ORIGINAL ARTICLE

Evaluation of T cells as carriers for systemic measles virotherapy in the presence of antiviral antibodies

HT Ong¹, K Hasegawa¹, AB Dietz², SJ Russell¹ and K-W Peng¹¹Molecular Medicine Program, Mayo Clinic College of Medicine, Rochester, MN, USA; ²Transfusion Medicine, Mayo Clinic College of Medicine, Rochester, MN, USA

Neutralizing antiviral antibodies (Abs) can hinder systemic virotherapy. Here, we used activated T cells as carriers to deliver oncolytic measles viruses (MV) to multiple myeloma xenografts in the presence of anti-MV antibodies (Abs). Virus-infected T cells expressing measles H/F fusogenic envelope glycoproteins could efficiently transfer MV infection by heterofusion, even after exposure to virus-inactivating anti-MV antisera. Severe-combined immunodeficiency (SCID) mice bearing subcutaneous or disseminated human myeloma xenografts were given MV-luciferase (MV-Luc) or MV-Luc-infected T cells intravenously. Indium111 labeling indicated that 1–2% of the virus-infected T cells trafficked to tumors. Preinfected T cells fused with tumor

cells in vivo and transferred MV-Luc to tumor xenografts where intratumoral viral spread was monitored non-invasively using bioluminescent imaging. In mice passively immunized with high titers of measles immune serum, intravenous virus and cell delivery were both inhibited. Decreasing the amount of measles immune serum given to mice permitted tumor infection by virus-infected T cells and cell-free virus. In conclusion, virus-loaded T cells may facilitate systemic measles virotherapy in the presence of antiviral Abs and they warrant further investigation as potential MV cell carriers.

Gene Therapy (2007) 14, 324–333. doi:10.1038/sj.gt.3302880; published online 19 October 2006

Keywords: oncolytic measles virus; multiple myeloma; T-cell carriers; anti-viral antibodies

Introduction

Multiple myeloma (MM) remains an incurable plasma cell malignancy that kills more than 15 000 people in the United States each year. The disease is disseminated throughout the bone marrow (BM) either as diffuse infiltrates of myeloma cells or as solid tumor nodules. Osteolytic bone lesions owing to increased osteoclast activity and elevations in paraprotein levels are characteristic of MM. Chemotherapy using corticosteroids, alkylating agents, proteasome inhibitors and thalidomide analogs prolongs survival but is not curative.¹ Thus, innovative MM targeted therapeutics are urgently needed.

Oncolytic viruses are attractive experimental therapeutics as they can potentially replicate and spread within tumors to directly induce tumor destruction or potentiate an antitumor immune response.² These viruses can be modified through genetic engineering to be tumor selective, be more potent through addition of therapeutic transgenes or tailored for non-invasive monitoring.³ However, efficient delivery of viruses/vectors to tumor sites remains a challenge. Accuracy and efficiency of delivery can potentially be achieved by retargeting vector tropism exclusively to tumor cells or tumor neovessels, thereby increasing vector selectivity

and decreasing uptake by non-target tissues. We have been developing the attenuated strain of measles virus (MV) as an oncolytic agent for cancer therapy and retargeting virus tropism.^{4,5} The virus uses its envelope glycoprotein, hemagglutinin (H), to attach to one of two cellular receptors, CD46 or SLAM (signaling lymphocyte activation molecule) and the fusion (F) glycoprotein to mediate viral–cell membrane fusion. Subsequent expression of MV-H and MV-F proteins on surfaces of infected tumor cells leads to extensive intercellular fusion (syncytia formation) and cell death. To enable non-invasive monitoring of the pharmacokinetics of virotherapy in patients, oncolytic MV has been modified to express a soluble marker peptide, human carcinoembryonic antigen (CEA), or the human sodium iodide thyroidal symporter, which concentrates radioiodine in the infected tumor sites.^{6,7} These viruses have potent antitumor activities in animal models and are being evaluated in phase I trials for patients with recurrent ovarian cancer, glioblastoma and multiple myeloma.^{8–10}

However, the presence of pre-existing anti-measles antibodies in vaccinated individuals can pose a major obstacle to systemic measles virotherapy. Indeed, the problem of neutralizing anti-viral Abs is relevant to all virus-based therapies as repeat dosing will likely be required to achieve significant therapeutic gains. To bypass circulating antiviral antibodies, we have chosen to first test MV in patients with ovarian cancer and glioma, as the diseases are amenable to intraperitoneal or intratumoral injection of the virus. On the other hand, patients with MM are severely immunocompromised, have low/non-existent levels of anti-measles antibodies

Correspondence: Dr K-W Peng, Molecular Medicine Program, Mayo Clinic College of Medicine, Guggenheim 18, 200 First Street SW, Rochester, MN 55905, USA.

E-mail: peng.kah@mayo.edu

Received 20 June 2006; revised 28 August 2006; accepted 28 August 2006; published online 19 October 2006

and systemic therapy is thus more likely to be therapeutically effective in these patients than in others.¹¹ Besides choice of delivery route, other strategies to evade or overcome the inhibitory effects of pre-existing antiviral antibodies can include surface modifications to mask the coat protein of viruses (eg polymer, polyethylene glycol) and use of cell carriers.^{12–15} Cells have served as ‘Trojan horses’ to deliver viruses, as virus production ‘factories’ nested in tumors, and to potentially protect viruses from neutralizing antibodies, although no studies have yet evaluated antitumor efficacy using cell carriers in the presence of pre-existing antiviral antibodies *in vivo*.^{14,16–23}

