

# Antibodies to Vascular Endothelial Growth Factor Enhance the Efficacy of Cancer Immunotherapy by Improving Endogenous Dendritic Cell Function<sup>1</sup>

Dmitry I. Gabrilovich,<sup>2</sup> Tadao Ishida,  
Sorena Nadaf, Joyce E. Ohm, and  
David P. Carbone

Department of Medicine and The Vanderbilt Cancer Center,  
Vanderbilt University School of Medicine, Nashville, Tennessee  
37232-6838

## ABSTRACT

Inadequate function of dendritic cells (DCs) in tumor-bearing hosts is one mechanism of tumor escape from immune system control and may compromise the efficacy of cancer immunotherapy. Vascular endothelial growth factor (VEGF), produced by most tumors, not only plays an important role in tumor angiogenesis but also can inhibit the maturation of DCs from hematopoietic progenitors. Here, we investigate a novel combination of antiangiogenic and immunotherapy based on this dual role of VEGF. Two s.c. mouse tumor models were used: D459 cells, expressing mutant human p53; and MethA sarcoma with point mutations in the endogenous murine p53 gene. Therapy with anti-mouse VEGF antibody (10  $\mu$ g i.p. twice a week over 4 weeks) was initiated when tumors became palpable. Treatment of established tumors with anti-VEGF antibody alone did not affect the rate of tumor growth. However, anti-VEGF antibody significantly improved the number and function of lymph node and spleen DCs in these tumor-bearing animals. To investigate the possible effects of this antibody on the immunotherapy of established tumors, tumor-bearing mice were immunized with DCs pulsed with the corresponding mutation-specific p53 peptides, together with injections of anti-VEGF antibody. Therapy with peptide-pulsed DCs alone resulted in considerable slowing of tumor growth but only during the period of treatment, and tumor growth resumed after the end of the therapy. Combined treatment with peptide-pulsed DCs and anti-VEGF antibody resulted in a prolonged and much more pronounced antitumor effect. This effect was associated with the

induction of significant anti-p53 CTL responses only in this group of mice. These data suggest that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer.

## INTRODUCTION

The success of cancer immunotherapy depends not only on the choice of appropriate tumor antigens and methods of antigen delivery but also on the ability of the patient's immune system to produce an effective and sustained response to immunization. Host professional antigen-presenting cells play a key role in this process by taking up vaccine antigens, processing and presenting them on class I and II MHC molecules, producing cytokines, and stimulating the production of activated antigen-specific cytotoxic effector cells. In addition, because tumor cells are inefficient stimulators of T-cell proliferation, the continuous presence of effective host antigen presentation may be needed to maintain an effective response. The most potent of these antigen-presenting cells is the tissue DC.<sup>3</sup> These cells are responsible for the induction of primary immune responses and are very effective in the stimulation of secondary responses as well (1).

The function of the immune system in tumor-bearing hosts is often seriously compromised (reviewed in Refs. 2 and 3). Defective antigen presentation by DCs is one of the mechanisms of immune dysfunction in cancer and is observed both in tumor-bearing mice and in cancer patients (4–9). We and others have demonstrated previously that defective DC differentiation caused by tumor-derived factors (10–15) contributes to the observed defective DC function in tumor-bearing hosts. One of these factors was found to be a VEGF (11). VEGF is induced by hypoxia and produced constitutively by almost all tumors. It directly stimulates the growth of vascular endothelial cells and the formation of tumor neovasculature (reviewed in Ref. 16). In animal models, we have shown that VEGF infusion results in a dramatic decrease in DC production associated with hyperproduction of immature myeloid cells (17). We have shown previously that neutralizing anti-VEGF antibody is able to abrogate the negative effects of tumor cell supernatants on DC differentiation from progenitors *in vitro* (11). This suggests that blockade of VEGF signaling may be an approach for improving DC function and hence immune system function and the efficacy of cancer immunotherapy in tumor-bearing hosts *in vivo*. Here, we tested this hypothesis in two experimental tumor models using mutated p53 as a defined tumor antigen: a poorly immunogenic

Received 3/25/99; revised 7/28/99; accepted 8/2/99.

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.

<sup>1</sup> Supported by NIH Grant CA76321 (to D. P. C.), pilot project funding (to D. I. G.) from Vanderbilt Cancer Center Core Grant CA68485, and American Cancer Society Institutional Grant IN 25-37.

<sup>2</sup> To whom requests for reprints should be addressed, at Cardinal Bernardin Cancer Center, Loyola University Medical Center, Building 112, Room 203, Maywood, IL 60153. Phone: (708) 327-3130; Fax: (708) 327-3238; E-mail: dgabril@luc.edu.

<sup>3</sup> The abbreviations used are: DC, dendritic cell; DCc, DCs pulsed with control peptides; DCsp, DCs pulsed with the D459 mutant p53-specific peptide; VEGF, vascular endothelial growth factor.

sarcoma (D459) expressing mutant human p53; and the relatively immunogenic MethA sarcoma expressing an endogenous mutant murine p53 (18–21). We show that concurrent administration of antibodies to VEGF improves both the function of host DCs in mice bearing these tumors and the antitumor efficacy of immunotherapy using specific mutant p53 peptides. These data thus may serve as proof of concept that this novel combined therapy with two classes of therapeutics may be of clinical utility.

## MATERIALS AND METHODS

### Animals

Female BALB/c and CBA mice, 6–8 weeks of age, were purchased from Harlan, Inc. (Indianapolis, IN) and were housed in specific pathogen-free units of the Division of Animal Care at Vanderbilt University Medical Center.

