A Vascular Endothelial Growth Factor Receptor-2 Inhibitor Enhances Antitumor Immunity through an Immune-Based Mechanism

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Abstract

Purpose: Given the complex tumor microenvironment, targeting multiple cellular components may be the most effective cancer treatment strategy. Therefore, we tested whether antiangiogenic and immune-based therapy might synergize by characterizing the activity of DC101, an antiangiogenic monoclonal antibody specific for vascular endothelial growth factor receptor-2 (VEGF-R2), alone and with HER-2/neu (neu)–targeted vaccination.

Experimental Design: Neu-expressing breast tumors were measured in treated nontolerant FVB mice and immune-tolerant neu transgenic (neu-N) mice. Neu-specific and tumor cell–specific immune responses were assessed by intracellular cytokine staining, ELISPOT, and CTL assays.

Results: DC101 decreased angiogenesis and increased tumor cell apoptosis. Although DC101 increased serum levels of the immunosuppressive cytokine VEGF, no evidence of systemic immune inhibition was detected. Moreover, DC101 did not impede the influx of tumor-infiltrating lymphocytes. In FVB mice, DC101 inhibited tumor growth in part through a T cell–dependent mechanism, resulting in both increased tumor-specific CD8+ T cells and tumor regression. Combining DC101 with neu-specific vaccination accelerated tumor regression, augmenting the lytic activity of CD8+ cytotoxic T cells. In tolerant neu-N mice, DC101 only delayed tumor growth without inducing frank tumor regression or antigen-specific T-cell activation. Notably, mitigating immune tolerance by inhibiting regulatory T cell activity with cyclophosphamide revealed DC101-mediated augmentation of antitumor responses in vaccinated neu-N mice.

Conclusions: This is the first report of DC101-induced antitumor immune responses. It establishes the induction of tumor-specific T-cell responses as one consequence of VEGF-R2 targeting with DC101. These data support the development of multitargeted cancer therapy combining immune-based and antiangiogenic agents for clinical translation.

Signaling pathways involved in transformation and tumor progression are key targets for cancer therapy. Because tumors are heterogeneous, with high mutation rates that activate compensatory pathways or facilitate acquired resistance to single therapies, multitargeted strategies may represent optimal cancer treatments.

Tumor immunotherapy can specifically target tumor-associated antigens, resulting in low toxicity and the potential for durable immunologic memory responses. Because tumor cells arise endogenously, most tumor antigens may be recognized as self. Consequently, tumor-specific T cell repertoires may be limited through mechanisms of central (thymic deletion) or peripheral tolerance [deletion, ignorance, anergy, or suppression by CD4+CD25+ regulatory T cells (Treg); ref. 1]. Because immunotherapy alone may not be successful against advanced disease, it is important to investigate combinatorial strategies for augmenting the potency of cancer vaccines.

Several studies support integrating tumor immunotherapy and antiangiogenic therapy (2–4). Angiogenesis, defined as the development of new vasculature from preexisting blood vessels, is involved in tumor growth and metastasis (5). The success of Bevacizumab (Avastin), the first Food and Drug
Administration–approved antiangiogenic therapy, shows the importance of targeting angiogenesis (6). However, antiangiogenic therapy often requires continuous drug administration, and residual tumor masses may be capable of regrowth (7). These findings reiterate the need for multitargeted therapy. Therefore, we sought to examine clinically relevant interactions between tumor immunotherapy and antiangiogenic therapy in the HER-2/neu (neu) mouse model.

The proto-oncogene neu is overexpressed in up to 25% of human breast cancers and confers a poor prognosis (8). Neu-N mice, derived from the parental FVB strain, express nontransferring rat neu cDNA under the control of the mammary-specific promoter mouse mammary tumor virus (9). Consequently, neu-N mice spontaneously develop mammary cancers and exhibit well-established peripheral immune tolerance to neu (10). Neu-specific whole-cell granulocyte macrophage colony-stimulating factor (GM-CSF)–secreting vaccines induce curative neu-specific immune responses in tumor-bearing FVB mice, with most neu-specific CD8+ T cells recognizing the MHC class I epitope RNEU420-429 (11). Conversely, neu-specific vaccines alone are ineffective in tolerant neu-N mice. We have shown that modulating immune checkpoints can improve tumor responses. Administering low-dose cyclophosphamide before vaccination abrogates the immunosuppressive effects of Tregs, leading to the expansion of high-avidity, RNEU420-429–specific CD8+ T cells and prolonged tumor-free survival in up to 30% of neu-N mice (12). Similar results were observed with OX40 (CD134) costimulation (13).

