

Systemic Combination Virotherapy for Melanoma with Tumor Antigen-Expressing Vesicular Stomatitis Virus and Adoptive T-cell Transfer

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Abstract

Oncolytic virotherapy offers the potential to treat tumors both as a single agent and in combination with traditional modalities such as chemotherapy and radiotherapy. Here we describe an effective, fully systemic treatment regimen, which combines virotherapy, acting essentially as an adjuvant immunotherapy, with adoptive cell transfer (ACT). The combination of ACT with systemic administration of a vesicular stomatitis virus (VSV) engineered to express the endogenous melanocyte antigen glycoprotein 100 (gp100) resulted in regression of established melanomas and generation of antitumor immunity. Tumor response was associated with *in vivo* T-cell persistence and activation as well as treatment-related vitiligo. However, in a proportion of treated mice, initial tumor regressions were followed by recurrences. Therapy was further enhanced by targeting an additional tumor antigen with the VSV-antigen + ACT combination strategy, leading to sustained response in 100% of mice. Together, our findings suggest that systemic virotherapy combined with antigen-expressing VSV could be used to support and enhance clinical immunotherapy protocols with adoptive T-cell transfer, which are already used in the clinic. *Cancer Res*; 72(18); 4753–64. ©2012 AACR.

Introduction

Oncolytic virotherapy is based on the concept that even low levels of a replication competent virus introduced into a tumor will result in rapid spread through, and lysis of, the tumor, with tumor-selective viral replication being possible through natural, or engineered, selectivity (1–4). Although preclinical, as well as several clinical, studies have shown highly encouraging results (1, 3, 5–7), virotherapy is still being developed for routine clinical use. Moreover, it is clear that there is also considerable potential value in the use of oncolytic viruses in combination with more conventional treatment modalities leading to enhancement of efficacy of each of the respective agents alone (8–14). In contrast, adoptive T-cell therapy is more well established in clinical use. Despite the technical challenges associated with isolation of T cells specific for tumor-associated antigens (TAA) from patients, their subsequent transfer has shown considerable efficacy against several types of cancer, especially when used in combination with regimens often associated

with conditioning of the patient for improved T-cell survival, persistence, and activation (15, 16).

We and others have shown that adoptive cell transfer (ACT; of both antigen-specific T cells and other cell types) can be used to enhance the delivery of oncolytic viruses, especially as a method to protect the viruses from neutralization in the circulation (17–20). There is also considerable potential to combine virotherapy with ACT as a means to improve the immunotherapy component of either, or both, modalities (21, 22). In this respect, we, and others, have shown that the negative strand, enveloped, RNA virus vesicular stomatitis virus (VSV) is a potent cytolytic agent *in vitro* against a wide range of tumor types (23–25). However, we have observed that, in immunocompetent, C57BL/6 mice bearing B16ova tumors, therapy mediated by intratumoral VSV is primarily mediated by the innate immune response to viral infection and does not require viral replication for tumor control (24, 26–28). On the basis of these studies, we hypothesized that it would be possible to exploit this potent immunogenicity of VSV as an adjuvant to improve the efficacy of ACT. Thus, we showed that intratumoral administration of VSV encoding a model TAA (OVA) led to the activation of adoptively transferred, ova-specific OT-I T cells. Similarly, when adoptive transfer of Pmel T cells, specific for the endogenous melanocyte differentiation antigen gp100 (against which full tolerance is in place in C57BL/6 mice) was combined with intratumoral treatment with VSV-hgp100, we observed very effective control of the local tumors that were directly injected with virus. We also observed, more significantly, effective treatment of distant disease that was not treated directly by virus injection (22), suggesting that

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VSV-hgp100 may act to enhance the antitumor efficacy of Pmel T cells independent of any directly oncolytic, and/or local immune stimulating, activities of the virus.

In light of these results (22), we proceeded toward our long-term goal of developing a systemic treatment in which no tumor has to be directly accessed for viral injections. Here we show that a combination of intravenous injections of Pmel T cells, followed by VSV-hgp100, resulted in regression of established B16 tumors and cures in a large proportion of mice. However, in some mice, regression was followed by recurrence, mimicking a common observation in patients. By targeting 2 TAAs simultaneously with the combination of VSV-TAA + ACT, we observed regression of tumors in all of the treated mice with long-term, recurrence-free survival over 100 days. Therefore, our data suggest that systemic virotherapy with antigen-expressing VSV could be used to support, and enhance, clinically useful protocols of immunotherapy with ACT.

Materials and Methods

Cell lines

B16(LIF) and B16ova melanoma cells (H2-K^b) (29) were grown in Dulbecco's Modified Eagle's Medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies), and L-glutamine (Life Technologies). B16ova cells were derived from a separate stock of B16 cells transduced with a cDNA encoding the chicken ovalbumin gene and were maintained in 5 mg/mL G418 (Mediatech) to retain the *ova* gene. All cell lines were free of *Mycoplasma* infection.

Mice

C57BL/6 mice (Thy 1.2⁺) were purchased from The Jackson Laboratory at 6 to 8 weeks of age. The OT-I mouse strain expresses a transgenic T-cell receptor, V α 2, specific for the SIINFEKL peptide of ovalbumin in the context of MHC class I, H-2K^b (30). OT-I breeding pairs were obtained as a gift from Dr. Larry Pease, Mayo Clinic. Pmel-1 transgenic mice express the V α 1/V β 13 T-cell receptor that recognizes amino acids 25 to 33 of gp100 (Pmel-17) presented by H2-D^b MHC class I (21). Pmel-1 breeding colonies were purchased from The Jackson Laboratory at 6 to 8 weeks of age.

Viruses

VSV-GFP and VSV-ova (Indiana serotype) were generated by cloning the cDNA for GFP or chicken ovalbumin, respectively, into the plasmid pVSV-XN2 as described previously (31). Plasmid pVSV-hgp100 was constructed by PCR amplifying the human gp100 cDNA, which was prepared from Mel-888 cells using forward (5'-ATCTCGAGATGGATCTGGTGCTAAAAA-GATGC-3') and reverse (5'-ATGCTAGCTCAGACCTGCTGCCCACT-3') primers. The gp100 PCR product was then digested and inserted into the *Xho*I and *Nhe*I sites of the VSV-XN2 vector (a gift from Dr. John Rose, Yale University, New Haven, CT). Recombinant VSV-hgp100 was recovered based on the method previously described (32, 33). Bulk amplification of plaque-purified VSV was carried out by infecting BHK-21 cells (multiplicity of infection = 0.01) for 24 hours. Filtered supernatants were subjected to 2 rounds of 10% sucrose (10% w/v) (Mediatech) cushion centrifugation at 27,000 r.p.m. for 1 hour at 4°C.

Viral titers were measured by standard plaque assay on BHK-21 cells (28, 31).

Adoptive transfer and tumor treatment

All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. To establish subcutaneous tumors, 5×10^5 B16 tumor cells in 100 μ L of PBS were injected into the flanks of C57BL/6 mice. Following tumor establishment, virus or PBS control (100 μ L) was administered intravenously.

Naive T cells were isolated from the spleens and lymph nodes of euthanized OT-I and Pmel-1 transgenic mice. Single cell suspensions were prepared by crushing tissues through a 100 μ m filter and red blood cells were removed by incubation in ACK buffer (distilled H₂O containing 0.15 mol/L NH₄Cl, 1.0 mmol/L KHCO₃, and 0.1 mmol/L EDTA adjusted to pH 7.2–7.4). CD8⁺ T cells were isolated using the MACS CD8a (Ly-2) microbead magnetic cell sorting system (Miltenyi Biotec). For adoptive transfer experiments, mice were intravenously administered naive Pmel, OT-I T cells or a combination of both following tumor establishment (1×10^6 total cells in 100 μ L PBS). Mice were examined daily for overall health and signs of autoimmunity. Tumor sizes were measured 3 times weekly using calipers and volume was calculated as $0.52 \times \text{width}^2 \times \text{length}$ (34). Mice were euthanized when tumor size was approximately 1.0×1.0 cm in 2 perpendicular directions.

