

# Antiangiogenic Agents Can Increase Lymphocyte Infiltration into Tumor and Enhance the Effectiveness of Adoptive Immunotherapy of Cancer

Rajeev K. Shrimali, Zhiya Yu, Marc R. Theoret, Dhanalakshmi Chinnasamy, Nicholas P. Restifo, and Steven A. Rosenberg

## Abstract

Adoptive cell transfer (ACT)-based immunotherapies can mediate objective cancer regression in animal models and in up to 70% of patients with metastatic melanoma; however, it remains unclear whether the tumor vasculature impedes the egress of tumor-specific T cells, thus hindering this immunotherapy. Disruption of the proangiogenic interaction of vascular endothelial growth factor (VEGF) with its receptor (VEGFR-2) has been reported to “normalize” tumor vasculature, enhancing the efficacy of chemotherapeutic agents by increasing their delivery to the tumor interstitium. We thus sought to determine whether disrupting VEGF/VEGFR-2 signaling could enhance the effectiveness of ACT in a murine cancer model. The administration of an antibody against mouse VEGF synergized with ACT to enhance inhibition of established, vascularized, B16 melanoma ( $P = 0.009$ ) and improve survival ( $P = 0.003$ ). Additive effects of an antibody against VEGFR-2 in conjunction with ACT were seen in this model ( $P = 0.013$ ). Anti-VEGF, but not anti-VEGFR-2, antibody significantly increased infiltration of transferred cells into the tumor. Thus, normalization of tumor vasculature through disruption of the VEGF/VEGFR-2 axis can increase extravasation of adoptively transferred T cells into the tumor and improve ACT-based immunotherapy. These studies provide a rationale for the exploration of combining antiangiogenic agents with ACT for the treatment of patients with cancer. *Cancer Res*; 70(15); 6171–80. ©2010 AACR.

## Introduction

Cell transfer immunotherapy and antiangiogenic therapy are two new biological approaches to the treatment of cancer. The adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) or lymphocytes genetically engineered to express antitumor T-cell receptors (TCR) can mediate the objective regression of cancer in up to 70% of patients with metastatic melanoma (1–4). The integrity of the tumor vasculature and the suppressive nature of the tumor microenvironment can play an important role in modulating the effectiveness of cell-based immunotherapies (5, 6), and antiangiogenic approaches can have a profound impact on both of these factors. Thus, in the current study, we explored the interactions and possible synergies between cell transfer and antiangiogenic therapies in a murine cancer model.

Vascular endothelial growth factor (VEGF), a proangiogenic factor secreted by various solid tumors including melanoma, has immunomodulatory effects, in part by di-

rectly suppressing various immune cells present in the tumor microenvironment (6). VEGF at concentrations similar to those found in cancer patients can contribute to tumor-associated immune deficiency and has been reported to negatively regulate antigen presentation by dendritic cells, shift mature dendritic cell populations to immature dendritic cell precursors, induce apoptotic pathways in CD8<sup>+</sup> T cells, and induce the activity of regulatory T cells (Tregs; refs. 6–12). VEGF can also alter the tumor endothelium, which can further disrupt the infiltration and function of tumor-infiltrating T cells (7).

Neutralizing antibodies against VEGF can block the immune-suppressive effect of tumor-derived supernatant on the function of mature dendritic cells (12). The administration of anti-VEGF antibody (bevacizumab) to 16 patients with colorectal cancer significantly decreased the number of immature dendritic cells in peripheral blood ( $P = 0.012$ ). In mixed lymphocyte reaction assays, anti-VEGF antibody could enhance the antigen-presenting capacity of dendritic cells (13). Immunotherapy with granulocyte-macrophage colony stimulating factor-secreting tumor cells in combination with VEGF inhibition enhanced the number of activated dendritic cells and tumor-infiltrating effector T cells and reduced the number of Tregs in the tumor microenvironment (14). Other antiangiogenic agents have been shown to inhibit tumor growth and microvessel density, and enhance the infiltration of leukocytes and CD8<sup>+</sup> cytotoxic T lymphocytes into tumor (15).

**Authors' Affiliation:** Surgery Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland

**Corresponding Author:** Steven A. Rosenberg, Surgery Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892-1201. Phone: 301-496-4164; Fax: 301-402-1738; E-mail: SAR@nih.gov.

doi: 10.1158/0008-5472.CAN-10-0153

©2010 American Association for Cancer Research.

Antiangiogenic agents have been used as a monotherapy or in combination with cytotoxic chemotherapy in both animal models and in humans, with variable results. Mouse anti-VEGF antibody, A4.6.1, was shown to suppress the growth of human rhabdomyosarcoma, glioblastoma, leiomyosarcoma, and various other tumors implanted in immunodeficient mice (16, 17). Other antiangiogenic agents targeting VEGFRs and small-molecule tyrosine kinase inhibitors have also been used effectively for tumor treatment in several preclinical and clinical models (18, 19). Anti-mouse VEGFR-2 antibody (DC101) treatment significantly suppressed the growth of primary murine Lewis lung, 4T1 mammary, and B16 melanoma tumors, and completely inhibited the growth of established epidermoid, glioblastoma, pancreatic, and renal human tumor xenografts (18). Monotherapy with anti-VEGF alone has not been successful in human clinical trials but when used in combination with chemotherapy have increased overall survival and/or progression-free survival in colorectal, breast, and lung cancer patients (19, 20). VEGF receptor kinase-selective multitargeted agents in combination with various chemotherapeutic agents are in different stages of clinical development (19, 20).

Inhibition of the VEGF/VEGFR-2 axis has been reported to normalize tumor vasculature, decrease interstitial fluid pressure, enhance oncotic pressure gradient, decrease hydrostatic pressure gradient across the tumor vasculature (21), and increase deeper penetration of high molecular weight tetramethyl rhodamine isothiocyanate (TRITC)-labeled bovine serum albumin (BSA; ref. 22). Based on the above observations, it has been postulated that judicious application of antiangiogenic agents can “normalize” the abnormal tumor vasculature, resulting in more efficient delivery of drugs and oxygen to targeted cancer cells (23).

The current study was thus proposed to test whether the addition of antiangiogenic agents could significantly enhance the antitumor therapeutic effect of cell transfer therapy. We tested this hypothesis in the pmel-1 TCR transgenic mouse model previously reported by our group (24), which models the use of adoptive cell therapy for the treatment of metastatic melanoma in the clinical setting.

## Materials and Methods

### Mice and cell lines

Pmel-1 TCR transgenic (24, 25) and C57BL/6 mice (The Jackson Laboratory) were bred, housed, and used according to the guidelines of the National Cancer Institute Animal Care and Use Committee. The V $\beta$ -13-pmel-1 TCR recognizes an epitope of the gp100 melanoma/melanocyte differentiation antigen present on the B16 melanoma, a spontaneous murine melanoma in C57BL/6 mice obtained from the National Cancer Institute tumor repository and maintained in culture (24).

