

# Active Immunization Against the Vascular Endothelial Growth Factor Receptor flk1 Inhibits Tumor Angiogenesis and Metastasis

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## Abstract

The vascular endothelial growth factor (VEGF) receptor fetal liver kinase 1 (flk1; VEGFR-2, KDR) is an endothelial cell-specific receptor tyrosine kinase that mediates physiological and pathological angiogenesis. We hypothesized that an active immunotherapy approach targeting flk1 may inhibit tumor angiogenesis and metastasis. To test this hypothesis, we first evaluated whether immune responses to flk1 could be elicited in mice by immunization with dendritic cells pulsed with a soluble flk1 protein (DC-flk1). This immunization generated flk1-specific neutralizing antibody and CD8<sup>+</sup> cytotoxic T cell responses, breaking tolerance to self-flk1 antigen. Tumor-induced angiogenesis was suppressed in immunized mice as measured in an alginate bead assay. Development of pulmonary metastases was strongly inhibited in DC-flk1-immunized mice challenged with B16 melanoma or Lewis lung carcinoma cells. DC-flk1 immunization also significantly prolonged the survival of mice challenged with Lewis lung tumors. Thus, an active immunization strategy that targets an angiogenesis-related antigen on endothelium can inhibit angiogenesis and may be a useful approach for treating angiogenesis-related diseases.

Key words: angiogenesis • antibody • cytotoxic T lymphocytes • cancer vaccine • tumor antigen

## Introduction

Tumor metastasis is the main cause for failure of conventional cancer therapy, necessitating the reevaluation of current cancer therapy strategies. Conventional approaches for cancer immunotherapy usually target antigens expressed by tumor cells and are aimed at eradicating tumor cells by direct or indirect immunological attack (1–3). Despite the identification of tumor-associated antigens and the demonstrated effectiveness of immunotherapy in many experimental animal tumor models, immunotherapy currently has limited clinical utility for human cancers. The limitations of cancer immunotherapy are thought to be due in part to poor immunogenicity, immune tolerance, and escape of tumor cells from immune surveillance through antigen modulation, decreased MHC expression by tumor cells, lack of costimulatory molecules, or secretion of immunosuppressive molecules by tumor cells (4, 5). One possible alternative to overcome these obstacles of conventional immunotherapy is to target the blood vessels nourishing the growing tumor cells rather than the tumor cells themselves.

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is a tightly regulated process important in fetal development and wound healing and in pathological conditions such as tumor growth and metastasis (6, 7). Vascular endothelial growth factor (VEGF)\* and its receptors fetal liver kinase 1 (flk1; murine) and KDR (human) play a critical role in regulating the process of normal and pathological angiogenesis. Targeted inactivation of the gene for VEGF or flk1 in mice results in embryonic lethality at day 7.5 due to severely impaired vascular development (8, 9). The importance of VEGF and flk1/KDR in tumor angiogenesis is exemplified in studies using a dominant-negative flk1 receptor (10), neutralization of VEGF by monoclonal antibodies (11), neutralizing flk1/KDR mAb (12, 13) or flk1/KDR kinase inhibitors (14), all of which were shown to inhibit angiogenesis and tumor growth. Moreover, overexpression of VEGF and KDR is strongly associated with invasion and metastasis in human malignant disease (15, 16). Therefore, overexpressed flk1/KDR could be a potential target for tumor immunotherapy.

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\*Abbreviations used in this paper: AP, alkaline phosphatase; DC, dendritic cell; flk1, fetal liver kinase 1; VEGF, vascular endothelial growth factor.

We have hypothesized that a therapeutically effective immune response can be elicited that targets flk1 expressed on tumor blood vessels and thus, inhibit tumor angiogenesis and growth. To test this hypothesis, we used a dendritic cell (DC) immunization strategy, which has been successfully used in mice to overcome immune tolerance to other antigens (17). In this report, we demonstrate that immunization with DCs pulsed with soluble flk1 induces neutralizing antibody and CD8<sup>+</sup> cytotoxic T cell responses, suppresses tumor angiogenesis, and strongly inhibits the development of metastasis in two mouse models.

## Materials and Methods

**Tumor Cell Lines.** Lewis lung carcinoma cell line D122-96 (H-2<sup>b</sup>) was provided by Dr. L. Eisenbach (Weizman Institute of Science, Rehovot, Israel). H5V endothelial cell line (H-2<sup>b</sup>), which expresses both flk1 and MHC class I, was provided by Dr. A. Vecchi (Istituto Mario Negri, Milan, Italy; reference 18). Mouse endothelial cell line bEND.3 (H-2<sup>d</sup>), which expresses flk1 (19), was obtained from Dr. T. Sato (University of Texas Southwestern Medical Center, Dallas, TX). Melanoma cell line B16 (H-2<sup>b</sup>), EL4 lymphoma cell line (H-2<sup>b</sup>), and the NK-sensitive YAC-1 cell line were all purchased from American Type Culture Collection. The cell lines were maintained in DMEM media (Invitrogen) containing 10% FCS (HyClone Laboratories).

**Animals.** Female C57BL/6 mice, 6–8 wk of age, were purchased from Harlan Sprague Dawley, Inc. and housed under pathogen-free conditions.

**Soluble flk1-AP Protein.** Construction of the expression plasmid vector Aptag-flk1, which contains the cDNA corresponding to the extracellular domain of flk1 fused to alkaline phosphatase (AP), was described previously (20). Soluble flk1-AP protein was purified using an anti-AP affinity chromatography, followed by a size exclusion chromatography. It was then subjected to SDS-PAGE, and shown as a single band with molecular weight of ~180 KD (>95% purity). The purified protein could be recognized by a flk1-specific monoclonal antibody, DC101 in Western blot. The binding of flk1-AP to VEGF was examined by an ELISA, and the protein was found to be active. The purified protein was tested for endotoxin using the Pyrogen<sup>®</sup> plus Limulus Amebocyte Lysate assay kit (BioWhittaker). All protein preparations used in animal studies contained <1.25 EU/ml of endotoxin.