Immune cell depletions were carried out by intraperitoneal injections of antibody to CD8 (0.1 mg/mouse; Lyt 2.43; BioXcell), CD4 (0.1 mg/mouse; GK1.5; BioXcell), Gr1 (0.2 mg/mouse; RB6-8C5; BioXcell), antibody to deplete natural killer (NK) cells (25 μ L/mouse; anti-asialo-GM-1; Cedarlane), or immunoglobulin G (IgG) control (0.2 mg/mouse; ChromPure Rat IgG; Jackson Immunoresearch) on days 3, 6, 10 posttumor cell implantation and then weekly until experiment completion. Fluorescence-activated cell sorting (FACS) analysis confirmed subset-specific depletions.

Reverse-transcriptase PCR

Tumors were excised from euthanized mice and dissociated to achieve single-cell suspensions. RNA was extracted using the Qiagen RNeasy Kit (Qiagen). cDNA was made from 1 μ g total RNA using the First Strand cDNA Synthesis Kit (Roche). A cDNA equivalent of 1 ng RNA was amplified by PCR with gene-specific primers. Expression of mouse GAPDH (*mgapdh*) was used as a positive control for both gp100 and OVA antigen expression. The following primers were used: OVA sense: CA-C AAGCAATGCCTTTTCAGA, OVA antisense: TACCACCTCTC-TGCCTGCTT, *mgp100* sense: CCAGCCATTGCTGCCACA, *mgp100* antisense: CCCGCTTGGCAGGACACAG, *mgapdh* sense: TCATGACCACAGTCCATGCC, *mgapdh* antisense: TC-AGCTCTGGGATGACCTTG.

Western blot analysis

Tumors were excised from euthanized mice and homogenized in Lamml's Lysis Buffer (distilled H₂O containing 125 mmol/L Tris, 2% SDS, 10% glycerol, pH 6.8). Proteins (50 μ g total) were separated by SDS-PAGE (12% Mini-PROTEAN TGX Gels; Bio-Rad) and transferred to 0.2 μ m nitrocellulose membranes

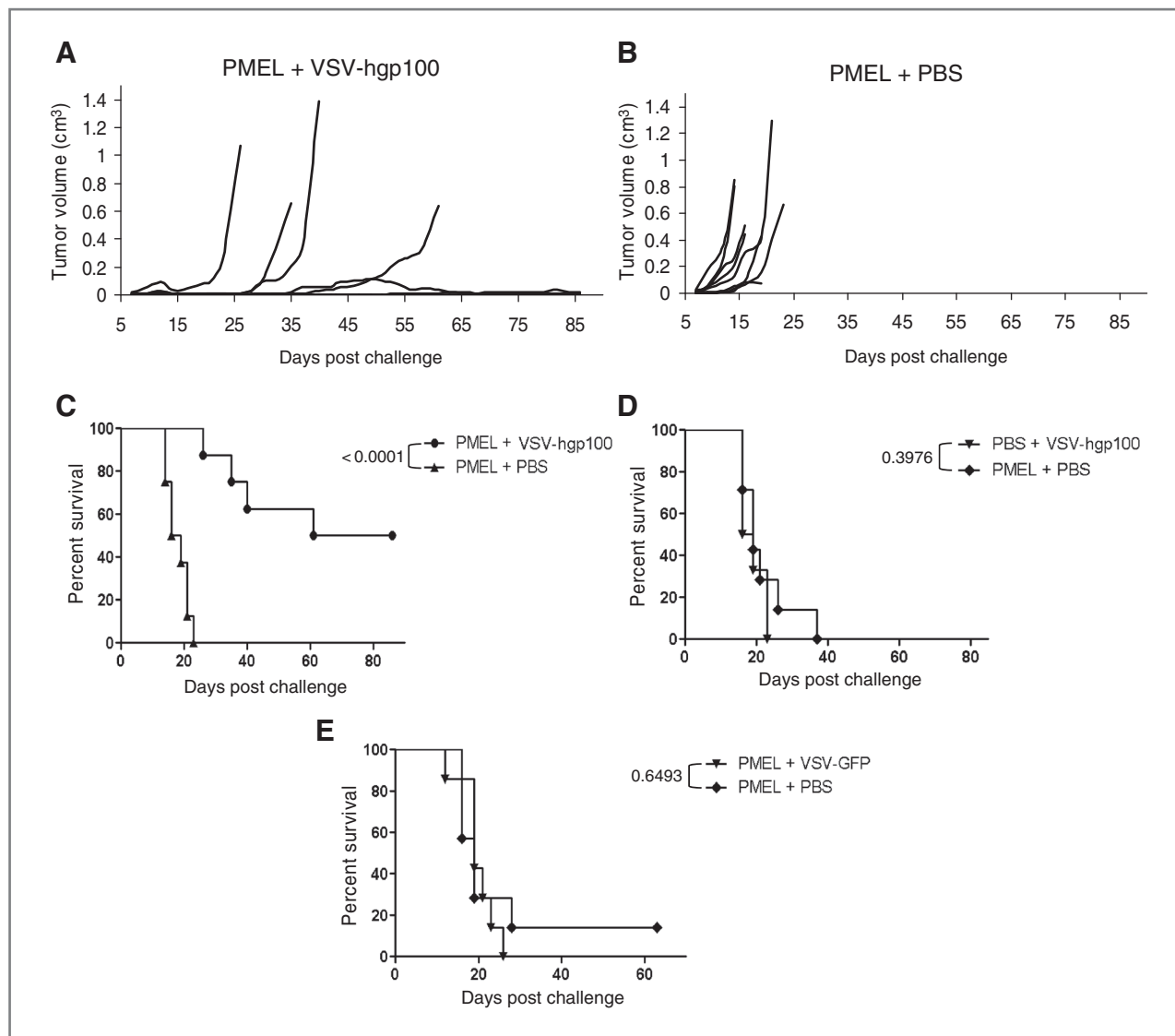


Figure 1. Adoptive transfer of Pmel T cells combined with systemic VSV-hgp100 has antitumor effects. Naive Pmel T cells (1×10^6 cells/100 μ L) or PBS (100 μ L) were adoptively transferred into C57BL/6 mice ($n = 7$) bearing subcutaneous B16ova tumors 0.2×0.2 cm in any diameter (day 5–7 posttumor cell implantation). Starting the next day, intravenous VSV-hgp100 (5×10^6 PFU/100 μ L), VSV-GFP (5×10^6 PFU/100 μ L), or PBS (100 μ L) was administered every other day for a total of 5 injections. An additional dose of T cells or PBS was given to surviving mice on day 20 followed by an intravenous injection of VSV or PBS on day 21. Growth of individual tumors (A, B) or overall survival (tumor less than 1.0 cm in any diameter; C–E) is shown.

(Bio-Rad). Membranes were incubated with the appropriate primary antibodies (Abcam) overnight at 4°C . Washed membranes were then incubated with horseradish peroxidase conjugated anti-rabbit (1:8000) or anti-donkey (1:2000) IgG (Abcam) for 1 hour. Finally, the membranes were coated with Pierce ECL Western Blotting Substrate (Pierce) and exposed.