### Cell Lines and Antibodies

Two tumor cell lines were used. The tumor cell line D459 was constructed by transfection of BALB/c 3T3 cells with EJ *ras* and a mutant human p53 expression vector. Details of this cell lines were described elsewhere (10, 18). MethA sarcoma cells were obtained from Dr. L. J. Old. This is a transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passaged as an ascitic tumor (21). The following antibody-producing hybridomas were obtained from American Type Culture Collection (Rockville, MD) and used as culture supernatants: anti-CD4 (L3T4, TIB-207), anti-CD8 (Lyt-2.2, TIB-210), and anti-MHC class II (I-A<sup>d,k</sup>, HB-120). Goat anti-mouse immunoglobulin was purchased from Sigma Chemical Co. (St. Louis, MO). FITC- and phycoerythrin-labeled antibodies used in flow cytometry were purchased from PharMingen (San Diego, CA): anti-CD11c, anti-CD86 (B7-2), and anti-I-A<sup>d</sup>. FITC- and phycoerythrin-conjugated IgG was used in control. Neutralizing goat anti-mouse VEGF antibody was purchased from R&D Systems (Minneapolis, MN). Control goat immunoglobulin was obtained from Sigma.

### Cell Preparation

Cells were prepared as described earlier (4). Briefly, a single-cell lymph node suspension was prepared from inguinal, axillary, and brachial lymph nodes by pressing the tissues through wire mesh. Cells were washed and then layered onto a metrizamide (Nygaard, Oslo, Norway) gradient (14.5 g plus 100 ml of RPMI 1640) and centrifuged for 10 min at 600 × *g*. Cells at the interface were washed once and resuspended in complete culture medium (RPMI 1640; Life Technologies, Inc., Gaithersburg, MD with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1 × 10<sup>-5</sup> M 2-mercaptoethanol, and 10% FCS; HyClone, Logan, UT). DCs were identified by their distinctive morphology and by labeling with N418 antibody. In control mice, the purity of DCs was >50%. For functional tests, DCs were additionally purified using a mixture of anti-T cells (TIB 207 and TIB 210) and anti-B cells (anti-mouse immunoglobulin) antibodies and complement (Low-Tox-M complement; Cedarline, Hornby, Ontario, Canada). Dead cells were removed by centrifugation on a Lympholyte-M gradient (Cedarline). The purity of this DC fraction was >90% with >95% viability. Pelleted cells from the

lymph node suspension were passed through nylon wool columns to obtain >90% pure T cells.

To obtain DCs from spleens, single-cell suspensions of splenocytes were incubated overnight in complete culture medium. The DC-containing fractions were enriched by centrifugation of nonadherent cells on metrizamide gradient. DCs were identified by their distinctive morphology and by labeling with N418 antibody. For functional tests, DCs were additionally purified as described above to obtain >80% purity.

For immunization, DCs were generated from bone marrow as described earlier (10). Briefly, bone marrow cells obtained from femurs and tibias were placed in six-well plates (Falcon, Becton Dickinson) at a concentration of 5–8 × 10<sup>5</sup> cells/ml in complete culture medium supplemented with 3 ng/ml recombinant murine granulocyte/macrophage-colony stimulating factor and 5 ng/ml recombinant murine interleukin 4 (R&D, Systems, Minneapolis, MN). After 4 days, one-half of the medium was removed after gentle swirling and replenished with an equivalent volume of fresh granulocyte/macrophage-colony stimulating factor and interleukin 4 supplemented media. Four to 5 days later, clusters of DCs were dislodged. Purity of DCs was >60% with a viability >90%.

### Tumor Induction and Immunization

Two hundred thousand D459 cells or 6 × 10<sup>5</sup> MethA sarcoma cells were injected s.c. into the shaved backs of BALB/c mice. These doses of tumor cells were selected after preliminary experiments to result in tumor formation in 100% of the mice. Palpable tumors were usually formed 7–10 days after tumor inoculation. DCs generated from the bone marrow of control healthy mice were pulsed for 2 h at 37°C with 10 μM of the appropriate peptides and then washed in PBS. DCs (1–2 × 10<sup>5</sup>) were injected i.v. Immunizations with peptide-pulsed DCs were started when tumors reached 5 mm in diameter (usually 12–14 days after tumor inoculation).

For the D459 tumor model, T1272 mutant p53 peptide (TYSALNKMFYQLAKTCPVQL) was used. This peptide contains a mutation of cysteine to tyrosine in position 135 corresponding to the mutant p53 gene expressed in D459 cells. An irrelevant p53 peptide STPPPGTRFRAMAIYKQS was used as a control. Immunization of control BALB/c mice with T1272, but not control peptide, elicits specific antimutant p53 CTL responses (4, 18, 19).

For the MethA sarcoma model, two peptides have been used. The peptide 234CM (KYICNSSCM), which contains isoleucine at position 3, corresponds to the sequence that results from a point mutation in codon 234 of p53. The peptide 234CW (KYMCNSSCM) is the wild-type counterpart of 234CM. Immunization of mice with 234CM, but not 234CW, resulted in potent specific CTL and antitumor immune responses (21). All peptides were purchased from QCB, Inc. (Hopkinton, MA).

### T-Cell Proliferation Assay

**Allogeneic Mixed Leukocyte Reaction.** DCs were obtained from lymph nodes or spleens as described above. Cells were irradiated (2500 cGy) and incubated in triplicate for 3 days with 5 × 10<sup>4</sup> T cells obtained from allogeneic CBA mice. Different DCs:T cell ratios (from 1:50 to 1:400) were used. The

cultures were pulsed overnight with 1  $\mu$ Ci of [ $^3$ H]thymidine (Amersham, Arlington Heights, IL). Cells were harvested using a cell harvester (Skatron Instruments, Sterling, VA). [ $^3$ H]Thymidine uptake was counted using a liquid scintillation counter.

