Simultaneous Targeting of Tumor Antigens and the Tumor Vasculature Using T Lymphocyte Transfer Synergize to Induce Regression of Established Tumors in Mice

Dhanalakshmi Chinnasamy, Eric Tran, Zhiya Yu, Richard A. Morgan, Nicholas P. Restifo, and Steven A. Rosenberg

Abstract

Most systemic cancer therapies target tumor cells directly, although there is increasing interest in targeting the tumor stroma that can comprise a substantial portion of the tumor mass. We report here a synergy between two T-cell therapies, one directed against the stromal tumor vasculature and the other directed against antigens expressed on the tumor cell. Simultaneous transfer of genetically engineered syngeneic T cells expressing a chimeric antigen receptor targeting the VEGF receptor-2 (VEGFR2; KDR) that is overexpressed on tumor vasculature and T-cells specific for the tumor antigens gp100 (PMEL), TRP-1 (TYRP1), or TRP-2 (DCT) synergistically eradicated established B16 melanoma tumors in mice and dramatically increased the tumor-free survival of mice compared with treatment with either cell type alone or T cells coexpressing these two targeting molecules. Host lymphodepletion before cell transfer was required to mediate the antitumor effect. The synergistic antitumor response was accompanied by a significant increase in the infiltration and expansion and/or persistence of the adoptively transferred tumor antigen–specific T cells in the tumor microenvironment and thus enhanced their antitumor potency. The data presented here emphasize the possible beneficial effects of combining antiangiogenic with tumor-specific immunotherapeutic approaches for the treatment of patients with cancer. Cancer Res; 73(11); 3371–80. ©2013 AACR.

Introduction

The adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) or peripheral blood T cells genetically engineered with conventional T-cell receptors (TCR) or chimeric antigen receptors (CAR) to recognize cancer antigens can result in durable objective regression in patients with a variety of cancer types including metastatic melanoma, sarcomas, lymphomas, and neuroblastoma (1–7). Preclinical and clinical studies have identified multiple inhibitory mechanisms evolved by tumors to escape from immunosurveillance including the maintenance of an immune-inhibitory tumor microenvironment (8–10). Furthermore, solid tumor cells can be heterogeneous; thus, inhibiting one target will affect some, but not all, tumor cells. Ongoing tumor angiogenesis can mediate immunosuppressive activity through downregulation of adhesion molecules on the vascular endothelium that are involved in leukocyte interactions and inhibit leukocyte extravasation into the tumor (11–14). However, most antiangiogenic cancer monotherapies selectively targeting tumor vasculature have limited clinical benefit for patients with advanced malignancies due to redundancies of angiogenesis factors and pathways (15–17). Recently, we have shown that adoptive transfer of T cells engineered with a CAR against VEGF receptor (VEGFR)-2, overexpressed in tumor vasculature, transiently inhibited tumor growth in mice with a variety of established tumor types. Studies in murine models of adoptive cell transfer (ACT) showed that tumor-specific T cells if administered with interleukin (IL)-2 and a vaccine that stimulates the transferred cells can result in tumor regression in mice but also rarely provide long-term cure and tumor-free survival (18, 19). Here, we show that simultaneous attack against specific cancer antigens and the tumor vasculature by coadministration of syngeneic T cells transduced with a vascular-specific anti-VEGFR2 CAR along with cells expressing a tumor-specific TCR resulted in a synergistic antitumor effect and prolonged tumor-free survival of mice with established cancers. These results open new possibilities for the application of this combination ACT for the treatment of a wide variety of cancer types.

Materials and Methods

Mice and cell culture assays

All animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of the NIH and were conducted under protocols approved by the Animal Care and Use Committee of the NCI (Bethesda, MD). Details of the...
mouse strains and cell culture conditions used in this article are described in Supplementary Methods.

Recombinant retroviral vectors, vaccinia viral vaccines

The recombinant retroviral vector constructs used in this study are schematically illustrated in Fig. 3A and are described in the corresponding figure legend and Supplementary Methods. Details of the molecular sequences and methods used to generate these retroviral constructs have been described elsewhere (20, 21–23). The recombinant vaccinia viral vaccines (rVV) expressing human gp100 (hgp100) or mouse TRP1 or TRP2 were used as vaccines in adoptive transfer experiments in this study were described elsewhere (18, 21–24).