### *In vitro* pmel-1 T-cell activation and adoptive cell transfer

Seven days before adoptive cell transfer (ACT), splenocytes from pmel-1 TCR transgenic mice were harvested, and depleted of erythrocytes by using ACK lysis buffer (GIBCO).

Splenocytes were further cultured in complete medium (RPMI 1640, 10% heat inactivated fetal bovine serum, penicillin streptomycin, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-mercaptoethanol) in the presence of 1  $\mu$ mol/L hgp<sub>25-33</sub> peptide and 30 IU/mL of rhIL-2 (Novartis Corp.; refs. 24, 25). On the day of ACT (day 0, D0), all tumor-bearing recipient mice received 500 cGy of sublethal radiation as a lymphodepleting regimen before cell transfer. *In vitro* activated pmel-1-Ly5.1 T cells ( $P; 1 \times 10^6$ ) were transferred intravenously along with the injection of  $2 \times 10^7$  plaque-forming units of recombinant vaccinia virus (V) expressing hgp100. All treated mice received an intraperitoneal injection of 600,000 IU of rhIL-2 twice daily for a total of six doses. In this article, groups of mice receiving all three of pmel-1-Ly5.1 cells (P), vaccine (V), and rhIL-2 (I) are referred to as the PVI group.

### Antibody administration and *in vivo* tumor treatment

Anti-mouse VEGF antibody (B20-4.1.1-PHAGE,  $\alpha$ -VEGF) was kindly supplied by Genentech, and rat anti-mouse VEGFR-2 (DC101) was kindly supplied by ImClone Systems. For tumor treatment experiments, antibodies were injected starting on the day of cell transfer (D0) and continued at an interval of 3 days. Rat serum IgG (Sigma) at an equivalent concentration was used as an isotope control (see figure and figure legends for antibody schedule and number of doses).

C57BL/6 female recipient mice (6–12 weeks of age) were injected subcutaneously with  $5 \times 10^5$  B16 melanoma cells, and tumors were allowed to grow for 12 to 18 days ( $n = 5$  mice per group in all tumor treatment experiments). On the day of cell transfer, experimental groups received either pmel-1-Ly5.1 T cells (P), vaccine (V), IL-2 (I), rat IgG,  $\alpha$ -VEGF, or DC101 alone or in various combinations (see figure and figure legends for more details). Tumors were measured using calipers, and the products of the perpendicular diameters were recorded. All experiments were done in a blinded fashion (experimental mouse groups were coded so the investigators measuring the tumor and analyzing the data had no knowledge of the identity of the treatment groups). Error bars represent SEM.

### Enumeration and flow cytometric analysis of adoptively transferred tumor-infiltrating pmel-1-Ly5.1 cells

For the studies of cell infiltration into the tumor, experimental groups received a single dose of antibody 2 days before (–D2) cell transfer (D0). Mice were euthanized on days 3, 4, 5, and 6 post cell transfers to collect spleen and tumor. Splenocytes were prepared as described above while total tumor cells were harvested by homogenizing the tumor into a single-cell suspension using the rubber end of a 3-cm<sup>3</sup> syringe and a 40- $\mu$ m filter cup. TILs were enriched by density gradient centrifugation (15,000  $\times$  g/20 min); counted by trypan blue exclusion; and analyzed by flow cytometry for the expression of CD3, Ly5.1, and V $\beta$ 13. Dead cells were excluded using propidium iodide (PI). We calculated the absolute number of pmel-1-Ly5.1 cells by multiplying the live cell count by the percentage of Ly5.1<sup>+</sup>PI<sup>–</sup> cells. Error bars represent the SEM, with four to five mice in each group.

### Measurement of mean tumor vessel area by CD31 immunofluorescence

B16 tumors were collected at various time points from experimental groups (two to five mice) receiving a single dose of rat IgG, DC101, or  $\alpha$ -VEGF alone and frozen in optimal cutting temperature (OCT) medium. Frozen sections were dried overnight after acetone treatment, washed with PBS, blocked with 10% normal goat serum, and stained with anti-CD31 antibody in 2.5% normal goat serum. After overnight incubation, sections were washed, treated with secondary goat anti-rat Cy5 antibody in 2.5% normal goat serum, and further treated with 4',6-diamidino-2-phenylindole-containing medium.

Tumor sections were scanned to identify areas of maximal vascular density, and five different images were obtained per tumor on a LSM 510 microscope. Zeiss LSM Image Software was used to exclude background fluorescence and to quantify the image area occupied by the Cy5 fluorescence marker. The vascular area is reported as the mean area of Cy5 fluorescence of the five images.

### Statistical analyses

We compared tumor growth slopes using the Wilcoxon rank-sum test, and the single-measurement comparisons between two groups were tested using unpaired *t* tests. Kaplan-Meier analysis was used to assess survival, and the log-rank (Mantel-Cox) test was used for comparison between the two groups.

## Results

### Administration of anti-VEGF antibody enhances the antitumor efficacy of cell transfer therapy and is dependent on prior lymphodepleting total body irradiation

In multiple preliminary experiments, administration of anti-VEGF antibody transiently inhibited growth of subcutaneous B16 melanomas that were  $\sim 50$  mm<sup>2</sup>, but had little or no antitumor activity on tumors that were  $>100$  mm<sup>2</sup>. An example of the efficacy of anti-VEGF antibody alone to treat 50 and 200 mm<sup>2</sup> tumors in the same experiment is shown in Fig. 1A and B. Significant ( $P = 0.009$ ) antitumor activity of anti-VEGF antibody was seen on small tumors ( $\geq 50$  mm<sup>2</sup>) whereas lesser impact was seen on large tumors ( $\sim 200$  mm<sup>2</sup>; see also Fig. 2). Prior published experiments showed that effective ACT of established B16 melanoma (irrespective of tumor size) required the administration of anti-GP100 transgenic pmel-1 T cells plus gp100 vaccine plus IL-2 administration after 5 cGy lymphodepleting total body irradiation (24, 25), referred to here as the PVI treatment. When small ( $\sim 50$  mm<sup>2</sup>) tumors were treated with anti-VEGF antibody, a profound antitumor effect was seen; when anti-VEGF antibody was added to PVI, there was little to no additive antitumor impact (Fig. 1C and D). To better mimic the treatment of human tumors, all subsequent experiments testing the impact of combined treatment with this antibody plus ACT used mice bearing tumors  $>100$  mm<sup>2</sup>.