Flow cytometry

Spleens, tumor-draining lymph nodes, and tumors were excised from euthanized mice and dissociated to achieve single-cell suspensions. Red blood cells were lysed as described above. Remaining cells were resuspended in PBS wash buffer containing 0.1% bovine serum albumin, and incubated with

directly conjugated primary antibodies for 30 minutes at 4°C . Cells were next washed and resuspended in 500 μ L PBS containing 4% formaldehyde. For intracellular IFN- γ staining, tumor cell suspensions were incubated for 4 hours with peptides of interest (5 μ g/mL) and Golgi Plug reagent (BD Biosciences). The samples were then fixed and stained using the Cytofix/Cytoperm kit from BD Biosciences according to the manufacturer's instructions. Flow cytometry data were analyzed using Flowjo software (Flowjo).

T-cell reactivation and IFN- γ ELISA

Spleens were excised from euthanized mice and dissociated to achieve single-cell suspensions. Red blood cells were lysed as

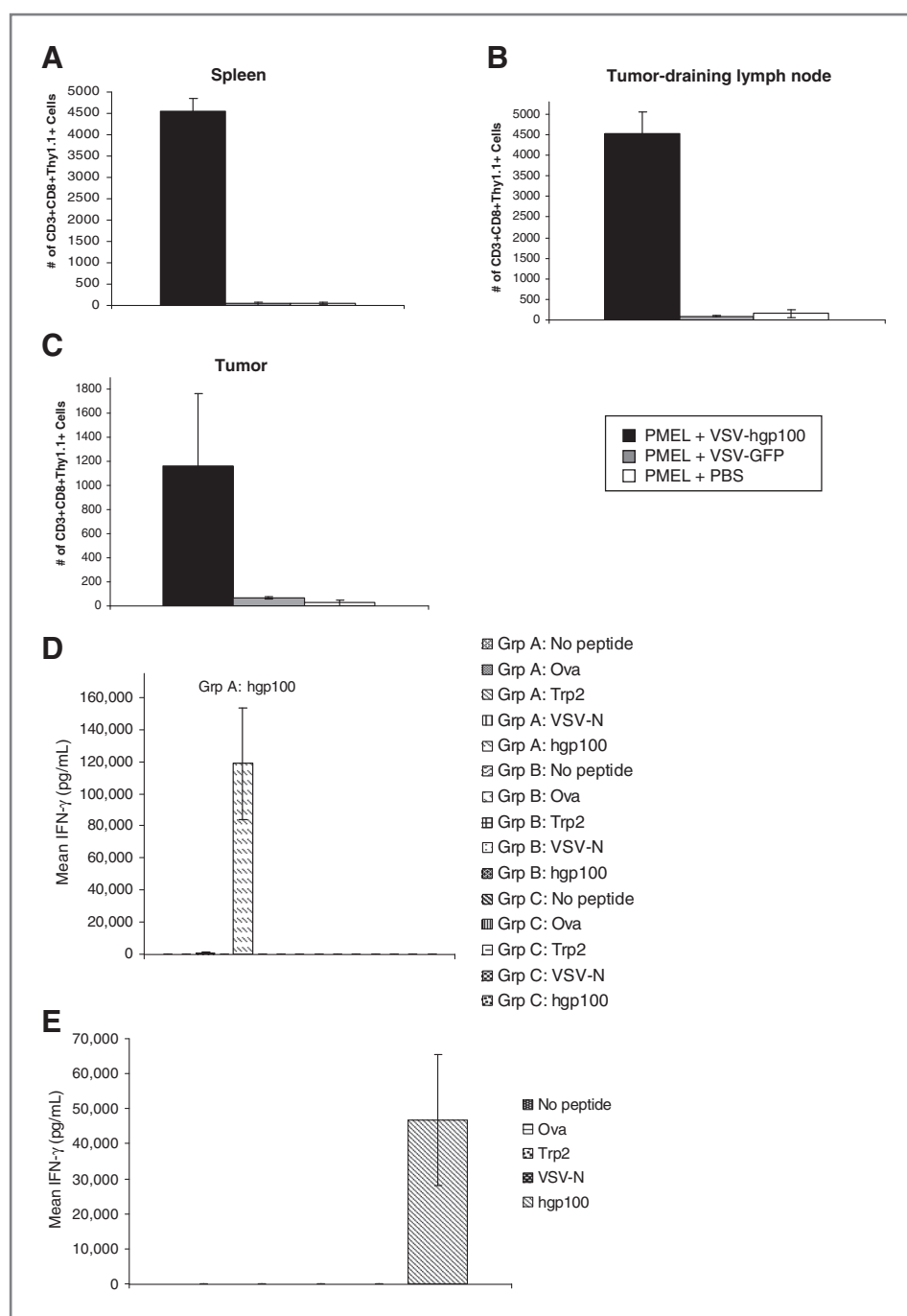


Figure 2. Combination VSV-hgp100 treatment results in accumulation and activation of Pmel T cells. Mice bearing subcutaneous tumors were treated as described in Fig. 1. Spleens, tumors, and TDLNs were harvested on day 12 ($n = 3$) and processed for FACS analysis (A–C) or T-cell reactivation assays (D, E). Number of CD3⁺CD8⁺Thy1.1⁺ Pmel T cells (per 100,000 sorted cells) in the spleens (A), TDLNs (B), or tumors (C) of treated mice. On the assumption that normal spleens and TDLNs of C57BL/6 mice contain 5×10^7 and 5×10^6 total cells, respectively, the total number of Pmel T cells recovered from each treatment can be estimated by multiplying the mean number of cells detected per 100,000 cells by either 500 (spleens) or 50 (TDLNs). D, antigen-specific, T-cell reactivation was assessed by pulsing splenocytes with no peptide (medium), ova, Trp2, VSV-N, or hgp100-specific peptides for 48 hours followed by IFN- γ ELISA. Group designations are as follows: Grp A = Pmel + VSV-hgp100, Grp B = Pmel + VSV-GFP, Grp C = Pmel + PBS. E, spleens were harvested from treated mice (as in A) that were tumor-free following the regimen (>60 days; $n = 8$). Splenocytes were pulsed with no peptide (medium), ova, Trp2, VSV-N, or hgp100-specific peptides for 48 hours followed by IFN- γ ELISA. Grp, group.

described above. Splenocytes were resuspended at 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (Gibco) + 5% FBS + 1% Pen-Strep + 40 μ mol/L 2-ME and pulsed with ova, hgp100, VSV-N, Trp2 specific peptides (2.5 μ g/mL) or medium for 48 hours. Supernatants were tested for IFN- γ production by ELISA as directed in the manufacturer's instructions (Mouse IFN- γ ELISA Kit, OptEIA; BD Biosciences).

The synthetic H-2D^b-restricted peptide hgp100_{25–33}: KVPR-NQDWL, and H-2K^b-restricted peptides OVA_{257–264}: SIINFEKL,

VSV-N_{52–59}: RGYVYQGL and Trp2_{180–188}: SVYDFVWL were synthesized at the Mayo Foundation Core Facility.

Statistics

Survival data from the animal studies were analyzed by the log-rank test using GraphPad Prism 5 (GraphPad Software). Two-sample, unequal variance Student t test analysis was applied for *in vitro* data. Statistical significance was determined at the level of $P < 0.05$.