Retroviral transduction of mouse T lymphocytes

The anti-VEGFR2 CAR (DC101 CAR) expressing retroviral vector supernatant was produced from a stable phoenix Eco producer clone as described previously (20). All other retroviral vector supernatants were generated in Platinum Eco packaging cell line stably expressing retroviral GAG and POL proteins as described elsewhere (20). CD3+ T cells were purified from the splenocytes of wild-type (WT) mice using Dynal mouse T cell negative isolation kit (Invitrogen Corp). WT T cells were stimulated for 24 to 48 hours with 2 μg/mL concanavalin A (conA; Sigma) and 1 ng/mL recombinant mouse IL-7 (R&D Systems) in mouse T-cell media as described in Supplementary Methods. Tg-Pmel-1 splenocytes were cultured for 2 days in the mouse T-cell media in the presence of 1 μmol/L hgp10025–33 peptide. Two days post stimulation T cells were transduced with retroviral vectors as previously described (20). Cultured cells were adoptively transferred 3 to 5 days posttransduction (>95% CD8+ T cells).

Transgene analysis by flow cytometry

Expression of the anti-VEGFR2 CAR (DC101 CAR) on retrovirally transduced mouse T cells was detected by flow cytometry with soluble mouse VEGFR2-hlgG.Fc protein (R&D Systems), followed by staining with a phycoerythrin (PE)-labeled goat anti-human IgG,Fc (α-hlgG.Fc) antibody (eBioscience) as described previously (20). Expression of SP6 CAR on transduced mouse T cells was detected as described previously (20). Transduction efficiency of Pmel TCR, TRP1 TCR, and TRP2 TCR in CD3+ T cells was determined by staining the cells with the fluorescein isothiocyanate (FITC)-conjugated antibodies against Vβ13, Vβ14, and Vβ3, respectively (BD Phamingen). Cells were also costained for CD3ε expression using allophycocyanin (APC)-conjugated rat anti-mouse CD3ε. Flow cytometric acquisitions were conducted on a FACSCalibur and analyzed with FlowJo software (TreeStar).

Cytokine release assays

Transduced mouse T cells were tested for specific reactivity against target cells using standard overnight coculture IFN-γ release assays as previously described (20). The MB49 (mouse bladder tumor cell line negative for the expression of gp100 antigen and VEGFR2) and MB49-Flk1 cells expressing full-length coding sequence of mouse VEGFR2 (20) were pulsed with 0 to 1 μmol/L hgp10025–33 or 1 μmol/L irrelevant influenza nucleoprotein peptide (NP) were used as targets.

Adoptive T-cell transfer

Six- to 8-week-old C57BL/6 mice (n = 5–9 for all groups) were injected subcutaneously with 5 x 105 B16 melanoma cells. After 10 to 14 days, they were irradiated with 5 Gy of total body irradiation (TBI) and treated intravenously with transduced or untransduced WT and/or Pmel T cells as indicated in the results. All treated mice received 2.2 x 105 IU recombinant human (rh)IL-2 twice a day intraperitoneally for 3 consecutive days. Where indicated mice also received a single dose of 2 x 107 plaque-forming units (PFU) of rVV coding for the relevant antigen (hgp100 or mouse TRP-1 or TRP-2) recognized by transfected T cells at the time of cell transfer to systematically modulate the intensity of antigen restimulation in vivo. All tumor measurements were conducted in a blinded, randomized fashion and conducted independently at least twice with similar results.

Enumeration of adoptively transferred T cells

Spleens and tumors from 3 mice in each treatment and control groups were harvested at the indicated time points, and single-cell suspensions were made by crashing the tissues through a 40-μm cell strainer. Splenocytes were obtained after red blood cell lysis. Dead cells were removed from the tumor suspensions by centrifugation with Lympholyte M. (Cedarlane Laboratories). Cells were then stained with FITC-conjugated anti-mouse Thy1.1 (CD90.1) and PE-labeled anti-mouse Ly5.1 antibodies (both from BD Biosciences). The absolute numbers of Ly5.1+ and Thy1.1+ cells were calculated by multiplying the absolute cell count by the total percentage of Ly5.1+ and Thy1.1+ cells. Fold changes in the absolute number of Thy1.1+ Pmel T cells in spleen or tumor tissues were calculated by dividing the absolute numbers of Thy1.1+ cells per spleen or per gram tumor of mice receiving a mixture of anti-VEGFR2 CAR–transduced T cells and Tg-Pmel T cells with that of mice treated with a mixture of SP6 CAR–transduced T cells and Tg-Pmel T cells.

