CD138+ myeloma cells were killed. In comparison, CD138- cells were resistant to VSV-hIFNβ oncolysis (Figure 6b). These results demonstrate the specific activity of VSV-hIFNβ against myeloma cell lines and primary myeloma cells. Viral hIFNβ expression enhances specificity of VSV oncolytic activity demonstrated by the diminished ability of VSV-hIFNβ to kill human bone marrow stromal cells. hIFNβ expression in human myeloma cells may, however, potentially attenuate VSV-IFNβ oncolytic activity depending on how significantly innate immune pathways are impaired in cancer cells and resultant responsiveness to IFN induction. Overall the results support the hypothesis that IFNβ expression enhances the specificity of VSV, exerting cytopathic activity against various types of human myeloma cell lines and in primary myeloma cells, with diminished activity in non-cancerous bone marrow stromal cells and CD138- cells.

DISCUSSION

Viral attenuation is utilized to decrease potential toxicity of oncolytic viral cancer therapies. Clinical studies testing oncolytic viruses have demonstrated limited therapeutic efficacy of systemically administered viral treatments, indicating that viral engineering utilized to enhance tumor specificity also debilitate viral replication and oncolysis. The use of VSV-IFNβ as a potential anticancer agent allows utilization of a viral vector with intact and functional viral genes that produces exogenous IFNβ. The results demonstrate that VSV-IFNβ retains robust viral replication and oncolytic potency in 5TGM1 myeloma cells while exhibiting diminished ability to kill non-cancerous cells.

There are various in vivo barriers to effective systemic oncolytic virotherapy. These include off-target sequestration, complement inactivation, cellular innate immunity, neutralizing antibodies and physical barriers such as tumor stroma that prevent effective viral propagation and spread. In our studies, therapeutic efficacy is achieved following a single, intravenous dose of VSV-mIFNβ in an immune-competent myeloma mouse model. These data indicate that sufficient virions of systemically administered VSV-IFNβ reach the tumor site, and selectively propagate within and kill myeloma cells to exert potent and specific antitumor activity. The absence of neurotoxicity indicates insubstantial off-target replication at this dose of VSV-IFNβ, although previous studies have demonstrated that IFNβ expression alleviates viral toxicity at high doses. The potent tumor-specific activity of intravenous VSV-IFNβ against myeloma tumors warrants further investigation into the processes and roles of viral extravasation, propagation and virus-induced cell death in mediating successful tumor destruction.

The results demonstrate that VSV-expressing mIFNβ induction delays myeloma progression and improves survival compared with VSV-expressing hIFNβ that is not functional in mouse tissues. This indicates that while IFNβ can alleviate potential VSV neurotoxicity, it also enhances VSV antitumor efficacy in myeloma bearing immune-competent mice. It will be important to further understand the basis of IFNβ-induced enhancement of therapeutic efficacy in mice with disseminated myeloma. IFNβ has been used as a therapy for the treatment of myeloma and tested against other malignancies with limited success and viral IFNβ expression may exert direct antitumor effects on myeloma cells or
the bone marrow microenvironment. Tumor histology indicates that mIFNβ expression does not affect early, but may promote late infiltration of immune cells within 5TGM1 myeloma tumors, suggesting that IFNβ could enhance the antitumor immune response.35 Further investigations are underway to delineate specific mechanisms by which IFNβ induction enhances VSV oncolytic efficacy against disseminated myeloma.

Currently used myeloma therapies have extended the life expectancy of patients diagnosed with myeloma, but are not curative. Refractory or relapsed myeloma evolve mechanisms to resist chemotherapies.36 The studies described here demonstrate the ability of VSV-IFNβ to target and destroy disseminated myeloma to delay myeloma progression and improve survival in mice. VSV-IFNβ destroys myeloma cells based on systematic weaknesses in cellular innate immunity that arise from various tumorigenic alterations.37 In addition, VSV-IFNβ infection results in (variably) reduced viability of human myeloma cell lines and primary patient samples. The potential ability of VSV-IFNβ to kill different types of myeloma cells and the rapidity of VSV-IFNβ oncolysis (>50% tumor destruction by 72 h post-treatment) reduces the likelihood that myeloma cells can escape or evolve to resist viral oncolysis, both features that favor the proposed utilization of VSV-IFNβ as a potential myeloma therapy.

Viral IFNβ induction can potentially either enhance VSV therapeutic efficacy against disseminated myeloma or attenuate viral propagation in target cells that respond to IFNβ. It will be important in future studies to test VSV-IFNβ efficacy in different immune-competent models of MM, firstly to validate that viral efficacy is not limited to this specific model of MM and additionally to shed further light into the role of viral IFNβ expression in successful tumor oncolysis. The data demonstrate overall that VSV-IFNβ is a potent oncolytic candidate for clinical use for myeloma treatment and a promising platform for further engineering to improve viral targeting, monitoring and therapy.

Figure 5. Vesicular stomatitis virus coding for interferon-β (VSV-IFNβ) oncolysis of orthotopic 5TGM1 myeloma. Bone marrows harvested 48 h post intravenous (i.v.) phosphate-buffered saline (PBS), VSV-murine IFNβ or VSV-human IFNβ administration processed for hematoxylin and eosin (H&E) (magnification × 200). Arrows indicate regions of cellular necrosis.

Figure 6. In vitro activity of recombinant vesicular stomatitis virus coding for interferon-β (VSV-IFNβ) in human myeloma cell lines. (a) Variable cytopathic activity of VSV-murine IFNβ (mIFNβ) versus VSV-human IFNβ (hIFNβ) in a panel of human myeloma cell lines. Oncolytic activity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 72 h post infection of myeloma cell lines at a multiplicity of infection (MOI) of 1.0. Results are plotted as the percentage of mock-infected cells. (b) VSV-hIFNβ cytopathic activity in primary myeloma samples from five separate patients. Patient bone marrow aspirates sorted into CD138+ myeloma (gray) and CD138− non-myeloma cells (shaded) infected with VSV-hIFNβ (MOI 1.0). Cell viability was assessed at 48 h post infection by flow cytometry analysis.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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