Neu overexpression correlates with increased angiogenesis and expression of vascular endothelial growth factor (VEGF). VEGF, which is produced by tumors and endothelial cells, can promote both angiogenesis (14) and immune suppression (15). VEGF signals in part via VEGF receptor-2 (VEGF-R2; Flk1/KDR), a transmembrane tyrosine kinase receptor whose expression is predominantly localized to vascular endothelial cells (14), and correlates with invasion and metastasis (16). Therefore, various VEGF-R2 inhibitors are being developed (17). One example is DC101, a rat monoclonal antibody specific for mouse VEGF-R2 that can significantly decrease tumor growth and angiogenesis (18, 19).

Given the importance of neu and VEGF in tumor progression, and their potential biological interactions, we investigated the bioactivity of DC101-mediated VEGF-R2 inhibition alone and combined with neu-specific vaccination. We first examined the effects of DC101 on tumor angiogenesis and apoptosis and then determined whether DC101 treatment might result in VEGF-mediated immune suppression or impaired T cell infiltration into tumors. We then characterized antitumor immunity and tumor regression after DC101 administration, comparing DC101-induced responses in nontolerized FVB mice and tolerized neu-N mice. Here, we show for the first time that DC101 can enhance antitumor responses by improving tumor-specific T-cell activity in the absence of immune tolerance.

### Materials and Methods

**Mice.** FVB mice were purchased from Harlan or Taconic Laboratories. Neu-N mice (9), provided by Dr. William Muller (McGill University, Montreal, Canada), were bred to homozygosity as verified by Southern blot (10). The colony was maintained at Harlan Biotech Center (Baltimore, MD).

Clone 100 T-cell receptor (TCR) transgenic mice, derived from FVB mice, express the high-avidity, RNEU420-429–specific TCR in the majority of peripheral CD8+ T cells. Briefly, mice were generated by cloning cDNA from high-avidity, RNEU420-429–specific T cell lines (12) into TCR α and β chain constructs, followed by ligation into corresponding TCR cassette vectors using previously described methods (20, 21). The linearized DNA fragments were microinjected into fertilized eggs, which were then implanted into pseudopregnant FVB mice in the Johns Hopkins Medical Institutions transgenic core. Flow-cytometric analysis of founder mice confirmed that >95% of peripheral CD8+ T cells contained Vγ4 sequences and 60% to 70% were also specific for RNEU420-429.

The generation of p56LK/GFP mice has been described (22). The pEFGP-C1 gene (Clonetech), ligated to the k<sub>c</sub> proximal promoter, was microinjected into fertilized FVB eggs. Flow-cytometric data confirmed >95% GFP+ T cells (data not shown).

Experiments were done with 8- to 12-week-old mice using AAALAC-compliant protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

**Cell lines and media.** The GM-CSF–secreting vaccine cell lines 3T3GM (mock) and 3T3neuGM (neu-specific), the NT2.5-neu expressing breast tumor cell line (derived from a spontaneous tumor explanted from a neu-N mouse), and the T2D<sup>th</sup> line were grown as previously described (10). The 3T3neuB7.1 and NT2.SB7.1 lines were produced by retroviral transduction with a human B7.1-encoding retrovirus (23). The pancreatic endothelial cell line MS-1 [American Type Culture Collection (ATCC)] was grown in ATEC medium [DMEM with 4 mMol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 5% fetal bovine serum (FBS)].

**RNA isolation, cDNA preparation and reverse transcription-PCR.** RNA was isolated (RNeasy Mini; Qiagen Inc.), followed by cDNA preparation [Superscript First-Strand Synthesis System for Reverse Transcription-PCR (RT-PCR); Invitrogen Corp.]. cDNA amplification used the following primers: VEGF sense, GAGATCCCTCAGGAGCGATCT, and antisense, GCATTAGGACGAGATATAAGA; VEGF-R2 sense, TACACATTCAGGACGATGTTG, and antisense, CTGGTTCC-TCCAATGGGATATCTTC (Invitrogen). Reaction conditions included a 50°C/5 min preincubation step, 94°C/1 min denaturation step, and 72°C/1 min annealing/extension step for 30 cycles. PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

**Antibody administration.** DC101 was supplied by Dr. Daniel Hicklin (ImClone Systems Inc., New York, NY). Non-specific polyclonal rat immunoglobulin G (IgG; Sigma-Aldrich, St. Louis, MO) served as a control. Antibodies were endotoxin free, as confirmed in-house by the Limulus amebocyte lysate test (Bio Whittaker, Walkersville, MD). Antibodies were diluted in 250 μL PBS and injected i.p. at 0.8 mg per mouse twice weekly as previously described (18, 19).