In this study, we explored the potential of using activated T cells as carriers for delivery of oncolytic MV to tumor sites. This approach should be highly feasible as natural measles virus infection is cell-associated, predominantly through lymphocytes and monocytes, and cell-free viremia has been undetectable.²⁴ In addition, expression of measles H and F fusogenic proteins on the infected cell carriers might facilitate transfer of the virus infection to tumor cells via heterologous cell fusion. Candidates for cell carriers include T cells, monocytes, tumor cells and mesenchymal stem cells. Out of these cell types, there is extensive human experience with the use of cytokine-activated T cells for adoptive transfer in immunotherapy. A typical protocol involves harvest of peripheral blood lymphocytes from the patient, CD3 isolation of the cells and expansion of the cells using various cytokine cocktail regimens.²⁵ While in culture, *in vitro* modifications to T cells have included transduction of cells to express tumor specific antigens or chimeric T cell receptors to facilitate tumor-specific cytolytic killing and induction of a tumor specific immune response.²⁶ In contrast to the immunotherapy strategy, we seek to use MV-loaded T cells as carriers to protect and deliver oncolytic measles virus to mice with multiple myeloma disease in the presence of antiviral antibodies (Abs) and achieve a favorable therapeutic outcome.

Results

Susceptibility of T cells to MV infection

Freshly isolated peripheral blood lymphocytes (PBLs) from 5 healthy donors were used immediately or activated by culture in media containing recombinant human interleukin-2 (IL-2) and phytohemagglutinin (PHA) for 1 to 2 weeks before use. We have previously shown that, in contrast to tumor cells, MV cytopathic effects in normal cells such as PBLs were significantly reduced with minimal (if any) intercellular fusion in MV-infected PBLs.⁸ Efficiency of MV-green fluorescent protein (GFP) infection of freshly isolated PBLs, as quantitated by flow cytometry analysis of GFP-positive infected cells, ranged from 10 to 20% at multiplicity of infection (MOI) of 1.0 (Figure 1a). Identity of the GFP-positive cells was determined by dual-color fluorescence activated cell sorter (FACS) using phycoerythrin (PE)-conjugated anti-CD3, anti-CD19 or anti-CD14 Abs. As shown in Table 1, CD3+ T cells were the predominant cell type infected by MV-GFP compared to CD19+ B cells or CD14+ monocytes. To increase the numbers of cells available for *in vivo* experiments and to potentially enhance cell susceptibility to MV infection, we activated

Figure 1 Efficient loading of measles virus requires activated T cells. (a) Freshly isolated or IL-2/PHA-activated (1 or 2 weeks) PBLs were infected with MV-GFP at an MOI of 1.0 and the percentage of GFP-positive MV-infected cells were analyzed by flow cytometry 48 h later. (b) Increase in the amount of input virus (MOI) resulted in a corresponding increase in numbers of infected T cells (activated for 1 week). Samples were obtained from five healthy donors and data represent the mean of three experiments (mean $\pm$ s.d.).

the PBLs in IL-2 and PHA for 1–2 weeks (Figure 1b). Expansion of PBLs using this cocktail regimen resulted in predominantly T cells (Table 1). Susceptibility of T cells maintained in IL-2/PHA medium for 1 or 2 weeks to MV infection was comparable and higher than freshly isolated cells (Figure 1a). As expected, efficiency of virus infection in the cells was increased by increasing the MOI, going up from 18% at MOI 0.5 to 50–60% at an MOI 4.0 (Figure 1b). All subsequent *in vitro* and *in vivo* experiments were performed at MOI of 1.0 using T cells from donor 1, which had been activated with IL-2/PHA for 1–2 weeks.

Transfer of MV infection from T cells to tumor cells

Transfer of MV from infected cell carriers to target cells can occur via release of progeny virus or heterologous fusion with the H/F-expressing infected cell carrier. The amount of virus output from infected T cells was determined by TCID₅₀ titrations (50% tissue culture infectious dose) on Vero cells and found to be below the limits of detection. As reported previously⁸ there was also minimal intercellular fusion between uninfected and infected T cells (data not shown). In contrast, mixing of MV-GFP-infected T cells with red DiI-labeled ARH-77 myeloma cells resulted in efficient transfer of MV infection, likely via heterofusion of the two cell types (Figure 2a–c). This resulted in ARH-77 syncytia that were

Table 1 Cell populations within freshly isolated or IL-2/PHA-activated (1 week) PBLs (mean $\pm$ s.d., $n = 3$ replicates)

	Freshly isolated			Activated		
	CD3	CD19	CD14	CD3	CD19	CD14
<i>Donor 1</i>						
% Cell type	60.48 $\pm$ 0.48	5.17 $\pm$ 0.35	0.96 $\pm$ 0.02	97.08 $\pm$ 0.22	0.72 $\pm$ 0.10	0.29 $\pm$ 0.09
% GFP+	13.15 $\pm$ 0.50	6.67 $\pm$ 1.65	5.21 $\pm$ 1.23	44.13 $\pm$ 0.60	ND	ND
<i>Donor 2</i>						
% Cell type	70.42 $\pm$ 0.01	8.93 $\pm$ 0.42	1.36 $\pm$ 0.29	95.54 $\pm$ 0.24	1.8 $\pm$ 0.14	0.54 $\pm$ 0.01
% GFP+	19.70 $\pm$ 0.65	8.15 $\pm$ 0.25	ND	35.18 $\pm$ 1.03	ND	ND
<i>Donor 3</i>						
% Cell type	67.99 $\pm$ 0.08	8.09 $\pm$ 0.20	1.00 $\pm$ 0.23	94.62 $\pm$ 0.18	0.89 $\pm$ 0.02	0.29 $\pm$ 0.06
% GFP+	13.79 $\pm$ 0.26	7.33 $\pm$ 0.52	ND	37.71 $\pm$ 0.40	ND	ND
<i>Donor 4</i>						
% Cell type	78.26 $\pm$ 0.16	9.25 $\pm$ 0.15	0.06 $\pm$ 0.08	96.40 $\pm$ 0.01	0.83 $\pm$ 0.07	0.07 $\pm$ 0.02
% GFP+	22.03 $\pm$ 0.91	6.53 $\pm$ 0.43	ND	38.65 $\pm$ 1.36	ND	ND
<i>Donor 5</i>						
% Cell type	78.83 $\pm$ 0.32	3.73 $\pm$ 0.04	0.64 $\pm$ 0.24	95.8 $\pm$ 0.01	0.7 $\pm$ 0.04	0.07 $\pm$ 0.01
% GFP+	18.2 $\pm$ 0.98	3.12 $\pm$ 0.08	0.01 $\pm$ 0.01	48.82 $\pm$ 2.11	ND	ND

Abbreviation: ND, not detectable.