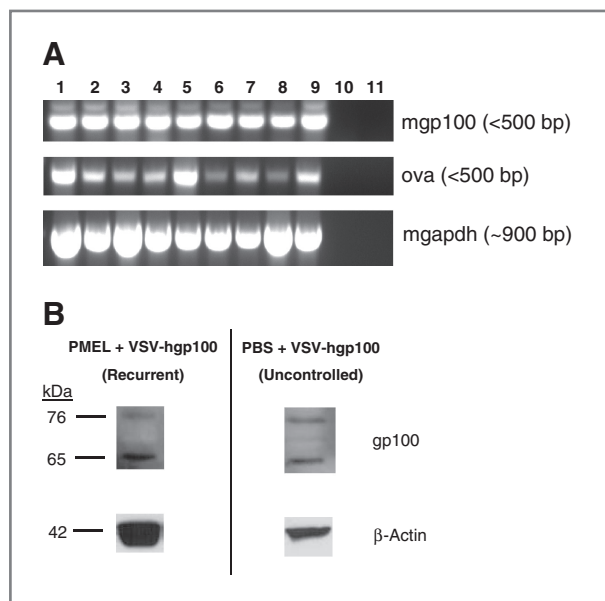


Figure 3. Loss of TAAs is not observed in recurrent tumors. **A**, tumor-bearing mice ($n = 7$) were treated as described in Fig. 1. When tumor size exceeded 1.0 cm in any diameter, the mice were sacrificed and their tumors excised ($n = 3$). RNA was isolated and tested for mgp100, ova, and mgapdh expression by reverse transcriptase PCR. Lane designations are as follows: 1–3 = Pmel + VSV-hgp100 treatment (recurrent tumors), 4–6 = PBS + VSV-hgp100 treatment (uncontrolled tumors), 7–9 = Pmel + PBS treatment (uncontrolled tumors), 10 = no cDNA control, 11 = no RNA control. **B**, mice were treated as in **A** with the exception that excised tumor tissue was homogenized and total protein was used for Western blot analysis. Protein samples were blotted with goat polyclonal anti-gp100 (1:250) or rabbit monoclonal anti- β -actin (1:1000) primary antibody. Shown blots are representative of all recurrent and uncontrolled tumor samples that were analyzed.

Results

Pmel T cells and systemic VSV-hgp100 mediate tumor regression

Treatment of mice bearing 5 to 7 days established B16ova tumors with adoptive transfer of Pmel T cells followed by systemic VSV-hgp100 led to initial tumor regression in most animals (Fig. 1A and B), with subsequent clearance and long-term survival of approximately 50% of mice ($P < 0.0001$; Fig. 1C). No antitumor effects were observed with Pmel T cells, or VSV-hgp100, alone (Fig. 1B–D) or with Pmel + VSV-GFP ($P = 0.6493$; Fig. 1E). Previously we have shown that the adoptive transfer of Pmel T cells alone have no therapeutic effect compared with PBS alone (22, 35). Therefore, in the current experiments, adoptive transfer of Pmel represent our negative control for tumor growth. Furthermore, increasing the dose of Pmel T cells by up to 1 log (10^7 cells per injection) did not increase therapy against B16ova tumors (not shown). The combination of Pmel + VSV-hgp100 was also significantly superior ($P < 0.0001$) to treatment with either Pmel or VSV-hgp100 alone against B16 (as opposed to B16ova) tumors (not shown).

Pmel T cells persist in mice treated with systemic VSV-hgp100

Consistent with the therapy observed in Fig. 1, treatment with Pmel T cells + VSV-hgp100 resulted in significant accumulation ($P < 0.0001$) of the adoptively transferred, Thy1.1⁺ Pmel T cells in spleens (Fig. 2A) and tumor-draining lymph nodes (TDLN; Fig. 2B) compared with mice treated with Pmel + VSV-GFP or Pmel T cells alone (Fig. 2A and B). Significant ($P < 0.05$) T-cell accumulation was also observed in the tumors of Pmel + VSV-hgp100 treated mice compared with controls (Fig. 2C; accumulation of adoptively transferred cells represents the situation wherever the total number of Pmel T cells detected in the spleens and TDLNs of mice, as corrected from the value per 100,000 cells sampled to the total number of cells per spleen/TDLN, exceeds that of the transfer of 1×10^6 cells per animal).

Spleens of mice treated with Pmel + VSV-hgp100 contained significantly elevated gp100-specific T-cell responses ($P < 0.0001$ compared with other treatment groups; Fig. 2D), presumably due to the persistence of the adoptively transferred cells. Furthermore, mice cured of tumors (>60 days) by the combination of Pmel + VSV-hgp100 retained a gp100-specific T-cell response (Fig. 2E). Interestingly, the gp100-specific response was the predominant T cell reactivity in these cured mice. We could detect ova-, and/or TRP-2-, specific T-cell responses in about one-third of mice cured of their tumors by Pmel + VSV-hgp100 treatment, at least 60 days posttreatment, although the responses were significantly less than the gp100-specific responses ($P < 0.0001$). The lack of consistency in the generation of this epitope spreading response, even within cured mice within the same experiment, makes its therapeutic significance unclear. It may also be possible that T-cell responses against tumor antigens other than those that we screened for are being raised in these mice, which contribute to the long-term tumor control.

Tumor recurrence is not associated with antigen loss

Although the majority of B16ova tumors in Pmel + VSV-hgp100 treated mice regressed to a point where they were barely palpable, some recurred and grew progressively, a situation that was not seen in control treated mice (Fig. 1A and B). However, in contrast to our experience using adoptive therapy with OT-I T cells to treat B16ova tumors (36), recurrent tumors from mice treated with Pmel + VSV-hgp100 retained expression of both gp100 and OVA antigens (Fig. 3A and B), suggesting that antigen loss was not the primary mechanism associated with tumor recurrence. However, despite continued expression of the target gp100 antigen at both RNA and protein levels, it may still be the case that recurrence was due to an inability of these tumors to successfully present the relevant epitopes of the protein due to loss of MHC, mutation of the epitopes, or other defects in antigen presentation.

Pmel + VSV-hgp100 therapy is dependent upon CD8⁺ and NK cells

Depletion of CD8⁺ and NK cells abrogated antitumor effects ($P = 0.0004$ and 0.0002, respectively; Fig. 4A and B), consistent with our previous studies (28), whereas depletion of CD4⁺ cells