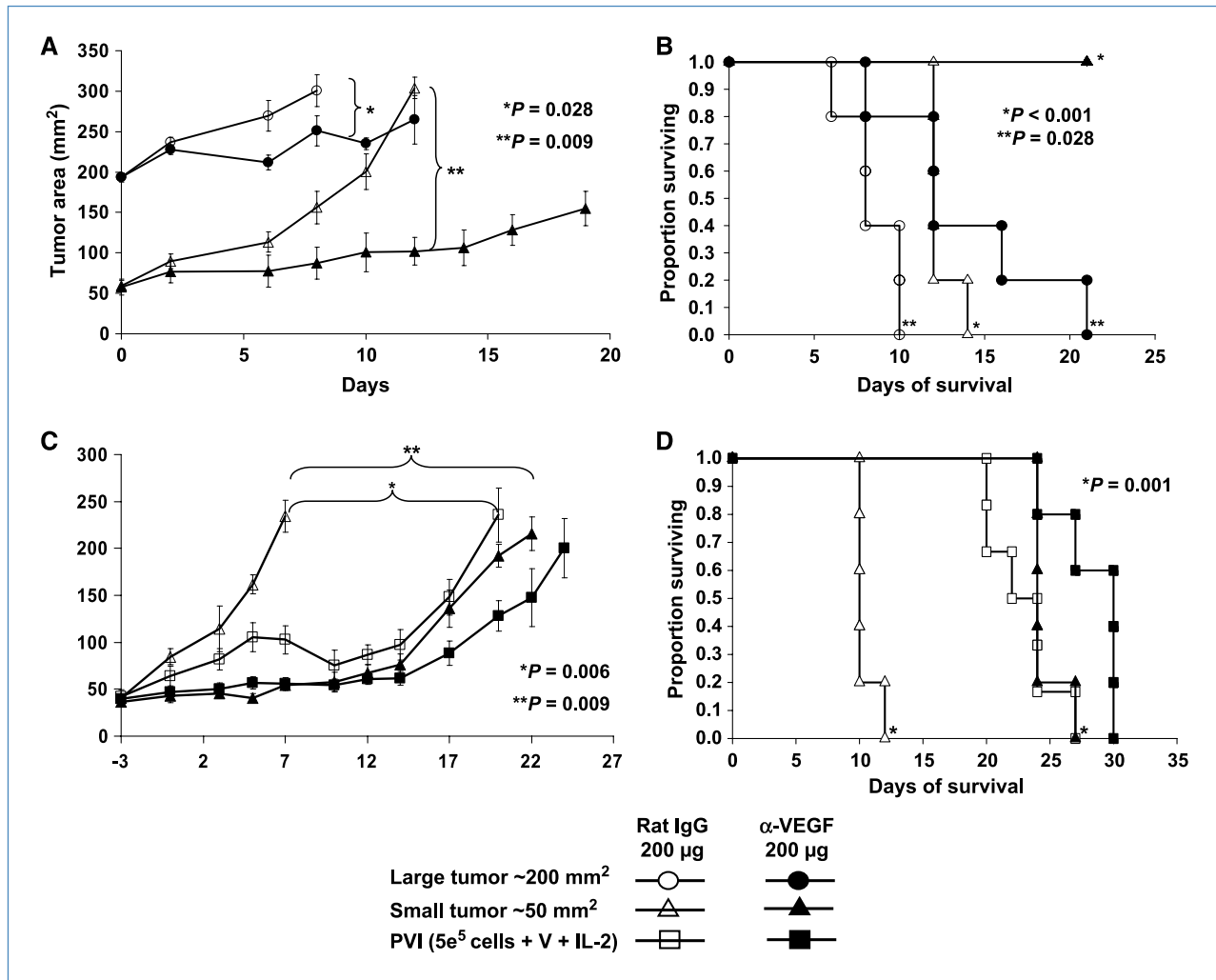
To determine whether the addition of  $\alpha$ -VEGF had any additive or synergistic impact on the therapeutic effect of ACT

in treating large established B16 tumors, we treated recipient mice with multiple doses of 100 or 200  $\mu$ g of  $\alpha$ -VEGF along with ACT immunotherapy. Two representative experiments combining ACT with the administration of anti-VEGF antibody are shown in Fig. 2. Increasing doses (four, six, and eight) of 100  $\mu$ g/dose  $\alpha$ -VEGF alone did not have an antitumor effect on large established B16 tumors nor did it prolong the survival of these mice when compared with the rat IgG control group (100  $\mu$ g/dose, eight doses). ACT with PVI alone exhibited significant antitumor activity and prolonged survival of mice (solid circles, Fig. 2A and B). Four doses of  $\alpha$ -VEGF had little effect when added to ACT, whereas six and eight doses of anti-VEGF showed significant ( $P = 0.009$ ) synergistic antitumor effects when added to ACT (Fig. 2A and B). In repeat experiments, eight doses of  $\alpha$ -VEGF consistently enhanced the antitumor effect of ACT, whereas lower dose numbers had a more variable impact.

We also investigated whether a prior lymphodepleting regimen was required for effective ACT when used in combination with  $\alpha$ -VEGF (Fig. 2C and D). Eight doses of 100  $\mu$ g/dose  $\alpha$ -VEGF alone did not have any antitumor effect on large B16 tumors when used in the presence or absence of 500 cGy radiation compared with corresponding rat IgG controls (Fig. 2C and D). ACT immunotherapy of large established B16 tumors using PVI in the absence of prior radiation failed to exhibit any antitumor effect when used with or without  $\alpha$ -VEGF (Fig. 2C). Concordant with our previous reports, host lymphodepletion by total body irradiation (500 cGy) significantly enhanced the antitumor effect of PVI. The addition of  $\alpha$ -VEGF to the PVI group receiving 500 cGy total body irradiation further enhanced the antitumor effect ( $P = 0.01$ ) and prolonged the survival of mice ( $P = 0.027$ ; Fig. 2C and D). Thus, host lymphodepleting conditioning before ACT is essential to achieve maximum antitumor activity and was further enhanced by the addition of  $\alpha$ -VEGF. Hence, in all our further experiments, we included 500 cGy of radiation as a host lymphodepleting regimen before ACT.