Figure 2 Virus-infected T cells, but not cell-free virus, were protected from the inhibitory effects of neutralizing antibodies. (a–c) Co-mixing of MV-GFP-infected T cells and red DiI-labeled ARH-77 cells. Photos were taken with (a) FITC, (b) Texas Red filters and (c) dual filter. Single GFP-positive (white arrow) T-cell and red ARH-77 cell (yellow arrow) are indicated. A syncytium (S), likely from heterofusion of MV-GFP-infected T cells with red ARH-77 cells, is indicated. (d) T cells were able to transfer MV infection to target cells over a period of 2 weeks. Aliquots of MV-CEA-infected T cells were added to fresh Vero cells at regular intervals for 2 weeks. Culture medium was harvested 48 h after cell-mixing and analyzed for CEA levels as a quantitative measurement of the extent of virus infection in Vero cells. Cell-free viruses were rapidly inactivated after 24 h incubation in culture medium. (e) Virus-infected T cells, but not cell-free MV, were protected from the inhibitory effects of measles immune serum. MV-GFP (1×10^4 TCID₅₀) or MV-GFP infected T cells (1×10^4 T cells, MOI 1.0) were exposed to increasing dilutions of measles immune serum for 30 min at 37°C, after which the T cells were pelleted and mixed with Vero cells. Anti-MV Abs-inactivated cell-free virus but not virus-infected T cells.

dual stained; red from red DiI-labeled T cells and green owing to transfer of the MV-GFP infection. To determine the time window where MV transfer could occur, T cells were infected with MV expressing a CEA soluble marker peptide (MV-CEA) and aliquots of pre-infected T cells were mixed with uninfected Vero cells daily. Transfer of virus infection from T cells to the Vero cell culture was monitored by measuring the amount of virally encoded CEA released by infected cells into the culture supernatant and plotted after subtracting the amount of CEA produced by infected T cells alone. As shown in Figure 2d, the CEA levels increased with time in Vero-T-cell co-cultures. Thus, MV-CEA-infected T cells were able to transfer MV infection to Vero cells over 2 weeks, with the peak period being between 1 and 7 days post-infection. In contrast, cell-free viruses suspended in culture media were inactivated after 24 h incubation at 37°C.

Virus-infected cells are protected from neutralizing anti-measles Abs

Sera from healthy volunteers served as a source of anti-measles Abs and titers were determined using an enzyme immunoassay (EIA, Diamedix Corporation, Miami, FL, USA) routinely performed by the Mayo Clinical Serology Laboratory to evaluate the immune status of subjects. A titer of >20 EU/ml is classified as positive for measles Abs. Measles immune serum from donor 1 with a high anti-MV titer of 200 EU/ml was used in all subsequent assays. Corresponding plaque reduction virus neutralization (250 plaque-forming unit MV) titer of the serum was 1:256.²⁷

MV-GFP virus or pre-infected T cells (18 h post-infection) were incubated with serum from donor 1 for 30 min at 37°C. The cells were then washed once with PBS and overlaid on Vero cells. As expected, there was a corresponding increase in virus infection with increasing dilution of the measles immune serum (Figure 2e). In contrast to cell-free virus, virus-infected cells were protected from the inhibitory effects of neutralizing antibodies and were able to transfer MV to the target cells post treatment with MV Abs. It is also important to note that compared to pre-infected T cell carriers, cell-free viruses were more efficient at infecting the target cells in the absence or very low titers of anti-MV Abs.

Multimodality imaging of delivery of MV-Luc by T cells to subcutaneous or disseminated myeloma xenografts

Two models of human MM, ARH-77 and KAS 6/1, were used to determine if MV-infected T cells could transfer the virus to subcutaneous ARH-77 or systemic KAS 6/1 myeloma disease disseminated in the BM of mice. In the first model, female 5–6 weeks old severe-combined immunodeficiency (SCID) mice bearing 0.5–1.0 cm subcutaneous ARH-77 tumors were given one intravenous tail vein injection of 1×10^6 TCID₅₀ MV or 1×10^6 preinfected T cells. The dose of virus used is therapeutically active in this model.⁸ The T cells had been incubated overnight with virus (MOI 1.0) and washed three times in cold phosphate-buffered saline (PBS) before infusion into the mice. Percentage of infected cells was typically in the range of 30–40%.

Serial gamma camera imaging using Indium111-oxine labeling indicated that the majority of MV-GFP-infected T cells initially arrested in the lungs and later disseminated

in the mice, localizing predominantly in the liver (Figure 3a and b). Mice were killed 6 h after cell infusion and the amount of radioactivity was measured in the major organs. As shown in Figure 3b, 40% of T cells were found in the liver, 12% in lungs, 2% in the spleen and 1.5% in the tumor. Based on the calculation that 1.5×10^6 cells were given initially to mice, it is estimated that there were 3.8×10^4 virus-infected T cells per gram of tumor tissue. These T cells have not been modified for tumor-targeting and their localization in these tumors is likely owing to passive accumulation.

