Abstract

Purpose: Induction of antitumor immune responses requires adequate function of dendritic cells. Dendritic cell defects in cancer patients have been implicated in tumor escape and the limited efficacy of cancer vaccines. Previous studies have shown that vascular endothelial growth factor (VEGF) plays a major role in abnormal dendritic cell differentiation and function in cancer. It has been proposed that inhibition of VEGF may result in improved immune responses. The goal of this study was to test this hypothesis.

Experimental Design: Fifteen patients with refractory solid tumors were enrolled into a phase I clinical trial of VEGF-Trap. Phenotype and function of different subsets of mononuclear cells were measured before and at different time points after the start of treatment.

Results: VEGF-Trap treatment did not affect the total population of dendritic cells, their myeloid or plasmacytoid subsets, myeloid-derived suppressor cells (MDSC), or regulatory T cells. It significantly increased the proportion of mature dendritic cells. However, that improvement was not associated with an overall increase in immune responses to various antigens and mitogens. A subset analysis revealed significant improvement in immune responses in patients who had no increase in the proportion of MDSC. An improvement in immune responses was absent in patients with an increase in the proportion of MDSC.

Conclusions: Inhibition of VEGF signaling may improve differentiation of dendritic cells in cancer patients. However, it was not sufficient to improve immune responses. This shows multifaceted nature of immune deficiency and points out to the need for complex approach to modulation of immune reactivity in cancer.
extracellular domains of human VEGF receptors 1 and 2 coupled with Fc portion of human IgG1. VEGF-Trap binds all VEGF-A isoforms and both placental growth factors 1 and 2. The main goal of VEGF-Trap therapy is to impair the tumor vasculature and prevent its critical role in tumor growth and dissemination. This broad spectrum of binding activity of VEGF-Trap may provide an additional immunologic benefit because placental growth factor exerts negative effects on dendritic cell differentiation similar to that of VEGF (17). We hypothesized that VEGF-Trap may reduce the negative effect of tumor on dendritic cell differentiation and thus improve immune response in cancer patients. If successful, this approach could be used to improve the effect of cancer vaccines. To test this hypothesis, we evaluated dendritic cells and immune function in cancer patients during a phase I trial of VEGF-Trap.

Materials and Methods

**Patient selection and treatment.** All patients eligible to participate in the treatment and companion studies were ≥18 years of age; had advanced, incurable, and progressive solid tumors; good performance status; a measurable tumor site suitable for dynamic contrast-enhanced magnetic resonance image; and signed Institutional Review Board–approved consent. All patients were enrolled at either Vanderbilt University Medical Center or Memorial Sloan-Kettering Cancer Center.

VEGF-Trap was supplied by Aventis Pharmaceuticals, Inc. in vials at 25 mg/mL for i.v. administration. The VEGF-Trap treatment dose ranged from 0.3 to 7.0 mg/kg (2.0-7.0 mg/kg) on patients who underwent immune evaluation as part of this study) over 1 h every 2 weeks. The initial tumor evaluation was at 8 weeks and treatment could continue if the disease was stable or responding with further disease assessments occurring every 8 weeks. Clinical response to the treatment was evaluated using Response Evaluation Criteria in Solid Tumors (18).

**Collection of blood samples.** Both bound and free VEGF-Trap were measured in plasma samples at days 15, 22, and 43, with free VEGF-Trap established as an indicator of complete ligand (VEGF) blockade. For evaluation of immunologic variables, peripheral blood was collected before the start of VEGF-Trap treatment on days 15, 29, and 57 after the start of treatment. Day 15 and day 57 samples were not obtained in all patients. Blood samples were shipped overnight to H. Lee Moffitt Cancer Center, and mononuclear cells (MNC) were isolated at each of these time points, cryopreserved in freezing medium containing 50% RPMI 1640, 40% fetal bovine serum, and 10% DMSO, and stored in liquid nitrogen for future analysis.

**Evaluation of cell phenotype.** To reduce interexperimental variations, MNCs collected at different time points from the same patient were thawed and analyzed at the same time. Peripheral blood MNCs were thawed, cultured overnight in complete culture medium (RPMI 1640 and 10% FCS), and then used for subsequent analysis. No additional cell purification was done. MNCs were cultured overnight before the analysis for two reasons: to allow for functional recovery of MNC after thawing and to allow for up-regulation of HLA-DR by immature dendritic cells. This helps to distinguish immature dendritic cells from abnormal MDSC not able to up-regulate MHC class II.