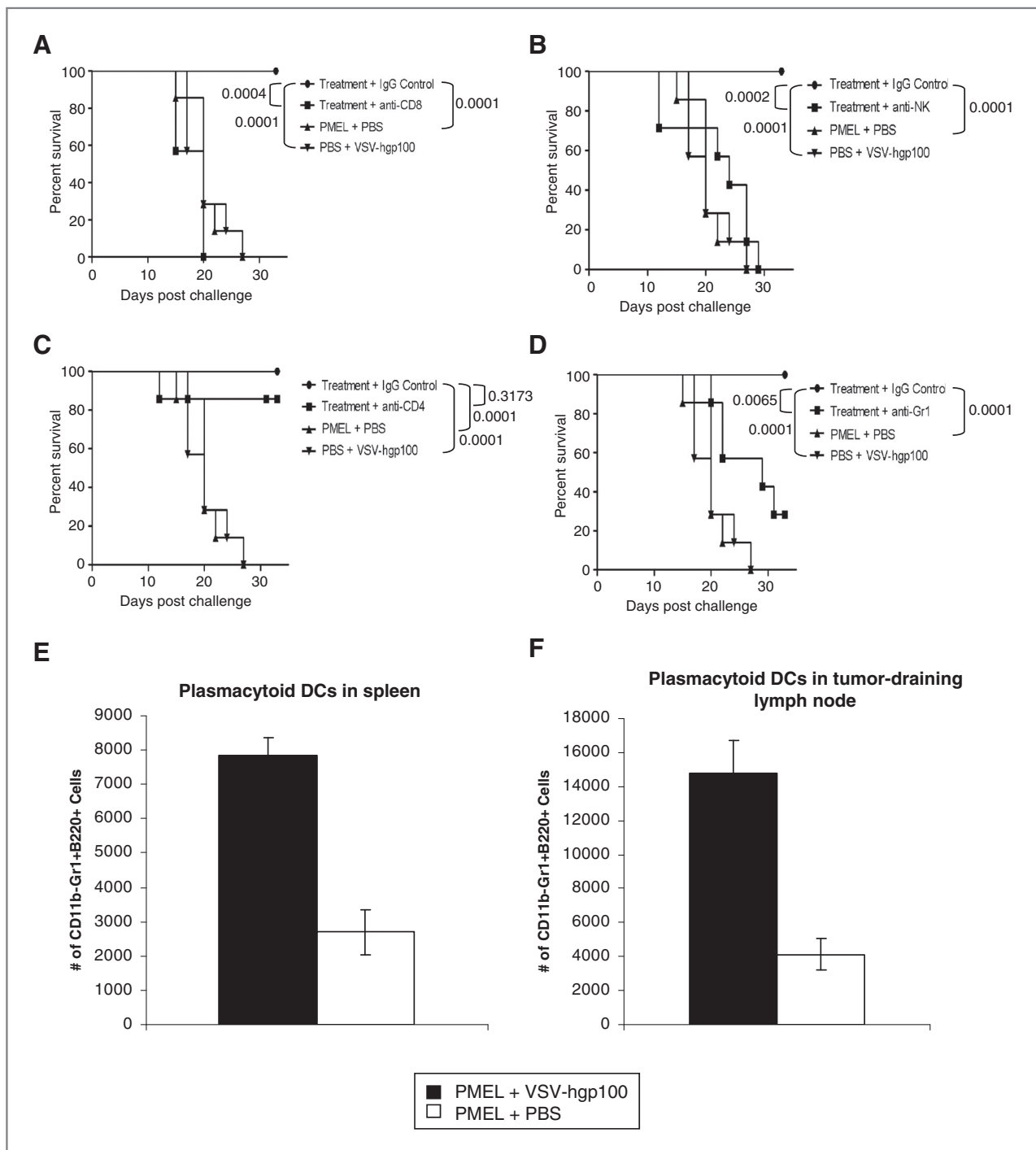


Figure 4. Influence of immune cell subsets on Pmel + VSV-hgp100 therapy. Mice ($n = 7$) bearing subcutaneous B16ova tumors were treated as described in Fig. 1 with the exception that mice were additionally administered CD8-depleting antibody (0.1 mg/mouse; A), NK-depleting antibody (25 μ L/mouse; B), Gr1-depleting antibody (0.2 mg/mouse; C), CD4-depleting antibody (0.1 mg/mouse; D), or IgG control antibody (0.2 mg/mouse; A-D) intraperitoneally starting on day 3 after tumor cell implantation. Overall survival (tumor less than 1.0 cm in any diameter) is shown. Experiments shown in A to D were run concurrently with the same control groups. E and F, spleens and TDNLs from combination treated mice were harvested on day 12 ($n = 3$) and processed for FACS analysis. Number of CD11b⁻Gr1⁺B220⁺ cells (per 100,000 sorted cells) is shown.

did not significantly change overall survival ($P = 0.3173$; Fig. 4C). Therapy was also significantly decreased following anti-Gr1 antibody treatment, compared with IgG control treated

mice ($P = 0.0065$; Fig. 4D), despite the fact that Gr1⁺ cell depletion was never complete in these experiments. Consistent with these depletion studies, the number of GR1⁺ pDCs in the

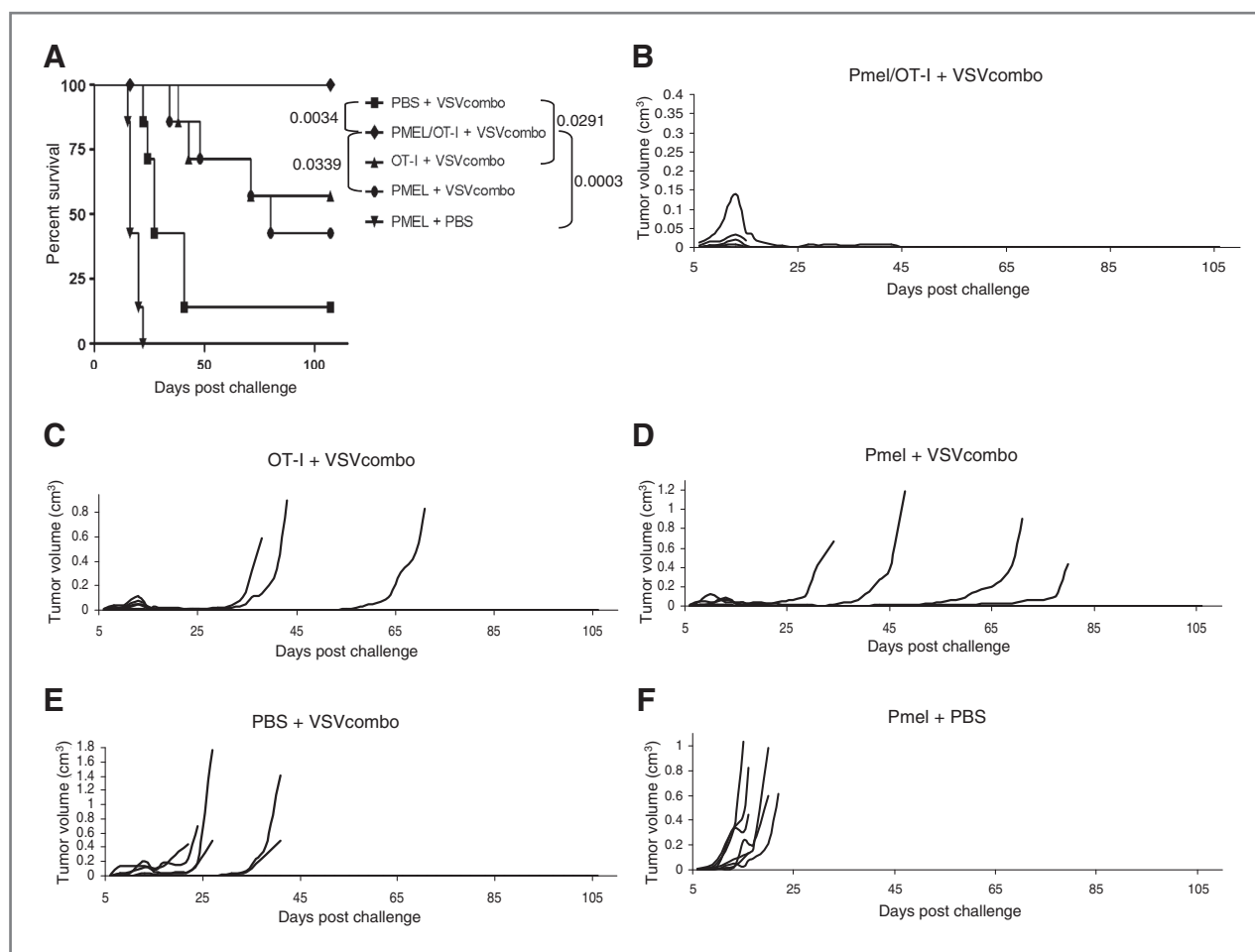


Figure 5. Therapy targeting more than one antigen enhances efficacy and inhibits tumor recurrence. C57BL/6 mice bearing subcutaneous B16ova tumors ($n = 7$) were treated with naive Pmel T cells (1×10^6 cells/100 μ L), naive OT-I T cells (1×10^6 cells/100 μ L), a combination of both naive Pmel and OT-I T cells (5×10^5 cells/100 μ L of each), or PBS (100 μ L) when tumors reached 0.2 \times 0.2 cm in any diameter (days 5–7 after tumor cell implantation). Five doses of combination VSV-hgp100 and VSV-ova (2.5×10^8 PFU/100 μ L each; labeled as VSVcombo) or PBS (100 μ L) were given intravenously every other day starting one day after adoptive T-cell transfer. On day 20, an additional dose of T cells or PBS was given followed by an intravenous injection of VSVcombo or PBS on day 21. Overall survival (tumor less than 1.0 cm in any diameter; A) or growth of individual tumors (B–F) is shown.

spleens and TDLNs of mice treated with Pmel + VSV-hgp100 were significantly elevated ($P < 0.001$) compared with mice receiving Pmel cells only (Fig. 4E and F). We also observed that the transferred Pmel T cells express GR1 (Supplementary Fig. S1), suggesting that the deleterious effects of GR1 depletion on therapy may also be attributable to partial depletion of the effector T-cell pool.