Statistical analysis

Tumor growth slopes were compared using Wilcoxon rank-sum test. P < 0.05 was considered significant. Student t tests were used to test for significant differences in enumeration assays. P ≤ 0.05 was considered significant.

Results

Anti-VEGFR2 CAR expression and ex vivo functional integrity of retrovirally engineered Tg-Pmel T cells

Anti-VEGFR2 CAR (DC101 CAR) transduction resulted in CAR expression in approximately 92% (range, 83%–95%) of T cells derived from WT mice (WT/DC101CAR) and 85% (range, 78%–92%) of Tg-Pmel T cells (Tg-Pmel/DC101CAR; Fig. 1A). Both the WT/DC101 CAR and the Tg-Pmel/DC101 CAR T cells specifically secreted IFN-γ when cocultured with the VEGFR2-expressing MB49-Flk1 cells, but failed to respond to VEGFR2-negative MB49 cells (Fig. 1B). Similarly, both the untransduced
and the anti-VEGFR2 CAR–transduced Tg-Pmel T cells secreted IFN-γ in response to MB49 tumor cells that were pulsed with hgp10027–33 peptide but not to those pulsed with an irrelevant peptide. These results suggest that anti-VEGFR2 CAR–expressing Pmel T cells (Tg-Pmel/DC101 CAR) retained their native TCR function while genetically modified to confer dual specificity through an MHC-unrestricted chimeric receptor. When the targeting specificities were present on different effector T cells (Tg-Pmel + DC101 CAR), they could independently recognize their target antigen and generate an IFN-γ response, which was greater than that obtained with effector T cells possessing both the targeting specificities.

**In vivo functional activity of anti-VEGFR CAR–transduced Tg-Pmel T cells**

We next treated groups of mice bearing 10- to 12-day-old B16 melanoma with different numbers of Tg-Pmel T cells engineered to express an anti-VEGFR2 CAR or a control vector or a mixture of Tg-Pmel T cells and the Wt open repertoire T cells transduced with an anti-VEGFR2 CAR or a control vector. As shown in Fig. 2A, tumors in groups receiving no treatment or 10⁷ empty vector–transduced T cells grew steadily and 10⁶ or 10⁵ anti-VEGFR2 CAR (DC101 CAR)–transduced T cells had little or no effect on B16 tumor growth. However, as shown previously (20), 10⁷ anti-VEGFR2 CAR–engineered open repertoire T cells mediated a significant antitumor effect compared with the no-treatment group and to the group treated with 10⁷ empty vector–transduced T cells (P = 0.002 and 0.001 respectively; Fig. 2A, left). The Tg-Pmel T cells mediated significant but transient tumor growth inhibition at all the dose levels tested compared with the no-treatment group (P = 0.01; 0.002; 0.002 for 10⁵, 10⁶, and 10⁷ T cells, respectively). Notably, transduction of the anti-VEGFR2 CAR into Tg-Pmel cells did not enhance the antitumor efficacy compared with Tg-Pmel or anti-VEGFR2 CAR T cells alone. In contrast, a synergistic antitumor effect mediating complete durable regression of tumors was seen in mice treated with a mixture of Tg-Pmel T cells and anti-VEGFR2 CAR–engineered open repertoire T cells (Tg-Pmel + DC101 CAR) at all cell doses studied compared with those treated with same number of...
Tg-Pmel or anti-VEGFR2 CAR–transduced WT T cells alone (P values are shown in Fig. 2A). Tumors in mice treated with a single dose $10^7$, $10^6$, or $10^5$ Tg-Pmel T cells mixed with $10^6$ but not with $10^6$ VEGFR2 CAR–transduced T cells regressed completely by days 15 to 21 posttreatment. These results showed that the presence of anti-VEGFR2 CAR T cells significantly enhanced the antitumor efficacy of tumor-specific TCR expressing T cells provided these 2 targeting molecules with different specificities were present on 2 different T cells rather than coexpressed on a single T cell. Furthermore, while administration of tumor antigen–specific rVV did not affect the antitumor efficacy of the anti-VEGFR2 CAR–transduced Tg-Pmel or the WT T cells, it significantly enhanced the tumor treatment effect ($P = 0.008$) and tumor-free survival mediated by the $10^6$ Tg-Pmel T cells administered in conjunction with $5 \times 10^6$ anti-VEGFR2 CAR–transduced WT T cells (Fig. 2B). These results suggest that the functionality of the tumor antigen–specific TCR was greatly reduced when they were enforced to possess dual specificity against a completely different target cell type, in this case, the VEGFR2-expressing cells in the tumor environment.

