Oncolytic Immunovirotherapy for Melanoma Using Vesicular Stomatitis Virus

Rosa Maria Diaz,1,2 Feorillo Galivo,1 Timothy Kottke,1 Phonphimon Wongthida,1 Jian Qiao, Jill Thompson,1 Mikael Valdes,3 Glen Barber,1 and Richard G. Vile 1,2,4

Abstract

Relatively little attention has been paid to the role of virotherapy in promoting antitumor immune responses. Here, we show that CD8+ T cells are critical for the efficacy of intratumoral vesicular stomatitis virus virotherapy and are induced against both virally encoded and tumor-associated immunodominant epitopes. We tested three separate immune interventions to increase the frequency/activity of activated antitumoral T cells. Depletion of Treg had a negative therapeutic effect because it relieved suppression of the antiviral immune response, leading to early viral clearance. In contrast, increasing the circulating levels of tumor antigen-specific T cells using adoptive T cell transfer therapy, in combination with intratumoral virotherapy, generated significantly improved therapy over either adoptive therapy or virotherapy alone. Moreover, the incorporation of a tumor-associated antigen within the oncolytic vesicular stomatitis virus increased the levels of activation of naïve T cells against the antigen, which translated into increased antitumor therapy. Therefore, our results show that strategies which enhance immune activation against tumor-associated antigens can also be used to enhance the efficacy of virotherapy.

Introduction

The primary goal of virotherapy of tumors using replication oncolytic viruses has been to promote vigorous viral replication and spread within tumors to cause direct cytotoxicity leading to tumor destruction (1, 2). Relatively little attention has been paid, however, to the role of virotherapy in promoting antitumor CD8+ T cell immune responses. This has been, in part, because many experiments using replicating viruses have, by necessity, been carried out in immunocompromised hosts using human xenografts (3–6). However, viral replication induces high levels of tumor cell killing. This might be expected to be an effective method by which tumor-specific antigens will be released and taken up by infiltrating antigen-presenting cells for cross-presentation to T cells for priming of (tumor) antigen-specific immune responses (7, 8). Indeed, the inflammatory milieu produced by viral replication might be expected to be strongly proinflammatory, with the release of potentially immunogenic intracellular cellular contents, ligation of Toll-like receptors on infiltrating macrophages designed to detect pathogenic infections, and cytokine release at the site of cell death (9). These factors should favor the recruitment, activation, and loading of antigen-presenting cells with tumor antigens, providing an opportunity for stimulatory (rather than tolerogenic) presentation of these antigens to T cells (10). Simultaneously, the proinflammatory effects of viral replication will inevitably also prime vigorous antiviral immune responses (11). It might also be expected that most viral proteins will be significantly more immunogenic than the majority of identified tumor antigens. This is likely because the precursor frequency of T cells potentially reactive against viral epitopes will be much higher than that of any nondeleted T cell populations which can recognize the self- or near–self-antigenic epitopes of most known tumor antigens (12).

Therefore, in a fully immunocompetent host, the therapeutic efficacy of local intratumoral virotherapy will be decided by multiple variables and in vivo interactions, many of which are not operative in experiments in animal models lacking complete immune systems. These variables include (a) the amount of viral replication inside the tumor, which will correlate with the levels of direct tumor cell destruction; (b) the immune-based effector mechanisms which will control viral spread both within, and outside of, the tumor, and will act to restrict viral-mediated tumor cell killing; and (c) the priming of antitumor immune effectors, which will contribute to immune-mediated tumor clearance both locally at the injected tumor site, as well as at distant sites of tumor growth. In addition, several reports have described the inclusion of additional immunostimulatory genes in viral vectors (13–19) or the use of attenuated viral mutants (20) to enhance immune-based clearance of infected tumors. However, relatively few studies have investigated the interactions between these three factors, as they apply to antigen-specific T cells, in fully immunocompetent hosts undergoing intratumoral virotherapy.

We used the vesicular stomatitis virus (VSV) as an oncolytic agent (21, 22) in the B16ova murine melanoma model. We chose this model because VSV replicates well in B16 cells, which can themselves be grown in immunocompetent C57Bl/6 mice (14). VSV is arthropod-borne, and the primary hosts are rodents, cattle, horses, and swine. Most patients living in non–endemic regions will themselves be grown in immunocompetent C57Bl/6 mice (14). VSV is arthropod-borne, and the primary hosts are rodents, cattle, horses, and swine. Most patients living in non–endemic regions will not have neutralizing titers to the virus. VSV has been successfully delivered to established tumors through systemic routes, including i.v. (3, 4, 14, 20, 23–26). With respect to the expected toxicities of the virus, VSV rarely infects humans, with only very mild, flu-like consequences, although a case of pediatric encephalitis possibly associated with VSV has been reported. Mice injected i.v. with a safety-enhanced VSV (24), or nonhuman primates injected intranasally with VSV, showed no significant toxicity (27), although neuroviral replication in both mice (24) and nonhuman primates (27) have been shown following intracranial delivery of the virus.
By using the B16ova model—in which B16 cells stably express the chicken ovalbumin protein as a surrogate tumor antigen—we were able to follow the development of immune responses against both the H-2K\(^{b}\)-restricted SIINFEKL epitope of ova, which is expressed only in the tumor and against the H-2K\(^{b}\)-restricted RGYVYQGL epitope of the VSV N protein, which is expressed only by the replicating virus. We intentionally used the ova antigen for these studies because no preexisting tolerance exists in C57Bl/6 mice for either the ova or N antigens; therefore, our results should, as far as possible, compare immune responses against antigens of similar immunogenicity in vivo. This would not be the case if we were to compare the priming of immune responses against a viral antigen and a truly self-antigen (such as the TRP-2 melanoma-associated antigen). In such a case, comparisons between immune responses generated by virotherapy would be confounded by large differences in the precursor frequencies of T cells potentially reactive against the two different classes of antigens.