**Antigen-specific T-Cell Proliferation.** DCs were infected with 250 HAU/ml influenza virus (strain PR8: A/Puerto Rico/8/34; American Type Culture Collection, Rockville, MD) for 2 h in serum-free medium. Cells were washed and incubated in triplicate with  $5 \times 10^4$  T cells obtained from syngeneic BALB/c mice for 4 days. Different DCs:T cell ratios (from 1:10 to 1:80) have been used. The cultures were pulsed overnight with 1  $\mu$ Ci of [ $^3$ H]thymidine (Amersham) and counted as described above.

### CTL Assay

Spleen cells ( $2 \times 10^6$ /ml) obtained from immunized mice were cultured with 10  $\mu$ M of specific T1272 peptide in 24-well plates for 7 days. After that time, cells were washed and incubated at different ratios with untreated or T1272 peptide pulsed  $^{51}$ Cr-labeled target P815 cells. After a 6-h incubation, supernatants were harvested, and radioactivity was measured using a gamma counter. The maximum and spontaneous release and the percentage of specific  $^{51}$ Cr release were determined as described (22).

### Statistical Analysis

Statistical analysis was performed with parametric and nonparametric methods using JMP statistical software (SAS Institute, Cary, NC).

## RESULTS

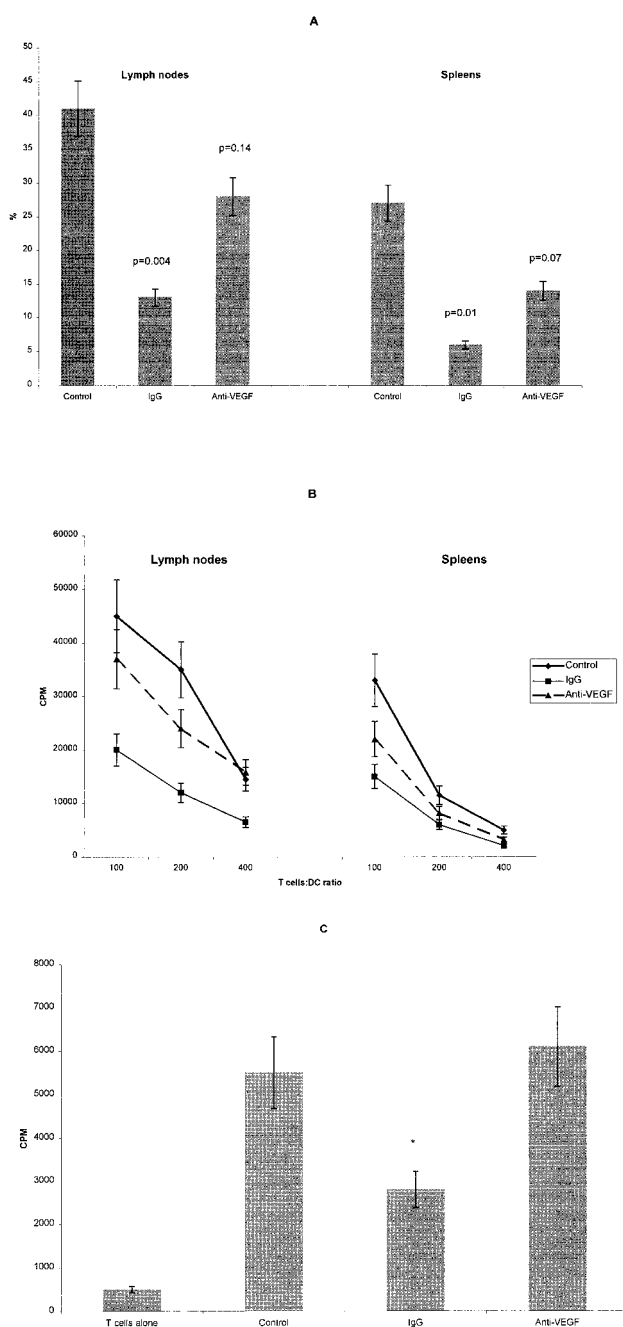
To estimate the *in vivo* level of VEGF production, mice were inoculated s.c. with  $2 \times 10^5$  D459 cells or  $6 \times 10^5$  MethA cells. The level of VEGF in plasma was measured using ELISA (R&D Systems) when tumors reached 1.5 cm in diameter. The plasma concentrations of VEGF in five mice bearing D459 tumors ranged from 100 to 300 pg/ml and in five mice with MethA sarcomas from 150 to 600 pg/ml. These concentrations are similar to those reported for other tumor models and in patients with cancer (23–25).

Anti-VEGF antibody was injected i.p. twice per week for 4 weeks. This schedule was selected based on data reported previously with anti-VEGF antibody (26, 27). Because direct anti-tumor effects of VEGF blockade could possibly complicate the interpretation of our data and tumor regression by any means may improve DC function, we tried to select a dose of antibody that was not able to directly affect the growth of the tumors used in these studies. Three doses of the antibody were tested: 2  $\mu$ g, 5  $\mu$ g, and 10  $\mu$ g per mouse. We tested whether anti-VEGF antibody was able to directly inhibit tumor growth at these doses. Mice were injected with D459 cells or MethA sarcoma cells, and when tumors reached 4–5 mm in diameter, treatment with anti-VEGF antibody was initiated. A control group of mice were treated with the same doses of goat IgG. In five independently performed experiments (four mice per group), anti-VEGF antibody at these doses did not measurably affect the growth of these established tumors (data not shown). The 10- $\mu$ g dose was used in all subsequent experiments. Four-week treatment of

MethA sarcoma-bearing mice with anti-VEGF antibody significantly reduced VEGF levels in plasma to  $88.6 \pm 11.4$  pg/ml from the  $422.7 \pm 140.4$  pg/ml detected in tumor-bearing mice treated with goat IgG ( $P < 0.01$ ). This level was still almost 10-fold higher than the VEGF level in control, tumor-free animals ( $8.6 \pm 4.5$  pg/ml;  $P < 0.001$ ).