### Anti-VEGF treatment enhances the infiltration of pmel-1 T cells into B16 tumors

A single dose of antiangiogenic agents (bevacizumab in humans and DC101 in mice) has been reported to lead to vascular normalization in tumors (21, 22). Treatment of tumor-bearing mice with DC101 resulted in a significantly deeper penetration of TRITC-labeled BSA molecules into the tumor (22). We thus sought to determine whether the antitumor therapeutic effect of the combination therapy of ACT plus anti-VEGF was associated with an enhanced pmel-1 T-cell infiltration into the B16 tumor. Ly5.1 pmel-1 T cells were infused into Ly5.2 mice to distinguish transferred from endogenous cells. A single dose of  $\alpha$ -VEGF (100, 200, or 500  $\mu$ g) given 2 days before ACT lead to significantly enhanced infiltration of pmel-1-Ly5.1 cells into tumors. In 12 consecutive experimental determinations of cells infiltrating into tumors on days 4 to 6 after cell transfer, in mice receiving anti-VEGF antibody 2 days before ACT, there were  $32.1 \pm 6.8\%$  (mean  $\pm$  SEM) Ly5.1 pmel-1 tumor-infiltrating T cells compared with  $14.8 \pm 5.7\%$  Ly5.1 pmel T cells in mice



**Figure 1.** Antitumor effect of anti-VEGF antibody alone and in combination with ACT. A, six doses of 200 µg anti-VEGF antibody had a substantial growth-inhibitory effect on small (50 mm<sup>2</sup>) established B16 tumors ( $P = 0.009$ ) and a lesser effect on large (100 mm<sup>2</sup>) tumors ( $P = 0.028$ ). Closed symbols are groups receiving α-VEGF; open symbols are groups receiving rat IgG. B, a significant impact was also seen on the survival of mice. C, four doses of 200 µg α-VEGF exhibited a substantial antitumor effect when given alone or in combination with ACT on small B16 tumors (50 mm<sup>2</sup>). D, a significant impact was also seen on the survival of mice.

receiving control rat IgG ( $p2 = 0.0051$ , paired  $t$  test). In 10 consecutive experimental determinations, the number of Ly5.1 pmel T cells was also increased in the anti-VEGF group compared with the rat IgG control ( $12.5 \pm 2.8 \times 10^5$  versus  $5.8 \pm 1.0 \times 10^5$ ,  $p2 = 0.034$ ). Total tumor cell suspensions were gated on live Ly5.1 cells. In mice treated with a single dose of 200 or 500 µg α-VEGF, we found significantly higher Ly5.1<sup>+</sup> cell (38.4%,  $P = 0.01$  and 43.0%,  $P = 0.003$ , respectively) infiltration into B16 tumor compared with mice receiving 500 µg of rat IgG (17%) as early as day 4 post ACT (Fig. 3A). In addition, we enumerated total live Ly5.1<sup>+</sup> cells in tumor and found significantly higher number of cells in mice treated with 200 or 500 µg α-VEGF ( $4.1 \times 10^5$  cells,  $P = 0.03$  and  $2.2 \times 10^5$  cells,  $P = 0.001$ ) when compared with mice receiving 500 µg of rat IgG control ( $6.1 \times 10^4$  cells; Fig. 3B). In a repeat experiment (Fig. 3C and D), we investigated T-cell infiltration on days 3, 4, 5, and 6

post ACT. Mice receiving a single dose of 200 or 500 µg α-VEGF exhibited a higher percentage and total number of live Ly5.1<sup>+</sup> cells in the tumor when compared with the control group (Fig. 3C and D). Thus, the enhanced antitumor effects in our combination therapy were associated with and may be explained by the ability of α-VEGF administration to enhance T-cell infiltration into the B16 tumor. Of interest, no increased cell infiltration was seen when anti-VEGF antibody was given on the day of cell infiltration, suggesting that the kinetics of the impact of a single dose of anti-VEGF on tumor vasculature relative to the time of cell infiltration is critical.

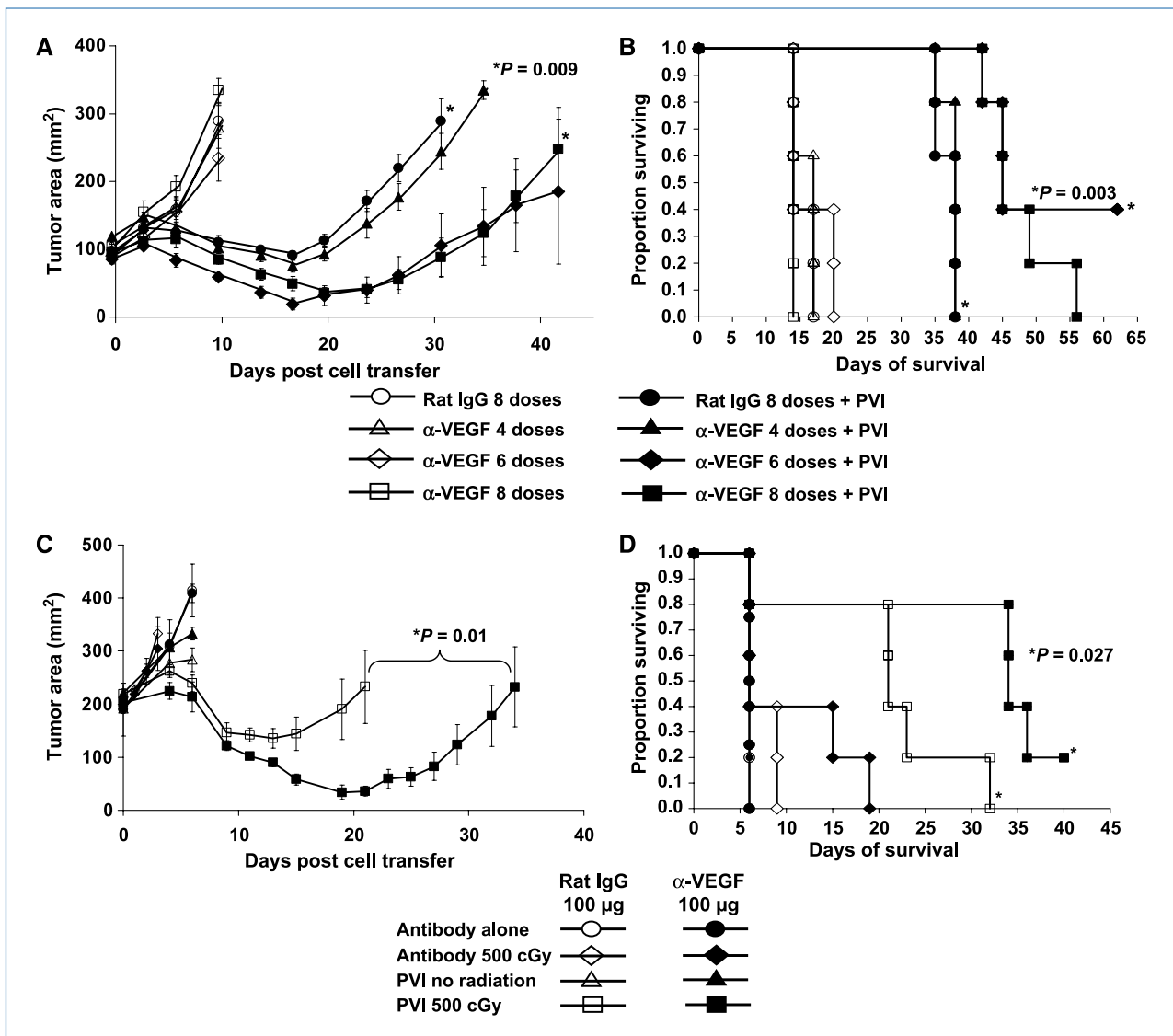
#### Effect of anti-VEGFR-2 antibodies along with ACT in treatment of B16 melanoma