**Recombinant flk1-His Protein.** The flk1 insert from the Aptag-flk1 vector was subcloned into the plasmid pET28a vector with a polyhistidine-encoding sequence at the amino-terminal (BD Biosciences/CLONTECH Laboratories, Inc.). The construct was verified by automatic sequence analysis. The recombinant flk1-His protein was expressed in *Escherichia coli*, purified from inclusion bodies by preparative electrophoresis, and assessed for purity by SDS-PAGE and binding to VEGF by ELISA.

**DC Generation.** DCs were generated from the bone marrow as described with modifications (21). Briefly, C57BL/6 mice were killed and bone marrow harvested from tibia and femurs. Bone marrow cells were depleted of existing T cells, B cells, macrophages, and granulocytes by incubation with a cocktail of antibodies including anti-CD4 (GK1.5), anti-CD8 (2.43), anti-Ia (B21-2), anti-B220 (RA3-3A1/6.1), and anti-Gr-1 (RB6-8C5/1; all from BD PharMingen), for 30 min at 4°C and then with rabbit complement (Accurate Chemical) for additional 30 min at 37°C. The remaining cells were cultured in 10% FCS supplemented RPMI 1640 in the presence of GM-CSF (20 ng/ml) and

IL-4 (50 ng/ml; PeproTech) at 37°C, 5% CO<sub>2</sub>, for 9 d. Nonadherent cells were then harvested and confirmed to be mature DCs by their morphology and phenotypic profile (CD40<sup>+</sup>, CD81<sup>+</sup>, CD86<sup>+</sup>, Ia<sup>+</sup>, and CD14<sup>-</sup>) on flow cytometric analysis.

**Antigen Pulsing of DCs and Immunization Protocol.** DCs were pulsed with antigen, as described previously (22). Briefly, DCs were washed twice in the serum-free medium AIM V (Invitrogen) and incubated with soluble flk1-AP protein or human AP (50 µg/ml) in AIM V for 16 h. The cells were then washed twice in AIM V before use for vaccination. For immunization, mice were injected intravenously with 5 × 10<sup>4</sup> flk1-AP-pulsed DCs, AP-pulsed DCs, or PBS (200 µl) per mouse at 8–10 d intervals.

**CTL Culture and Assay.** Mice were immunized three times with DCs pulsed with flk1-AP (DC-flk1), DCs pulsed with AP (DC-AP), or PBS as described above. CTL response was assessed as described previously (23). Briefly, spleen cells were prepared from immunized mice (two mice per group) and restimulated with DCs pulsed with flk1-AP (at 100:1 effector/stimulator ratio) in a 24-well plate for 5 d in RPMI 1640 (Invitrogen) with 10% FCS. The CTL activity was tested in a 4-h <sup>51</sup>Cr release assay against a panel of target cells including flk1<sup>+</sup> H5V endothelial cells, flk1<sup>-</sup> D122-96 tumor cells, flk1-AP-pulsed DCs, AP-pulsed DCs, and NK-sensitive YAC-1 cells. The percentage cytotoxicity was calculated using the formula: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

**Detection of Antibody Response.** Mice were immunized three times with flk1-AP-pulsed DCs, AP-pulsed DCs, or PBS as described above. Blood samples were collected from mice before and 7 d after vaccinations. Anti-flk1 antibody in the sera was detected by ELISA. Briefly, a 96-well plate was incubated with 200 ng/well of flk1-His protein overnight at 4°C. After three washes with 0.1% Tween in PBS, 2% BSA was added to the plate and incubated at room temperature for 1 h. Diluted sera were then added to wells and incubated for 1 h. Wells were washed three times and then incubated with 100 µl goat anti-mouse peroxidase for 1 h. Wells were washed three times and then incubated with 50 µl of 3,3', 5,5'-tetra-methylbenzidine (TMB) substrate (Kirkegaard and Perry Lab, Inc.) for 15 min. The reaction was stopped by adding 50 µl of 1 M phosphoric acid and wells read at 450 nm on a microtiter plate reader. For flk1-VEGF blocking assays, wells were coated with 100 ng of recombinant human VEGF<sub>165</sub> (provided by Dr. P. Kussie, ImClone) overnight at 4°C. Wells are blocked as described above and then incubated for 1 h at room temperature with 100 ng of flk-AP that had been preincubated for 2 h with various concentrations of sera from immunized mice. Wells were washed and incubated with p-nitrophenyl phosphate (PNPP; Sigma-Aldrich). Color was developed for 30 min at room temperature and was then read at 405 nm on a microtiter plate reader.

**Cell-based Ligand-binding Competition Experiments.** Cell-based ligand-binding assays were performed as described with modifications (24). Flk1-expressing bEND.3 cells (10<sup>5</sup> per well) were grown in 24-well plates in DMEM 10% FCS for 48 h. Cells were washed three times with binding buffer (DMEM with 0.1% BSA). Pooled immune sera from DC-flk1-immunized mice or from DC-AP-immunized mice and unlabeled recombinant human VEGF<sub>165</sub> were serially diluted in binding buffer as indicated. In a total volume of 400 µl/well, cells were incubated with the samples for 4 h at 4°C. Cells were then washed with binding buffer and incubated with 10 nCi [<sup>125</sup>I]VEGF (Amersham Pharmacia Biotech) in 400 µl binding buffer per well for 1 h. After incubation, cells were washed using cold PBS with 0.1% BSA. Cells were harvested by adding 200 µl of 0.5 M NaOH. The ra-

radioactivity bound to the cells was determined in a Wizard 1470 gamma counter (PerkinElmer).