In addition, we investigated three different immune-based manipulations by which the frequency and/or activity of activated T cells specific for the tumor-associated antigen could be increased at the site of the tumor undergoing virotherapy with VSV. In this way, we hoped to change the balance which normally exists between direct viral-mediated cytotoxicity to the tumor, immune-mediated control of virus replication, and immune-mediated tumor clearance, in favor of increased tumor destruction.

Materials and Methods

Cells. Murine B16ova melanoma cells (H-2K\(^{b}\)) were derived from B16 cells by transduction with a cDNA encoding the chicken ovalbumin gene (28). Cell lines were grown in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (v/v) FCS (Life Technologies) and l-glutamine (Life Technologies). All cell lines were monitored routinely and found to be free of Mycoplasma infection.

OT-I mice are transgenic mice in which the T cells recognize the SIINFEKL peptide from the chicken ovalbumin protein in the context of H-2K\(^{b}\) (29). For the preparation of naïve OT-I T cells, spleen and lymph nodes from OT-I transgenic mice were combined and crushed through a 100-μm filter to prepare a single cell suspension. RBC were removed by a 2-min incubation in ACK buffer (sterile distilled H\(_2\)O containing 0.15 mol/L NaCl, 1.0 mmol/L KHCO\(_3\), and 0.1 mmol/L EDTA adjusted to pH 7.2-7.4). When indicated, CD8\(^{+}\) T cells were isolated using the MACS CD8o (ly-2) Microbead magnetic cell sorting system (Miltenyi Biotec, Auburn, CA) and stained with CFSE dye (Molecular Probes, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For preparation of activated OT-I T cells, single cell suspensions from spleen and lymph nodes were adjusted to 1.0 × 10\(^{6}\) cells/mL in Iscove's modified Dulbecco's medium plus 5% FCS, 10\(^{-5}\) mol/L of 2-ME, 100 units/mL of penicillin, and 100 μg/mL of streptomycin and stimulated with 1 μg/mL of SIINFEKL peptide and 50 μg/mL of human interleukin 2 (Mayo Clinic Pharmacy). Every 2 to 3 days, one-half of the medium was replaced and fresh medium containing 50 μg/mL of interleukin 2. For use in vivo, nonadherent and loosely adherent cells were harvested following one activation cycle of 3 to 5 days and viable cells were purified by density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories) according to the manufacturer's instructions. More than 90% of the cells expressed the V\(_{α}\)\(_{2}\) chain of the transgenic OT-I T-cell receptor.

VSV-GFP and VSV-ova were generated by cloning the appropriate cDNAs into the plasmid pSVS-XN2, as described in ref. 14. VSV-GFP is referred to as VSV. Monoclonal VSVs were obtained by plaque purification on BHK-21 cells. Concentration and purification were done by sucrose gradient centrifugation. Virus stock titers were measured by standard plaque assays of serially diluted samples on BHK-21 cells (14).

In vivo studies. All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 weeks of age. To establish s.c. tumors, 5 × 10\(^{5}\) B16ova cells in 100 μL of PBS were injected into the flank of mice. Viral injections (50 μL) were done intratumorally at days 7, 9, and 11 after tumor establishment. Immune cell depletions were done by i.p. injections (0.1 mg/mouse) of anti-CD8 (LYT 2.43) and anti-CD4 (GK1.5), both from the Monoclonal Antibody Core Facility, Mayo Clinic (Rockchester, MN); anti–natural killer (NK) cells (anti-asialo-GM-1; Cedarlane) and IgG control (ChromPure Rat IgG; Jackson Immunoresearch, West Grove, PA) at day 4 after tumor implantation and then weekly thereafter. For Treg depletion, 0.5 mg of PC-61 antibody (Monoclonal Antibody Core Facility, Mayo Clinic) per mouse was given i.p. 4 days after tumor implantation and 3 days before the first viral injection. Fluorescence-activated cell sorting analysis of spleens and lymph nodes confirmed subset-specific depletions.