Uninfected or MV-GFP-infected T cells were labeled with DiI and infused intravenously into tumor-bearing mice. Fluorescence microscopy of freshly harvested whole tissues confirmed the presence of red DiI-labeled T cells in the organs examined; liver, lungs, spleen (data not shown) and tumor (Figure 3c–i). Single small red cells were seen as well as larger aggregates, likely as a result of fusion between the tumor cells and the red MV-GFP-infected T cells (Figure 3e and f). In contrast, no GFP-positive cells or 'red' syncytia were observed in tumors of mice that received uninfected red T cells (Figure 3c and d). Higher resolution analysis of tumor sections using confocal microscopy confirmed the presence of GFP-positive 'red' syncytia, likely owing to fusion of DiI-labeled MV-GFP-infected T cells with ARH-77 tumor cells (Figure 3g–i).

Delivery of measles virus encoding firefly luciferase (MV-Luc) by cell carriers and subsequent intratumoral viral spread was monitored intensively using bioluminescent imaging in a mouse bearing a subcutaneous ARH-77 xenograft and given one intravenous (i.v.) dose of MV-Luc-infected T cells (1×10^6). As shown in Figure 4, there was a rapid increase in luciferase activity which peaked at day 7, declined from day 9 to 18, suggesting either tumor regression and/or decline of the virus infection. Subsequent to the decline, we detected an increase in luciferase activity from day 20 to 35, likely owing to re-growth of the tumor and 'rekindling' of the virus infection. It is also interesting to note that the signal is now shifted to the left of the mouse, suggesting tumor re-growth on that side and the mouse was euthanized thereafter owing to tumor burden (Figure 4a).

The potential of preinfected T cells to deliver MV-Luc to disseminated myeloma disease was also evaluated in a systemic human myeloma model. After i.v. administration, KAS 6/1 cells seed to the BM and proliferate as tumor nodules, eventually replacing the marrow and causing hind limb paralysis of the mice. In advanced disease, the tumors 'erupt' from the marrow and form nodules in the interscapular region, paraspinal muscles and liver. To enable monitoring of tumor burden in this systemic model, we transduced the KAS 6/1 cells with a lentiviral vector to express the soluble β -chain of human chorionic gonadotropin (bhCG) so that total tumor burden can be quantitated by measuring plasma levels of bhCG.²⁸ As shown in Figure 4b, both virus and virus-infected cells were able to traffick to and spread the virus infection within the tumors. When we plotted total tumor burden (plasma bhCG levels) against viral gene expression (luciferase activity), we found a very good correlation between total tumor burden in the mice and virus gene expression ($r^2 = 0.92$) with a significant P -value of <0.0001.

Figure 3 Biodistribution of virus-infected T cells in SCID mice bearing subcutaneous ARH-77 xenografts. (a) Gamma camera image of 1×10^6 Indium111-oxine-labeled MV-GFP preinfected T cells in the SCID mouse at 30 min and 6 hr post-intravenous tail vein administration. The predominant signals are in the lungs and liver. (b) Relative biodistribution of Indium-111 labeled MV-GFP-infected T cells in mice ($n=3$) bearing subcutaneous ARH-77 tumors. (c–i) High-resolution analysis of red Dil-labeled T cells in ARH-77 tumor xenografts. Freshly isolated tumors from mice given (c, d) uninfected or (e, f) MV-GFP-preinfected T cells were sliced into 3 mm sections and photographed using a fluorescence microscope equipped with green (b, e) or red (c, f) filters. In contrast to (e), no GFP-positive cells were seen in tumors of mice given (c) uninfected cells. Abundant single red (white arrows) T cells were seen in (d) and (f). Yellow arrows point to syncytia (f) resulting from heterologous fusion of MV-GFP-infected T cells and ARH-77 cells. (f–h) Tumor sections from mice that received MV-GFP-infected Dil-labeled T cells were examined using confocal microscopy. Colocalization (yellow) of the red syncytia resulting from fusion of red Dil-labeled MV-GFP-infected T cells with tumor cells was apparent. Nuclei are stained blue.

Systemic delivery of MV-Luc was inhibited by anti-MV antibodies

To test the impact of antibodies on MV virotherapy, SCID mice bearing ARH-77 xenografts were passively

immunized by intraperitoneal (i.p.) administration of 0.5 ml of serum from donor 1 containing 200 EU/ml of anti-MV Ab (plaque neutralization titer of 1:256). Three to five hours later, mice received one i.v. injection of

Figure 4 Delivery of MV-Luc by preinfected T cells to tumor xenografts. (a) Mouse with a subcutaneous ARH-77 xenograft was given 1×10^6 preinfected T cells and Fluc gene expression was monitored by regular imaging. The number of days (d) post-administration of MV-Luc-infected T cells is indicated. (b) Systemic KAS 6/1 human multiple myeloma. Mice bearing disseminated disease were given 1×10^6 TCID₅₀ MV-Luc or 1×10^6 preinfected T cells, at 4 and 6 weeks post-myeloma cell implantation. Bioluminescent imaging of virally encoded firefly luciferase signal in treated mice at day 3 and 16 after virus or cell therapy. (c) Tumor burden is concordant with levels of viral gene expression. Levels of tumor marker bhCG from plasma of mice with disseminated KAS 6/1 tumors were plotted against levels of viral luciferase gene expression (photons/s/cm²). The correlation value is $r^2=0.92$ and $P<0.0001$, indicating a significant relationship between the two parameters.

1×10^6 TCID₅₀ MV-Luc ($n=10$ mice) or 1×10^6 preinfected T cells ($n=10$ mice). Measles-naïve mice were also treated with the same number of virus ($n=10$ mice) or cells ($n=10$ mice).

No significant luciferase signals were seen in measles naïve mice given MV-Luc at 30 min after virus infusion (Figure 5a). In mice given MV-Luc-infected T cells, luciferase signals were detected in the lungs owing to arrest of T cells in the pulmonary circulation (Figure 5a). However, at day 8, there were intense signals in tumors of both groups of animals. Intratumoral luciferase activity was quantitated (Figure 5b) and found to be comparable in mice that received cell-free ($n=5$) or cell-bound virus ($n=5$). In contrast to measles-naïve mice, no luciferase activity was detected in all 10 immunized mice at day 8 in immunized mice (Figure 5c). Delivery of virus via preinfected T cells also did not result in transfer and spread of MV in these immunized mice (Figure 5c). There were luciferase activity in the lungs of mice that received infected T cells at 30 min post-infusion but not at day 8. These data suggest that, in agreement with *in vitro* results in Figure 2 where infected cells were treated with anti-MV sera, neutralizing antibodies did not lead to the destruction of virus-infected cells *in vivo* but virus delivery using cell carriers and spread were inhibited by anti-MV antibody.