**Alginate In Vivo Angiogenesis Assay.** An alginate bead assay was designed to measure in vivo angiogenesis induced by tumor cells (25). Lewis lung tumor cells were suspended in a 1.5% solution of sodium alginate and added drop by drop into a swirling 37°C solution of 250 mM calcium chloride. Alginate beads were formed containing  $\sim 5 \times 10^4$  tumor cells per bead. Mice were anesthetized and four beads implanted subcutaneously through an incision made on the dorsal side. Incisions were closed with surgical clips. After 12 d, mice were injected intravenously with 100  $\mu$ l of FITC-dextran solution (20 mg/ml). Animals were killed after 20 min, beads removed, and incubated overnight at room temperature in 1 ml of buffer (1 mM Tris-HCl, pH 8). The beads were ground briefly with a hand-held mixer and an additional 1 ml of buffer was added. Samples were then vortexed and centrifuged at 1,500 rpm for 5 min. Fluorescence of the sample supernatants was quantitated against a standard curve of FITC-dextran.

**B16 Metastasis Model.** Mice were immunized three times with flk1-AP-pulsed DCs, AP-pulsed DCs, or PBS as described above. 10 d after the last immunization, mice were injected intravenously with  $10^6$  B16 cells. Mice were killed based on the metastatic death in the control groups. Tumor load was assessed by counting the tumor nodules on the lung surface.

**Lewis Lung Metastasis Model.** Mice were immunized three times with flk1-AP-pulsed DCs, AP-pulsed DCs, or PBS as described above. 10 d after the last immunization, mice were challenged with an intrafootpad injection with  $2 \times 10^5$  of D122-96 tumor cells. When tumors reached  $\sim 5$  mm in diameter, the tumor-bearing leg was surgically removed. Mice were killed based on the metastatic death in the control groups. Tumor load was assessed by counting the tumor nodules on the lung surface. In separate experiments, mice were monitored daily for survival.

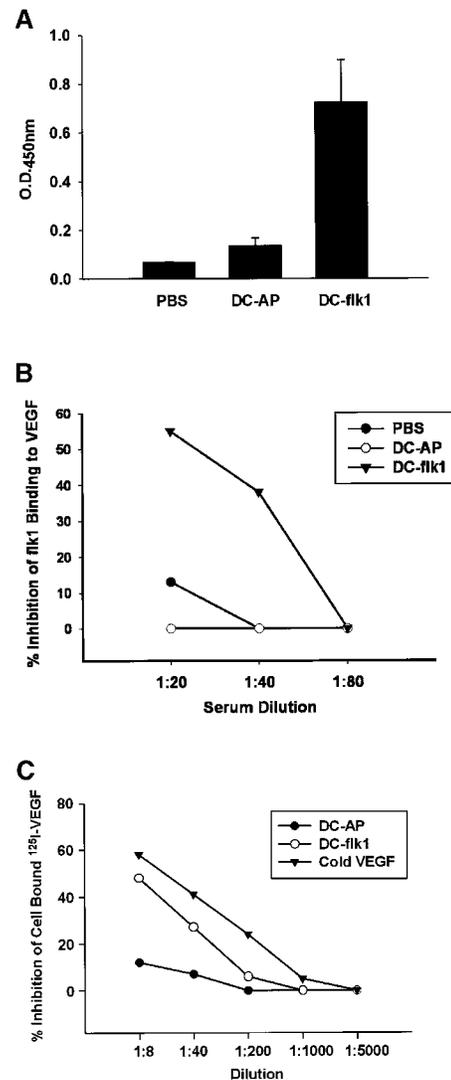
**In Vivo T Cell Depletion Experiment.** 1 d before DC-flk1 immunization, mice received intraperitoneal injection of 0.5 mg of either anti-CD4 (GK1.5), or anti-CD8 (clone 116), or control rat IgG (Jackson ImmunoResearch Laboratories). To ensure complete depletion of respective T cell population, one mouse from each group was killed the next day and splenocytes were analyzed by FACS<sup>®</sup> after staining with FITC-conjugated anti-CD4 (L3T4) and PE-conjugated anti-CD8 (Ly2; BD PharMingen). The same depletion procedure was repeated every 2 wk to prevent recovery of depleted T cell populations. The mice were immunized three times with DCs pulsed with flk1-AP and then challenged with Lewis lung tumor as described in the Lewis lung metastasis model. Tumor load in the lungs were compared among the groups.

**Mouse Pregnancy Experiment.** To test whether flk1 immunization could affect mouse pregnancy by interfering with prenatal angiogenic process, female mice were immunized with DCs pulsed with flk1-AP or PBS as described above. In an additional control, another group of mice were treated with intraperitoneal injections of the anti-flk1 antibody DC101 (800  $\mu$ g per injection, twice weekly for 30 d; reference 12). Mice were then mated with males and monitored daily for signs of pregnancy. Number of pups of each delivery was recorded. The pups were also carefully examined for signs of sickness and abnormality.

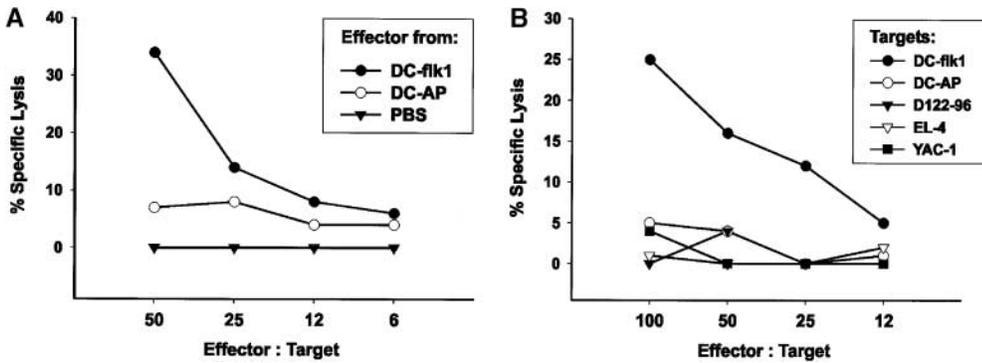
**Wound Healing Experiment.** 10 d after the last immunization with flk1-AP-pulsed DCs, a full thickness wound, including the panniculus carnosus, was excised from the dorsum of each mouse. A 1.60-cm<sup>2</sup> circular defect was outlined 2.0 cm from the nape of the animal's neck using a fine-tipped marking pen. The defect was created by elevating the skin and panniculus carnosus in the center of the outlined defect using forceps, followed by excision

of the outlined area using microdissecting scissors. Wound area was measured twice weekly. 15 d after this excision, mice were killed, and scar tissues were removed for histological examination.