For adoptive transfer experiments, mice were given activated OT-1 T cells i.v. (10\(^{7}\) cells in 100 μL per injection) starting 8 days after tumor establishment. For survival studies, tumor diameters were measured thrice weekly in two dimensions using calipers, and mice were killed when tumor size was ~1.0 × 1.0 cm in two perpendicular directions.

Histopathology of tumor sections. Tumors were harvested and fixed in 10% formalin in PBS, then paraffin-embedded, and sectioned. H&E-stained sections were prepared for analysis of tissue destruction and gross infiltrate. A pathologist examining H&E sections, blinded to the experimental design, scored the degree of necrosis.

ELISPOT and ELISA analysis for IFN-γ secretion. Spleens or tumor-draining lymph nodes were harvested from mice at the indicated times. For ELISA, a million cells were plated in 24-well plates and incubated at 37°C with the indicated peptides. Cell-free supernatants were collected after 48 h and tested by specific ELISA for IFN-γ (BD OptElia IFN-γ; BD Biosciences, San Diego, CA). For ELISPOT assays, 1 × 10\(^{6}\) cells were plated into each well of a 96-well plate in triplicates and were restimulated for 48 h at 37°C under different conditions (all peptides were at 5 μg/mL). Peptide-specific IFN-γ-positive spots were detected according to the manufacturer's protocol (Milabtech Inc., Cincinnati, OH) and were quantified by a computer-assisted image analyzer.

The synthetic, H-2K\(^{b}\)-restricted peptides hgp100\(_{25-33}\), KVPQRNQDWL, TRP-2\(_{160-168}\), SVYDFFVWL, ova SIINFEKL, and VSV N protein-derived RGYVYQGL were synthesized at the Mayo Foundation Core facility. We used the altered ligand from hgp100, as opposed to the murine epitope, because it has been shown to be presented more effectively in the context of H-2K\(^{b}\)-restricted murine dendritic cells.

Flow cytometry and INF-γ intracellular staining assay. For analysis of phenotype, 1 × 10\(^{6}\) cells were washed in PBS containing 0.1% bovine serum albumin (wash buffer), resuspended in 50 μL of wash buffer, and exposed to directly conjugated primary antibodies for 30 min at 4°C. Cells were then washed and resuspended in 500 μL of PBS containing 4% formaldehyde. Cells were analyzed by flow cytometry and data were analyzed using CellQuest software (BD Biosciences). For intracellular staining, single cell suspensions were prepared from tumors harvested (three mice per group) at the indicated times. INF-γ production in response to antigen was then measured by incubation with peptides (5 μg/mL) in the presence of Golgi Plug reagent for 4 h. Cells were then stained, fixed, and permeabilized for intracellular staining using a Cytofix/Cytoperm kit from BD Biosciences according to the manufacturer's instructions. All antibodies were obtained from BD Biosciences (San Diego, CA).

Statistics. Survival data from the animal studies was analyzed using the log-rank test (30), and the two-sample unequal variance Student's t test analysis was applied for in vitro assays. Statistical significance was determined at P < 0.05.

Results

Host CD8 and NK cells are required for the efficacy of oncolytic therapy. A protocol was established in which three direct intratumoral injections of VSV into 7-day established B16ova...
tumors was consistently significantly therapeutic compared with heat-inactivated virus (Fig. 1C–E; \( P = 0.0001 \)) or PBS (data not shown).

As expected, histologic examination of tumors indicated significant levels of necrosis associated with ongoing virotherapy (Fig. 1A). In addition, we observed very extensive leukocytic infiltration into VSV-treated tumors (Fig. 1B). These data suggested that some of the therapies seen in Fig. 1A might be contributed by host-derived immune effectors reactive against either viral antigens expressed in infected tumor cells, and/or against tumor-associated antigens.

In order to understand the role of these immune effectors in \textit{in vivo} therapy, virotherapy was done in mice previously depleted of CD4+, CD8+, or NK cells (Fig. 1C–E). Interestingly, depletion of CD4+ T cells alone (no intratumoral VSV) significantly slowed the growth of B16ova tumors (\( P = 0.002 \)). However, we did not observe a significant difference in the rate of tumor growth in animals treated with VSV but lacking CD4+ T cells compared with animals with CD+ cells intact (\( P = 0.29 \)), suggesting that CD4+ T cells are not important in the antitumor effects (Fig. 1C). In contrast, depletion of neither CD8+ nor NK cells alone delayed tumor growth relative to the control immunoglobulin-treated animals (Fig. 1D and E). However, the addition of VSV intratumoral injections were also unable to delay tumor growth in CD8 or NK-depleted animals, to the extent that such injections were effective in fully immunocompetent animals (nondepleted versus CD8+-depleted, \( P = 0.024 \); versus NK depleted, \( P = 0.0247 \); Fig. 1D and E). Taken together, these data indicate that the therapeutic effects of direct intratumoral injections of this oncolytic virus are contributed not only by the direct cytolytic effects of viral replication, but to a very significant extent, also by host-derived immune effectors.