Antibodies to VEGFRs also represent attractive antiangiogenic agents. DC101 (rat anti-mouse VEGFR-2) has been

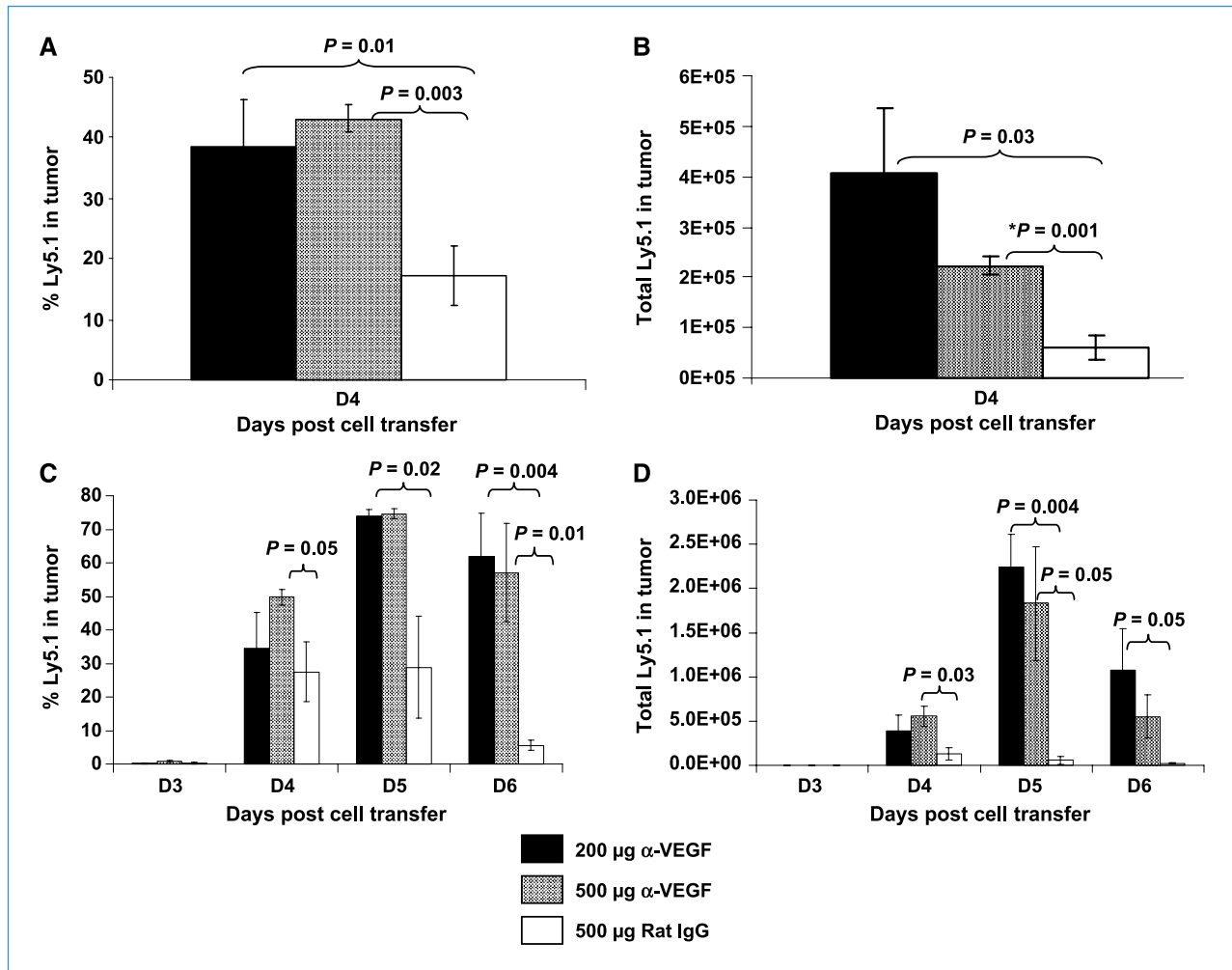
successfully used in various preclinical models as a monotherapy or combination therapy for tumor treatment (18, 22). We thus also screened the impact of DC101 administration along with ACT on established B16 tumor. In mice bearing small tumors (25–50 mm<sup>2</sup>), three doses of DC101 alone (800 µg/dose) had some antitumor effect and prolonged the survival of mice compared with the rat IgG control (Fig. 4A and B). The addition of DC101 to ACT exhibited a significant ( $P = 0.013$ ) additive antitumor effect (Fig. 4A), while no significant additive impact was observed on prolonging survival (Fig. 4B). In mice bearing

large tumors (~100 mm<sup>2</sup>), higher doses of DC101 (six doses, 800 µg/dose) alone had no antitumor effect (Fig. 4C). When DC101 was added to ACT, we observed a small but reproducible therapeutic impact (Fig. 4C,  $P = 0.028$ ) but no effect on survival (Fig. 4D). Thus, we next investigated whether treatment of tumor-bearing mice with a single dose of DC101 2 days before ACT augments enhanced cell infiltration in to B16 tumors.

In contrast to our results using anti-VEGF administration, data from eight consecutive experimental determinations revealed no impact of the administration of the DC101



**Figure 2.** Combined therapeutic effect of anti-VEGF antibody with ACT on large B16 tumor treatment. A, the administration of four, six, and eight doses of 100 µg of anti-VEGF antibody alone had no impact on the growth of these large established B16 melanomas. A synergistic antitumor effect was seen when six and eight doses of anti-VEGF antibody was given in conjunction with ACT ( $P = 0.009$ ). B, survival of mice was also improved ( $P = 0.003$ ). C, eight doses of 100 µg of α-VEGF alone had no impact on tumor growth but synergized with ACT ( $P = 0.01$ ). The impact of ACT with and without antibody administration was dependent on the administration of 500 cGy total body irradiation before the cell transfer. D, survival of mice correlated with the antitumor growth effect.