**Histology.** Lung samples and scar tissues were fixed overnight in 10% zinc formalin at 4°C, embedded in paraffin, and sectioned at 5  $\mu$ m onto saline-coated slides. Hematoxylin and eosin stain-



**Figure 1.** Flk1-specific neutralizing antibody induced by vaccination with DCs pulsed with soluble flk1 protein. (A) Mice were immunized three times with flk1-AP-pulsed DCs (DC-flk1), AP-pulsed DCs (DC-AP), or PBS. Postimmunization sera (1:100 dilution) were analyzed for anti-flk1 antibody using ELISA in plates coated with flk1-His protein. Mice immunized with DC-flk1 exhibited significantly higher levels of anti-flk1 antibody compared with control groups. (B) Serially diluted sera from immunized mice were coinubated with soluble flk1-AP protein and then tested for binding to VEGF by ELISA. Results indicated that sera from DC-flk1-immunized mice blocked binding of VEGF to soluble flk1 receptor. (C) Serially diluted sera from immunized mice were incubated with flk1-expressing bEND.3 cells at 4°C for 4 h in a total volume of 0.4 ml. In parallel, the cells were incubated with a decreasing amount of unlabeled VEGF (serially diluted from a 400 ng/ml solution). After incubation, cells were washed and incubated with 10 nCi [<sup>125</sup>I]VEGF for 1 h at room temperature. The cells were washed and counted in a  $\gamma$  counter. Results suggested that sera from DC-flk1-immunized mice blocked binding of VEGF to flk1 expressed at the surface of endothelial cells.



**Figure 2.** Flk1-specific CTL responses induced by vaccination with DC-flk1. (A) Mice were immunized three times with DC-flk1, DC-AP, or PBS. Splenocytes were harvested and restimulated in vitro with flk1-AP-pulsed DCs for 5 d. CTL activity against flk1<sup>+</sup> endothelial cells was assessed in a standard <sup>51</sup>Cr release assay using H5V endothelial cell line as target. Higher CTL activity was detected in DC-flk1-immunized mice compared with DC-AP-immunized or PBS control mice.

(B) T cells cultured from DC-flk1-immunized mice were further analyzed for their antigen specificity in the <sup>51</sup>Cr release assay using the following targets: flk1-AP-pulsed DCs, AP-pulsed DCs, flk1-negative tumor cell lines Lewis lung (D122-96) and EL4, and YAC-1. Results indicated that CTLs induced in DC-flk1-immunized mice were specific to flk1 antigen and showed no cytotoxicity against flk1-negative tumor cell lines.

ing was performed. Scar tissues from the wound healing study were additionally stained with a Masson's trichrome kit (Richard Allen Scientific) according to manufacturer's instructions.

**Statistic Analysis.** All data including tumor counts and vessel measurement were analyzed using Student's *t* test with Sigma-Stat v.2.03.

## Results

**Immune Tolerance to Self-flk1 Antigen Can Be Broken by Immunization with DCs Pulsed with Soluble flk1.** C57BL/6 mice were immunized three times at 8–10 d intervals with DCs pulsed with soluble flk1-AP protein (DC-flk1), DCs pulsed with a control protein alkaline phosphatase (DC-AP), or with vehicle alone (PBS).

Sera were collected from immunized mice and tested for anti-flk1 antibody by ELISA in plates coated by flk1-His protein. A strong antibody response was generated in DC-flk1 vaccinated mice (Fig. 1 A). In contrast, anti-flk1 antibody was detected in only 1 of 10 mice from DC-AP group and in none of the PBS group. Antibody titer was significantly higher in all mice vaccinated with DC-flk1 compared with prevaccination sera (data not shown). Notably, the immune sera from DC-flk1 group inhibited the binding of soluble flk1 receptor to VEGF by ELISA, whereas the immune sera from DC-AP or PBS groups did not show any significant inhibition (Fig. 1 B), demonstrating the presence of a neutralizing anti-flk1 antibody. The specificity and neutralizing activity of the anti-flk1 antibody was further evaluated in a [<sup>125</sup>I]VEGF binding assay using flk1<sup>+</sup> bEND.3 cells (Fig. 1 C). Immune sera from DC-flk1 group strongly inhibited the binding of [<sup>125</sup>I]VEGF to the native flk1 receptor on bEND.3 cells, compared with control sera from DC-AP group.

To analyze the cellular immune response in mice immunized with DC-flk1, splenocytes from the mice were harvested 10 d after the second boost immunization and further stimulated in vitro by cocultivation with flk1-AP-pulsed DCs for 14 d, followed by testing in a 4-h <sup>51</sup>Cr release assay for CTL activity against flk1-positive and flk1-negative targets. Splenocyte cultures from mice immunized

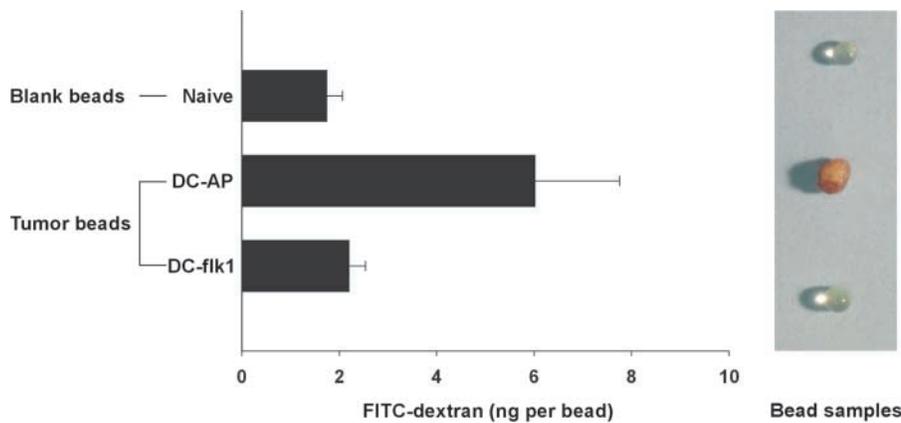
with DC-flk1 showed a significantly higher level of CTL activity against the flk1-positive endothelial cell line H5V, compared with DC-AP or PBS groups (Fig. 2 A). To determine whether this CTL response was specific for flk1, T cells from the DC-flk1 group were also tested against flk1-AP-pulsed DCs, and AP-pulsed DCs as targets. Only the DC-flk1-AP but not the DC-AP target cells were lysed by these T cells, indicating the specificity for flk1 antigen (Fig. 2 B). Moreover, these T cells did not lyse Lewis lung carcinoma or EL-4 lymphoma tumor cells, or the NK-sensitive YAC-1 cell line. These results demonstrate that both flk1-specific humoral and cellular immune responses can be induced in mice by immunization with DC-flk1.