Targeting 2 TAAs enhances therapy

Because Pmel + VSV-hgp100 treatment led to some instances of recurrence (Fig. 1A), we hypothesized that applying additional immune-selective pressure against the tumor would enhance tumor clearance and prevent tumor regrowth. Consistent with this hypothesis, adoptive transfer of both OT-I and Pmel T cells, combined with systemic VSV-ova and VSV-hgp100 (VSVcombo), induced regression of established tumors in all treated mice ($P = 0.0034$ compared with VSVcombo alone, $P = 0.0003$ compared with Pmel

T cells alone; Fig. 5A) with no recurrence observed up to 105 days posttumor challenge (Fig. 5B). Treatment with either OT-I + VSVcombo or Pmel + VSVcombo resulted in trends toward survival benefit compared with control regimens, although significance ($P < 0.05$) was not reproducible (Fig. 5A). In addition, recurrence was observed in a proportion of mice treated with OT-I + VSVcombo and Pmel + VSVcombo therapies (Fig. 5C and D). Neither VSVcombo, nor Pmel T cells, alone had significant therapy against B16ova tumors (Fig. 5A, E, and F).

T-cell responses following Pmel/OT-I + VSVcombo treatment

Enhanced accumulation of both Pmel and OT-I T cells was observed in spleens, TDLNs, and tumors of mice treated with Pmel/OT-I + VSVcombo compared with control groups (P at least < 0.05 ; Fig. 6A–C). Significant gp100-specific, splenic T-cell responses ($P < 0.001$) were present

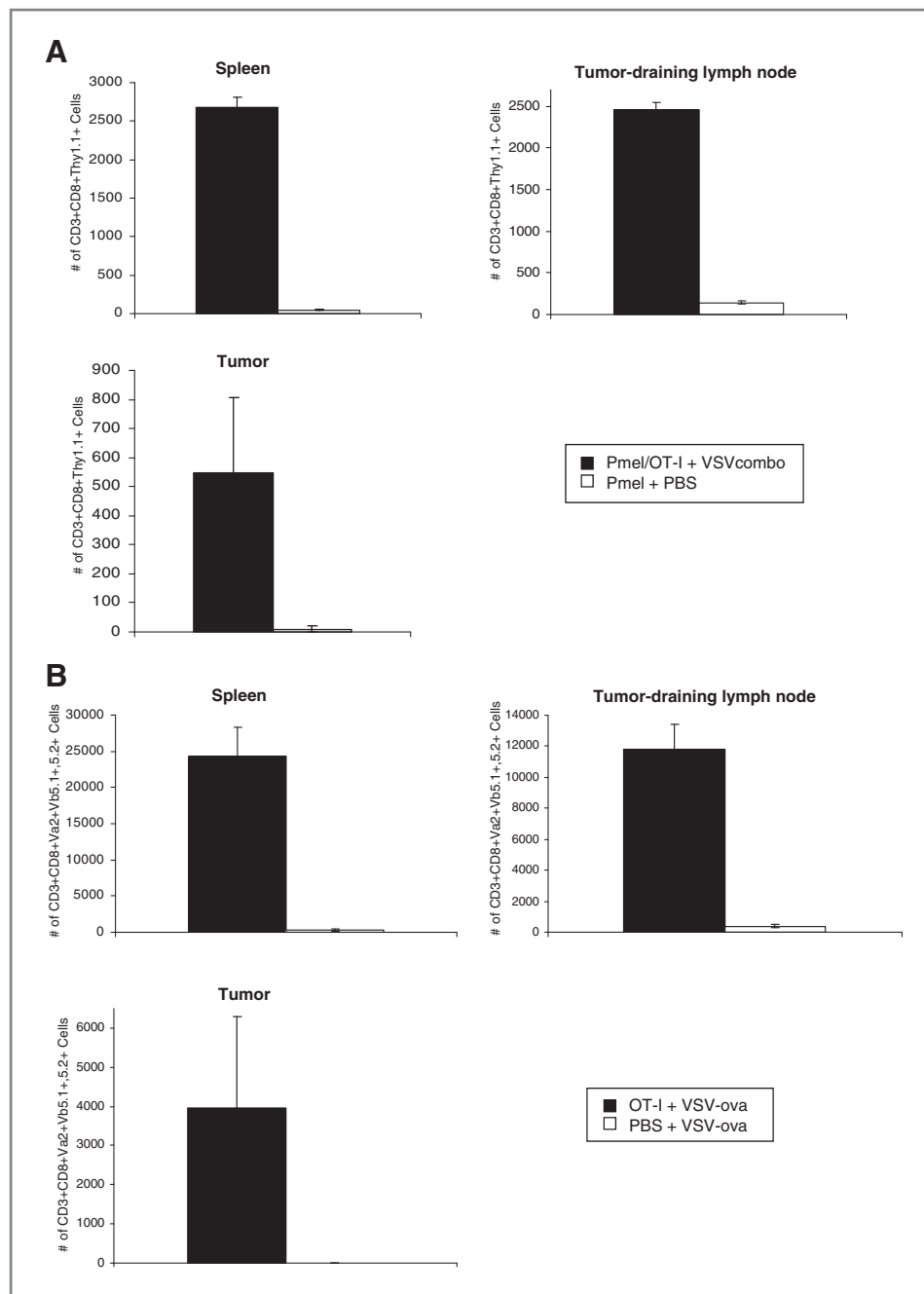


Figure 6. Accumulation and activation of T cells following dual-TAA targeted therapy. Tumor-bearing mice were treated as described in Fig. 5. Spleens, tumors, and TDLNs were harvested on day 12 ($n = 3$) and processed for FACS analysis. A, number of $CD3^+CD8^+Thy1.1^+$ Pmel T cells (per 100,000 sorted cells) in the spleens, TDLNs, or tumors of treated mice.