Cells were labeled with the antibodies for 40 min on ice and analyzed the same day by flow cytometry. The cocktail of lineage-specific antibodies included phycoerythrin-conjugated antibodies against CD3, CD19, CD56, and CD14 (all from BD PharMingen). In addition, we used peridinin chlorophyll protein–conjugated anti-HLA-DR antibody, antigen-presenting cell–conjugated anti-CD33 and CD11c antibodies, and FITC-conjugated anti-CD86, CD83, and CD40 antibodies (all from BD PharMingen). Anti-GITR antibody was obtained from R&D Systems and anti-CD123 were obtained from R&D Systems and Miltenyi, respectively. Anti-GITR antibody was obtained from R&D Systems. The phenotype of the cells was evaluated by multicolor flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). At least 25,000 cells were collected from each variable to obtain reliable data. Analysis of the samples was carried out essentially as described elsewhere (19).

**Allogeneic mixed leukocyte reaction.** T cells were purified from a leukocyte-enriched buffy coat from healthy donors obtained from Florida Blood Services using T-cell enrichment columns (R&D Systems) according to the manufacturer’s instructions. Irradiated (25 Gy) patients’ MNCs were incubated with $10^7$ donors’ T cells at MNC to T-cell ratios of 1:1, 1:2, 1:4, and 1:8. Assays were done in round-bottomed 96-well plates in triplicates. $[^1]H$thymidine (GE Healthcare)}
was added (1 μCi/well) on day 4 and the cells were harvested after additional 18 h. Thymidine uptake was measured using a liquid scintillation counter (Packard Instrument). T cells in culture medium alone served as a background control.

**Evaluation of T-cell function.** Nonirradiated patients' MNCs were cultured in round-bottomed 96-well plates (10^5 MNCs/well) for 4 days in culture medium with either 0.1 μg/mL tetanus toxoid (List Biological Labs) or 5 μg/mL phytohemagglutinin (PHA; Sigma). These concentrations of tetanus toxoid and PHA were selected after initial testing. [3H]thymidine (1 μCi/well) was added 18 h before cell harvest. Thymidine uptake was measured using a liquid scintillation counter. MNC in culture medium without any stimulation served as background controls. T-cell response to stimulation with immobilized anti-CD3 antibody was assessed in round-bottomed 96-wells plates coated overnight at 4°C with 1 μg/mL anti-CD3 antibody (BD Pharmingen) diluted in 1× Dulbecco’s PBS. Excess of unbound antibody was washed off with Dulbecco’s PBS. MNCs (10^5 per well) were incubated for 5 days and proliferation was evaluated as described above. To measure response to influenza virus, 10^5 MNCs were incubated with influenza virus (A/PR/8/34) in 100 μL of serum-free RPMI 1640 for 2 h and then 100 μL of RPMI 1640 containing double concentration of serum were added. T-cell proliferation was measured as described above, and all measurements were done in triplicates.

**Statistical analysis.** Data from a phase I clinical trial were summarized using descriptive statistics and tabular representation. Phenotype and function of different subsets of MNCs measured prior and at 4 weeks were evaluated using an independent t test assuming unequal variances to compare between two groups (i.e., controls versus pretreatment cancer patients) and a paired t test to compare two correlated groups (i.e., precancer and postcancer patients). All P values were two sided unless specified as a one sided for some subset analyses and declared significant at 5% level. No multiple comparisons adjustment was considered due to the exploratory nature of this study.

**Results**

Between February 2005 and January 2006, 21 patients were consented and enrolled onto this companion trial as part of the phase I treatment trial of VEGF-Trap. Paired samples (pretreatment and posttreatment day 29) were available on 15 of these individuals. No maximal tolerated dose of VEGF-Trap was determined and these 15 patients were assigned to several dose levels as dose (number of patients): 2.0 mg/kg (3), 3.0 mg/kg (3), 4.0 mg/kg (3), 5.0 mg/kg (2), and 7.0 mg/kg (4) administered every 2 weeks. Patients' demographic and clinical...
characteristics with their assigned dose levels are presented in Table 1. Clinical response to the treatment was evaluated beginning at 8 weeks of therapy. Of the 15 patients, 4 patients had >20% increase in tumor size by Response Evaluation Criteria in Solid Tumors, which meet the criteria for progressive disease and include one patient who had a rapid decline in performance status due to disease progression. Ten patients had stable disease, and 1 patient had partial response (32% decrease in tumor size by Response Evaluation Criteria in Solid Tumors). As controls, samples from six healthy donors (ages 32-45) were obtained at H. Lee Moffitt Cancer Center and Research Institute.