DC-flk1-immunized mice appeared generally healthy, gained normal weight, and no gross toxicity was observed. However, breeding experiments revealed a striking effect of DC-flk1 immunization on pregnant female mice. Female mice were immunized on days 0, 10, and 20 and mated with nonimmunized male mice 10 d after the final immunization. Only 20% of DC-flk1-immunized mice became pregnant, compared with a 70% pregnancy rate in the control group. Moreover, the mean litter size was significantly smaller in the DC-flk1 group (2 vs. 6) compared with the PBS control group (Table I). The small number of

**Table I.** Inhibition of Female Reproductive Function After Vaccination with DC-flk1

Treatment <sup>a</sup>	No. of pregnancies	Mean litter no.
PBS	7/10	6
DC-flk1 immunization	2/10	2
DC101 treatment	3/10	3

<sup>a</sup>Female mice were immunized with flk1-AP-pulsed DCs or vehicle alone as described above. In an additional control group, mice were treated with intraperitoneal injections of the anti-flk1 antibody DC101 (800 μg per injection, twice weekly for 30 d) as a control. Mice were then mated with males and monitored daily for signs of pregnancy. Number of pups of each delivery was also recorded.

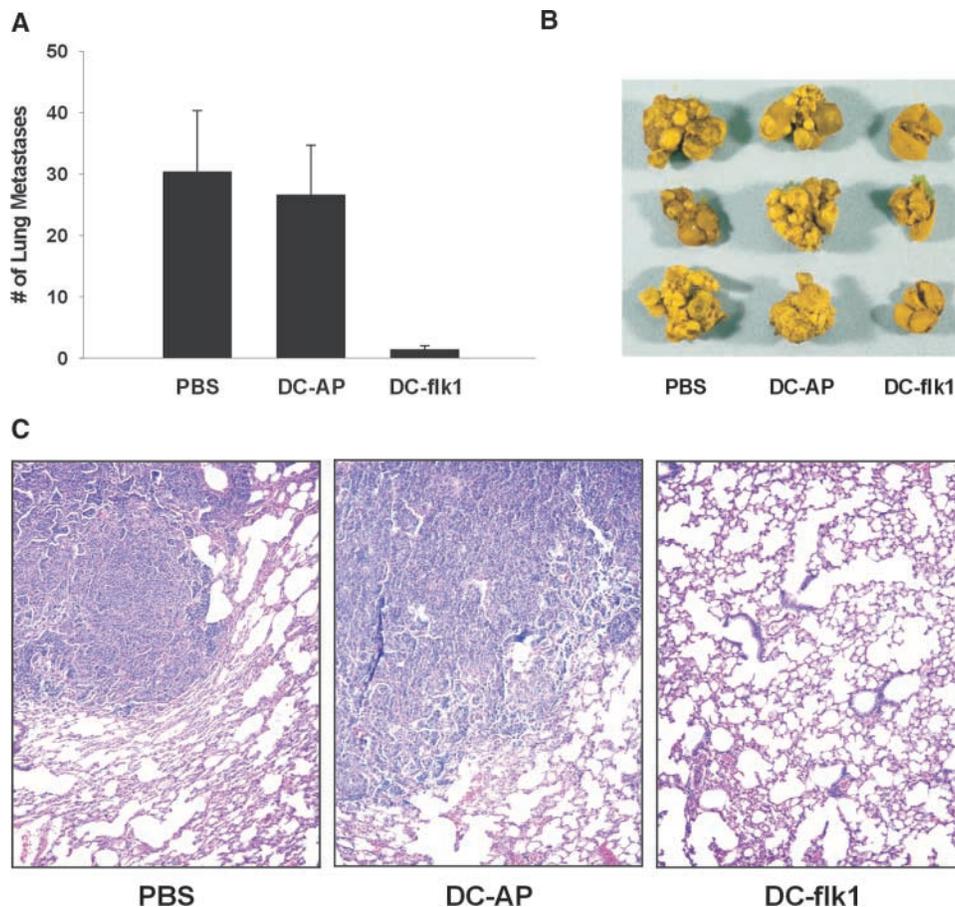


**Figure 3.** DC-flk1 vaccination inhibits tumor-induced angiogenesis. Mice were immunized three times with DC-flk1, or DC-AP. Alginate beads containing  $5 \times 10^4$  Lewis lung tumor cells were then implanted subcutaneously into the mice. 12 d later, mice were injected intravenously with FITC-dextran. Beads were then surgically removed and FITC-dextran quantitated. Mean  $\pm$  SE of  $n = 12$  mice/group. In a negative control, beads containing no tumor cells (blank) were implanted in naive mice. On the right panel, pictures of representative samples of beads from each group were shown.