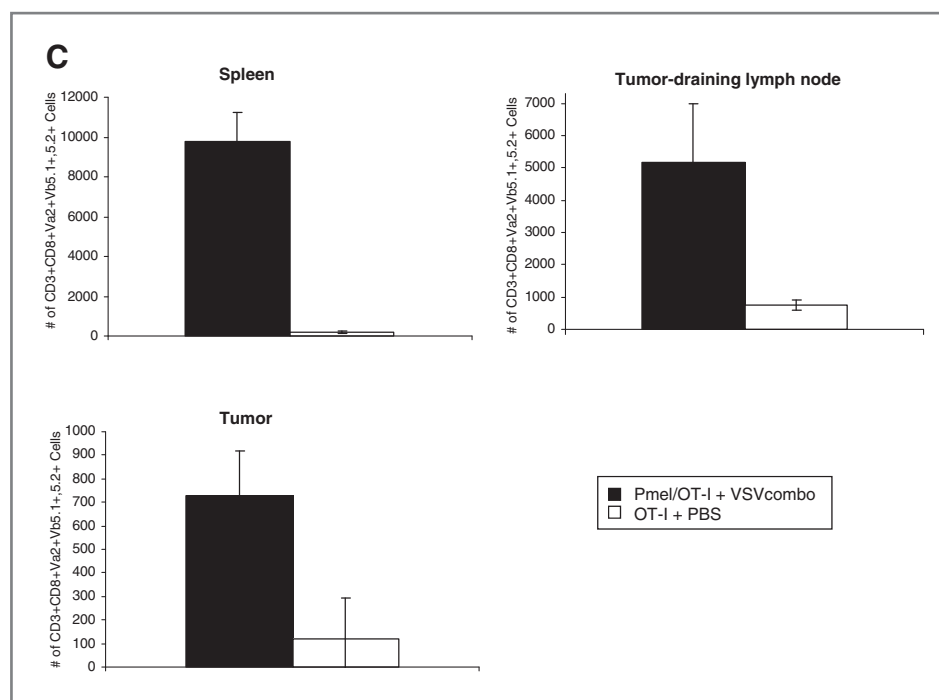
in mice receiving Pmel + VSVcombo or Pmel/OT-I + VSVcombo therapy (Fig. 7A). Anti-ova responses predominated over gp100 responses in mice treated with OT-I, or Pmel/OT-I T cells and VSVcombo ($P < 0.0001$), consistent with the foreign nature of OVA in C57BL/6 mice (Fig. 7A). Significant anti-ova responses, as measured by intracellular $CD8^+$ T-cell IFN- γ staining, were also present in the tumor following treatment with OT-I + VSVcombo ($P = 0.0073$) and Pmel/OT-I + VSVcombo ($P = 0.011$; Fig. 7B top). However, gp100-specific, tumor-infiltrating lymphocytes were also detected after treatment with Pmel + VSVcombo

($P = 0.0022$ compared with no peptide control; Fig. 7B bottom). Although both intravenous VSV-ova and VSV-hgp100 generated effective endogenous T-cell responses to their respective antigens, we observed only minimal (anti-ova) or no (anti-gp100) endogenous T-cell responses following adoptive transfer of either T-cell population alone (not shown; refs. 22, 28, 36).

Autoimmunity correlates with therapy

Mice that received Pmel + VSVcombo, OT-I + VSVcombo, or Pmel/OT-I + VSVcombo therapy developed

Figure 6. (Continued) B and C, number of CD3⁺CD8⁺Vα2⁺Vβ5.1, 5.2 TCR⁺ T cells (per 100,000 sorted cells) in the spleens, TDLNs, or tumors of treated mice.



whisker whitening suggestive of treatment-related autoimmunity (Supplementary Fig. S2A). The mice were otherwise phenotypically normal and showed no overt signs of toxicity. A proportion of mice in the T cell + VSVcombo treatment groups also developed patches of white fur between their hind limbs (Supplementary Fig. S2B). Mice receiving control treatments (systemic VSV, Pmel T cells, OT-I T cells) retained normal whisker, tail and coat coloring (Supplementary Fig. S2C and not shown). Loss of whisker and/or fur pigmentation was also associated with Pmel + VSV-hgp100 treatment, but not OT-I + VSV-ova treatment (not shown). Furthermore, only those mice in which response to therapy was observed developed whisker or coat whitening. Taken together, these data suggest that direct, effective targeting of gp100 was a major requirement for development of autoimmunity.

Discussion

We show here that virotherapy with VSV expressing TAA can support adoptive transfer of TAA-specific T cells to induce long-term, recurrence-free cure of established tumors following systemic therapy. We started these studies with a model in which intravenous treatment with neither VSV (VSV-GFP or VSV-hgp100), nor T cells (Pmel), alone had any significant therapeutic effect (Fig. 1B–E). However, when used in combination (Pmel + VSV-hgp100), about 50% of mice were reproducibly cured without recurrence (Fig. 1). Similarly, persistence, accumulation, and activation of transferred Pmel T cells was only observed in mice treated with VSV-hgp100 as opposed to VSV-GFP or PBS (Fig. 2). Therefore, although systemic VSV-hgp100 alone was unable to break tolerance to

the gp100 antigen at sufficient levels to activate antitumor immunity, providing a pool of exogenous, naive T cells before virus administration resulted in the generation of a potent antitumor response.

We used VSV expressing the altered-self, human gp100 protein, instead of the mouse protein, based upon the rationale that Pmel T cells bind the human peptide/MHC class-I complex with greater avidity than the mouse peptide/MHC class-I complex, despite the peptides differing in only the 3 amino-terminal residues (21, 22, 37). This difference in avidity in the priming stage of Pmel T cells facilitates superior recognition of, and activation by, the murine gp100 as expressed on the target tumor cells (21), consistent with our observation that a VSV expressing mgp100 was less effective in combination with Pmel than was VSV-hgp100 (not shown). The number of injections of VSV encoding TAA correlated with magnitude of the antitumor T-cell response and therapy (38) and, although single injections can still have reduced therapeutic effects, multiple injections were necessary to achieve reproducible therapy between experiments. These data further emphasize the potential value of developing this approach with altered self antigens/epitopes encoded by VSV in combination with T cells that may have low-affinity T-cell receptors against truly self, endogenous TAA (21, 38).

Our data here are consistent with a model in which VSV acts both as a source of TAA to antigen presenting cells, and as a potent adjuvant leading to their activation *in vivo*, thereby mediating immunostimulatory presentation of TAA to the transferred T cells. In this respect, we consistently observed that adoptive transfer of naive T cells was therapeutically superior to preactivated T cells—which are used in most

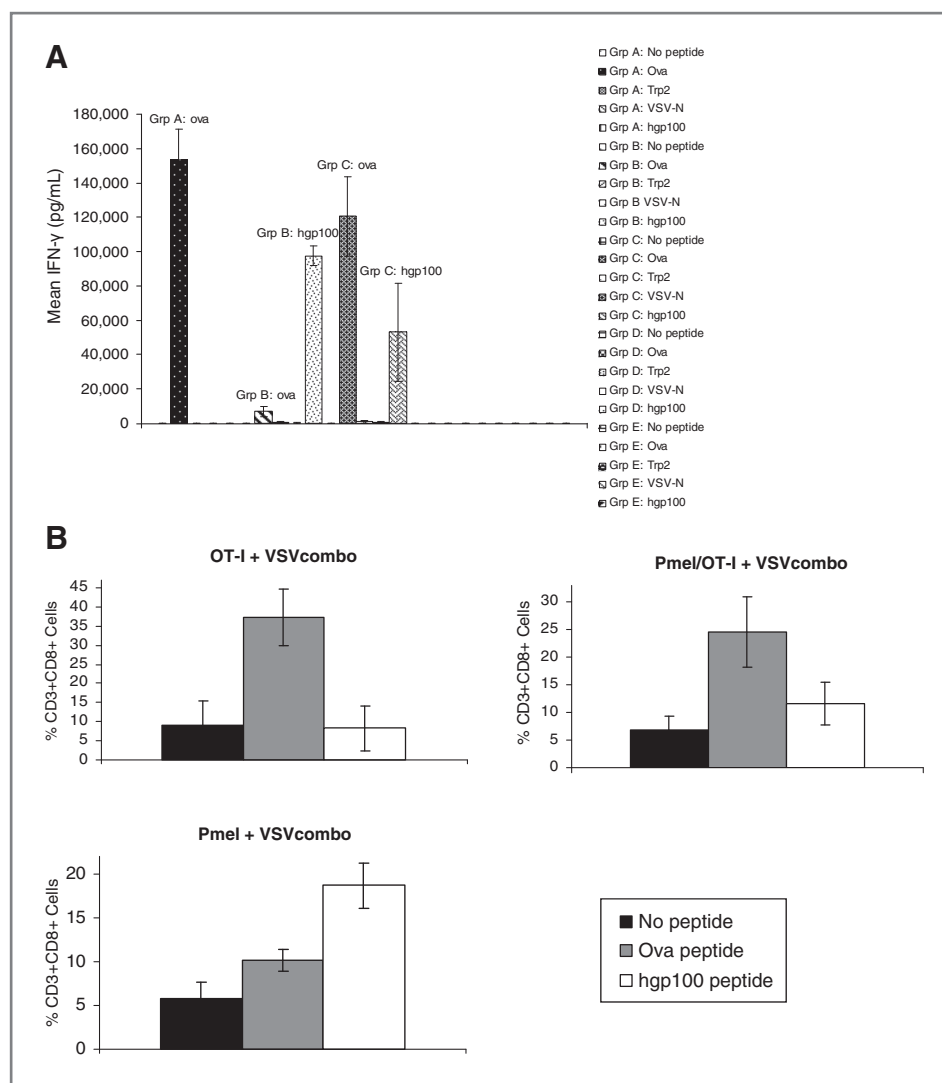


Figure 7. Antigen-specific T-cell infiltration into tumors of combination treated mice.