Both bound and free VEGF-Trap were measured in plasma samples at days 15, 22, and 43, with free VEGF-Trap established as an indicator of complete ligand (VEGF) blockade (data not shown). In all patients included in the immune evaluations at dose levels 2.0 to 7.0 mg/kg, complete ligand blockade was achieved. Preliminary analyses of tumor blood flow and volume by dynamic contrast-enhanced magnetic resonance imaging scans showed that the VEGF-Trap reduces tumor vascular perfusion and permeability, which is consistent with its role in blocking VEGF (data not shown).

**Effect of VEGF-Trap treatment on phenotype of dendritic cells and immune function in cancer patients.** MDSC were defined as Lin-HLA-DR-CD33+ cells (Fig. 1A). Total population of dendritic cells was defined as Lin-HLA-DR+ cells, myeloid dendritic cells as Lin-HLA-DR+CD11c+CD123- and plasmacytoid dendritic cells as Lin-HLA-DR+CD11c+CD123+ cells (Fig. 1B). Mature dendritic cells were defined as Lin-HLA-DR+ that expressed CD86, CD40, CCR7, or CD83 markers (Fig. 1B). Consistent with previously reported observations (20, 21), cancer patients showed substantially lower proportion of total dendritic cells, myeloid dendritic cells, and mature dendritic cells than healthy donors (all \( P < 0.05 \); Fig. 2A, C, and D), whereas >5-fold higher proportion in the MDSC (\( P = 0.016 \); Fig. 2B). Treatment with VEGF-Trap did not significantly affect the proportion of dendritic cells, myeloid dendritic cells, or MDSC for the cancer patients (\( P > 0.05 \); Fig. 2A-C). In contrast, 4 weeks of VEGF-Trap treatment significantly improve the proportion of mature dendritic cells. The presence of dendritic cells expressing CD86, CD40, CCR7, and CD83 receptors was restored toward the level in control (\( P > 0.05 \); Fig. 2D). This level remained relatively stable after 2 months of treatment.

Several assays were used to evaluate immune function in these patients. Allogeneic mixed leukocyte reaction (MLR) is a hallmark of dendritic cell activity and it showed significantly lower value in cancer patients at each of the four MNC to T-cell ratio [1:1, 1:2, 1:4, and 1:8; all \( P < 0.05 \); Fig. 3A, right (MLR in healthy donors) and Fig. 3A, left (MLR in cancer patients)]. Treatment with VEGF-Trap did not significantly improve the ability of MNC to stimulate allogenic T cells.
Comparing with healthy donors, cancer patients had low response to tetanus toxoid, PHA, and influenza virus ($P < 0.01$; Fig. 3B). Treatment with VEGF-Trap did not improve those responses ($P > 0.05$; Fig. 3B). Consistent with previous results (22), cancer patients had normal level of T-cell response to stimulation with immobilized anti-CD3 antibody (Fig. 3B). VEGF-Trap treatment also did not affect this response ($P > 0.05$). Thus, despite significant improvement in the presence of mature dendritic cells, VEGF-Trap may not affect T-cell responses for cancer patients as a whole population.

Association between the presence of regulatory T cells, suppressive MNCs (MDSC), clinical responses to VEGF-Trap treatment, and its effect on immune system. We and others have previously shown a critical role of MDSC in antigen-specific immune suppression in cancer (reviewed in refs. 23, 24). Elimination of these cells substantially improved immune responses in tumor-bearing mice and cancer patients (19, 25–27). This study showed that VEGF-Trap did not affect the proportion of these cells (Fig. 3B). However, the analysis of changes in the MDSC population in VEGF-Trap–treated patients showed the existence of two different groups. In nine patients, the proportion of MDSC was either decreased or remained stable during the treatment, whereas in six patients it was increased (>50% over pretreatment level; Fig. 4A, left and right, respectively). We investigated the possibility that the changes in MDSC might influence the effect of VEGF-Trap on immune responses. VEGF-Trap treatment resulted in similar
increase in the proportion of mature dendritic cells in both these groups \( (P > 0.05); \text{data not shown} \). No differences in the total population of dendritic cells as well as their myeloid or plasmacytoid subsets were found \( (P > 0.05); \text{data not shown} \). Thus, the percentage of MDSC was not associated with the effect of VEGF-Trap on maturation of dendritic cells. In the six patients who had increase in the proportion of MDSC after the treatment with VEGF-Trap, no changes in any of functional tests were found \( \text{allogeneic MLR, PHA, tetanus toxoid, and influenza virus with } P = 0.22, 0.41, 0.84, \text{and } 0.17 \text{, respectively; Fig. 4B} \). In sharp contrast, in the nine patients who had either decreased or unchanged levels of MDSC, the treatment with VEGF-Trap resulted in significant increase in allogeneic MLR \( (P = 0.009) \), PHA \( (P = 0.024) \), and influenza virus-induced T-cell proliferation \( (P = 0.024; \text{Fig. 4B}) \). Tetanus toxoid-induced T-cell proliferation showed improvement, but it was not statistically significant \( (P = 0.11; \text{Fig. 4B}) \). This was not due to a correlation with clinical tumor response because, among the 11 patients with regression or stable disease, 4 \( (36\%) \) showed increased percentage in MDSC, whereas for the 4 patients with clinically progressive disease, 2 \( (50\%) \) showed increased MDSC.