Notably, in mice receiving a mixture of $10^6$ Tg-Pmel and $5 \times 10^5$ anti-VEGFR2 CAR–engineered T cells, tumor growth was significantly inhibited without the administration of tumor antigen–specific viral vaccine compared with the control groups receiving $10^6$ Tg-Pmel T cells or $10^6$ WT T cells mixed with $5 \times 10^6$ empty vector–transduced WT T cells without rVV administration or rVV alone without T cells or any treatment ($P = 0.01, 0.007, 0.003, 0.009$, respectively) and also to $10^6$ WT
Adoptive T-cell Therapy Targeting Tumor and Tumor Vasculature

Figure 3. Retroviral transduction of mouse T lymphocytes. A, schematic representation of tumor antigen–specific TCR-expressing recombinant retroviral vectors used in this study. In the MSGV1 retroviral vectors expressing the codon optimized Pmel TCR (Vα1, Vβ13), TRP-2 TCR (Vα3.2, Vβ14), or TRP-1 TCR (Vα12.1, Vβ3) recognizing the murine gp100, tyrosinase-related protein-1 (TRP-1), or tyrosinase-related-protein-2 (TRP-2) antigens, respectively, the corresponding Vα and Vβ cDNAs were fused together using the picornavirus foot-and-mouth disease virus 2A sequence. LTR, long terminal repeat; PA2, polyadenylation signal; SD, splice donor; SA, splice acceptor. B, enriched splenic CD3⁺ T cells from C57BL/6 mice were stimulated for 2 days with ConA and IL-7 and then transduced with an empty or anti-VEGFR2 CAR retroviral vector. The next day, cells were transduced with retroviral vector expressing one of the TCRs or left untransduced and analyzed by FACS 2 days later. Cells were also costained for CD3ε expression using APC-conjugated rat anti-mouse CD3ε. FACS data showing the percentage of cells in gated CD3 ε⁺ viable T cells are presented. Data are representative of 2 independent experiments.

T cells mixed with 5 × 10⁶ empty vector–transduced Wt T cells transferred together with rVVs (P = 0.007; Fig. 2B). However, their tumor treatment effect was comparable or equal to groups receiving 10⁷ Tg-Pmel T cells mixed with 5 × 10⁶ empty vector–transduced Wt T cells and rVVs or 10⁶ Wt T cells mixed with 5 × 10⁶ anti-VEGFR2 CAR–transduced Wt T cells administered in conjunction with or without rVV expressing hgp100 antigen. Importantly, the tumor treatment efficacy was significantly enhanced in mice treated with a mixture of Tg-Pmel T cells and anti-VEGFR2 CAR T cells in conjunction with rVVs compared with group receiving the same T-cell mixture but no vaccine (P = 0.008) and resulted in complete tumor regression and long-term tumor-free survival of mice (Fig. 2B).

In vivo tumor treatment efficacy of combination therapy using anti-VEGFR CAR and/or tumor antigen–specific TCR–transduced open repertoire T cells

Next we evaluated whether the results obtained in our previous experiments were restricted to Tg-Pmel T cells or could be extended to open repertoire T cells genetically engineered to express an MHC class I or II–restricted antigen–specific TCR. We used MSGV1-based retroviral vectors expressing MHC class I–restricted TCRs recognizing the gp100 or TRP-2 antigens or an MHC class II–restricted TCR recognizing the TRP-1 antigen (Fig. 3A). All 3 antigens are widely expressed on the B16 mouse melanoma. We transduced C57BL/6 splenocytes with an empty vector or an anti-VEGFR2 CAR vector and/or one of the retroviral constructs expressing a TCR shown in Fig. 3A. On day 3 posttransduction, the anti-VEGFR2 CAR–expressing T cells were cotransduced with retroviral vectors expressing one of the TCRs, the coexpression of the Pmel TCR, TRP-1 TCR, and TRP2 TCR was detectable in 54% to 75% of cells (Fig. 3B).

