

Antiangiogenic Therapy Impedes Infiltration by CD4+ and CD8+ Cells Into an Early Colon Tumor

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Background: While the majority of angiogenesis studies have focused on the late stages of cancer, the emergence of neovascularization in colon tumorigenesis has been observed an earlier stage than expected. Recent reports implied that early angiogenesis might be a defense mechanism to stimulate the natural clearance of microadenomas during colon tumorigenesis. However, little is known about how early angiogenesis affects the natural clearance of tumors.

Methods: Spontaneous colon tumors were developed in adenomatous polyposis coli conditional knockout mice with Cre recombinase adenovirus administration. Vascular endothelial growth factor (VEGF) antagonist, DC101, was administered to determine the effect of early angiogenesis and then infiltration of immune cells into tumor and concentration of cytokines were evaluated.

Results: The continuous administration of the VEGF receptor 2 antagonist DC101 in the mouse models impeded the infiltration by CD4+ and CD8+ cells into the tumor region. Furthermore, the administration of the VEGF antagonist decreased the amounts of anti-tumoral cytokines such as interleukin (IL)-6 and IL-10.

Conclusions: We revealed that newly formed vessels during tumorigenesis can be channels for particular anti-tumoral immune cells. Our results may confer insight for the clinical development of an efficient antiangiogenic therapeutic manual and a timely chemoprevention to suppress tumor growth.

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Key Words: Colon tumorigenesis, Angiogenesis, Antiangiogenic therapy

INTRODUCTION

Colorectal cancer is one of the most common cancers and leading causes of death in the world.¹ Despite the understanding of the molecular pathology of this disease and advancements in the area of colonoscopy screening, only minor improvements of mortality have been achieved.² Angiogenesis is a distinctive characteristic of cancer. While the inhibition of angiogenesis has been a mainstream strategy to treat patients with cancer, the provision of antiangiogenic therapy with standard chemotherapy has demonstrated only a modest survival benefit; furthermore, recent clinical studies showed that antiangiogenic therapy for colorectal cancer could lead to a more invasive tumor growth.^{3,4} In

a recent report, we implied that early angiogenesis might contribute to an anti-tumoral effect.⁵ In that study, we suppressed early angiogenesis with vascular endothelial growth factor (VEGF) antagonists like DC101 and sunitinib, thereby delaying the natural clearance of sporadic tumors; however, the intrinsic mechanism of this phenomenon is still unknown.⁵

A number of immune cells are related to both tumor growth and suppression. Macrophages, known for enhancing the proliferation of a tumor under inflammatory conditions that can be related or unrelated to the cancer,⁶ also secrete cytokines that are considered a direct cause of a tumor's acquisition of malignant properties. Alternatively, CD4+ and CD8+ cells appear to be responsible for restraining tumor growth^{7,8} especially in the colon

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and reduced tumor growth has been observed in interleukin (IL)-10 knockout mice.⁷ The microenvironment surrounding a tumor seems to be an indispensable factor in the capability of a tumor to persist.

Cancer is a genetic disease that is the result of alterations that disturb normal gene functioning. In colon cancer, adenomatous polyposis coli (*Apc*) and Kirsten rat sarcoma viral oncogene homolog are critical genetic contributors to the development of the disease. Using genetically engineered mice, Hung et al.⁹ showed that spontaneous mutations of the *Apc* gene can mimic the conditions of human colorectal cancer, both upon onset and during drug treatment. Further, the cancerous condition of the tumor can be expressed using a green fluorescent protein (GFP) signal, so we used a confocal side-view endomicroscope capable of visualizing the mucosa in the descending colon to observe tumor growth and the vasculature around the tumor from early tumorigenesis onward. Previously, we had intensively used an ex vivo histological method for our analysis, but it is a slide-based method that provides only static information from specific time points. Our newly established system overcame this limitation, so it was therefore possible to investigate dynamic events to observe aspects of the phenomenon that we had not been able to see before; furthermore, the isolation of the colon tumor and the subsequent experiments revealed a new function of early angiogenesis during colon tumorigenesis.

Based on the in vivo imaging data on vascular change during tumorigenesis, here our study reveals how early angiogenesis controls infiltration of immune cells into colon adenoma and which subset of immune cells is critically affected by anti-angiogenic therapy.