\textbf{Oncolytic virotherapy primes CD8+ T cells specific for tumor antigens.} Consistent with a critical role for CD8+ T cells in VSV virotherapy, we observed both a general increase in immune presentation capacity in the tumor-draining nodes (Fig. 2A), as well as a significant accumulation of CD8+ T cells at the tumor site 1, 3, and 7 days following the last intratumoral injection of VSV (Fig. 2B). Despite the importance of NK cells to therapy, as evidenced by depletion studies (Fig. 1E), we did not see any significant increased accumulation of NK cells in treated tumors (data not shown). Intracellular staining for IFN-\( \gamma \) showed that intratumoral VSV therapy primes CD8+ T cells with specificity for a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Intratumoral virotherapy of B16ova tumors in immunocompetent mice requires host immune effectors. C57Bl/6 mice (seven per group) bearing 7-d s.c. tumors were injected intratumorally every 2 d with 5 \( \times \) 10^5 plaque-forming units (pfu) of heat-inactivated virus (HI-VSV) or VSV (50 \( \mu \)L in PBS) for a total of three injections. Three days following the final viral injection, tumors were harvested, fixed in formalin, processed for paraffin sections, and stained for H&E. Levels of necrosis (A) and leukocytic infiltration (B) in the tumors were scored by an independent pathologist blinded to the treatments. Histopathologic gradings for necrosis, degeneration, and apoptosis: grade 1, <10% tissue involvement; grade 4, >50% tissue involvement. Gradings for leukocytic infiltration: grade 1 to 2, occasional to few small aggregates; grade 3, multiple aggregates or areas of leukocytes. C to E, 0.1 mg of depleting [anti-CD4 (C), anti-CD8 (D), or anti-NK (E)] or control antibodies as shown were given i.p. 4 d after tumor implantation (n = 8) and every week for the duration of the experiment. Viruses were injected intratumorally on days 7, 9, and 11 after tumor implantation. Tumor sizes were monitored every other day. Points, means of at least two independent experiments.}
\end{figure}
defined viral epitope (derived from the VSV N protein), which can be recovered from the treated tumors (Fig. 2C). However, CD8+ T cells specific for an epitope derived from the ova protein, which is exclusively expressed within, and by, the tumor were also detected in the tumor 7 days after the last viral injection (Fig. 2C). Replication-competent virus was required for these effects because no such virus- or tumor-specific cells were primed by heat-inactivated VSV. These results indicate that viral cytolysis allows antigens expressed exclusively in the tumor to be released in vivo, leading to priming of tumor-specific CD8+ T cells. Depletion of Treg decreases the efficacy of virotherapy. Both antiviral and antitumor T cell responses are susceptible to suppression by a population of putative suppressor cells in the CD4+CD25+ compartment known as regulatory T cells (Treg; refs. 31–33). Viral therapy resulted in a greater number of CD4+CD25+ T cells within the tumors ($P = 0.0158$) and a lower number in the tumor-draining lymph nodes ($P = 0.0193$) as compared with heat-inactivated virus–treated mice (data not shown). Therefore, we investigated how the depletion of Treg would affect the balance between the antitumor and antiviral effector populations. Far from relieving the suppression of antitumor CD8+ T cell responses, antibody-mediated depletion of Treg prior to viral replication in the tumors did not significantly increase the antitumor efficacy of VSV treatment relative to mice in which CD4+CD25+ cells were not depleted (Fig. 3A), and led to significantly fewer tumor regressions (2 of 10 tumors regressed in PC61-treated animals 7 to 10 days following virus injection, compared with 8 of 10 regressions in control-treated animals in the experiment in Fig. 3A). We confirmed that the single i.p. injection of the anti-CD25 antibody PC61 depleted CD4+ CD25+ cells in both spleens (data not shown) and tumor-draining lymph nodes of treated mice (Fig. 3B). Consistent with this loss of therapy, the antiviral cell–mediated response was significantly enhanced in the lymph nodes of Treg-depleted animals compared with nondepleted animals ($P = 0.0017$; Fig. 3C), although there was no difference in the levels of the anti-ova response, consistent with our deprivation of Treg. A, up-regulation of MHC class II molecules after viral therapy. One day after the last viral infection (as described in Fig. 1), tumor-draining lymph nodes were harvested and analyzed for expression of MHC class II (Ia$^d$) by flow cytometry (B). S.c. B16ova tumors were treated as described in Fig. 1. Tumor samples were harvested at 1, 3, and 7 d following the last viral injection. The percentage of CD8+ T cells, relative to the total number of lymphocytes in the tumors, as assessed by flow cytometry. Statistics were done by using a two-tailed Student’s $t$ test analysis. C, mice (three per group) were treated as described in Fig. 1. Seven days after the last viral injection, tumors were harvested, and cells were incubated with medium alone, the H2-Kd–restricted SIINFEKL, or the H2-Kd–restricted RGYVYQGL epitope from the VSV N protein to stimulate infiltrating cells. CD8+ T cells expressing intracellular IFN-γ recovered from these cultures. Points, means of at least two independent experiments.