Adoptive transfer of a mixture of T cells transduced with an antigen–specific TCR and the anti-VEGFR2 CAR induced a pronounced synergistic inhibitory effect on tumor growth and increased the tumor-free survival of mice compared with treatment with only one of these transduced T cells or those cotransduced to express both of the TCR and the CAR (Fig. 4). Although the 10⁵ TCR or 5 × 10⁶ anti-VEGFR2–transduced T cells transiently controlled the tumor growth, the treatment effect was ineffective beyond 3 weeks. Empty vector–transduced T cells and low numbers (10⁵) of anti-VEGFR2 CAR–transduced T cells failed to control tumor growth similar to the no-treatment group.

Coadministration of anti-VEGFR2 CAR–transduced T cells enhanced the effective infiltration and persistence of the adoptively transfected tumor–specific T cells in the tumor

Next we studied whether the enhanced synergistic antitumor effect of combination therapy with tumor–specific T cells and the anti-VEGFR2 CAR T cells was due to their increased infiltration and/or persistence at the tumor site. Mice bearing 10-day-old B16 melanoma were lymphodepleted by 5-Gy TBI before treatment and intravenously injected with either 10⁶ untransduced Wt or Tg-Pmel T cells expressing the congenic marker Thy1.1 mixed with 5 × 10⁶ Ly5.1 marker–positive T cells engineered with an irrelevant control CAR (SP6 CAR specific for a synthetic hapten) or an anti-VEGFR2 CAR together with vaccinia virus vaccine expressing the gp100 antigen. Single-cell preparations of spleen and tumor samples obtained from 3 mice in each group on 3, 6, and 9 days post–T-cell transfer were analyzed by flow cytometry to determine the total number of adoptively transferred Thy1.1⁺ and Ly5.1⁺ T cells. Representative fluorescence-activated cell sorting (FACS) data showing the percentage of viable Thy1.1⁺ and Ly5.1⁺ T cells in spleen and tumor tissues of one mouse in each group are presented in Fig. 5. The average absolute numbers of viable Thy1.1⁺ and Ly5.1⁺ T cells in spleen and tumor (normalized to per gram tumor) tissues of 3 mice in each group are presented in Fig. 6A. Both the percentage and number of Tg-Pmel T cells (Thy1.1⁺) in spleen and tumor were increased at
transduced T cells were adoptively transferred into B16 tumors in mice and increased their tumor-free survival. Enriched splenic engineered mouse T cells induced regression of established syngeneic tumors in mice and increased their tumor-free survival. Enriched splenic CD8+ T cells obtained from C57BL/6 mice were stimulated for 2 days with ConA and IL-7 and then transduced with an empty or anti-VEGF2 CAR retroviral vector. The next day, cells were transduced with retroviral vector expressing one of the TCRs or left untransduced. Two days later, transduced T cells were adoptively transferred into B16 tumor–bearing C57BL/6 mice (5 mice per group) as indicated in the figure. Animals received 5-Gy TBI before T-cell transfer and concurrently received vaccine and rhIL-2 as described in legend for Fig. 2A. Serial, blinded tumor measurements were obtained and the products of perpendicular diameters were plotted ±SEM. The data shown are representative of 2 independent experiments.

all time points if they were cotransferred with the anti-VEGF2 CAR–engineered T cells (Ly5.1+) but not with T cells expressing a control SP6 CAR (Ly5.1+).

In our ACT model, we administered vaccinia virus expressing hgp100 vaccine to systematically modulate the intensity of antigen restimulation of Pmel T cells, which resulted in similar systemic distribution in the spleen and tumor tissues. Even though the percentage values of Pmel in spleen and tumor tissues on day 6 post-ACT are close, there was a 10-fold increase in the total number of transferred Pmel T cells in tumor compared with spleen, suggesting that vaccine induced an increase in their proliferative capacity and likely facilitated their rapid expansion during a second stimulation when they encountered the target antigen on the tumor. Unlike Pmel T cells, the activation and proliferation of DC101 CAR T cells is solely dependent on the target antigen VEGFR2 expressed by the tumor vasculature, which resulted in accumulation of DC101 CAR T cells in the tumor but not in spleen.