**Immunohistochemistry.** OCT-coated tumors (Tissue-Tek, VWR) were snap frozen and sectioned at 5 μm at the JHMI Pathology Core. Sections were stained with H&E or retained for immunohistochemistry using reagents and protocols from BD Biosciences. Vascularization and apoptosis were analyzed with antibodies specific forPECAM/CD31 andcleaved caspase-3, respectively (BD PharMingen), and slides were developed with 3,3′-diaminobenzidine (Dako Incorporated). Images were captured under light microscopy (E800, Nikon) at 20× magnification. Quantification of PECAM staining was done by digital densitometry using MetaMorph software via TopHat analysis (Molecular Devices Corp.).

**VEGF ELISA.** NT2.5 cells were seeded at 10<sup>6</sup> cells/ml in a six-well plate, and supernatant was harvested 24 h later. Blood was harvested from mice by cardiac puncture, and serum was collected by centrifugation. Supernatant and serum were analyzed by VEGF ELISA (R&D Systems).

**Supplementary Material.**

Antibodies, flow cytometry (fluorescence-activated cell sorting) analysis, and tetramer staining. Anti–CD3-FITC, anti–CD62L-FITC, anti–B7.1-FITC, anti–B7.2-FITC, anti–Gr1-FITC, anti–CD8-CyCh, anti–CD4-PE, anti–CD11c-PE and anti–Mac1-PE were used to stain leukocytes (BD Pharmingen). NT2.5 cells were stained with serially diluted serum harvested from tumor-bearing mice, followed by a PE-labeled anti-mouse IgG antibody (BD Pharmingen). The H-2Dk RNEU420-429 tetramer was constructed and used to stain splenic T cells as previously described (12, 24). Fluorescence-activated cell sorting data was collected using a BD FACScan with CellQuest software (BD Bioscience) and analyzed with FlowJo software (TreeStar, Inc.).

Tumor treatment experiments and chemotherapy. FVB mice were challenged s.c. with 5 × 10⁶ NT2.5 tumor cells in the right mammary fat pad, followed by vaccination 14–28 days later. Neu-N mice were challenged with 5 × 10⁶ NT2.5 cells and vaccinated 3 days later. Vaccine cells were irradiated before s.c. injection in both hind limbs and the left front limb. Cyclophosphamide (Bristol-Myers Squibb) was injected i.p. at 100 mg/kg 1 day before the vaccine. Doxorubicin (Gensia Sior) was injected i.v. at 5 mg/kg 7 days after vaccine. Doses and timing for tumor cells, vaccinations, and chemotherapy have been previously optimized and reported (10, 25). Mice were monitored for tumor growth and onset twice weekly. Tumor growth was determined by measuring tumor diameter in two perpendicular dimensions with calipers. Tumor size was calculated by multiplying these two measurements. Mean tumor size for an experimental group included only those mice with measurable tumors.

Ex vivo antigen presentation assay. Tumor-bearing FVB mice received a 2-week course of DC101 or rIgG, followed by vaccination. One week postvaccination, splenic dendritic cells (DC) were harvested, purified over a Nycodenz gradient (Accurate Chemical), depleted of CD3⁺ and CD19⁺ cells (Dynal Biotech, Invitrogen), and enriched for CD11c⁺ cells (Milltenyi Biotech). Conventional DCs were isolated from Clone 100 TCR transgenic mice by negative selection (Dynal Biotech). The percent of antigen-specific CD8⁺ T cells is given as the percent of IFN-γ⁺ cells in the irrelevant NP118-126 sample subtracted from that in the neu-specific RNEU420-429 sample.

ELISPOTs. CD8⁺ splenic lymphocytes were purified by negative selection (Dynal Biotech). About 10⁵ CD8⁺ T cells were incubated in duplicate with 10⁵ target cells (NT2.5B7.1 cells stimulated with IFN-γ for 2 days) at 37°C overnight on precoated IFN-γ–specific ELISPOT plates and developed according to manufacturer’s protocols (R&D Systems). IFN-γ–secreting CD8⁺ T cells were enumerated using the Immunospot counter (Cellular Technology, Ltd.). The average number of spots in control wells was subtracted from the average number of spots in each well containing both CD8⁺ T cells and targets.

In vivo depletions. CD4⁺ and CD8⁺ T cells were continuously depleted using GK1.5 and 2.43 antibodies, respectively, as previously described (10), except that filtered, diluted ascites was used. Natural killer cells and macrophages were depleted by twice weekly i.p. injections of α-asialo GM1 (Wako Chemical) and carrageenan (Sigma-Aldrich), respectively. Greater than 95% depletion was confirmed by flow cytometry.