**Figure 3.** Effect of anti-VEGF antibody on pmel-1-Ly5.1 cell infiltration into B16 tumor following ACT. A, the percentage of infiltrating Ly5.1<sup>+</sup>PI<sup>-</sup> cells into B16 melanoma on day 4 post cell transfer was significantly increased in mice receiving 200 or 500 µg anti-VEGF antibody compared with mice receiving 500 µg rat IgG. B, the total number of infiltrating Ly5.1<sup>+</sup>PI<sup>-</sup> cells was also increased in mice receiving 200 and 500 µg α-VEGF compared with mice receiving 500 µg rat IgG. C and D, a repeat experiment evaluating the infiltration of transferred Ly5.1 pmel-1 cells on days 3, 4, 5, and 6 gave a similar result. Thus, prior administration of α-VEGF to B16 tumor-bearing mice receiving ACT significantly enhances tumor infiltration of the adoptively transferred pmel-1 cells.

antibody on the percentage of live Ly5.1 cells infiltrating into the tumor of mice 4 to 6 days after cell administration ( $p = 0.33$ , paired *t* test). We could, however, detect an impact on vessel area within tumors after DC101 administration. The mean tumor vessel area of mice receiving a single dose of DC101 remained relatively stable for at least 7 days, whereas vessels increased significantly in mice receiving rat IgG (Fig. 5A). Later time points were not tested. In a repeat experiment, mice receiving rat IgG showed a significantly increased ( $P = 0.0003$  and  $P = 0.001$ , respectively) mean tumor vessel area on days 3 and 4 (CD31 immunofluorescence staining) compared with mice receiving DC101 (Fig. 5B). Figure 5C represents a blinded, microscopic observation made on days 0, 3, and 4 in the first experiment. Tumor vasculature on days 3 and 4 was less in mice receiving DC101 than in those receiving rat IgG. In a preliminary experiment

done with α-VEGF and rat IgG, a similar pattern was seen (data not shown).

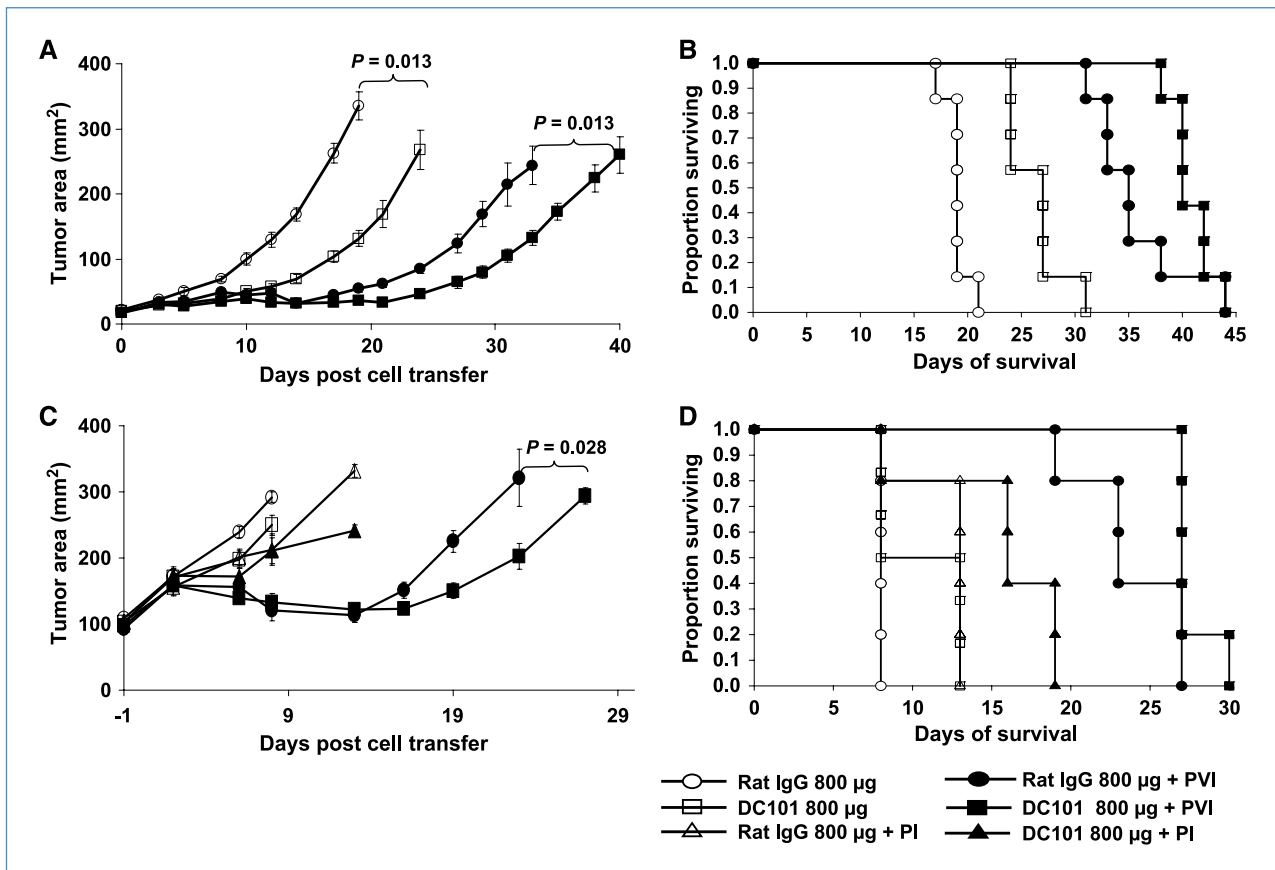
## Discussion

The adoptive transfer of antitumor T cells (ACT) can mediate tumor regression in both murine models and in patients with metastatic melanoma (1–4). A lymphodepleting preparative regimen applied before the adoptive transfer profoundly enhances the therapeutic effects of the transferred cells in part by depleting immune regulatory elements such as T regulatory cells and by eliminating competition of the transferred cells for homeostatic cytokines (1–4, 24, 26). Immunosuppressive factors produced by the tumor and the integrity of the tumor vasculature can affect the activity of lymphocytes and limit the extravasation of lymphocytes