Notably, on day 3 posttherapy, the absolute number of Thy1.1–Tg-Pmel T cells was 25.3- and 12.7-fold more in the spleen and tumor tissues, respectively, if the mice were coadministered with anti-VEGF2 CAR–transduced T cells compared with those treated together with control CAR (SP6 CAR)–transduced T cells (Fig. 6B). Interestingly, the anti-VEGF2 CAR T cells (Ly5.1+) failed to persist both in the spleen and in the tumor tissues if they were cotransferred with Tg-Pmel T cells but not with the Wt T cells, despite their effective infiltration on day 3 posttransfer. These results suggest that the anti-VEGF2 CAR–expressing T cells facilitated the trafficking and early infiltration of the tumor-specific T cells and enhanced their persistence at the tumor site.

Discussion

ACT using T cells reactive with tumor antigens can mediate the regression of established tumor masses in both mouse models and in the human (1, 18, 19, 25–29). In the human, ACT using TILs that target tumor antigens following a lymphodepleting regimen can mediate the regression of widely metastatic melanoma in 50% to 70% of patients including up to 40% of patients who exhibit durable complete responses (1, 7, 25, 26, 30–32). ACT using T cells genetically engineered to express either conventional TCRs or CARs can mediate tumor regression in patients with melanoma, lymphomas, synovial cell sarcomas, and neuroblastoma (2–6). These studies have provided definitive evidence that T cells targeting specific tumor antigens can mediate tumor destruction.

Therapeutic attempts to target tumor vasculature using antiangiogenic agents have had limited success in the treatment of tumors in both mouse models and in the human (15, 17, 33–36) possibly due to the multiple redundant pathways involved in angiogenesis (15, 17). Multiple studies have shown that antiangiogenic agents can remodel the tumor vasculature and improve drug penetration into tumors (37, 38) and also enhance active immunotherapy through increasing the infiltration of immune cells into tumors (39). In a prior study, we showed that the administration of an anti-VEGF antibody could improve the infiltration of adoptively transferred anti-gp100 transgenic T cells into a growing B16 melanoma tumor mass and could enhance the antitumor effects of this T-cell transfer (14). In a recent study, low-dose TNF-α treatment was shown to mediate inflammatory vessel remodeling and enhanced the infiltration of CDS+ effector cells and increased survival of spontaneous pancreatic neuroendocrine tumor–bearing RIP1-Tag5 transgenic mice (40). In a similar study, low-dose anti-VEGF2R antibody therapy was shown to enhance the antitumor efficacy of T-cell activation induced by a whole cancer cell vaccine therapy in a CDS+ T-cell–dependent manner in both immunotolerant and immunogenic murine breast cancer models (41). We have developed an alternate approach to target the tumor vasculature using lymphocytes genetically engineered to express a CAR that targets VEGFR2 on tumor vasculature. ACT using these genetically modified lymphocytes could inhibit the growth of 5 different vascularized murine tumors in 2 different mouse strains (20). T cells transduced with this anti-VEGF2 CAR exhibited durable and increased infiltration of T cells into tumor, which correlated with their antitumor effect. The ability to use ACT to target tumor antigens expressed on tumor cells as well as the tumor vasculature led to the current study attempting to simultaneously target these 2 tumor elements.

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<th>Figure 4. Simultaneous transfer of anti-VEGF2 CAR- and TCR–engineered mouse T cells induced regression of established syngeneic tumors in mice and increased their tumor-free survival. Enriched splenic CD8+ T cells obtained from C57BL/6 mice were stimulated for 2 days with ConA and IL-7 and then transduced with an empty or anti-VEGF2 CAR retroviral vector. The next day, cells were transduced with retroviral vector expressing one of the TCRs or left untransduced. Two days later, transduced T cells were adoptively transferred into B16 tumor–bearing C57BL/6 mice (5 mice per group) as indicated in the figure. Animals received 5-Gy TBI before T-cell transfer and concurrently received vaccine and rhIL-2 as described in legend for Fig. 2A. Serial, blinded tumor measurements were obtained and the products of perpendicular diameters were plotted ±SEM. The data shown are representative of 2 independent experiments.</th>
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**P = 0.008**