**Figure 2.** CD8+ T cells specific for both viral- and tumor-associated antigens were induced by VSV virotherapy. A, up-regulation of MHC class II molecules after viral therapy. One day after the last viral infection (as described in Fig. 1), tumor-draining lymph nodes were harvested and analyzed for expression of MHC class II (Ia$^d$) by flow cytometry (B). S.c. B16ova tumors were treated as described in Fig. 1. Tumor samples were harvested at 1, 3, and 7 d following the last viral injection. The percentage of CD8+ T cells, relative to the total number of lymphocytes in the tumors, as assessed by flow cytometry. Statistics were done by using a two-tailed Student’s $t$ test analysis. C, mice (three per group) were treated as described in Fig. 1. Seven days after the last viral injection, tumors were harvested, and cells were incubated with medium alone, the H2-Kd–restricted SIINFEKL, or the H2-Kd–restricted RGYVYQGL epitope from the VSV N protein to stimulate infiltrating cells. CD8+ T cells expressing intracellular IFN-γ recovered from these cultures. Points, means of at least two independent experiments.
observations that anti-ova responses require longer time periods to become detectable (see Fig. 2C). In addition, the levels of virus which could be recovered from tumors in mice depleted of Treg were consistently lower than those from tumors from nondepleted mice (Fig. 3D). Interestingly, this was not attributable to increased levels of neutralizing antibodies to the virus as these remained unchanged between depleted and nondepleted mice (data not shown). Moreover, CD8+ T cells accumulated more rapidly (1 day after the last viral injection) at the tumor site in PC61-depleted mice as compared with control mice (P = 0.0027; data not shown). Taken together, these results show that Treg-mediated suppression is predominately active at the level of the antiviral, rather than the antitumor T cell response, and that by removing these suppressive mechanisms, the efficacy of virotherapy is not improved.

Combination of adoptive T cell therapy with virotherapy. Because tumors undergoing virotherapy with VSV contain higher numbers of CD8+ T cells than control injected tumors, we hypothesized that virotherapy may synergize with adoptive transfer of activated antigen-specific cells. The OT-I mouse strain is on a C57BL background (H2-Kb) and expresses a transgenic T cell receptor Vα2 specific for the SIINFEKL peptide in the context of MHC class I H2-Kb (29). A single i.v. injection of activated OT-I T cells, between the two intratumoral virus treatments, significantly enhanced the efficacy of either virotherapy (P = 0.014) or adoptive T cell therapy (P = 0.0007) alone (Fig. 4A). To understand the mechanism of this combinatorial effect, we measured the trafficking of the adoptively transferred OT-I T cells to the tumor site. Surprisingly, we were unable to detect any reproducible, significant difference in the numbers of OT-I cells reaching tumors injected with PBS, heat-inactivated virus, or VSV (Fig. 4B). Thus, our initial hypothesis that the inflammatory milieu created by ongoing tumor destruction by VSV would enhance T cell recruitment to tumors was not correct. However, the OT-I T cells in tumors injected with VSV were significantly more active, as measured by IFN-γ production, than were OT-I T cells in tumors injected with either PBS (P = 0.03) or heat-inactivated virus (P = 0.04; Fig. 4C). Therefore, the enhanced therapy seen with the combination of virotherapy and adoptive T cell therapy can be explained, at least in part, by enhanced IFN-γ production by the

---

**Figure 3.** Treg depletion inhibits virotherapy with VSV. A, mice (10 per group) were injected i.p. with 0.5 mg of PC61, or control antibody, 4 d after tumor implantation and 3 d before initiation of viral therapy as in Fig. 1. Tumor sizes were measured every other day and survival was plotted using a Kaplan-Meier survival curve. B, tumor-draining lymph nodes were harvested from three additional mice per group 1 d after the last viral injection, and cells were analyzed by flow cytometry for CD4 and CD25. C, three additional mice per group were treated as described in (A). Draining lymph nodes were harvested 1 day after the last viral injection, pulsed with the indicated peptides, and the supernatants were analyzed for IFN-γ by ELISA. D, tumors from mice treated as in (A) above were harvested (three mice per group) 1 d after the last viral injection, weighed and homogenized, and used in a standard plaque assay to measure viral titers.
T cells that reach the tumor, rather than by an increased number of T cells trafficking to the tumor site.

All four mice cured of their B16ova tumors by the combination of VSV virotherapy and OT-1 adoptive T cell therapy in Fig. 4A survived rechallenge with a lethal dose of B16ova cells 53 days following the first initial viral injection, indicating the generation of long-term immunologic memory. Consistent with these findings, mice cured long term (92 days) of B16ova tumors by combination intratumoral VSV/adoptive OT-1 therapy contained significantly higher frequencies of active ova-specific T cells by ELISPOT analysis than did the single mouse cured of its tumor by VSV alone (Fig. 4D). Of particular interest, all of these mice now contained higher frequencies of T cells specific for the tumor-associated antigen than for the VSV-encoded viral antigen. This situation represents a reversal of the situation following treatment with the virus in the absence of adoptive T cell therapy, in which the frequency of nucleoprotein-reactive T cells was greater than that of ova-reactive T cells (Fig. 2C).