B16ova-bearing mice were treated as described in Fig. 5. Spleens (A) and tumors (B) were harvested on day 12 ($n = 3$). A, antigen-specific T-cell reactivation was assessed by pulsing splenocytes with no peptide (medium), ova, Trp2, VSV-N, or hgp100-specific peptides for 48 hours followed by IFN- γ ELISA. Group designations are as follows: Grp A = OT-I + VSVcombo, Grp B = Pmel + VSVcombo, Grp C = Pmel/OT-I + VSVcombo, Grp D = Pmel + PBS, Grp E = OT-I + PBS. B, single-cell suspensions of tumors were pulsed with no peptide (medium), ova, hgp100-specific peptides for 1 hour and stained for intracellular IFN- γ . Percentages were determined relative to the CD3⁺CD8⁺ T-cell population in the tumors. Grp, group.

current adoptive transfer studies. We believe that this is because of the enhanced ability of naive T cells to proliferate and survive in the host following immunostimulatory presentation of the cognate antigen by the appropriate VSV-TAA, compared with activated T cells that may undergo more rapid activation-induced cell death upon encountering VSV-presented antigen. Depletion of at least a proportion of Gr1⁺ cells resulted in decreased therapy (Fig. 4D), suggesting that Gr1⁺ pDCs are involved in VSV-mediated TAA presentation, consistent with our previous studies using VSV-TAA for intratumoral injection (27) as well as other reports where VSV was used to prime ova-specific responses (39). Therapy with Pmel + VSV-hgp100 was also dependent upon both CD8⁺ T cells and NK cells (Fig. 4). Although the relative importance of host-derived CD8⁺ T cells, compared with the adoptively transferred Pmel T cells, was not resolved by these depletion studies, a role for host-derived NK cells was clear. Therefore, it seems probable that immunostimulatory presentation of TAA by host antigen-presenting cells (APC; such as Gr1⁺ pDC) is

promoted by VSV-TAA to immune effectors (such as NK cells) in addition to the adoptively transferred T cells. However, depletion of Gr1⁺ cells will also affect a variety of other cell types *in vivo*—including myeloid-derived suppressor cells (MDSC). Although we would predict that depletion of MDSCs would have an overall positive impact on therapy, rather than the decreased effect that we observed in Fig. 4D, further studies will be required to dissect the roles of each particular Gr1⁺ cell subset in the mechanisms associated with the therapy reported here.

Consistent with a role of antigen-nonspecific effectors, such as NK cells, in the combination VSV-TAA/adoptive T-cell therapy, recurrent tumors that developed in a proportion of mice that were initially well controlled by Pmel + VSV-hgp100 all retained appreciable levels of expression of the target gp100 antigen (Fig. 3). This is in contrast to our experience with ACT therapy for B16ova tumors with OT-I T cells in which recurrent tumors escape the T-cell pressure by losing expression of OVA (36). Because

recurrence following apparently effective primary therapy is a major clinical problem, we therefore hypothesized that applying a second immunologic therapy, which may operate through additional mechanisms, would reduce the chances of escape and recurrence. Consistent with this hypothesis, targeting both gp100 and OVA resulted in enhanced efficacy and completely prevented tumor recurrence (Fig. 5). This improved efficacy was associated with a shift in the specificity of the persistent T-cell responses in treated/cured mice with ova-specific T-cell responses predominating in Pmel/OT-I + VSVcombo treated mice (Figs. 6 and 7). Therefore, it is possible that the effective, curative therapy that we observed with the Pmel/OT-I + VSVcombo treatment may be attributed to a strong reactivity against the immunogenic OVA antigen—a situation that will not be reflected in patients, whose tumors will express only self, or near-self antigens. Nonetheless, significant anti-gp100 responses were also achieved in these cured mice (Fig. 7). To mimic the clinical situation more closely—in which patient T cells with lower affinity to their cognate antigens are likely to be recovered—we have recently developed an approach to target a broad range of near-self antigens expressed from VSV, which leads to the activation of polyclonal populations of T cells (38, 40).

Mice in which tumors regressed following treatment with both tumor-specific T cells and systemic VSV-TAA developed whisker and coat whitening (Supplementary Fig. S2). Autoimmunity was associated with targeting of the gp100 antigen as mice receiving OT-I + VSV-ova retained whisker and coat pigmentation regardless of treatment outcome. Therefore, further development of this approach will require careful monitoring for toxicity associated with development of autoimmunity, especially as further TAA are added to the VSV-expressed repertoire.

On the basis of our previous studies on the antitumor, innate immune activating effects of VSV, we believe that VSV acts as an effective adjuvant for the expression of TAA to adoptively transferred, TAA-specific T cells by activation of APC through MyD88-, Type I-, and Type III IFN-mediated signaling (24, 26–28). Interestingly, a strategy targeting gp100 expressed by B16 tumors using a recombinant fowlpox virus encoding the human gp100 peptide ligand along with Pmel T cells required concomitant injection of interleukin-2 (IL-2) for optimal therapy (also accompanied by more marked autoimmune vitiligo; 21). In our studies, we consistently observed a trend toward

improved efficacy with the use of naive Pmel and/or OT-I T cells compared with activated T cells. These preliminary studies suggest that VSV may directly activate T cells through Toll-like receptor-mediated mechanisms, which may partly replace the need for adjuvant IL-2 and comparison of VSV and pox-mediated expression of TAA is currently underway.

In summary, we describe here a completely systemic treatment regimen in which two ineffective individual therapies were combined to generate potent antitumor effects. In our model of 5 to 7 day-established B16 tumors, further improvements were obtained by targeting 2 antigens with the adoptive T-cell/VSV-TAA strategy that led to recurrence-free cures of all treated mice. In the clinical setting, administration of multiple VSV-TAA may be able to activate polyclonal populations of adoptively transferred T cells targeting a range of different antigens, each of which alone has low affinity and activity against patient tumors, without the need for additional supportive cytokine or conditioning regimens. Therefore, by providing both efficient delivery of TAA to APC, as well as highly immunostimulatory activation *in vivo*, we believe that systemic administration of VSV-TAA represents a potentially valuable addition to current clinical protocols of adoptive T-cell therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Rommelfanger, R.G. Vile

Development of methodology: D. Rommelfanger, P. Wongthida, R.G. Vile

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Rommelfanger, R.M. Diaz, K.M. Kaluza

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Rommelfanger

Writing, review, and/or revision of the manuscript: D. Rommelfanger, R.G. Vile

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Rommelfanger, J. Thompson, T. Kottke

Study supervision: J. Thompson, R.G. Vile

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Rommelfanger, J. Thompson, T. Kottke

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Systemic Combination Virotherapy for Melanoma with Tumor Antigen-Expressing Vesicular Stomatitis Virus and Adoptive T-cell Transfer

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