Degree of inhibition is dependent on Ab titers

To determine if virus delivery using cells is at all possible in the presence of anti-MV Ab and if the degree of inhibition is correlated with Ab dose, varying amounts (500, 200, 75 or 25 μ l) of measles-immune serum from donor 1 (titer 200 EU/ml) were diluted with saline to a final volume of 0.5 ml and administered i.p. to tumor-bearing mice. Control mice given the same amount of sera were euthanized, and sera from two to three mice were pooled and the titer of human anti-MV IgG was measured using a clinical (Diamedix) assay. The corresponding anti-MV Ab titers in mice that received 500, 200 and 75 μ l of serum were 108 EU/ml, 64 EU/ml and 14 EU/ml, respectively. A titer of >20 EU/ml in humans is considered measles immune. The average titers of anti-MV Ab in healthy individuals and myeloma patients are 100 ± 59.17 EU/ml ($n=10$) and 36.58 ± 35.99 EU/ml ($n=10$), respectively.¹¹ As shown in Figure 5d and e, the amount of virus expression in the tumors was inversely correlated to the amount of anti-measles antibody administered. At the highest Ab concentration (500 μ l or 108 EU/ml), virus and cells were neutralized or unable to fuse with the tumor cells. It is also apparent that virus-infected cell carriers and cell-free virus were able to overcome lower titers of anti-MV Ab to result in detectable luciferase expression in the tumors (Figure 5e). At lower Ab dosing (75 μ l or 14 EU/ml), viral gene expression was comparable using either method of delivery and the increases in luciferase activity were comparable to measles-naïve mice (Figure 5e).

Discussion

Oncolytic measles viruses derived from the attenuated Edmonston vaccine lineage have promising antitumor activities and two recombinant MVs expressing transgenes to enable non-invasive monitoring of the pharma-

Figure 5 Inhibition of MV therapy by virus or virus-infected cells is inversely correlated with anti-MV titers. (a) Representative luciferase images of measles-naïve mice bearing backpack ARH-77 xenografts that were given 1×10^6 TCID₅₀ MV-Luc (virus) or 1×10^6 MV-Luc-infected T cells and imaged 30 min and day 8 post-therapy. Note luciferase signals from MV-Luc-infected T cells, which localized in the lungs at 30 min. (b) Increase in intratumoral luciferase signals over time in measles naïve mice that received virus ($n=5$) or infected cells ($n=5$) was comparable. Error bars represent s.d. (c) Cell-free virus or cell-bound virus were inhibited by anti-MV Ab in passively immunized mice (+500 μ l serum). No signals were detected at day 8 for both treatment groups ($n=10$ mice per group). (d) Tumor-bearing mice were given no (0) or varying doses (25, 75, 200 or 500 μ l) of measles immune serum before to administration of 1×10^6 TCID₅₀ MV-Luc or 1×10^6 preinfected T cells. Bioluminescent images of mice at day 3 post-therapy. (e) Quantitative measurement of increases in intratumoral viral gene expression (luciferase activity) of treated mice over time from the day of virus or cell infusion (d0).

cokinetics of viral gene expression are being evaluated in phase I clinical trials for patients with ovarian cancer, glioma and multiple myeloma.^{8–10} The appeal of atte-

nuated MV as an oncolytic agent are: (1) large bystander killing owing to virus-induced cytopathic effects of intercellular fusion (syncytia); (2) MV tropism can be

efficiently retargeted to tumor cells/neovessels through display of single-chain antibodies on the H attachment protein; (3) its excellent safety record in humans with millions of doses of vaccine routinely administered to preschool children throughout the world. Indeed, the success of these vaccination programs results in a high prevalence of pre-existing anti-viral antibodies in patients who pose a major challenge for use of MV in cancer therapy.

Neutralizing anti-viral antibodies can inhibit virus delivery to the target site, preventing virus infection and subsequent viral spread. In addition, the role of cytotoxic T lymphocytes (CTLs) in virotherapy remains to be addressed; these CTLs could inhibit efficacy by removing virus-infected T cells or tumor cells but could also improve efficacy by harnessing the immune system to attack the infected tumor. To first address the issue of delivery in the presence of antibodies, we demonstrated here that systemically administered T cells can act as carriers to deliver MV to subcutaneous and disseminated human myeloma nodules in SCID mice. While previous studies have reported successful delivery of viruses to tumors using cell carriers and superior therapeutic efficacy compared to cell-free virus, no studies have reported on the utility of cell carriers for oncolytic viruses in the presence of pre-existing anti-viral antibodies *in vivo*.^{14,16–23} Here, we compared free virus or virus-infected cells for systemic virotherapy in tumor-bearing mice that were passively immunized with measles-immune serum and found that neither delivery strategy was able to overcome high titers of anti-viral antibodies. Indeed, both delivery and subsequent intratumoral viral spread were inhibited at high anti-viral titers (Peng, data not shown). When measles-immune serum were serially diluted and given to mice, both delivery methods transferred MV infection and intratumoral viral spread was detectable. Indeed, protection of humans from infection by wild-type MV is dependent on Ab titers. A titer of >120 but <1052 from the plaque reduction neutralization assay protects against classic but not against mild clinical infections and those of >1052 indicate full protection against infection by wild-type MV.²⁹ It is thus apparent that virotherapy could be optimally therapeutic when combined with strategies that deplete or cause a transient decrease in pre-existing antiviral Abs. A possible Ab depletion strategy is plasmapheresis which can potentially deplete more than 90% of the immunoglobulins after 4–5 cycles.³⁰ This gives a 2–3 week time window for virotherapy to proceed before build-up of immunoglobulin levels.