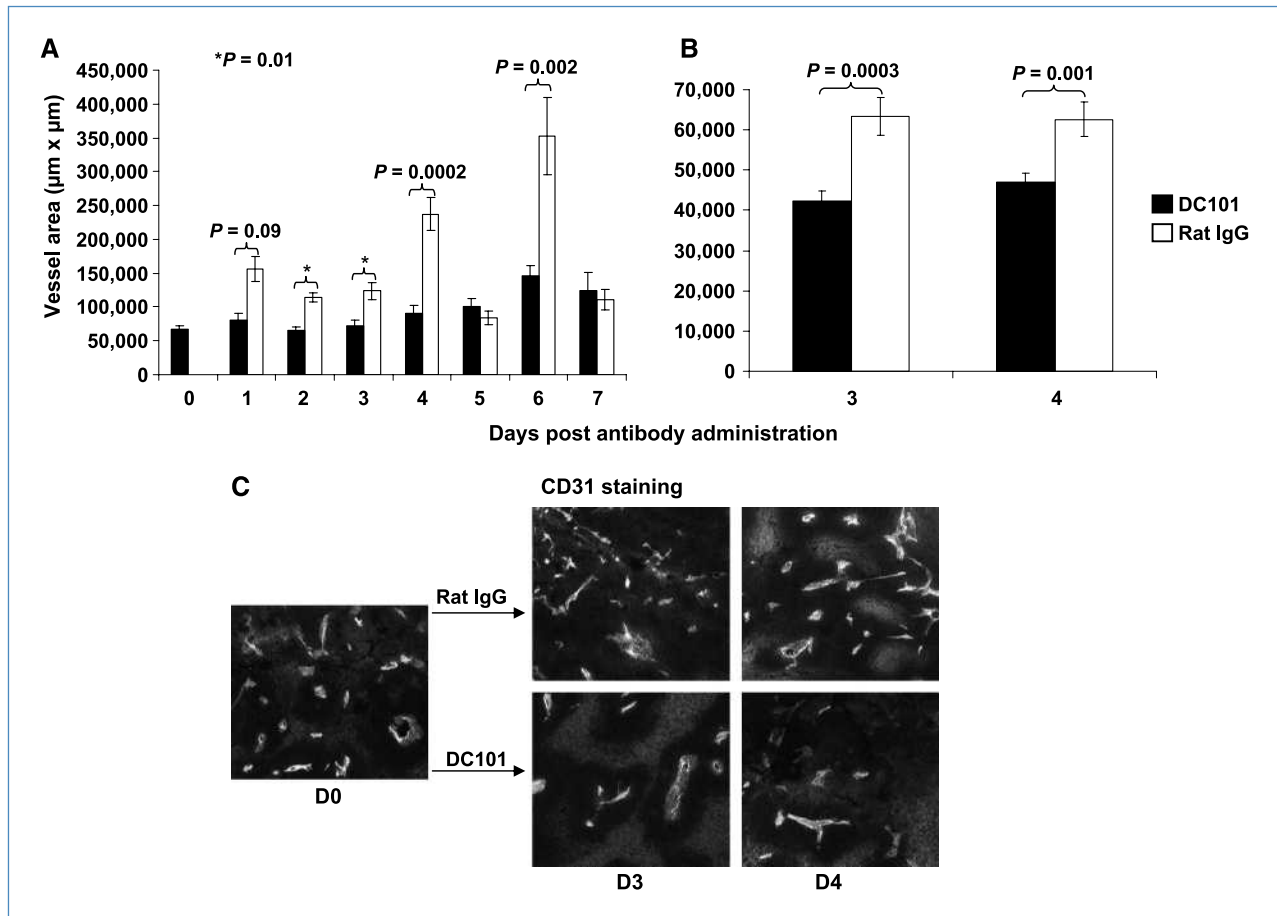
from tumor vessels into the tumor stroma. VEGF produced by tumor cells can mediate local immune suppression by interfering with the function of antigen-presenting cells and can stimulate the growth of tumor vasculature (6–12). In murine models, inhibition of VEGF or its interaction with its predominant receptor VEGFR-2 can overcome the immunosuppressive activity of VEGF (12). Interruption of the VEGF/VEGFR-2 axis can also normalize the tumor vasculature and enhance the extravasation of intravascular components into the tumor stroma. The administration of angiogenesis inhibitors can upregulate endothelial adhesion molecules in tumor vessels and increases the number of tumor infiltrating leukocytes (15). Several receptors, such as endothelin B, have been identified as a molecule that can affect lymphocyte infiltration into tumors (27). Several studies have shown that blockade of VEGF or VEGFR-2 can enhance the therapeutic effect of cancer vaccines and increase the numbers of vaccine-induced T cells that infiltrate into tumor (14, 28).

Despite these effects of VEGF blockade, antiangiogenic agents have had minimal clinical impact when used alone, an observation that is probably due to the redundancy of angiogenic pathways. However, antiangiogenic treatments have exhibited modest clinical effectiveness when administered in conjunction with chemotherapy (19–21, 29). This impact has been attributed in large part to the ability of antiangiogenic drugs to increase extravasation of chemotherapy agents into the tumor. The therapeutic effectiveness of ACT therapy in cancer patients as well as the ability of antiangiogenic agents to alter tumor vasculature in ways that could potentially increase the availability of tumor to the transferred T cells thus led us to explore the combination of these approaches in mice with large established B16 melanoma.

We thus evaluated antibodies against VEGF and VEGFR-2 for their ability to enhance ACT. Although these antibodies could inhibit the growth of small tumors (~50 mm<sup>2</sup>), they had little impact on larger tumors that were ≥100 mm<sup>2</sup> (Fig. 1). In an attempt to more closely mimic the treatment



**Figure 4.** Impact of rat anti-mouse VEGFR-2 antibody (DC101) on tumor treatment and cell infiltration in B16 tumor-bearing mice following ACT. A and B, three doses of 800 µg DC101 antibody mediated a modest growth-inhibitory effect on small established B16 melanoma compared with rat IgG alone. An additive effect of the antibody was seen when combined with ACT ( $P = 0.013$ ). Open symbols are groups receiving antibody alone; closed symbols are groups receiving antibody with ACT (PVI group). C and D, six doses of 800 µg DC101 had no impact on the growth of large (100 mm<sup>2</sup>) B16 melanoma but mediated a modest tumor-inhibitory effect when added to ACT. In accordance with our previous results, vaccination with recombinant vaccinia virus expressing hgp100<sub>25-33</sub> peptide was required to observe the tumor-inhibitory impact of ACT. Mice receiving PI (without vaccination) exhibited no antitumor effect with or without anti-VEGFR-2 antibody administration.



**Figure 5.** Antivascular effect of a single dose of anti-VEGFR-2 antibody (DC101) on B16 tumor vasculature. A and B, mean tumor vessel area of B16 tumors in mice receiving a single dose of 800 µg DC101 was decreased compared with mice receiving rat IgG on various days post antibody administration. Results for each time point are the mean of five images for each section/mice and two or five mice in each group (A and B, respectively). Two separate experiments are shown. C, representative microscopic sections from mice receiving DC101 antibody or control rat IgG. Vessel area was determined by a blinded measurement of CD31 expression. At 3 and 4 days after DC101 administration, a significant decrease in tumor vessels was seen compared with mice receiving rat IgG.

of human tumors, we confined our studies to the treatment of larger established vascularized B16 melanomas. When treating these larger tumors, the addition of anti-VEGF antibody synergized with ACT therapy. The combination was more effective than either modality used alone (Fig. 2). Prior studies have shown that a lymphodepleting regimen is an essential component of ACT therapy in this mouse model (24). In our studies, little therapeutic impact of ACT with or without anti-VEGF antibody was seen in immunocompetent mice, whereas profound combined effects were seen in mice pretreated with 500 cGy whole body irradiation. Multiple doses of anti-VEGF antibody given every 3 days were essential to observe this synergistic therapeutic effect in conjunction with ACT. A dose of 100 µg anti-VEGF antibody was as effective as higher doses, in accordance with prior reports indicating that in mice the 50 to 100 µg dose was optimal for mediating antivascular effects (16). The dose and schedule of antiangiogenic agents is a critical factor in the development of effective combination therapies (19, 20).