Chromium-release assays. Splenic lymphocytes were isolated by Ficoll gradient centrifugation (Amersham Bioscience), and their lytic activity against NT2.5B7.1 and 3T3neuB7.1 target cells was tested in triplicate in a standard 4-h chromium-release assay as previously described (12). The percent specific lysis was determined using the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

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Isolation and characterization of tumor-infiltrating lymphocytes. Tumors from p56c-srcGFP FVB mice were prepared as described above. Unstained sections were visualized by fluorescence microscopy (E800, Nikon) at 20× magnification. GFP⁺ cells were quantified using MetaMorph software via TopHat analysis. Tumors from FVB mice were weighed and digested into single-cell suspensions with hyaluronidase, collagenase type IV and trypsin (Life Technologies, Invitrogen) using standard methods. Cells were plated for 2 to 3 h to allow tumor cells to adhere, and then the T cell–enriched supernatant was collected. Cells were counted, analyzed by flow cytometry, and normalized to tumor weight.

Statistical analysis. A Student’s t-test was applied to determine the statistical significance of differences between treatment groups, with P < 0.05 being significant. Analyses were done using GraphPad Prism, version 3.0a for Macintosh (GraphPad Software). All experiments were repeated at least twice, with 5 to 20 mice per group depending on the study end point.

Results

DC101 inhibits tumor growth, decreases angiogenesis, and increases tumor cell apoptosis in neu-expressing tumors. We first examined the expression of VEGF and VEGF-R2 in FVB and neu-N mice. The neu-overexpressing NT2.5 breast tumor cell line secreted VEGF at ~150 pg/10⁶ cells/24 h (data not shown). VEGF-R2 mRNA was not detected in these cells, but was measurable in normal mammary tissue and seemed to be increased in transplanted and spontaneous tumors (Fig. 1A).

These explanted tumor samples contain both tumor and surrounding endothelial cells, suggesting that VEGF-R2 is up-regulated within the microenvironment of neu-expressing tumors in vivo.

The administration of DC101 to tumor-bearing FVB mice significantly inhibited the growth of neu-expressing tumors (Fig. 1B). Tumors from DC101-treated mice were necrotic (Fig. 1C). Immunohistochemistry showed that DC101 reduced angiogenesis and increased tumor cell death (Fig. 1C).

Specifically, the amount of endothelial cell–specific PECAM staining was decreased by 81% (P = 0.006) in tumors treated for 2 weeks with DC101 as compared with rIgG. These data confirm the known antiangiogenic activity of DC101.

DC101 treatment does not induce VEGF-mediated immune suppression or impede the influx of tumor-infiltrating lymphocytes. Although VEGF levels often correlate with tumor burden, DC101 treatment increases systemic VEGF levels, even when tumor growth is inhibited (26). This increase may be due to reduced receptor-mediated endocytosis of VEGF as DC101 blocks VEGF-R2 or increased tumor-derived VEGF to compensate for diminished signaling. Although the VEGF ELISA assay has some cross-reactivity with circulating DC101, we found serum VEGF levels increased by almost 5-fold in DC101-treated tumor-bearing FVB mice, consistent with a bona fide elevation in VEGF (Fig. 2A).

Although elevated VEGF levels correlate with increased levels of T cell–suppresserivate immature myeloid cells (MSC), (27), MSC were not significantly expanded in DC101-treated FVB mice (Fig. 2B). Similarly, although VEGF can inhibit the maturation of DCs (28–30), DC101-induced VEGF did not inhibit DC maturation as measured by the expression of the costimulatory markers B7.1 and B7.2 (data not shown). Furthermore, activated DCs harvested from vaccinated FVB mice pretreated with DC101 or rIgG were equally able to stimulate the proliferation of naïve RNEU420-429–specific clonotypic CD8⁺ T cells.
cells from TCR transgenic mice (Fig. 2C). The frequency and avidity of CD8+ T cells specific for RNEU420-429 were also comparable between the two groups (Fig. 2D and E). Therefore, whereas DC101 administration increased VEGF levels in vivo, DC-dependent immune priming was not impaired, and no evidence of functional CD8+ T cell suppression was detected.

We next analyzed tumor-infiltrating lymphocytes (TIL) to determine if T cells could still effectively traffic to DC101-treated tumors. Two weeks after the DC101 treatment of tumor-bearing p56<sup>lck</sup>GFP FVB mice (alone or before vaccination), increased numbers of TIL were detected by fluorescence microscopy (Fig. 3A and B). GFP<sup>+</sup> T cells were not found in the kidney (an irrelevant control), demonstrating that T cells traffic to the tumor after DC101 treatment. Flow-cytometric analysis revealed greater numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the tumors of FVB mice treated with DC101 (Fig. 3C). These observations show that DC101 administration does not restrict, and may facilitate, T cell trafficking to the tumor. Therefore, DC101 treatment does not negatively impact functional host immune responses or T cell trafficking.