As myeloma results from clonal expansion of malignant plasma cells, it is often associated with suppression of normal B-cell function, leading to profound hypogammaglobulinemia with very low titers of anti-measles antibodies. Indeed, efficacy of vaccination of myeloma patients against influenza, *Streptococcus pneumoniae* and *Haemophilus influenza* type B is poor, and only a small percentage of patients achieved protective antibodies owing to their impaired immune status.³¹ Indeed, we previously determined that the average anti-MV Ab titers in myeloma patients (36.58 ± 35.99 EU/ml, $n = 10$) were lower than in healthy individuals (100 ± 59.17 EU/ml, $n = 10$).¹¹ This is an appealing feature that should favor measles virotherapy in this cohort of patients, especially as the current study demonstrates that cell-free virus can

be delivered to and successfully spread in tumors at low titers of Ab. The kinetics of virus neutralization is then a mathematical equation between speed of neutralization, speed of virus trafficking to the tumors, numbers of viruses administered and numbers of neutralizing antibodies; these complex systemic interactions in space and time will require further *in vivo* studies.

Based on data from this study, we envisage that the ideal protocol for systemic measles virotherapy would combine antibody depletion strategies coupled with the use of virus-loaded cell carriers for delivery of MV infection to tumor sites. Using indium111 labeled cells, we estimated that 1–2% of the injected T cells reached the tumor nodules, a level comparable to that of systemically administered antibodies.³² Localization of these T cells in tumors is likely owing to passive accumulation. However, expression of leukocyte function adhesion factor-1, which promotes adherence to endothelial cells, is increased in measles infected monocytes and lymphocytes and could aid in localization of virus-infected cell carriers to tumors.³³ As chemokines have been shown to enhance T-cell localization in the tumors, a potential strategy to enhance localization is via *in vitro* modification of T cells to express chemokine receptors.^{34,35} Owing to the logistics and mandatory validation of all clinical products and procedures, the value of elaborate *in vitro* modifications to the cell carriers needs to be carefully considered.

The type of cells to use as carriers will play an important role in determining the outcome of the therapy. Ideally, the virus should be able to amplify in the infected cell carrier and generate a large burst size at the tumor site. This increases the local concentration of viruses at the tumor site and may tip the neutralization equation in the favor of the virus. The use of cytokine-induced killer cells with vaccinia virus is an elegant pairing owing to the high tropism of vaccinia for T cells but also potentially high progeny output.¹⁴ Natural measles viremia is cell-associated and cell-free virus is undetectable in the blood of infected individuals.²⁴ The wild-type virus uses SLAM receptor found on activated T cells, B cells and monocytes.³⁶ It is thought that in wild-type virus infection, infected immune cells carry the virus for dissemination to distant sites. In our study, the T cells were activated by high levels of PHA and IL-2 expansion and more studies are required to evaluate if T cells are the optimal cell carrier for MV. The attraction of T cells as carriers is the extensive human experience with adoptive T-cell transfer, chimeric T-cell receptor engineering to enhance antitumor immunity and well-established *ex vivo* expansion regimens that yield high numbers of T cells for systemic infusion. However, as reported previously normal cells such as PBLs are significantly less susceptible to MV infection compared to tumor cells,^{8,37} and would limit the efficiency of virus loading or amplification in the PBLs. Other possible cell carriers include the use of irradiated tumor cells,¹⁸ which are highly susceptible to MV infection and support high levels of MV amplification, mesenchymal stem cells as they reportedly contribute to the stroma of tumors and thus would traffick to tumors.²³ These cell types should be examined as potential carriers for MV in combination with strategies (e.g. plasmapheresis) to transiently deplete pre-existing Abs to achieve optimal therapeutic outcome.

Materials and methods

Cell culture and viruses

Vero African green monkey kidney cells were maintained in Dulbecco's modified Eagles's medium (DMEM) containing 5% fetal bovine serum (FBS). Human ARH-77 and KAS 6/1 cells were maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% FBS. The KAS 6/1 media was supplemented with 1 ng/ml interleukin 6 (R&D Systems, Minneapolis, MN, USA). KAS 6/1 cells were also transduced with a HIV-1-based lentiviral vector expressing the β -chain of human chorionic gonadotropin (bhCG) to generate KAS-bhCG to allow non-invasive monitoring of tumor growth *in vivo* through analysis of levels of soluble peptide in the blood of the animals.²⁸ Blood from healthy volunteers were collected in heparinized tubes, layered on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 800 g for 30 min. The buffy coat was isolated and maintained in serum-free T-cell expansion medium (Sigma, St Louis, MO, USA). After monocyte depletion using plastic adherence, PBLs were expanded and activated in serum-free T-cell expansion medium containing 20–25 U/ml interleukin-2 (IL-2; R&D Systems) and 5 μ g/ml PHA (Sigma). MV-GFP, MV-Luc, MV-CEA were propagated in Vero cells as described previously.⁶ Briefly, cells were infected with viruses at an MOI of 0.02 for 2 h at 37°C after which standard medium was added. When more than 80% of the cell monolayer was fused into syncytia, the cells were scraped into reduced serum medium, Opti-MEM (Gibco, Long Island, NY, USA), and subjected to two freeze-thaw cycles to release the cell-associated viruses. The viral supernatant was clarified by centrifugation and frozen at –80°C until analysis. Virus titers were determined by TCID₅₀ titrations on Vero cells as described previously.⁶

Anti-measles antibody titers

Serum was collected using clot tubes from healthy volunteers and aliquoted. The aliquots of serum were frozen at –30°C until use. Anti-measles antibody titers were determined by Mayo Central Clinical Laboratory using the Diamedix EIA assay. Positive titers ranged from >20 to 119.5 EU/ml. Plaque neutralization assays were performed as described previously. For plaque reduction virus neutralization assays, serial dilutions (4-fold) of serum were incubated with MV-GFP (250 TCID₅₀) for 1 h at 37°C in 5% CO₂ and then plated in triplicate onto confluent Vero cells in 96-well plates.²⁷ After 1 h of incubation, inocula were removed and cells were cultured in 100 μ l of DMEM containing 5% FBS. The numbers of plaques were counted at day 4 post infection. The dilution that prevents cytopathic effects in at least two out of three wells corresponds to the neutralizing titer.