The mechanism of action of the anti-VEGF-mediated anti-tumor activity is a matter of some controversy. Although the inhibition of growth of tumor vessels seems to play a role, there is considerable evidence that normalization of the properties of the tumor vasculature can substantially enhance drug penetration into tumors (22, 23). Administration of antiangiogenic agents can sustain a hydrostatic pressure gradient across the tumor vasculature by decreasing interstitial fluid pressure and increasing pericyte coverage of tumor endothelium. Antiangiogenic agents can thus enhance infiltration of labeled BSA from the vasculature into the tumor stroma (22). To study the mechanism of action of the combination of cell transfer therapy with anti-VEGF therapy, we explored whether this combination treatment could have an impact on the extravasation of transferred antitumor T cells into the tumor. These experiments clearly showed an increased percentage ( $p2 = 0.0051$ ) and absolute number ( $p2 = 0.034$ ) of transferred T cells into tumor that was even apparent at the lowest dose of anti-VEGF used (100 µg per dose; Fig. 3).



The effectiveness of anti-VEGF administration to improve ACT led us to investigate the effects of the administration of the anti-VEGFR-2 antibody DC101. As was the case for the use of anti-VEGF antibody, the administration of anti-VEGFR-2 antibody could mediate an antitumor effect on small tumors (50 mm<sup>2</sup>), but there was little to no impact on large tumors (Fig. 4). There was, however, a modest additive effect of anti-VEGFR-2 antibody in conjunction with ACT. In these experiments, there was no increase in infiltration of the adoptively transferred cells into tumor ( $p = 0.33$ ; Fig. 4E), possibly accounting for the lesser impact of this antibody compared with anti-VEGF. This may also be, in part, due to the ability of anti-VEGF antibody to block the binding of VEGF to both VEGFR-1 and VEGFR-2, whereas anti-VEGFR-2 antibody could only block VEGFR-2 binding. In these experiments, the anti-VEGFR-2 antibody had some impact on angiogenesis because it was able to limit the growth of tumor vasculature following a single dose of antibody (Fig. 5).

It should be noted that the ability of anti-VEGF antibody to inhibit tumor growth in conjunction with cell transfer required the administration of multiple doses of antibody (Fig. 2A and B) despite the demonstration that increased infiltration of the transferred lymphocytes could be seen after a single dose of antibody. This implies that prolonged impact of anti-VEGF antibody on the tumor vasculature is required

to observe the antitumor effect. Successful synergy of anti-VEGF antibody with chemotherapy in humans also requires prolonged anti-VEGF administration (29). Anti-VEGF antibody may also be manifesting antitumor effects in conjunction with cell transfer by inhibiting the known immunosuppressive impact of VEGF (6–11).

The studies reported here show that agents capable of blocking the VEGF/VEGFR-2 axis can enhance the antitumor activity of adoptively transferred antitumor T cells. The therapeutic effectiveness of ACT in patients with metastatic melanoma and the ready availability of clinically approved antiangiogenic agents provide a rationale for the exploration of this combination therapy in the treatment of human cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We thank Douglas Palmer, Lindsay Garvin, Robert Reger, and David Jones for helping us with the animal studies; Arnold Mixon and Shawn Farid for help with flow cytometry; and Don White for statistical analysis.

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.

Received 01/15/2010; revised 05/14/2010; accepted 05/26/2010; published OnlineFirst 07/14/2010.

### References

- Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol* 2009;21:233–40.
- Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–4.
- Dudley ME, Yang JC, Sherry R, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008;26:5233–9.
- Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006;314:126–9.
- Rosenberg SA. Overcoming obstacles to the effective immunotherapy of human cancer. *Proc Natl Acad Sci U S A* 2008;105:12643–4.
- Ohm JE, Carbone DP. VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res* 2001;23:263–72.
- Mulligan JK, Day TA, Gillespie MB, Rosenzweig SA, Young MR. Secretion of vascular endothelial growth factor by oral squamous cell carcinoma cells skews endothelial cells to suppress T-cell functions. *Hum Immunol* 2009;70:375–82.
- Folkman J. Angiogenesis. *Annu Rev Med* 2006;57:1–18.
- Johnson BF, Clay TM, Hobeika AC, Lyerly HK, Morse MA. Vascular endothelial growth factor and immunosuppression in cancer: current knowledge and potential for new therapy. *Expert Opin Biol Ther* 2007;7:449–60.
- Ohm JE, Gabrilovich DI, Sempowski GD, et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood* 2003;101:4878–86.
- Gabrilovich D, Ishida T, Oyama T, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages *in vivo*. *Blood* 1998;92:4150–66.
- Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996;2:1096–103.
- Osada T, Chong G, Tansik R, et al. The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. *Cancer Immunol Immunother* 2008;57:1115–24.
- Li B, Lalani AS, Harding TC, et al. Vascular endothelial growth factor blockade reduces intratumoral regulatory T cells and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. *Clin Cancer Res* 2006;12:6808–16.
- Dirx AE, oude Egbrink MG, Castermans K, et al. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors. *FASEB J* 2006;20:621–30.
- Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993;362:841–4.
- Gerber HP, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. *Cancer Res* 2005;65:671–80.
- Prewett M, Huber J, Li Y, et al. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res* 1999;59:5209–18.
- Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* 2006;3:24–40.
- Crawford Y, Ferrara N. VEGF inhibition: insights from preclinical and clinical studies. *Cell Tissue Res* 2009;335:261–9.
- Willet CG, Boucher Y, di Tomaso E, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004;10:145–7.
- Tong RT, Boucher Y, Kozin SV, et al. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a

- pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* 2004;64:3731–6.
23. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307:58–62.
  24. Overwijk WW, Theoret MR, Finkelstein SE, et al. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 2003;198:569–80.
  25. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 2005;115:1616–26.
  26. Wrzesinski C, Paulos CM, Kaiser A, et al. Increased intensity lymphodepletion enhances tumor treatment efficacy of adoptively transferred tumor-specific T cells. *J Immunother* 2010;33:1–7.
  27. Buckanovich RJ, Facciabene A, Kim S, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat Med* 2008;14:28–36.
  28. Manning EA, Ullman JG, Leatherman JM, et al. A vascular endothelial growth factor receptor-2 inhibitor enhances antitumor immunity through an immune-based mechanism. *Clin Cancer Res* 2007;13:3951–9.
  29. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–42.