Virus encoding a tumor antigen potently activates T cell responses. We hypothesized that it would be possible to exploit the potent antiviral T cell response by engineering the oncolytic virus to express an antigen which is not unique to the virus but is also shared by the tumor. Therefore, we used a virus expressing the ovalbumin protein. We confirmed that VSV-ova is both equally cytopathic to, and supports similar levels of viral replication in, B16ova cells in vitro as compared with the parental VSV (data not shown). To test the hypothesis that the VSV-ova virus would activate naïve T cells against the target ova antigen better than the parental VSV, we adoptively transferred naïve OT-I T cells into mice bearing B16ova tumors treated intratumorally with VSV viruses. OT-I cells proliferated in all treated groups, as evidenced by the dilution of CFSE, indicating that the ova antigen could access the lymph nodes from B16ova tumors (Fig. 5A). However, mice treated with intratumoral VSV-ova showed maximal levels of in vivo proliferation of the adoptively transferred OT-I cells (Fig. 5A). Only intratumoral injection of replication-competent viruses (VSV-GFP and VSV-ova) could activate adoptively transferred OT-I T cells, as measured by IFN-γ release (Fig. 5B). However, intratumoral VSV-ova activated the adoptively transferred OT-I significantly more than VSV-GFP in both the tumor-draining lymph nodes
(Fig. 5B) and in the treated tumors (Fig. 5C). Moreover, we were able to detect activated OT-I T cells in the contralateral lymph nodes only after intratumoral injection of VSV-ova (data not shown). Taken together, these data show that the expression of an antigen within the VSV virus that is shared with the tumor greatly enhances the activation and migration of naive T cells against that antigen compared with when the antigen is expressed solely by the tumor cells.

The ability of VSV-ova to prime naive T cell responses to the tumor-associated ova antigen translated into increased therapy of B16ova tumors compared with virotherapy with VSV alone \((P = 0.0009; \text{Fig. 6A})\). In addition, ELISPOT analysis of splenocytes from treated mice confirmed that although intratumoral VSV-ova activated similar numbers of antiviral-specific T cells, significantly more endogenous SIINFEKL-specific T cells were generated by VSV-ova compared with VSV-GFP \((P < 0.001; \text{Fig. 6B})\).

**Discussion**

Understanding the immunogenic effects of virotherapy will be important in manipulating the *in vivo* interactions between viral replication, immune control of viral spread, and immune control of tumor growth in favor of increased tumor clearance. Here, we monitored the generation of T cell responses against only a single viral antigen and a single tumor antigen. Given the large number of potentially highly immunogenic dominant, and subdominant, viral epitopes throughout the VSV genome, the antiviral T cell immune response is likely to predominate considerably over the antitumor T cell immune response. We showed that host CD8+ and NK cells are critical for the therapy of intratumoral viretreatment with VSV. This finding confirms that simple viral replication and direct tumor cell killing cannot solely explain the therapeutic efficacy of intratumoral virotherapy in an immunocompetent host. Indeed, the dependence on an intact CD8+ T and NK cell compartment for therapy with VSV implies that studies of oncolytic virotherapy, developed in immunodeficient hosts, may not be measuring the factors that will truly determine efficacy in human patients. Because we observed antigen-specific T cell responses against both viral- and tumor-associated antigens, it may be that the CD8+ therapy results from direct T cell–mediated killing of tumor cells expressing either, or both, of these target antigens. Alternatively, it may be that therapy is contributed by nonspecific effects in which activated T cells (in which the specificity may not be important) secrete cytokines, such as IFN-γ, at the tumor site leading to enhanced tumor cell killing. Experiments in mice genetically deficient for T cell effector cytokines will help to resolve these questions.

Given our results that the efficacy of VSV virotherapy depends on CD8+ T cells, we investigated methods by which the frequency/activity of activated T cells (particularly, although not necessarily exclusively) against the tumor-associated antigen might be increased at the tumor site undergoing active virotherapy with VSV.