Infection assays

Freshly isolated and IL-2/PHA-activated T cells were infected with MV-GFP at various MOI (0.5, 1, 2 and 4) for 2 h at 37°C. At the end of the incubation, the virus was removed and the cells were maintained in IL-2-supplemented serum-free T-cell expansion medium in the presence of fusion inhibitory peptide to allow analysis of single infected GFP-positive cells by flow cytometry at 48 h post-infection. For analysis of PBL- subsets infected

with MV-GFP, cells were collected 48 h post-infection and labeled with PE-conjugated monoclonal antibodies against CD3, CD14 and CD19 (BD Biosciences, Pharmingen, San Diego, CA, USA). Cells were incubated with the antibodies for 30 min at 4°C. After staining, the cells were analyzed on a FACSCalibur flow cytometer (BD, San Jose, CA, USA) using the CellQuest software (BD).

In vivo experiments: imaging studies

All procedures involving animals were reviewed and approved by the Mayo Clinic Animal Use and Care Committee. The trafficking of T cells post-infusion into mice was monitored using a radioactive method or fluorescent method. T cells (2×10^6) were incubated with 100 μ l of Indium111 oxine (200 μ Ci) for 20 min at room temperature after which the cells were washed and resuspended in 100 μ l PBS before i.v. injection via the tail vein into mice. Mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) and imaged using a micro SPECT-CT imaging machine (X-SPECT, Gamma Medica, Northridge, CA, USA). MV-GFP-preinfected T cells were also incubated with Dil (Molecular Probes, Eugene, Oregon, USA) for 5 min at 37°C, washed and infused i.v. into mice. Forty-eight hours later, mice were euthanized and organs harvested to determine the biodistribution and heterologous fusion of red fluorescent T cells with tumor xenografts using a fluorescence microscope. The transfer of MV-Luc infection from the infected T cells to tumor sites were monitored using luciferase imaging techniques. Mice were injected intraperitoneally with 3 mg/kg of D-luciferin (Xenogen Corporation, Alameda, CA, USA) and 10 min later, anesthetized via isoflurane inhalation. Mice were placed on the imaging platform and images were acquired between 15 and 30 min after luciferin injection. Images were analyzed using the Xenogen Living Systems 2.50 software.

Tumor models

Five-week-old female CB.17 ICR SCID mice (Harlan-Sprague-Dawley, Indianapolis, IN, USA) were irradiated (150 cGy) 1 day prior to tumor cell implantation. ARH-77 cells were implanted subcutaneously as back-pack or flank tumors. When tumors have reached more than 0.5 cm in diameter, the mice were given 1×10^6 TCID₅₀/100 μ l MV-Luc or 1×10^6 T cells/100 μ l PBS i.v. via the tail vein. For the disseminated myeloma model, KAS-bhCG cells (2×10^6 cells/100 μ l) were injected i.v. through the tail vein into irradiated SCID mice. Mice received 2 doses of 1×10^6 MV-Luc or 1×10^6 T cells/100 μ l PBS intravenously via the tail vein at 4 and 6 weeks after cell seeding. The T cells were infected with MV-Luc (MOI 1.0) the day before and left in culture overnight. Mice were imaged using the Xenogen Bioluminescent IVIS 200 Imaging System at regular intervals after virus or cell therapy. Total tumor burden in the systemic model was monitored by harvesting blood from the mice at regular intervals via the retro-orbital route and measuring bhCG levels by Mayo Central Clinical Laboratory, Bayer Healthcare, Terrytown, NY, USA (Bayer Centaur Immunoassay) in the plasma. For passive immunization studies, mice were given i.p. injection of saline or varying amounts of measles immune serum from donor 1 (anti-MV Ab titer 200 EU/ml or 1:256 plaque reduction virus neutralization titer) diluted in saline to a final volume of

500 μ l. Three to five hours later, mice were given i.v. injections of virus or virus-infected cells.

Acknowledgements

This work was supported by the JARI Foundation for Myeloma Research and the Mayo Foundation.