Fig. 1. DC101 inhibits tumor growth, decreases angiogenesis, and increases apoptosis within tumors of FVB mice. A, RT-PCR was conducted with gene-specific primers for VEGF-R2 and GAPDH. Lane 1, normal mammary tissue; lane 2, transplanted tumor; lane 3, spontaneous tumor; lane 4, NT2.5 tumor cells; lane 5, MS-1 endothelial cells; lane 6, no cDNA. B, FVB mice (n = 20 per group) were tumor challenged and 2 wks later began receiving rlgG or DC101 biweekly for 3 wks (horizontal arrow). C, tumors from FVB mice were prepared for histologic examination 2 wks after antibody administration as described in (B). Representative samples of mice treated with rlgG (top) or DC101 (bottom) are shown with H&E staining or immunohistochemistry to detect endothelial cells (PECAM/CD31) or apoptotic cells (cleaved caspase-3), at 20× magnification. PECAM staining was quantified using MetaMorph software. Numbers (mean ± SD) are shown as the density values acquired from three samples per group.

Fig. 2. DC101 administration increases serum VEGF levels, but does not cause immune suppression or inhibit vaccine-induced immunity in FVB mice. A, FVB mice were tumor challenged and then given rlgG or DC101 biweekly beginning 2 wks later. At various time points (n = 5 per group), serum VEGF levels were quantified by ELISA. *, P < 0.05. B, splenocytes were harvested from mice in (A) and analyzed by flow cytometry using antibodies specific for immature myeloid cell markers Gr1 and Mac1*. *, P < 0.05. C, tumor-bearing FVB mice (n = 5 per group) received a 2-wk course of DC101 or rlgG, followed by neu-specific vaccination (3T3neuGM) or mock vaccination (3T3GM). Splenic DCs harvested 1 wk after vaccination were cocultured with naive CFSE-labeled RNEU420-429-specific CD8<sup>+</sup> T cells from Clone 100 TCR transgenic mice for 4 d. T-cell proliferation was analyzed by CFSE dilution. Representative samples from each group are shown. D, additional FVB mice (n = 5 per group) were treated as described in (C). Splenic T cells were isolated 2 wks after vaccination, and reactivity to the immunodominant RNEU420-429 epitope was determined by IFN-γ ICS. E, splenic T cells were also stained with CD8, CD62L, and D<sup>+</sup>-RNEU420-429 tetramer. Histograms show representative samples from each group, with gating on the CD8<sup>+</sup>, CD62L<sup>+</sup>, and D<sup>+</sup>-RNEU420-429 tetramer. Black solid line, 1:5 tetramer dilution; gray line, 1:50 tetramer dilution; shaded, 1:500 tetramer dilution.
The DC101-mediated antitumor effect was dependent on both subsets (Fig. 4A). Natural killer cell or macrophage depletion did not impact the antitumor effect of DC101 (data not shown). After discontinuing DC101 treatment, tumors resumed growth only in T cell–depleted mice (Fig. 4A). Collectively, these data show that DC101 can facilitate T cell–dependent tumor regression in addition to mediating an antiangiogenic effect (Fig. 1C).

DC101-mediated T cell responses were also investigated. We found a significant expansion of tumor-specific CD8+ and CD4+ T lymphocytes in FVB mice treated for 1 to 3 weeks with DC101 compared with rIgG (Fig. 4B and C). Withdrawing DC101 after a 3-week treatment period revealed that only immune-competent FVB mice completely rejected their tumors (data not shown). Upon rechallenge, one-half of these cured mice remained tumor free, with most of them developing RNEU420-429–specific CD8+ T cell responses almost as well as neu-vaccinated FVB mice (Fig. 4D). Antitumor antibody titers in mice treated with rIgG or DC101 were similar (data not shown). Although B cells may contribute, these data suggest that DC101 induces T cell–mediated tumor rejection and tumor-specific immune responses in nontolerant tumor-bearing hosts.

Combining DC101 with neu-specific vaccination in FVB mice is effective. Because DC101 alone augmented antitumor responses in FVB mice, we examined DC101 in combination with neu-targeted vaccination. Tumors regressed faster in neu-vaccinated mice pretreated for 2 weeks with DC101 compared with rIgG-treated neu-vaccinated controls (Fig. 5A, P = 0.004). To equalize the extent of tumor burden at the time of vaccination, antibody treatment was alternatively begun the day of vaccination. Here, there was a significant difference in the rate of tumor regression between mock-vaccinated mice and neu-vaccinated mice in the setting of DC101 treatment (Fig. 5B, P < 0.001). However, the difference between the rIgG- and DC101-treated mice in the setting of neu-targeted vaccination was not statistically significant (Fig. 5B, P = 0.086). This may be due to the ability of the neu-specific vaccine to induce robust antitumor immune responses in FVB mice. Nevertheless, neu-vaccinated mice did seem to reject tumors sooner in the setting of DC101 treatment because the time to reach 50% tumor free was shorter (18 versus 25 days posttreatment, respectively).