In the first strategy, we investigated how the depletion of Treg would affect virotherapy. Our data shows that Treg-mediated...
treatment with either VSV-GFP (see Fig. 1) or with VSV-ova (50 μL in PBS) for a total of three injections. Tumor sizes were measured every other day and survival with time since tumor seeding (9). Our data shows that the combination of adoptive T cell therapy with intratumoral virotherapy leads to significantly more tumor cures than either treatment alone. However, apparently, the mechanism of action of this combinatorial therapy was not through increased trafficking of the OT-I T cells to the tumor site, as we had first hypothesized. In contrast, intratumoral virotherapy with VSV acts as an effective stimulus to enhance the activity of adoptively transferred, tumor antigen–specific T cells. One of the most significant differences between the combination of adoptive T cell therapy with VSV intratumoral virotherapy, compared to treatment with VSV, was that the addition of adoptive T cell transfer promoted the survival and/or differentiation of memory effector T cells specific for a tumor-expressed antigen over long periods of time in vivo. Whether these ova-specific memory T cells were derived from the input OT-I T cells, or from endogenously activated ova-specific T cells, is unclear, and will be resolved using adoptive transfer of OT-I T cells into congenic Thy1.1/Tyhi.12 strains of C57Bl/6 mice. Whatever the mechanism turns out to be, the combination of VSV and adoptively transferred T cells leads to the long-term population of the tumor-cured animals with T cells specific for tumor antigens at greater frequencies than for the viral antigens. This repopulation of cured animals is sufficient to confer long-term memory against tumor rechallenge, suggesting that this may be an effective treatment protocol to treat not only local, viral-injected tumors, but also distant or recurring tumors. Studies to investigate the ability of the combination of adoptive therapy with virotherapy to enhance the treatment of metastatic disease, including tumors that have not been injected with virus, are currently under way.

We also observed that the T cell response to virally encoded antigens predominates over the response to an antigen of similar potential immunogenicity expressed only within the tumor cells. Therefore, we hypothesized that it would be possible to exploit the potent antiviral T cell response by using an oncolytic virus expressing an antigen which is also shared by the tumor. In this way, the potent antiviral response could also be harnessed, at least in part, to become an antitumor response. We showed that expression of the ova antigen within the VSV greatly enhances the activation and migration of naïve T cells against that antigen compared with when the antigen is expressed solely by the tumor cells. Moreover, intratumoral therapy with VSV-ova was significantly more effective than with VSV, an effect that correlated with significantly elevated frequencies of ova-reactive T cells. It may be that these effects are due to the increased quantity of ova antigen, or a better quality of the presentation of the ova antigen, when it is expressed solely within the tumor cells. Therefore, we hypothesized that it would be possible to exploit the potent antiviral T cell response by using an oncolytic virus expressing an antigen which is also shared by the tumor. In this way, the potent antiviral response could also be harnessed, at least in part, to become an antitumor response. We showed that expression of the ova antigen within the VSV greatly enhances the activation and migration of naïve T cells against that antigen compared with when the antigen is expressed solely by the tumor cells. Moreover, intratumoral therapy with VSV-ova was significantly more effective than with VSV, an effect that correlated with significantly elevated frequencies of ova-reactive T cells. It may be that these effects are due to the increased quantity of ova antigen, or a better quality of the presentation of the ova antigen, when it is included in the VSV virus compared with when it is encoded solely within the tumor cells. Certainly, the levels of antigen available can influence the balance between tolerogenic or stimulatory presentation by antigen-presenting cells (36, 37). However, in the mice in which VSV intratumoral injection cures those tumors, we would expect high concentrations of ova antigen to be released and available for cross-priming of T cells, although we still did not see the elevated levels of activated T cells that were observed using VSV-ova. It seems more likely that the pathways of suppression is predominantly active at the level of the antiviral, rather than the antitumor, T cell response. By removing this suppression in vivo, antiviral responses were enhanced, leading to earlier clearance of virus from tumors and lower levels of virus-mediated cytoxicity. Similar results were reported in a model of acute herpes simplex virus infection in which mice depleted of Treg before infection showed 2- to 3-fold elevations of virus-specific CD8+ and CD4+ T cell responses (32). However, our results contrast somewhat with other reports in which depletion of Treg using low-dose cyclophosphamide increased the efficiency of virotherapy, although cyclophosphamide has multiple other effects on the antiviral immune response in addition to Treg depletion (34, 35). There are multiple possible reasons for these differences. In particular, the mechanisms of action and specificities of different subsets of Treg are still under investigation, and which subsets are depleted by relatively crude drug- or antibody-based regimens remains to be clarified. Nonetheless, by inference, our results suggest that strategies designed to enhance Treg activity, especially against antiviral T cell responses, in the context of ongoing virotherapy may prove beneficial to antitumor efficacy.