References

- Rajkumar SV. Novel approaches to the management of myeloma. *Oncology (Williston Park)* 2005; **19**: 621–625.
- Russell SJ. Replicating vectors for cancer therapy: a question of strategy. *Semin Cancer Biol* 1994; **5**: 437–443.
- Thorne SH, Hermiston T, Kirn D. Oncolytic virotherapy: approaches to tumor targeting and enhancing antitumor effects. *Semin Oncol* 2005; **32**: 537–548.
- Nakamura T, Russell SJ. Oncolytic measles viruses for cancer therapy. *Expert Opin Biol Ther* 2004; **4**: 1685–1692.
- Nakamura T, Peng KW, Harvey M, Greiner S, Lorimer IA, James CD *et al*. Rescue and propagation of fully retargeted oncolytic measles viruses. *Nat Biotechnol* 2005; **23**: 209–214.
- Peng KW, Facticeau S, Wegman T, O’Kane D, Russell SJ. Non-invasive *in vivo* monitoring of trackable viruses expressing soluble marker peptides. *Nat Med* 2002; **8**: 527–531.
- Dingli D, Peng KW, Harvey ME, Greipp PR, O’Connor MK, Cattaneo R *et al*. Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. *Blood* 2004; **103**: 1641–1646.
- Peng KW, Ahmann GJ, Pham L, Greipp PR, Cattaneo R, Russell SJ. Systemic therapy of myeloma xenografts by an attenuated measles virus. *Blood* 2001; **98**: 2002–2007.
- Peng KW, TenEyck CJ, Galanis E, Kalli KR, Hartmann LC, Russell SJ. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res* 2002; **62**: 4656–4662.
- Phuong LK, Allen C, Peng KW, Giannini C, Greiner S, TenEyck CJ *et al*. Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. *Cancer Res* 2003; **63**: 2462–2469.
- Dingli D, Peng KW, Harvey ME, Vongpunsawad S, Bergert ER, Kyle RA *et al*. Interaction of measles virus vectors with Auger electron emitting radioisotopes. *Biochem Biophys Res Commun* 2005; **337**: 22–29.
- Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Therapy* 2001; **8**: 341–348.
- Croyle MA, Chirmule N, Zhang Y, Wilson JM. PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum Gene Ther* 2002; **13**: 1887–1900.
- Thorne SH, Negrin RS, Contag CH. Synergistic antitumor effects of immune cell-viral biotherapy. *Science* 2006; **311**: 1780–1784.
- Harrington K, Alvarez-Vallina L, Crittenden M, Gough M, Chong H, Diaz RM *et al*. Cells as vehicles for cancer gene therapy: the missing link between targeted vectors and systemic delivery? *Hum Gene Ther* 2002; **13**: 1263–1280.
- Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992; **256**: 1550–1552.
- Ram Z, Culver KW, Oshiro EM, Viola JJ, DeVroom HL, Otto E *et al*. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
- Coukos G, Makrigiannakis A, Kang EH, Caparelli D, Benjamin I, Kaiser LR *et al*. Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. *Clin Cancer Res* 1999; **5**: 1523–1537.
- Raykov Z, Balboni G, Aprahamian M, Rommelaere J. Carrier cell-mediated delivery of oncolytic parvoviruses for targeting metastases. *Int J Cancer* 2004; **109**: 742–749.
- Jevremovic D, Gulati R, Hennig I, Diaz RM, Cole C, Kleppe L *et al*. Use of blood outgrowth endothelial cells as virus-producing vectors for gene delivery to tumors. *Am J Physiol Heart Circ Physiol* 2004; **287**: H494–H500.
- Garcia-Castro J, Martinez-Palacio J, Lillo R, Garcia-Sanchez F, Alemany R, Madero L *et al*. Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors. *Cancer Gene Ther* 2005; **12**: 341–349.
- Cole C, Qiao J, Kottke T, Diaz RM, Ahmed A, Sanchez-Perez L *et al*. Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. *Nat Med* 2005; **11**: 1073–1081.
- Komarova S, Kawakami Y, Stoff-Khalili MA, Curiel DT, Pereboeva L. Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* 2006; **5**: 755–766.
- Griffin D. Measles virus. In: Knipe D, Howley P, Griffin D, Lamb R, Martin M, Roizman B, Strauss S (eds). *Field’s Virology*. Lippincott Williams & Wilkins: Philadelphia, 2001, pp 1401.
- Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer* 2003; **3**: 666–675.
- Riviere I, Sadelain M, Brentjens RJ. Novel strategies for cancer therapy: the potential of genetically modified T lymphocytes. *Curr Hematol Rep* 2004; **3**: 290–297.
- Ratnam S, Gadag V, West R, Burris J, Oates E, Stead F *et al*. Comparison of commercial enzyme immunoassay kits with plaque reduction neutralization test for detection of measles virus antibody. *J Clin Microbiol* 1995; **33**: 811–815.
- Shih IM, Torrance C, Sokoll LJ, Chan DW, Kinzler KW, Vogelstein B. Assessing tumors in living animals through measurement of urinary beta-human chorionic gonadotropin. *Nat Med* 2000; **6**: 711–714.
- Chen RT, Markowitz LE, Albrecht P, Stewart JA, Mofenson LM, Preblud SR *et al*. Measles antibody: reevaluation of protective titers. *J Infect Dis* 1990; **162**: 1036–1042.
- Watts A, Foley A, Awwad M, Treter S, Lambrigts D, Buhler L *et al*. Plasma perfusion by apheresis through a gal immunoadfinity column successfully depletes anti-Gal antibody: experience with 275 aphereses in baboons. *Transplant Proc* 2000; **32**: 860.
- Robertson JD, Nagesh K, Jowitt SN, Dougal M, Anderson H, Mutton K *et al*. Immunogenicity of vaccination against influenza, Streptococcus pneumoniae and Haemophilus influenzae type B in patients with multiple myeloma. *Br J Cancer* 2000; **82**: 1261–1265.
- Adams GP, Schier R, McCall AM, Simmons HH, Horak EM, Alpaugh RK *et al*. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res* 2001; **61**: 4750–4755.
- Nanan R, Chittka B, Hadam M, Kreth HW. Measles virus infection causes transient depletion of activated T cells from peripheral circulation. *J Clin Virol* 1999; **12**: 201–210.
- Gough M, Crittenden M, Thanarajasingam U, Sanchez-Perez L, Thompson J, Jevremovic D *et al*. Gene therapy to manipulate effector T cell trafficking to tumors for immunotherapy. *J Immunol* 2005; **174**: 5766–5773.
- Kershaw MH, Teng MW, Smyth MJ, Darcy PK. Supernatural T cells: genetic modification of T cells for cancer therapy. *Nat Rev Immunol* 2005; **5**: 928–940.
- Tatsuo H, Ono N, Tanaka K, Yanagi Y. SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 2000; **406**: 893–897.
- Ong HT, Timm MM, Greipp PR, Witzig TE, Dispenzieri A, Russell SJ *et al*. Oncolytic measles virus targets high CD46 expression on multiple myeloma cells. *Exp Hematol* 2006; **34**: 713–720.