*P = 0.009*
In these experiments, we used the anti-VEGFR2 CAR that we previously described (20). The specific tumor antigen reactive T cells we used included the transgenic Pmel cells reactive with the gp-100 antigen, as well as WT cells transduced with the anti-gp100 TCR, the anti-TRP-1 TCR, or the anti-TRP-2 TCR. Mice treated with a mixture of T cells transduced with an anti-VEGFR2 CAR and the tumor antigen–specific TCR exhibited a superior antitumor effect compared with those treated with T cells expressing only one of these targeting molecules or those modified to coexpress both targeting molecules. These effects were synergistic, as the dose of the anti-VEGFR2 CAR cells had a moderate impact when used alone but could help mediate complete tumor regression when combined with the antigen-specific cells that alone had only a transient impact on tumors (Fig. 2A, middle and right).

Furthermore, our study showed increased numbers of adoptively transferred tumor antigen-specific T cells within the tumor if they were coadministered with T cells targeted against the tumor vasculature. However, it is not known for certain whether the increased numbers of transferred antigen–specific T cells within the tumor were because of increased infiltration, increased proliferation, or increased retention in the tumor after the initial entry, although it is likely that all factors played a role. Even though the SP6 CAR–transduced T cells reach the tumor similar to anti-VEGFR2 CAR–cotransduced T cells at the earlier time point, they failed to proliferate and persist due to the absence of antigenic stimulation. Our results showed that provision of a CAR with a predefined specificity against VEGFR2 facilitated their retention at the tumor site and conferred proliferative potential.

Of interest was our finding that the coexpression of the anti-VEGFR2 CAR and antigen–specific receptors on the same cell was not effective in mediating tumor regression, despite the demonstration in vitro that both receptors were expressed on the cells and were functionally competent in vitro. It is possible that if anti-VEGFR2 CAR and tumor-specific TCRs are engaged one after the other, with several hours delay, T cells with dual receptors may be in a refractory state after recognition one of the targets. In a clinical trial in patients with neuroblastoma treated with Epstein–Barr virus–specific T cells engineered with a CAR against the tumor-associated antigen GD2, cells expressing both receptors survived longer than T cells that expressed the same chimeric receptor alone (42). However, our ability to study whether these T cells with dual specificity could...
CAR Pmel T cells adoptively transferred in conjunction with anti-VEGFR2 tissues. B, fold changes in the absolute numbers of Thy1.1 cells. Furthermore, immuno-highly correlated with the level of VEGFR2 expressed on target endothelial cells tested. The amount of IFN-γ secreted was significantly limited to the myeloid suppressor cells and regulatory T cells (Treg) that are known to express VEGFRII (36, 43–48). Notably, other studies have shown that the effect of antiangiogenic drugs such as sunitinib and anti-VEGF antibodies was mediated partly through the elimination of VEGFRII-expressing myeloid suppressor cells and Tregs in the tumor environment in addition to targeting the tumor endothelial cells (13, 49, 50).

No significant adverse effects were seen in mice treated with T cells engineered with an anti-VEGFR2 CAR in this and previous studies (20) under conditions where significant antitumor effects were seen. In this study, no signs of morbidity or mortality were seen in animals treated simultaneously with genetically engineered T cells expressing the anti-VEGFR2 CAR and a tumor antigen–specific TCR except those receiving mixture of TRP2 TCR–transduced T cells and anti-VEGFR2 CAR–engineered T cells. Notwithstanding these and previous observations (20), the potential adverse effects of antiangiogenic immunotherapy have to be carefully considered in future experiments.

In conclusion, using an established mouse model of melanoma, we have shown antitumor effects of a dual-targeting adoptive therapy strategy of simultaneously attacking the tumor and tumor vasculature using genetically modified T cells. The strategy is particularly meaningful for the treatment of solid tumors with known antigenic signatures and may encourage the clinical application of combined immunotherapy and antiangiogenic therapy in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: D. Chinnasamy, E. Tran, N.P. Restifo, S.A. Rosenberg
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Writing, review, and/or revision of the manuscript: D. Chinnasamy, E. Tran, Z. Yu, R.A. Morgan, N.P. Restifo, S.A. Rosenberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Chinnasamy, E. Tran, N.P. Restifo, S.A. Rosenberg
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