MATERIALS AND METHODS

1. Animal preparation

The *Apc* conditional knockout mice were kindly gifted by Dr. Raju Kucherlapati (Harvard Medical School, Boston, MA, USA). The *Apc* inactivation was initiated with the administration of Cre recombinase adenovirus (adeno-Cre), as previously described.⁹ DC101 were purchased from Sigma Aldrich (St. Louis, MO, USA). The mice were orally administered 0.9% saline. For DC101 treatment, the mice were intraperitoneally injected with 40 mg/kg.

All of the animal experiments were performed in compliance with institutional guidelines and were approved by the subcommittee on research-animal care at Wonkwang University.

2. Immunofluorescent Staining

The mice were orally given 0.9% saline or sunitinib (40 mg/kg) once every 3 days for 4 weeks. Isolated tissue sections were paraffinized, whereas deparaffinized tissues were blocked with 3% H₂O₂ (hydrogen peroxide) in methanol to inactivate the endogenous peroxidases. The slides were firstly washed with phosphate-buffered saline and incubated for 20 minutes in a protein-blocking solution that was supplemented with 4% normal bovine serum albumin; they were then incubated overnight at 4°C with primary antibodies against CD31 (Invitrogen, Waltham, MA, USA), F4/80, CD11c, CD335, CD4, and CD8; lastly, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The vessel density was analyzed using ImageJ 2.0 (public domain).

3. Optical imaging

The mice were starved for 24 hours before imaging to eliminate any strong auto-fluorescence signals from excrement and digested food. Prior to in vivo imaging, the colons were cleaned by injecting 0.5 mL of 0.9% saline via an enema using a rubber-tipped needle. The mice were anesthetized with an intraperitoneal injection of ketamine–xylazine (90 mg–9 mg/kg body weight). The mice were placed on the heated plate of a motorized XYZ translation stage. For vasculature imaging, tetramethylrhodamine (TAMRA) dextran conjugates (5 µg/µL, 2,000,000 MW; Invitrogen) were intravenously injected. Images were typically acquired within a time range of 5 minutes to 40 minutes after the injection. eGFP-expressing cells were visualized by excitation at 491 nm, and were detected through a bandpass filter at 502 nm to 537 nm (Semrock, Rochester, NY, USA). TAMRA was imaged by excitation at 532 nm and detected through a bandpass filter at 562 nm to 596 nm (Semrock). At each time point, vascular parameters including vascular density, average diameter, and branch number were analyzed, and the degree of deviation from the normal hexagonal structure was calculated with a spatial autocorrelation analysis using the MATLAB and ImageJ programs. A previously validated image-analysis method was used to measure the size of the tumor.¹⁰

4. Enzyme-linked immunosorbent assay

Colonic tissue samples were obtained from the polyp regions in 6 *Apc* mice (DC101-treated group n = 3, untreated group n = 3) and from the normal regions of 6 wild-type mice (DC101-treated group n = 3, untreated group n = 3). The protein levels of the colonic IL-6, IL-10, and VEGF in the tissue homogenates were

measured using enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-6, IL-10, and VEGF were purchased from R&D Systems (Minneapolis, MN, USA); the experiments were performed according to the manufacturer's instructions. Three tumors of similar volume were randomly selected and homogenized in 300 μ L of a Tris-HCl buffer that contained protease inhibitors (Sigma). After centrifugation for 30 minutes, the supernatant was quantified and assessed using the ELISA test.

RESULTS

1. Observation of early angiogenesis

As there were several reports that angiogenesis in colon tumorigenesis emerges at an earlier stage compared with other tissues, we confirmed vascular change by monitoring the colon of a single *Tie2*-GFP mouse after an azoxymethane (AOM) injection. To investigate vascular leakage, we injected rhodamine-dextran into the mouse and performed an image analysis. Irregular changes in the GFP region were observed 4 weeks after the AOM injection, while the vessels were simultaneously perfused with

rhodamin fluorescence; the rhodamin region overlapped well with the GFP region to mark the *Tie2* expression. Between the two different methods that are commonly used to check vasculature, we found that noticeable differences were exhibited following a single injection of carcinogen that were presumably due to vascular leakage and irregular blood perfusion around the microadenoma (Fig. 1). Our observation is consistent with previous reports that describe abnormal vasculature during tumorigenesis in other genetically engineered mouse models.¹¹