**Anti-VEGF Antibody Improves the Number and Function of DCs in Tumor-bearing Mice.** To investigate the effect of anti-VEGF antibody on DC function, mice were inoculated with  $2 \times 10^5$  D459 cells. Treatment with anti-VEGF antibody was initiated when tumors reached 4–5 mm in diameter (usually 10–12 days after tumor inoculation). Mice were treated with goat IgG or goat anti-mouse VEGF antibody, i.p. twice per week for 4 weeks. After that time (when tumors had reached a size of 300–400 mm $^2$ ) mice were sacrificed, DC fractions were isolated from lymph nodes and spleen, and cells were analyzed morphologically, phenotypically, and functionally. DC fractions isolated from tumor-bearing mice expressed lower levels of MHC class II $^+$  and B7-2 $^+$  cells. This was in agreement with observations published previously (4, 6, 8). To quantify the presence of typical DCs in lymph nodes and spleen, cells were labeled with CD11c and B7-2 antibodies. Double-positive cells represent a population of relatively mature DCs. The proportion of morphologically distinct and CD11c $^+$  B7-2 $^+$  DCs were analyzed. Tumor-bearing mice treated with IgG had a 3-fold reduced fraction of DC in lymph nodes and an almost 5-fold reduction in the spleen compared with control animals. This decrease was much less pronounced in tumor-bearing mice treated with anti-VEGF antibody (Fig. 1A). This decrease in the proportion of DCs in tissues resulted in a dramatically reduced ability of these cells to stimulate allogeneic T cells (Fig. 1B) and antigen-specific primary T-cell response (Fig. 1C). Treatment of mice with anti-VEGF antibodies significantly improved these functions (Fig. 1, B and C). Although the level of T-cell stimulation in these mice was still lower than in control animals, those differences were not statistically significant.

**Anti-VEGF Antibody Improves the Efficacy of Cancer Immunotherapy.** We then asked whether improved DC function resulted in more effective immunotherapy. We tested the effect of anti-VEGF antibody on the effectiveness of immunotherapy of the poorly immunogenic D459 tumor. Tumor cells ( $2 \times 10^5$ ) were injected s.c. into the shaved back of mice. When tumors reached 4–5 mm in diameter, mice were split into four groups. Each group included 10 mice (two independent experiments with five mice per group). A control group was treated with DCc and treated with goat IgG. The second group had mice treated with DCc and anti-VEGF antibody. The third group was immunized with T1272 (DCsp), along with goat IgG. The fourth group was treated with DCsp and anti-VEGF antibody. DCs ( $1-2 \times 10^5$  cells) were injected i.v. on days 0, 4, and 7 after the start of the treatment. Anti-VEGF antibody or goat IgG (10  $\mu$ g/mouse) was injected i.p. twice per week for 4 weeks. Tumor growth was significantly ( $P < 0.05$ ) slower in mice immunized with the specific peptide (DCsp) during the first 2 weeks of the treatment (Fig. 2A). However, in mice treated with DCsp and control IgG, it resumed quickly after that. These results are consistent with previously reported data that the effects of immunotherapy of poorly immunogenic tumors is often transient (10, 19, 28). Combined treatment of DCsp and anti-VEGF



**Fig. 1** Effects of anti-VEGF antibody on the presence and function of DCs in tumor-bearing mice. **A**, effects of anti-VEGF antibody on the presence of DCs in lymph nodes and spleens. Lymph nodes and spleens were obtained from control tumor-free mice (*Control*), D459 tumor-bearing mice after 4 weeks of treatment with either goat IgG (*IgG*), or anti-VEGF antibody (*Anti-VEGF*). The presence of DCs was determined based on morphological criteria and the surface expression of CD11c and B7-2 markers. The cumulative results of three experiments are shown. *P*s demonstrate differences from control (tumor-free) group. **B**, effects of anti-VEGF antibody on the ability of DCs from tumor-bearing mice to stimulate allogeneic T-cell proliferation. Purified DCs were obtained from lymph nodes or spleens as described in "Materials and Methods." Cells were irradiated (2500 cGy) and incubated in triplicates for 3 days with  $5 \times 10^4$  T cells obtained from allogeneic CBA mice. The cultures were pulsed overnight with  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine

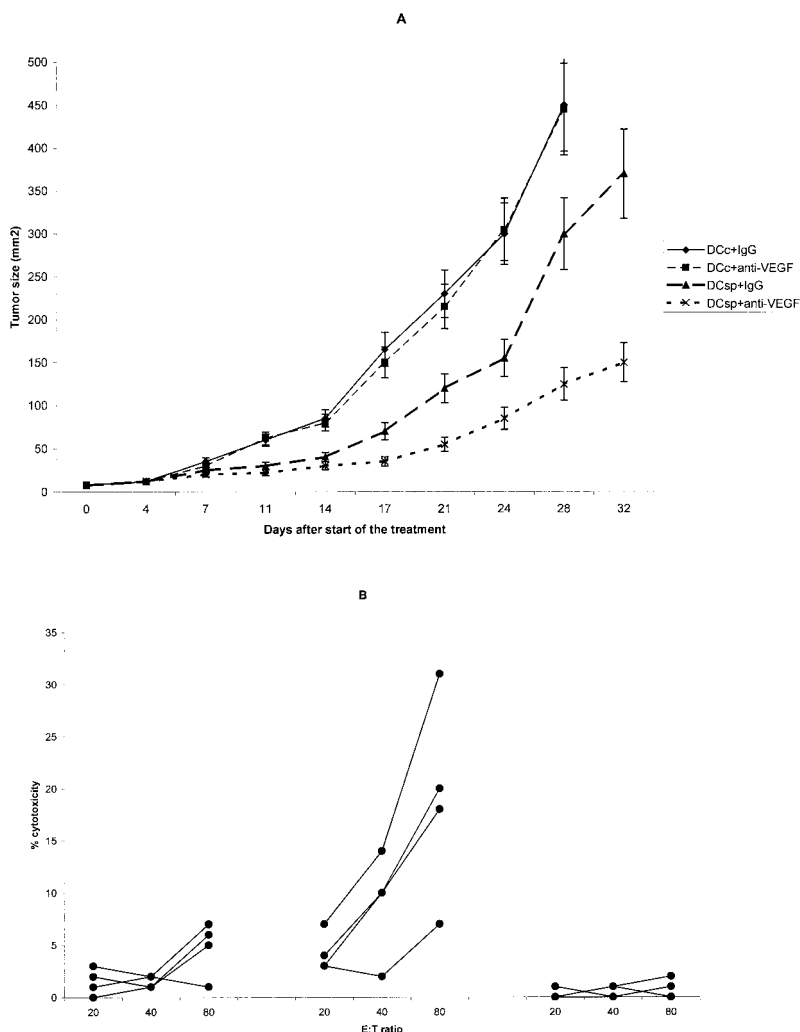
antibody resulted in a more pronounced and much longer lasting decrease in tumor growth rate. This effect persisted during the entire period of observation (32 days; Fig. 2A).

We asked whether this effect was associated with an improved p53-specific CTL response. Spleen cells obtained from mice on day 28 of the treatment were restimulated with the mutant p53-specific T1272 peptide, and CTL activity was measured against unpulsed or T1272 peptide-pulsed P815 target cells. CTL activity was measured in four mice/group. The level of nonspecific killing (nonpulsed targets) was subtracted in each case. As expected, mice from the control group (DCc+IgG) did not demonstrate specific CTL activity (Fig. 2B). Very little CTL activity was found in immunized mice treated with control IgG (DCsp+IgG). However, three of four mice in the group immunized with the specific peptide and treated with anti-VEGF antibody (DCsp+aC-VEGF) demonstrated significant levels of CTL activity (Fig. 2B).

We then studied the effect of anti-VEGF antibodies in another tumor model (the relatively immunogenic MethA sarcoma). We tested the effect of a large number of tumor cells ( $6 \times 10^5$ ). At this dose, tumor was developed in 100% of mice without any episodes of spontaneous tumor regression. The treatment was initiated when tumors reached 4–5 mm in diameter (within 6–8 days after tumor inoculation). Mice were divided into four groups. The treatment was essentially the same as for the D459 tumor model, except that the MethA mutant 234CM p53 peptide was used in DCsp group, and the corresponding wild-type 234CW p53 peptide was used in the DCc group. Tumor growth was almost blocked in mice immunized with the specific peptide (DCsp) during the first 2 weeks of treatment. However, 10 days after the end of therapy, tumor growth resumed (Fig. 3A). Tumor growth in mice receiving combined therapy with anti-VEGF antibody was significantly slower than in the DCsp+IgG group, and survival was significantly prolonged ( $P < 0.05$ ). The experiments described previously were repeated with  $2.5 \times 10^5$  MethA sarcoma cells. Tumor growth in mice immunized with specific peptide was significantly slower than in DCc-immunized animals. By day 22, the average tumor size in the group treated with DC CM234+IgG was only one-third of that in groups treated with DCc+IgG or anti-VEGF antibody alone (Fig. 3B). The addition of anti-VEGF antibody dramatically improved the effectiveness of this immunotherapy. In three of five mice, the tumor completely regressed, and tumor growth in the fourth mouse was considerably slowed. Only one mouse from this group exhibited a tumor similar in size to those in the DC CM234+IgG group (Fig. 3B).

and harvested using a cell harvester. [ $^3\text{H}$ ]Thymidine uptake was counted using a liquid scintillation counter. Typical results of one of three performed experiments are shown. **C**, effects of anti-VEGF antibody on the ability of DCs to stimulate antigen-specific T-cell proliferation. DCs were infected with influenza virus for 2 h in serum-free medium. Cells were washed and incubated in triplicate with  $5 \times 10^4$  T cells obtained from syngeneic BALB/c mice for 4 days. Data from a DC:T cell ratio of 1:20 is shown. The cultures were pulsed overnight with  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (Amersham) and counted as described above. Typical results of one of three experiments are shown. \*, statistically significant differences ( $P < 0.05$ ) from control. A–C: bars, SE.

**Fig. 2** Anti-VEGF antibody improves the effectiveness of immunotherapy in the D459 tumor model. **A**, effects of anti-VEGF antibody on tumor growth. D459 cells ( $2 \times 10^5$ ) were inoculated s.c. When tumors reached 4–5 mm in diameter (day 0), mice were treated with DCs pulsed with the specific or control peptides in addition to control IgG or anti-VEGF antibody as described in the text. DCs ( $1-2 \times 10^5$ ) were injected i.v. on days 0, 4, and 7. Ten  $\mu\text{g}$  of IgG or anti-VEGF antibody was injected i.p. twice a week from day 0 to day 26. Each group included 10 mice. Bars, SE. **B**, CTL responses in mice treated with anti-VEGF antibody. Spleen cells ( $2 \times 10^6/\text{ml}$ ) obtained from treated mice on day 28 were cultured with 10  $\mu\text{M}$  of specific T1272 peptide in 24-well plates for 7 days. After that time, cells were washed and incubated in duplicate at different ratios with untreated or T1272 peptide-pulsed,  $^{51}\text{Cr}$ -labeled target P815 cells. After a 6-h incubation, supernatants were harvested, and radioactivity was measured using a gamma counter as described in "Materials and Methods." Individual results from four mice in each group are presented. The background level of killing (3–7%; unpulsed P815 target cells) was subtracted in each case.