Because we had already observed that tumors undergoing virotherapy with VSV contained higher numbers of (endogenous) CD8+ T cells than control-injected tumors, we hypothesized that virotherapy may synergize with adoptive transfer of activated antigen-specific cells by providing a local environment in which inflammatory tumor cell killing may act as a potent attractant for the adoptively transferred T cells (9). Our data shows that the combination of adoptive T cell therapy with intratumoral virotherapy leads to significantly more tumor cures than either treatment alone. However, apparently, the mechanism of action of this combinatorial therapy was not through increased trafficking of the OT-I T cells to the tumor site, as we had first hypothesized. In contrast, intratumoral virotherapy with VSV acts as an effective stimulus to enhance the activity of adoptively transferred, tumor antigen–specific T cells. One of the most significant differences between the combination of adoptive T cell therapy with VSV intratumoral virotherapy, compared to treatment with VSV, was that the addition of adoptive T cell transfer promoted the survival and/or differentiation of memory effector T cells specific for a tumor-expressed antigen over long periods of time in vivo. Whether these ova-specific memory T cells were derived from the input OT-I T cells, or from endogenously activated ova-specific T cells, is unclear, and will be resolved using adoptive transfer of OT-I T cells into congenic Thy1.1/Tyhi.12 strains of C57Bl/6 mice. Whatever the mechanism turns out to be, the combination of VSV and adoptively transferred T cells leads to the long-term population of the tumor-cured animals with T cells specific for tumor antigens at greater frequencies than for the viral antigens. This repopulation of cured animals is sufficient to confer long-term memory against tumor rechallenge, suggesting that this may be an effective treatment protocol to treat not only local, viral-injected tumors, but also distant or recurring tumors. Studies to investigate the ability of the combination of adoptive therapy with virotherapy to enhance the treatment of metastatic disease, including tumors that have not been injected with virus, are currently under way.

We also observed that the T cell response to virally encoded antigens predominates over the response to an antigen of similar potential immunogenicity expressed only within the tumor cells. Therefore, we hypothesized that it would be possible to exploit the potent antiviral T cell response by using an oncolytic virus expressing an antigen which is also shared by the tumor. In this way, the potent antiviral response could also be harnessed, at least in part, to become an antitumor response. We showed that expression of the ova antigen within the VSV greatly enhances the activation and migration of naïve T cells against that antigen compared with when the antigen is expressed solely by the tumor cells. Moreover, intratumoral therapy with VSV-ova was significantly more effective than with VSV, an effect that correlated with significantly elevated frequencies of ova-reactive T cells. It may be that these effects are due to the increased quantity of ova antigen, or a better quality of the presentation of the ova antigen, when it is included in the VSV virus compared with when it is encoded solely within the tumor cells. Certainly, the levels of antigen available can influence the balance between tolerogenic or stimulatory presentation by antigen-presenting cells (36, 37). However, in the mice in which VSV intratumoral injection cures those tumors, we would expect high concentrations of ova antigen to be released and available for cross-priming of T cells, although we still did not see the elevated levels of activated T cells that were observed using VSV-ova. It seems more likely that the pathways of

Figure 6. Virotherapy with a viral-encoded tumor antigen. A, C57BL/6 mice (eight per group) bearing 7-d s.c. tumors were injected intratumorally every 2 d with 5 × 10^6 pfu of heat-inactivated virus (HI-VSV), VSV, or VSV-ova (50 μL in PBS) for a total of three injections. Tumor sizes were measured every other day and survival with time since tumor seeding (bottom). B, splenocytes were recovered and pulsed with ova-derived SIINFEKL, VSV-derived RGYVYQGL, or with B16-derived epitopes from the TRP-2 (TRP-2,150–158) SVYDFFVWL or gp100 (hgp100a,33–35, KVPRNQDWSL) peptides. IFN-γ production from these splenocytes was measured 48 h later by ELISPOT.
antigen presentation of viral-associated antigens in infected cells enhances the immune response to such antigens compared with the response induced to endogenously expressed cellular antigens from the same infected cells.

In summary, we have shown here that viral oncolysis leads to the priming of T cell responses against both viral- and tumor-associated antigens. Moreover, these CD8+ T cell responses are critical for the therapy of viral oncolysis in the immunocompetent host. Whereas depletion of Treg inhibits virotherapy by allowing greater viral clearance, the combination of adoptive T cell therapy with intratumoral virotherapy lead to significantly enhanced cures and repopulation of the host with long-term effector memory T cells. Finally, encoding a tumor antigen within the virus allows the potent antiviral T cell response to be harnessed to become, at least in part, an antitumor T cell response. Therefore, we believe that the future design of combination therapies involving oncolytic viruses should take into account the therapeutic potential of augmenting their ability to serve as both dual cytotoxic and immunotherapeutic agents for the treatment of established tumors. Studies to determine the effects of viral oncolysis on breaking tolerance to self-tumor antigens are currently under way.

Acknowledgments

Received 10/26/2006; revised 12/12/2006; accepted 1/10/2007.

Grant support: Mayo Foundation and NIH grants ROI CA085931, 1ROI CA04180, and 1ROCA107082.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors thank Toni L. Higgins for expert secretarial assistance and Gisella Caceres for experimental assistance.

References

11. Yewdell JW, Haeryfar SMM. Understanding presenta-
13. Fernando M, Porosnicu M, Markovic D, Barber GN.
Oncolytic Immunovirotherapy for Melanoma Using Vesicular Stomatitis Virus

Rosa Maria Diaz, Feorillo Galivo, Timothy Kottke, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/6/2840

Cited articles
This article cites 35 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/6/2840.full.html#ref-list-1

Citing articles
This article has been cited by 30 HighWire-hosted articles. Access the articles at:
/content/67/6/2840.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.