Regulatory T cells \( \text{(Treg; defined as } CD4^+CD25^+\text{GITR}^+ \text{cells)} \) are capable of suppressing immune responses and are proposed to play an important role in immune defects in cancer. We evaluated the kinetics of this cell population during VEGF-Trap treatment. Before start of the treatment, cancer patients had significantly increased proportion of Treg compared with control healthy donors \( (P = 0.0012; \text{Fig. 5A}) \). During VEGF-Trap treatment, the proportion of these cells further increased in all cancer patients \( (P = 0.048) \). No association between the levels of MDSC and the presence of Treg was found \( \text{data not shown} \). Based on the kinetics of change in the Treg population, all patients were split into two groups. One group included six patients who had increased proportion of Treg during VEGF-Trap treatment \( (>50\% \text{ above background level}) \). The other group included seven patients who had stable or decreased levels of Treg \( \text{Fig. 5B}) \). Before the therapy, no differences between these groups were found \( (P = 0.22) \). VEGF-Trap increased the proportion of mature dendritic cells in primarily patients who had increased proportions of Treg. However, the differences did not reach significant level \( (P > 0.05); \text{data not shown} \). No differences between the groups were found in the level of immune responses before and after the VEGF-Trap treatment \( (P > 0.05) \text{ in all tests; Fig. 5C} \).

**Discussion**

It is known that tumors via tumor-derived factors affect differentiation of dendritic cells, which results in decreased
presence of mature dendritic cells and accumulation of MDSCs (reviewed in ref. 1). One of the first factors implicated in abnormal dendritic cell differentiation in cancer was VEGF. The involvement of VEGF in tumor-induced defects in dendritic cell differentiation was shown initially by in vitro (6) and in mice in vivo (28, 29). Administration of neutralizing VEGF-specific antibody to tumor-bearing mice improved dendritic cell differentiation and increased the number of mature dendritic cells (30, 11). In recent years, correlative clinical data have supported an important role of VEGF in dendritic cell defects in cancer. The expression of VEGF negatively correlated with dendritic cell numbers in the tumor tissue and peripheral blood of patients with different types of cancer (12, 16, 21, 31–35). All these data provided strong rationale for using inhibition of VEGF signaling as means to improve dendritic cell differentiation and immune function in cancer patients.

The VEGF-Trap is a specific antagonist that binds and inactivates circulating VEGF in the bloodstream and in the extravascular space (36). The VEGF-Trap is a fusion protein consisting of human VEGF receptor extracellular domains fused to the Fc portion of human IgG1. Clinical testing of the VEGF-Trap in cancer patients aims at interfering with the growth of primary and metastatic tumors by reducing tumor vascularity and diminishing the abnormal leakiness of tumor vessels and possible “normalization” of the tumor vasculature. It is currently in phase I/II clinical trials and soon will enter pivotal phase III clinical trials. We used this opportunity to investigate its potential effect on dendritic cell differentiation and immune function in cancer patients. All patients enrolled in this study had advanced-stage cancer and profound defects in immune function, decrease in the presence of dendritic cells, and increase in MDSC and Tregs. These data are consistent with well-established phenomenon of immune suppression observed in advanced-stage cancer patients. VEGF-Trap treatment reduced tumor vascular perfusion in these patients, which was consistent with its role in blocking VEGF.

Treatment with VEGF-Trap did not affect the total population of dendritic cells, MDS, or MDSC but significantly increased the proportion of mature dendritic cells. Thus, these data for the first time in clinical setting suggested that block of VEGF signaling indeed improved dendritic cell differentiation. We expected that this should result in improved T-cell response to antigen-specific and nonspecific stimulation. To investigate immune function in cancer patients, we used several different assays. Consistent with previously reported observations (19, 22), cancer patients did not have defects in anti-CD3-induced T-cell proliferation but their ability to respond to recall antigens, PHA, and stimulate allogeneic T cells was severely impaired.