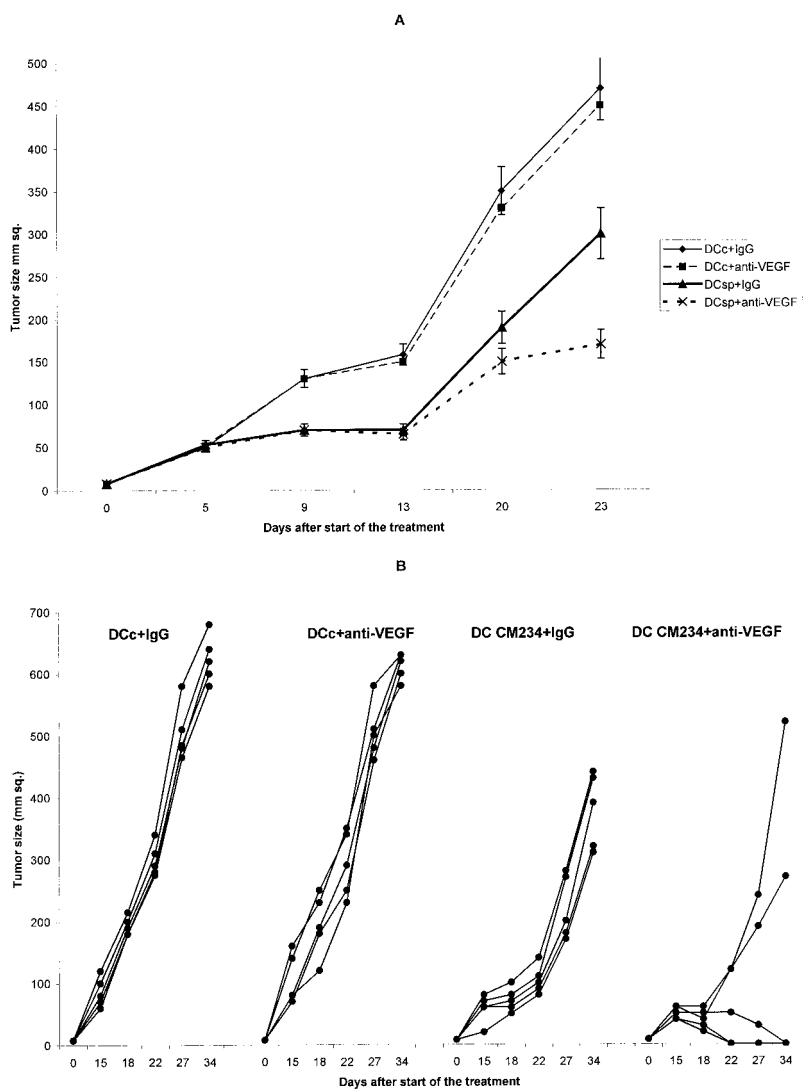


## DISCUSSION

Defects in the immune system in tumor-bearing hosts contribute greatly to immune nonresponsiveness to tumors. Defective function of professional antigen-presenting cells, DCs, is one of the factors responsible for this immune nonresponsiveness. We and others have previously demonstrated that tumor-derived factors may affect the normal process of DC differentiation from progenitor cells, resulting in the production of immature cells that are not able to effectively stimulate antitumor immune responses (10–15). VEGF is one of the factors implicated in these defects (11, 17, 29). VEGF is produced by almost all tumors and is the major factor responsible for the formation of tumor neovasculature (reviewed in Ref. 16). *In vitro* data suggested that blockade of VEGF receptors on hematopoietic progenitors may improve the function of DCs in tumor-bearing hosts. In this study, we for the first time address the question whether these *in vitro* findings can be reproduced *in vivo* and whether blockade of VEGF effects may, in turn, result in enhanced antitumor responses to immunotherapy. To test this hypothesis, we selected two animal tumor models: the poorly

immunogenic D459 tumor and the relatively immunogenic MethA sarcoma. Both of these tumors produce moderate levels of VEGF.

There are several possible approaches to blocking VEGF interaction with its receptors. One of these is via neutralizing anti-VEGF antibody. Treatment with anti-human VEGF antibody is able to significantly delay the growth of several human tumor xenografts in nude mice (30). It was especially effective against microtumors and metastases (26, 27, 31). Studying DC function in nude mice bearing human tumors is difficult, because DC function in nude mice might be abnormal (32). Therefore, we used syngeneic mouse tumors and anti-mouse VEGF neutralizing antibody. This antibody, at a dose of 10  $\mu\text{g}/\text{mouse}$  i.p. twice a week over 4 weeks, decreased the VEGF level in the plasma of tumor-bearing mice almost 5-fold. However, at this dose, soluble ligand was not completely eliminated. We observed that the level of VEGF in our tumor-bearing mice was still almost 10-fold higher than in non-tumor-bearing control mice at the selected dose of antibody. At this dose, anti-VEGF antibody did not affect the growth of established tumors. We