Significant increases in CTL activity against NT2.5 tumor cells and 3T3neu target cells were detected in neu-vaccinated mice that also received DC101 (Fig. 5C and D). Interestingly, whereas immune responses specific for RNEU420-429 remained unchanged after combination therapy (Fig. 2D and E), enhanced T cell activity was seen against whole tumor cells and full-length neu (Fig. 5C and D). This suggests that combining DC101 and vaccination may induce a more heterogeneous repertoire of T cells, recognizing tumor-specific epitopes other than RNEU420-429. Together, these data show that DC101 can be effectively incorporated into a tumor vaccine regimen, leading to improved tumor-specific immune responses.

DC101-induced antitumor responses are only detected in neu-N mice after inhibition of Tregs with immune modulating doses of cyclophosphamide. We next evaluated DC101 activity in neu-N mice, which exhibit profound peripheral immune tolerance to neu and thus represent a more clinically relevant model. DC101 treatment of tumor-bearing neu-N mice inhibited angiogenesis, induced apoptosis, and increased serum VEGF levels without inducing T cell–depleted mice (day 0: 33.9 ± 6.06 mm²; day 21: 31.07 ± 7.04 mm²; P = 0.20). Selective depletion of CD4+ and/or CD8+ T cells revealed that the DC101-mediated antitumor effect was dependent on both subsets (Fig. 4A). Natural killer cell or macrophage depletion did not impact the antitumor effect of DC101 (data not shown). After discontinuing DC101 treatment, tumors resumed growth only in T cell–depleted mice (Fig. 4A). Collectively, these data show that DC101 can facilitate T cell–dependent tumor regression in addition to mediating an antiangiogenic effect (Fig. 1C).

DC101 inhibits tumor growth through a T cell–dependent mechanism and enhances antitumor immune responses in FVB mice. Potential immune-based antitumor effects of DC101 were first examined in nontolerant FVB mice, where the neu tumor antigen is highly immunogenic. Systemic DC101 given for 3 weeks significantly reduced the size of established tumors (day 0: 28.1 ± 5.5 mm²; day 21: 18.8 ± 5.8 mm²; P < 0.05; Fig. 4A). Notably, DC101 stabilized tumor growth without inducing regression in T cell–depleted mice (day 0: 33.9 ± 6.06 mm²; day 21: 31.07 ± 7.04 mm²; P = 0.20). Selective depletion of CD4+ and/or CD8+ T cells revealed that...
levels without affecting MSCs or DCs (data not shown). Moreover, the rate of tumor growth was significantly reduced in tumor-bearing DC101-treated neu-N mice compared with rIgG-treated controls (Fig. 6A).

Fig. 4. DC101 treatment inhibits tumor growth by a T cell–dependent mechanism in FVB mice. A, the experiment in Fig. 1B, where tumor-bearing FVB mice received antibodies for 3 wks, was repeated in the setting of T cell depletion. Additional groups (n = 10) were included, where before receiving DC101, CD4+ and CD8+ T cells were depleted either individually (CD4+ and CD8+) or together (CD4+/CD8+). The data shown represent the averaged values for DC101-treated mice in the presence and absence of combined CD4 and CD8 depletion (two replicates). The individual CD4 and CD8 depletions were done once with the second evaluation of combined depletion. In one experiment, the group of mice treated with DC101 was followed to cure, with 100% tumor rejection (data not shown). B and C, splenic T cells were isolated from tumor-bearing FVB mice at 1, 2, and 3 wks after antibody administration (n = 5 per group). The CD8+ and CD4+ effector T cells (B and C, respectively) were then incubated with NT2.5.B7.1 target cells for an IFN-γ ELISPOT. Symbols, individual mice; line, average. N.D. = not detected. D, DC101-treated FVB mice that completely rejected tumor (data not shown) were rechallenged and followed for 1 mo. At that time, half of the mice had developed tumors (striped columns), and half remained tumor free (solid columns). Splenic T cells were then isolated, and reactivity to RNEU420-429 was determined by IFN-γ ICS. Controls included FVB mice given a neu-specific vaccine (3T3neuGM) or a mock vaccine (3T3GM).

Fig. 5. Combining DC101 and neu-specific vaccination improves antitumor responses and CTL activity in FVB mice. A, FVB mice (n = 10 per group) were tumor challenged and 2 wks later received a 2-wk course of DC101 or rIgG (horizontal arrow), followed by vaccination (3T3neuGM, vertical arrow). B, FVB mice (n = 10 per group) were tumor challenged and 2 wks later were vaccinated and began receiving either DC101 or rIgG on the same day. C and D, 2 wks postvaccination, splenic effector T cells were isolated from mice in A (n = 5) and used in a 4-h chromium-release assay with NT2.5.B7.1 cells (C) or 3T3neuB7.1 cells (D) as targets to detect tumor-specific and neu-specific lysis, respectively. 3T3 cells, lacking the neu antigen, were used as a negative control.* P < 0.05.
In contrast to DC101-induced tumor regression in FVB mice, tumors in neu-N mice continued to grow slowly during DC101 treatment (day 0: 23.7 ± 4.7 mm²; day 21: 33.7 ± 6.4 mm²; \( P < 0.05 \)) and then upon discontinuing DC101 (Fig. 6A). Also, unlike FVB mice, tumor-specific CD8+ T cells did not significantly expand in DC101-treated neu-N mice (data not shown), and there was no difference in tumor growth in DC101-treated neu-vaccinated and mock-vaccinated neu-N mice (Fig. 6B).