![Figure 5](image_url)

*Fig. 5.* Association between the levels of Tregs and the effects of VEGF-Trap on immune responses. A, level of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>) in the total population of treated patients. Columns, mean; bars, SD. *, statistically significant differences from control level in one-tailed t test (P < 0.05). B, patients were split into two groups based on the changes in the proportion of Tregs 4 wks after the start of VEGF-Trap treatment: seven patients with unchanged or decreased proportion of Tregs and six patients with increased proportion of Tregs (>50% above pretreatment level). Columns, mean; bars, SD. C, functional tests in patients divided based on changes in Tregs level.
impaired. Overall, VEGF-Trap did not improve any of these immune responses. These data suggested that improved dendritic cell maturation was not sufficient to increase T-cell responses in cancer patients. It seems that other MNCs could negate possible positive effect of VEGF-Trap treatment. The most direct experiment to answer this question would be to sort dendritic cells from patients’ samples and then test their functional activity. However, in a framework of the current trial, we had access only to frozen cells and the number of cells was not sufficient to do such experiments. Despite this limitation, we nevertheless were able to address this question by analyzing available data. First, we asked whether the effect of VEGF-Trap was associated with clinical outcome. The functional tests showed no differences between patients with stable disease and/or partial response and those with progressive disease. Although these data were obtained in relatively small number of patients, it indicated that lack of VEGF-Trap effect on immune function was probably not due to negative effect of increased tumor burden and suggested that other MNCs could be an important factor able to negate the effect of VEGF-Trap.

Previous studies have shown a potentially important role of Tregs in tumor-associated immune suppression. We measured the presence of Treg by staining with anti-CD4, CD25, and GITR antibodies. As expected, the proportion of Treg was substantially increased in cancer patients. Individual analysis showed that half of the patients had a stable or decreased levels of Tregs 4 weeks after start of the treatment and the other half had substantially increased proportion of these cells. The pretreatment levels of Tregs were similar between these two groups. We hypothesized that if Tregs play a major role in why VEGF-Trap did not improve antigen-specific immune responses, then patients with stable level of Tregs would have better immune responses than those with increased proportion of these cells. However, it was not the case. Thus, either Tregs did not play a prominent role in suppression of immune responses in these patients or, more likely, increased pretreatment levels of Tregs were sufficient to inhibit T-cell function in all patients, which made them nonresponsive to VEGF-Trap therapy. Similar analysis was done with MDSC. Patients were split into two groups based on the changes in MDSC levels during the treatment. Changes in MDSC did not affect the VEGF-Trap effect on the proportion of mature dendritic cells. Patients who had increased proportion of MDSC during the treatment showed no improvement in any of assays studied. In contrast, patients with stable or decreased levels of MDSC showed significant improvements in allogeneic MLR, PHA, and influenza virus–induced T-cell proliferation as well as strong trends in improvement of tetanus toxoid–induced T-cell proliferation. The ability of MDSC to suppress allogeneic and antigen-specific immune responses in cancer patients has been described previously (20, 37). Elimination of these cells with all-trans-retinoic acid substantially improved immune responses in cancer patients (19). VEGF-Trap did not affect the amount of these cells in peripheral blood of cancer patients. It seems that increased presence of these cells negates any positive effect VEGF-Trap could exert through dendritic cell maturation. Direct experiments with sorting different cell populations before and after the treatment with VEGF-Trap can probably clarify the role of different cell populations in this process. However, such experiments will require additional patients treated with VEGF-Trap as is being planned in future phase II/III studies. Thus, our study has concluded that inhibition of VEGF signaling in cancer indeed may improve differentiation of dendritic cells. VEGF-Trap is not the only method of treatment able to increase the proportion of dendritic cells. Recent study in patients with peritoneal carcinomatosis or mesothelioma has shown that treatment with FLT3L resulted in significant increase in dendritic cells (38). However, in this study, increased presence of mature dendritic cells was not sufficient to improve antigen-specific immune responses in cancer patients in the presence of increased MDSC. It seems that MDSC could play a major role in preventing the improvement in the immune responses. These data illustrate the complex nature of immune suppression present in cancer patients and necessitate further search for therapeutics or use of therapeutics in combinations able to improve immune function in cancer patients.

References

Vascular Endothelial Growth Factor-Trap Overcomes Defects in Dendritic Cell Differentiation but Does Not Improve Antigen-Specific Immune Responses

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