**Fig. 3** Anti-VEGF antibody improves effect of immunotherapy in MethA sarcoma tumor model. MethA sarcoma cells [ $6 \times 10^5$  (A) or  $2.5 \times 10^5$  (B)] were inoculated s.c. When tumors reached 4–5 mm in diameter, mice were treated with DCs pulsed with specific or control peptides in addition to control IgG or anti-VEGF antibody as described in the text. DCs ( $1-2 \times 10^5$ ) were injected i.v. on days 0, 4, and 7 after the start of the treatment. Ten  $\mu\text{g}$  of IgG or anti-VEGF antibody were injected i.p. twice a week from days 0 to 26. Each group included five mice. Summarized results from all mice (A) or results from each separate mouse (B) are presented. Bars, SE.

deliberately did not escalate the dose of the antibody and did not try to achieve therapeutic effect of antibody treatment alone. The absence of a direct effect on the tumor growth rate eliminated the possible confounding effects of other factors related to tumor size on DC function. We reasoned, however, that if the plasma level of VEGF was closely associated with DC defects, a 5-fold drop in VEGF concentration would be sufficient to see improvement in DC function and improve the effectiveness of immunotherapy.

A 5–6-week exposure to tumor resulted in a significant decrease in the fraction of DCs in spleen and lymph nodes. This was accompanied by a reduced ability of these cells to stimulate allogeneic and antigen-specific T-cell proliferation. This is in agreement with previously reported data about defective DC function in tumor-bearing hosts (4, 9). We have shown previously that a decreased proportion of mature DCs in tissues, but not decreased function of mature cells, was responsible for the defects in the total population of DCs (15). Although anti-VEGF antibodies were not able to completely abrogate this effect, they

significantly reduced it. Thus, the results of these experiments confirmed the hypothesis that blocking VEGF would improve the differentiation of DCs and therefore increase their function.

In this study, the systemic effects of improved DC function alone were not associated with evident spontaneous immunity induction or direct antitumor effects of anti-VEGF antibody. The reason for this is unknown but may be explained by higher local concentrations of tumor-derived VEGF at the tumor, the site of natural antigen uptake by DC. When functional antigen-loaded DC are introduced i.v., immunity induction may be more efficient.

We asked in this study whether these observed effects of anti-VEGF antibody on endogenous DC function would result in a more effective antitumor immune response to tumor-specific immunotherapy. Mice with established poorly immunogenic D459 tumors were treated with DCs pulsed with specific p53 peptide. In our previous experiments, this treatment resulted in a significant delay of tumor growth. However, the growth delay lasted only for a short time, and tumor growth resumed 1

week after the end of the treatment (4, 19). This indicated that the host immune system was not able to sustain the induced antitumor immune response. The same results were observed in this study in mice immunized with specific peptide-pulsed DCs and control IgG. In contrast, in immunized mice treated with concurrent anti-VEGF antibody, the tumor size was smaller, and tumor growth was suppressed much longer than in immunized mice treated with control IgG. This effect was associated with sustained CTL responses only in this group of mice. Almost exactly the same effect was observed with MethA sarcoma tumors when a large number of tumor cells ( $6 \times 10^5$ ) were used, and a significant percentage of cures were observed with a lower dose of MethA sarcoma cells.

The exact mechanism of the positive effect of anti-VEGF antibody on immune response in tumor-bearing hosts is not clear. As discussed above, we have shown that VEGF inhibits the maturation of DC hematopoietic progenitors. It is possible that VEGF directly inhibits the function of mature immune cells or those of other lineages (macrophages, DCs, or T or B cells), but there is no evidence in the scientific literature for this. It is also possible that VEGF may inhibit the function of immune cells indirectly, via soluble factors released by activated endothelial cells or macrophages. All of these mechanisms are presently under investigation.

These data strongly suggest that inhibition of VEGF signaling may not only be effective in blocking tumor angiogenesis mediated by VEGF but also can improve the function of DCs in tumor-bearing hosts and the effectiveness of specific immunotherapy.