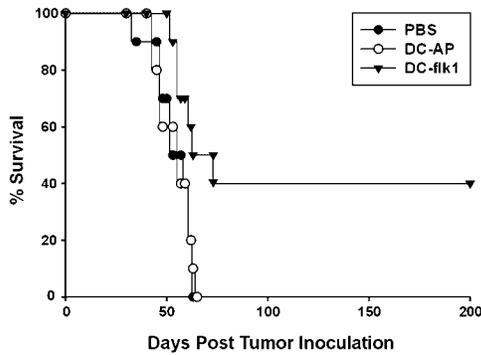
pups from the DC-flk1-immunized mice were either still-born or died within 10 d after birth and were found to have massive hemorrhage in all organs. Similar results were observed in a group of female mice treated with the neutralizing anti-flk1 monoclonal antibody DC101 (12). These data indicate an inhibitory effect of DC-flk1 immunization on fetal development.

*Inhibition of Tumor Angiogenesis In Vivo by DC-flk1 Vaccination.* To determine whether the anti-flk1 immune responses could inhibit tumor-induced angiogenesis, we conducted an in vivo angiogenesis assay using alginate

beads. Lewis lung tumor cells encapsulated alginate beads were implanted subcutaneously in DC-flk1-vaccinated mice. Growth factors produced by the encapsulated tumor cells induced vascularization of the beads, which was then measured by uptake of FITC-dextran. Beads removed from control mice after 12 d were extensively vascularized whereas beads from DC-flk1-immunized mice were essentially avascular (Fig. 3). Quantitative analysis of tumor cell-induced vascularization decreased by 65% in mice vaccinated with DC-flk1 compared with DC-AP group ( $P < 0.01$ ).



**Figure 4.** Lewis lung tumor metastasis can be inhibited by DC-flk1 vaccination. (A) Mice were immunized three times with DC-flk1, DC-AP, or PBS and then challenge with Lewis lung tumor cells intra-footpad. The primary tumor was surgically removed when it reaches  $\sim 5$  mm in diameter. Mice were killed based on the metastatic death in the control groups, and lungs were weighed and assessed for tumor load.  $P < 0.01$  by Student's  $t$  test. (B) Pictures of representative lung samples from each group. (C) Histological examination (H&E staining) of lung samples from each group. Original magnification:  $\times 100$ .

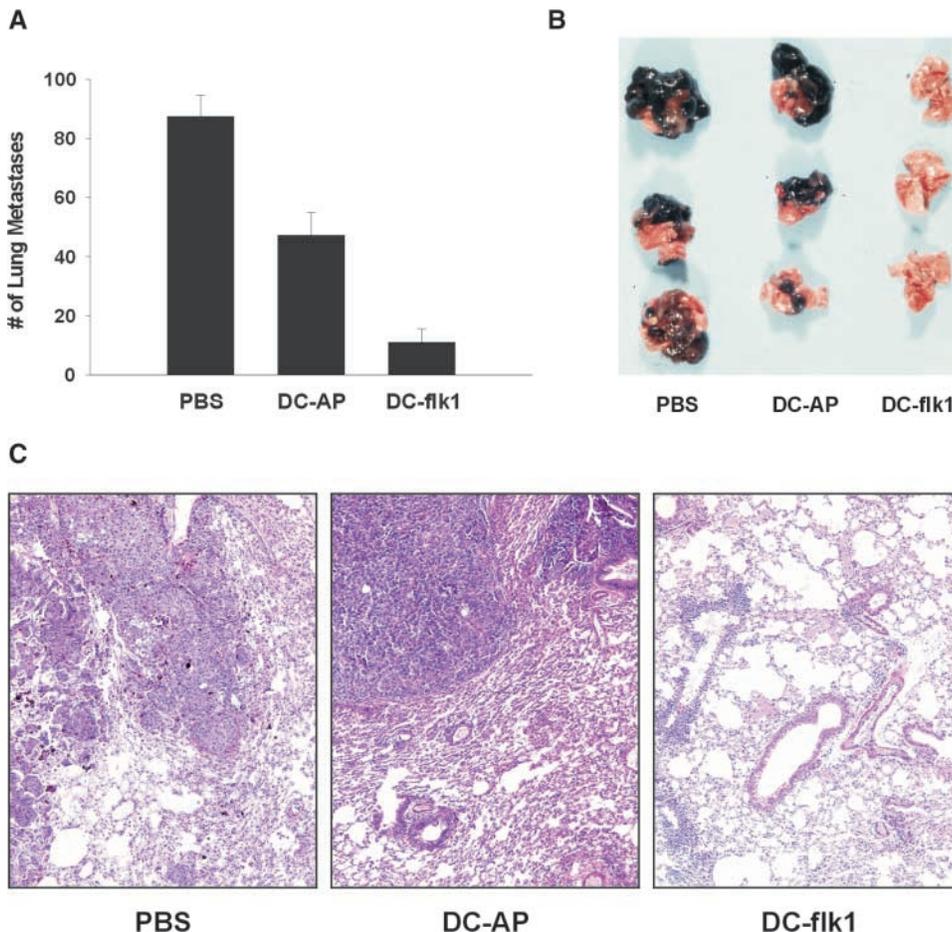


**Figure 5.** Survival advantage in mice immunized with DC-flk1 after a tumor challenge. Animals ( $n = 10$ ) were immunized with DC-flk1, DC-AP, or PBS. The experiment is similar to that described Fig. 3 A, except that the animals were not killed but were monitored for survival. The data is expressed as percentage of survival as function of time. Survival for the mice immunized with DC-flk1 was significantly prolonged over the DC-AP and PBS controls.