One strategy used to overcome tolerance in neu-N mice is immune modulation with low-dose cyclophosphamide and doxorubicin (25). Cyclophosphamide selectively depletes cycling CD4+CD25+ Tregs, leading to improved tumor-free survival and vaccine-induced neu-specific T cell activation in vaccinated neu-N mice (12). With each chemotherapeutic agent given as a single dose 1 week apart, it is unlikely that endothelial cells are selectively targeted by this timed sequential chemotherapy (as described in some metronomic chemotherapy regimens; ref. 31).

Depleting Tregs before vaccination could uncover the ability of DC101 to improve the efficacy of neu-specific vaccination in neu-N mice. We found that combining DC101 and chemotherapy-modulated neu-specific vaccination resulted in the highest tumor-free survival rate to date in vaccinated neu-N mice at 40% compared with 10% with chemotherapy-modulated neu-specific vaccination alone (\( P = 0.022 \)) or 0% with DC101 and chemotherapy-modulated mock vaccination (\( P < 0.05 \); Fig. 6C). Although immune responses to the neu antigen were not significantly improved after incorporating DC101 into the chemotherapy-vaccine regimen (data not shown), the enhanced in vivo antitumor response did correlate with a small but statistically significant increase in CTL activity specific for NT2.5 tumor cells (Fig. 6D). These observations suggest that DC101 combined with immune modulation and vaccination in neu-N mice could augment the activity of tumor-specific T cells overall. These data also provide evidence that, in the appropriate context (i.e., absence of immune tolerance), DC101 can be effectively integrated with vaccination, enhancing both tumor-free survival and antitumor immune responses.

**Discussion**

The data presented here support three new findings. First, DC101 alone can induce T cell–mediated antitumor immune responses leading to tumor rejection in the absence of immune tolerance; both CD4+ and CD8+ T cells contribute to this effect. Second, combining DC101 and tumor-specific vaccination can induce greater immune responses than either therapy alone. Third, DC101 does not induce systemic VEGF-mediated immune suppression or inhibit the infiltration of TIL in this model. Importantly, this is the first study to systematically

![Fig. 6](https://www.aacrjournals.org/clin-cancer-research/article-pdf/13/13/3957/3184792/clin-cancer-research-2007-07-01.pdf)

**Fig. 6.** DC101 enhances antitumor responses in neu-N mice only when immune tolerance is modulated by elimination of Tregs. A, tumor-bearing neu-N mice \( (n = 20 \) per group) received rlgG or DC101 biweekly for 3 wks (horizontal arrow). B, Neu-N mice \( (n = 10 \) per group) were tumor challenged, and 3 d later, mice were vaccinated and received rlgG or DC101 biweekly for the duration of the experiment. C, Neu-N mice \( (n = 10 \) per group) were tumor challenged, vaccinated, and given antibody as described in (B). However, immune modulation with low-dose cyclophosphamide and doxorubicin was also added \( (C/D) \). Listed next to each curve is the proportion of mice that remained tumor free at the end of the experiment (day 50). D, at the end of the experiment, splenic effector T cells were isolated and used in a 4-h chromium-release assay with NT2.5B7.1 cells as targets. *, \( P < 0.05 \).
examine tumor-specific immune responses after DC101 treatment and thus reveals the induction of an immune response as a novel antitumor activity mediated by DC101.

Most studies examining the therapeutic activity of DC101 have tested the antibody against human tumor xenografts in immune-deficient mice (32–35). These models show potent antiangiogenic effects, but are inherently unsuitable for detecting the immune activating potential of DC101. For example, whereas DC101 reduces the growth rate of tumors in nude mice compared with controls, most tumors are still slowly growing during and upon withdrawing DC101 treatment (19, 33). Improved tumor-free survival was recently described when DC101 was combined with DC-based vaccination in immune-competent mice; however, immune responses were not examined (36).

In our experiments, immune-competent mice are receiving the rat monoclonal antibody DC101. Tumors in FVB mice regress both during and after DC101 treatment, suggesting that any neutralizing antibodies specific for DC101 present do not impact its antitumor effect.