## REFERENCES

- Banchereau, J., and Steinman, R. M. Dendritic cells and the control of immunity. *Nature (Lond.)*, 392: 245–252, 1998.
- Ioannides, C., and Whiteside, T. T cell recognition of human tumors: implication for molecular immunotherapy of cancer. *Clin. Immunol. Immunopathol.*, 66: 91–106, 1993.
- Kavanaugh, D. Y., and Carbone, D. P. Immunologic dysfunction in cancer. *Hematol. Oncol. Clin. N. Am.*, 10: 927–951, 1996.
- Gabrilovich, D., Ciernik, F., and Carbone, D. P. Dendritic cells in anti-tumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. *Cell. Immunol.*, 170: 101–110, 1996.
- Tas, M., Simons, P., Balm, F., and Drexhage, H. Depressed monocyte polarization and clustering of dendritic cells in patients with head and neck cancer: *in vitro* restoration of this immunosuppression by thymic hormones. *Cancer Immunol. Immunother.*, 36: 108–114, 1993.
- Nestle, F. O., Burg, G., Fah, J., Wrone-Smith, T., and Nickoloff, B. J. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am. J. Pathol.*, 150: 641–651, 1997.
- Chaux, P., Moutet, M., Faivre, J., Martin, F., and Martin, M. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation. *Lab. Invest.*, 74: 975–983, 1996.
- Chaux, P., Favre, N., Martin, M., and Martin, F. Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int. J. Cancer*, 72: 619–624, 1997.
- Gabrilovich, D. I., Corak, J., Ciernik, I. F., Kavanaugh, D., and Carbone, D. P. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin. Cancer Res.*, 3: 483–490, 1997.
- Gabrilovich, D. I., Nadaf, S., Corak, J., Berzofsky, J. A., and Carbone, D. P. Dendritic cells in anti-tumor immune responses. II. Dendritic cells grown from bone marrow precursors, but not mature DC from tumor-bearing mice are effective antigen carriers in the therapy of established tumors. *Cell. Immunol.*, 170: 111–120, 1996.
- Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D., and Carbone, D. P. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.*, 2: 1096–1103, 1996.
- Steinbrink, K., Wolf, M., Jonuleit, H., Knop, J., and Enk, A. H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.*, 159: 4772–4780, 1997.
- Menetrier-Caux, C., Montmain, G., Dieu, M. C., Bain, C., Favrot, M. C., Caux, C., and Blay, J. Y. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage-colony-stimulating factor. *Blood*, 92: 4778–4791, 1998.
- Jasani, B., Navabi, H., Adams, M., Evans, A., and Mason, M. Serum dependent down-regulation of CD1a expression and specific CTL priming activity in human dendritic cells. *Proc. Am. Assoc. Cancer Res.*, 30: 611, 1998.
- Ishida, T., Oyama, T., Carbone, D., and Gabrilovich, D. I. Defective function of Langerhans cells in tumor-bearing animals is the result of defective maturation from hematopoietic progenitors. *J. Immunol.*, 161: 4842–4851, 1998.
- Ferrara, N., and Smyth, D. T. The biology of vascular endothelial growth factor. *Endocr. Rev.*, 18: 4–25, 1997.
- Gabrilovich, D. I., Ishida, T., Oyama, T., Ran, S., Kravtsov, V., Nadaf, S., and Carbone, D. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages *in vivo*. *Blood*, 92: 4150–4166, 1998.
- Yanuck, M., Carbone, D. P., Pendleton, D., Tsukui, T., Winter, S. F., Minna, J. D., and Berzofsky, J. A. Mutant p53 tumor suppressor protein is a target for peptide-induced CD8<sup>+</sup> cytotoxic T-cells. *Cancer Res.*, 53: 3257–3261, 1993.
- Gabrilovich, D. I., Cunningham, H. T., and Carbone, D. P. IL-12 and mutant p53 peptide-pulsed dendritic cells for the specific immunotherapy of cancer. *J. Immunother.*, 19: 414–418, 1997.
- Noguchi, Y., Chen, Y., and Old, L. A mouse mutant p53 product recognized by CD4<sup>+</sup> and CD8<sup>+</sup> T cells. *Proc. Natl. Acad. Sci. USA*, 91: 3171–3175, 1994.
- Noguchi, Y., Richards, E. C., Chen Y-T., and Old, L. Influence of interleukin 12 on p53 peptide vaccination against established MethA sarcoma. *Proc. Natl. Acad. Sci. USA*, 92: 2219–2223, 1995.
- Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghten, R., Cornette, J., DeLisi, C., Moss, B., Germain, R. N., and Berzofsky, J. A. An immunodominant epitope of the HIV gp160 envelope glycoprotein recognized by class I MHC molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA*, 85: 3105–3109, 1988.
- Takano, S., Yoshii, Y., Kondo, S., Suzuki, H., Maruno, T., Shirai, S., and Nose, T. Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. *Cancer Res.*, 56: 2185–2190, 1996.
- Kondo, S., Asano, M., Matsuo, K., Ohmori, I., and Suzuki, H. Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. *Biochim. Biophys. Acta*, 1221: 211–214, 1994.
- Dirix, L. Y., Vermeulen, P. B., Pawinski, A., Prove, A., Benoy, I., De Pooter C., Martin, M., and Van Oosterom, A. T. Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. *Br. J. Cancer*, 76: 238–243, 1997.
- Borgstrom, P., Bourdon, M. A., Hillan, K. J., Sriramarao, P., and Ferrara, N. Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma microtumors *in vivo*. *Prostate*, 35: 1–10, 1998.
- Borgstrom, P., Hillan, K. J., Sriramarao, P., and Ferrara, N. Complete inhibition of angiogenesis and growth of microtumors by anti-

vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer Res.*, *56*: 4032–4039, 1996.

28. Mayordomo, J. I., Loftus, D. J., Sakamoto, H., De Cesare, C. M., Appasamy, P. M., Lotze, M. T., Storkus, W. J., Appella, E., and DeLeo, A. B. Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. *J. Exp. Med.*, *183*: 1357–1365, 1996.

29. Oyama, T., Ran, S., Ishida, T., Nadaf, S., Kerr, L., Carbone, D., and Gabrilovich, D. I. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor- $\kappa$ B activation in hematopoietic progenitor cells. *J. Immunol.*, *160*: 1224–1232, 1998.

30. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Fillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature (Lond.)*, *362*: 841–844, 1993.

31. Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A., and Ferrara, N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J. Clin. Investig.*, *95*: 1789–1797, 1995.

32. Grabbe, S., Gallo, R. L., Lindgren, A., and Granstein, R. D. Deficient antigen presentation by Langerhans cells from athymic (*nu/nu*) mice: restoration with thymic transplantation or administration of cytokines. *J. Immunol.*, *151*: 3430–3439, 1993.



# Clinical Cancer Research

## Antibodies to Vascular Endothelial Growth Factor Enhance the Efficacy of Cancer Immunotherapy by Improving Endogenous Dendritic Cell Function

Dmitry I. Gabrilovich, Tadao Ishida, Sorena Nadaf, et al.

*Clin Cancer Res* 1999;5:2963-2970.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/5/10/2963>

**Cited articles** This article cites 31 articles, 14 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/5/10/2963.full#ref-list-1>

**Citing articles** This article has been cited by 47 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/5/10/2963.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/5/10/2963>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.