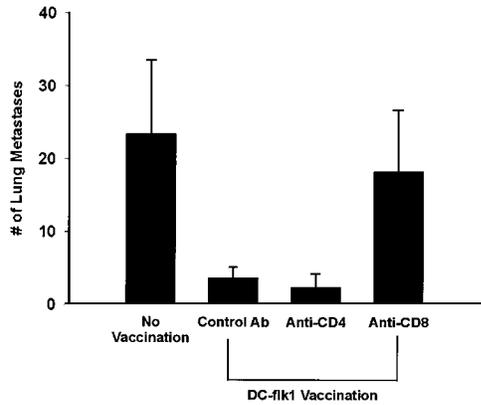
*DC-flk1 Vaccination Inhibits Lewis Lung Pulmonary Metastasis.* To determine whether active immunization against flk1 could inhibit tumor metastasis, we tested immunized mice in a metastasis model of Lewis lung carcinoma. Mice immunized with flk1-AP-pulsed DCs developed significantly reduced numbers of metastases in the

lungs ( $P < 0.01$ ) compared with mice immunized with either AP-pulsed DCs or PBS (Fig. 4, A and B). Most significantly, 6 of 10 mice were found to be tumor-free in the DC-flk1-immunized group, whereas all mice in the two control groups developed extensive pulmonary metastases. Macroscopic observations were further confirmed by microscopic examination of lung samples showing significant reduction or complete absence of tumor cells in the lungs of DC-flk1-immunized mice, whereas extensive tumor infiltration was found in lungs of control groups (Fig. 4 C). In addition to examination of tumor metastasis in immunized mice, a similar experiment was conducted to evaluate the effect of flk1 immunization on survival. As shown in Fig. 5, while all mice had died of tumor by day 63 in both control groups, 40% mice in the DC-flk1 group survived over 200 d. The other 60% of DC-flk1 group died almost as quickly as the control mice, suggesting that it was “all or none” protection.

*DC-flk1 Vaccination Inhibits Metastasis Induced by B16 Melanoma.* DC-flk1 immunization was also tested in a B16 melanoma model. After three immunizations with flk1-AP-pulsed DCs at days 0, 10, and 20, mice were challenged with an intravenous injection of B16 tumor cells. As shown in Fig. 6, DC-flk1 immunization significantly inhibited the formation of lung metastases compared with PBS or DC-AP control groups ( $P < 0.01$ ). Interestingly,



**Figure 6.** Inhibition of B16 tumor metastasis by DC-flk1 vaccination. (A) Mice were immunized with DC-flk1, DC-AP, or PBS, before challenged with intravenous injection of B16 tumor cells. Mice were killed 30 d after tumor inoculation and the lungs were assessed for tumor load.  $P < 0.01$  by Student's  $t$  test. (B) Pictures of representative lung samples from each group. (C) Histological examination of lung samples from each group. Original magnification:  $\times 100$ .



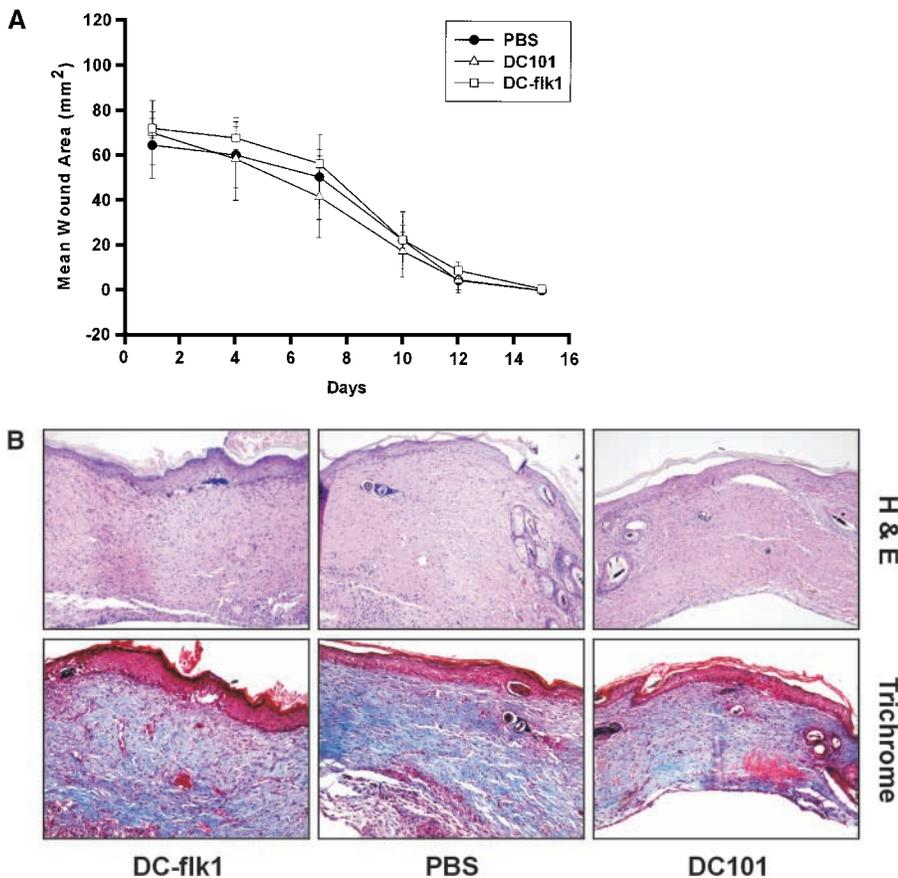
**Figure 7.** CD8<sup>+</sup> CTL play a major role in antitumor response induced by DC-flk1. Mice were treated with anti-CD4, CD8, or control Abs before they were immunized DC-flk1. In an additional control group, mice were not immunized. Mice were then challenged with Lewis lung tumor intrafootpad. The primary tumor was surgically removed when it reached ~5 mm in diameter. Mice were killed based on the metastatic death in the control groups.  $P < 0.01$  by Student's *t* test.

DC-AP group also showed a certain level of metastatic inhibition compared with PBS group.

*CD8<sup>+</sup> T Cells Play a Critical Role in Antitumor Response Induced by DC-flk1 Vaccination.* To determine which T cell population (i.e., CD4, CD8, or both) was responsible for the antitumor activities, we conducted an *in vivo* T cell

depletion assay in mice vaccinated with flk1-AP-pulsed DCs in the Lewis lung metastatic model (23). Mice were treated with either anti-CD4, anti-CD8, or control Ab before vaccinations with DC-flk1 and again 2 wk later. Depletion of respective T cell population was confirmed by FACS<sup>®</sup> analysis of splenocytes of one mouse from each group. The antitumor effect of vaccination against flk1 was abrogated in CD8-depleted but not in CD4-depleted mice (Fig. 7). These results suggest that the CD8 population is mainly responsible for the T cell-mediated anti-flk1 immune response.