Our model system offers the opportunity to examine immune responses in both the absence (FVB mice) and presence (neu-N mice) of immune tolerance against the neu tumor antigen. Here, we show for the first time that DC101 can induce T cell–dependent tumor regression in immune-competent FVB mice (which do not exhibit antigen-specific immune tolerance). The strong immunogenicity of the neu antigen in FVB mice might lead to effective antigen presentation, thereby augmenting T cell activation and promoting tumor regression. In contrast, the ability of DC101 to augment neu-specific immune responses may be minimal in neu-N mice due to preexisting mechanisms of immune tolerance. In fact, DC101 merely slowed tumor growth, but induced neither frank tumor regression nor tumor-specific immune responses in neu-N mice. Moreover, Treg depletion with cyclophosphamide given before vaccination was required to allow DC101 to enhance vaccine-induced tumor regression responses in neu-N mice. This response correlated with very modest, but statistically significant, increases in tumor-specific CTL-mediated lysis. The impact of combined therapy on tumor-free survival in neu-N mice suggests that leveraging very small therapeutic gains in the setting of multitargeted therapy can result in a clinically meaningful therapeutic effect.

Notably, the ability of DC101 to mediate durable tumor regression in FVB mice seems to be T cell–dependent. DC101 treatment alone resulted in the significant expansion of tumor-specific CD8+ T cells in immune-competent, non tolerant FVB mice. When DC101 was given to neu-vaccinated tumor-bearing FVB mice, there was enhanced lytic activity against tumor cells and cells expressing full-length neu protein, but an equivalent response to the immunodominant epitope RNEU420-429. This suggests that heterogeneous epitopes from neu, and possibly other tumor antigens, may be presented to enhance DC101-mediated antitumor responses. Currently, we are working to identify alternate epitopes of neu that may be recognized. Finally, although the tumor-specific antibody titer did not change with DC101 treatment, it may also be possible that DC101 modulates the repertoire of the tumor-specific humoral immune response.

These data show that, in addition to its direct antiangiogenic activity, DC101 requires functional T cells to induce tumor-specific immune activation. Consistent with these data, the antiangiogenic agents endostatin and angiotatin can also enhance the antitumor response in immune-competent mice as compared with immune-deficient mice (37, 38). These observations together illustrate that the host immune system can contribute to the antitumor activity of antiangiogenic therapy.

At least two possible mechanisms may explain DC101-mediated immune activation. First, DC101 may enhance the cross-presentation of multiple neu-derived T cell epitopes by inducing tumor cell apoptosis (39). In fact, presentation of tumor-specific antigens after apoptosis has been shown to result in cross-priming both in vitro and in vivo (40–43). Alternatively, CD8+ T cells may be primed directly by DC101-treated tumors. DC101 could induce up-regulation of costimulatory molecules or cytokines within the tumor microenvironment to improve direct priming. This has been shown for other cancer therapies such as low-dose melphalan, mitomycin C, and gamma-irradiation (44, 45). Further studies are now under way to explore the mechanism of DC101-mediated immune activation in FVB and neu-N mice.

We did not find evidence for VEGF-related immune suppression with DC101 treatment (15, 28). Others have shown that the administration of anti-VEGF neutralizing antibodies enhanced the effectiveness of tumor-specific immunotherapy by improving DC activity in murine models (46). High VEGF levels did not inhibit DCs in our model, consistent with data showing that DCs can exhibit different degrees of sensitivity to VEGF-mediated inhibition depending on the context of their stimulation (47). Moreover, in our model, the size of the immature myeloid cell population correlated with tumor burden rather than VEGF concentration. Because multiple cytokines regulate myeloid cell differentiation and expansion (27), activation of immune responses in DC101-treated mice might induce differentiation, whereas tumor-derived factors could promote expansion in rlgG-treated mice. It is also possible that the serum VEGF lacks bioactivity or the ability to bind VEGF-R1, which has been shown to mediate DC suppression (30).

Additionally, we hypothesized that T cells could still infiltrate DC101-treated tumors despite the inhibition of neovascularization because tumor regression was improved in DC101-treated mice. Evaluating the TIL revealed that DC101 does not inhibit, and may actually improve, T cell infiltration into the tumors of treated FVB mice. These data are consistent with recent work showing that DC101 can promote vascular normalization, where immature vessels are pruned and the remaining ones are strengthened (48). Normalization of aberrant tumor-associated vessels has been shown to increase the delivery of chemotherapeutic agents (49) and improve the trafficking of adoptively transferred T cells (50).

In conclusion, we show for the first time that DC101, in addition to its established antiangiogenic activity, can also elicit effective tumor-specific, T cell–mediated immune responses in the absence of immune tolerance. We also provide evidence that DC101 can be strategically combined with tumor immunotherapy to improve both tumor-free survival and tumor-specific immune responses. These data support the development of multitargeted treatments combining active vaccination and antiangiogenic therapy for clinical translation.

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References