*Wound Healing Is Not Affected by DC-flk1 Vaccination.* As described above, breeding experiments indicated an effect of DC-flk1 immunization on prenatal development. To further examine whether active immunization against flk1 has an effect on normal physiological angiogenesis, we analyzed wound healing in immunized mice using an excisional cutaneous wound model. Full-thickness wounds were created on the dorsum of control or DC-flk1-immunized mice 10 d after three immunizations on days 0, 10, and 20. Wound areas were measured twice weekly until they were completely healed. No significant difference in wound healing was observed between DC-flk1-immunized and control mice (Fig. 8 A). Microscopic examination of wound tissues revealed no difference in wound epithelialization, vascularity or collagen deposition (Fig. 8 B).



**Figure 8.** Cutaneous wound healing is not affected by DC-flk1 vaccination. A full-thickness excisional wound was created in DC-flk1 and untreated mice. Wound areas were measured twice weekly until wounds were completely healed (A). Wound scar tissues at day 15 from both groups were analyzed by histology (B). Top: hematoxylin and eosin staining; bottom: trichrome staining for collagen. Original magnification:  $\times 100$ . No difference was found between these tissues.

## Discussion

By using DCs to break tolerance to self-antigen, we demonstrate that active immunization against the VEGF receptor flk1 can inhibit tumor angiogenesis and metastasis in mice. We chose flk1 as the molecular target for these studies because of its recognized importance in angiogenesis and tumor growth. These results provide strong proof of concept that an active immunization approach against an angiogenic target can be effectively used for tumor therapy. Our data are consistent with those of other studies that have used antagonists of the VEGF pathway to inhibit tumor-associated angiogenesis (10–14). Recently, Wei et al. reported that cross-immunization with xenogeneic endothelial cells was effective in inducing an endothelial cell-specific immunity and protecting mice from tumor challenge. In their study, peptides within  $\alpha v$  integrin and EGFR-2 were identified as the possible antigen epitopes (26). Plum et al. showed that administration of a liposomal FGF-2 peptide vaccine led to abrogation of FGF-mediated angiogenesis and tumor development in mouse models (27). These studies suggest that angiogenesis-directed immunotherapy approach may potentially extend to other angiogenic targets such as VEGF, the Tie2 receptor (28), angiopoietin-1 (29, 30),  $\alpha v \beta 3$  (31), and VE-cadherin (32).

We detected a strong CTL response after DC-flk1 immunization. It is likely that flk1-specific CTLs exert antitumor effect by destroying endothelial cells in the neovasculature surrounding tumor nodules. Notably, flk1-specific neutralizing antibody was also present in the sera of immunized mice. The antibody effectively blocked the binding of VEGF to native flk1 at the surface of endothelial cells. Blockade of the VEGF-flk1 pathway could play an important role in the suppression of angiogenesis observed in DC-flk1-immunized mice. In the *in vivo* T cell depletion experiment, depletion of CD8<sup>+</sup> T cells abrogated the antitumor effect in the Lewis lung metastasis model whereas CD4<sup>+</sup> T cell depletion had a marginal effect. This result suggested a dominant role of CD8<sup>+</sup> CTLs in the antitumor activity while the role of flk-1 neutralizing antibody could not be excluded. This finding is consistent with the notion that antitumor immunity depends on CD8<sup>+</sup> T cells in many experimental tumor models (33). Given that an anti-flk1 neutralizing antibody response occurred in DC-flk1-immunized mice, however, we were surprised that CD4<sup>+</sup> T cell depletion did not affect the antitumor activity. One possible explanation for this observation is that the antibody induced by DC-flk1 immunization recognized a flk1 epitope that is CD4 independent. In the study by Wei et al., CD4<sup>+</sup> T cells and antibody were reported to be important in the antitumor activity induced by xenogenic endothelial cell vaccine (26). Plum et al. found that an anti-FGF-2 antibody was induced after immunization with liposomal FGF-2 peptide vaccine (27). The importance of CD8<sup>+</sup> versus CD4<sup>+</sup> T cell immunity in these studies may have been determined by the use of different target antigens, adjuvants, and routes of immunization.

Flk1 is expressed in normal vascular endothelium albeit in

lower levels compared with that in tumor vasculature and is important in fetal development (9, 34–37) and in physiological angiogenesis such as wound healing (34, 38, 39). One important observation in our studies was the adverse effect of DC-flk1 immunization on mouse pregnancy. This finding is consistent with the role of angiogenesis in multiple aspects of female reproductive function and fetal development. In non-pregnant adult mice, however, vaccination did not induce observable toxicity. Wound healing was not affected in the immunized mice. This result is compatible with the report that the angiogenesis inhibitor vasostatin does not impair wound healing at tumor-inhibiting doses (40). The lack of toxicity suggests a different sensitivity of malignant tumor growth than physiologic wound healing to inhibition of angiogenesis. Another possible explanation is that the level of expression of flk1 in quiescent normal vascular endothelium may be too low to be recognized by flk1-specific CTLs.

As an antiangiogenesis therapy, active immunization affords the opportunity to elicit a sustained effect in chronic disease settings. In contrast, antagonist drug therapies must be administered for long periods of time. In our experiments, the anti-flk1 immune response was monitored as long as 20 wk after the last booster immunization and was found to gradually diminish after the last immunization but could be restored to a therapeutic level with 1–2 booster immunizations (data not shown). Thus, the immune response against an angiogenesis-related target (and any effects associated with the immune response) may be short lived but could be recalled by repeated immunizations. In the context of immunotherapy, this approach benefits from the advantages of targeting an antigen expressed on non-transformed cells, *i.e.*, endothelial cells. Endothelial cells do not bear the problems associated with targeting antigens on tumor cells, such as genetic instability, mutations, or loss of antigen expression. Another advantage is that one single antiangiogenesis vaccine may be used against multiple tumor types. This notion is supported by this study and others (26, 27). In conclusion, angiogenesis-targeted immunotherapy offers the potential for a new approach to treatment of cancer and other angiogenesis-related diseases.

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