2. Responsiveness of early angiogenesis upon application of antiangiogenic therapy

To investigate the role of early angiogenesis, we tried to suppress the new formation of vessels with antiangiogenic therapy. First, we tested whether the DC101 VEGFR2 antagonist inhibited early angiogenesis by performing time-lapse confocal endomicroscopy on *Apc* mice treated with DC101 (30 mg/kg, once every 3 days) for a duration of 10 weeks. In vivo images showed an apparent recover in vascular diameter and tortuosity after the DC101 treatment (Fig. 2A). While the vasculature appeared like hexagonal matrix implying normal condition at week 0, admini-

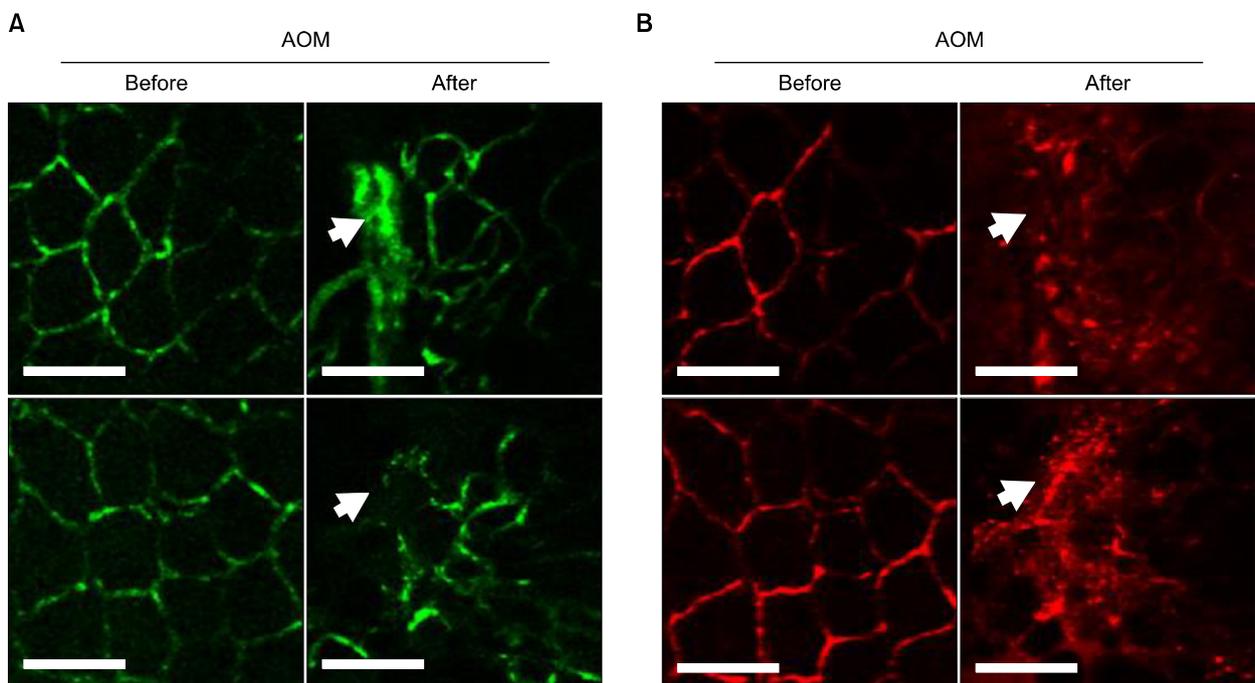


Figure 1. Development of disharmonized region between the *Tie2*-labeling endothelial cells and perfused vessels during angiogenesis. (A) To induce a colon tumor in the *Tie2*-green fluorescent protein (GFP) mouse, azoxymethane (AOM, Sigma Aldrich, St. Louis, MO, USA) was injected intraperitoneally at 5 mg/kg. One week later, the mice were given 3% dextran sulfate sodium (DSS; MP Biochemicals, Solon, OH, USA) in drinking water for 5 days, followed by normal drinking water. The colons of the mice were imaged using side-view confocal endomicroscopy at week 8 after the DSS treatment. (B) Intravenously-injected rhodamin-dextran conjugates (500 μ g/100 μ L, 2,000,000 MW; Invitrogen, Waltham, MA, USA) were used for the imaging of the perfused vessels. The arrows indicate a significantly disharmonized area between the *Tie2*-GFP and the perfused rhodamin-dextran in the tumor region. Scale bars: 100 μ m.

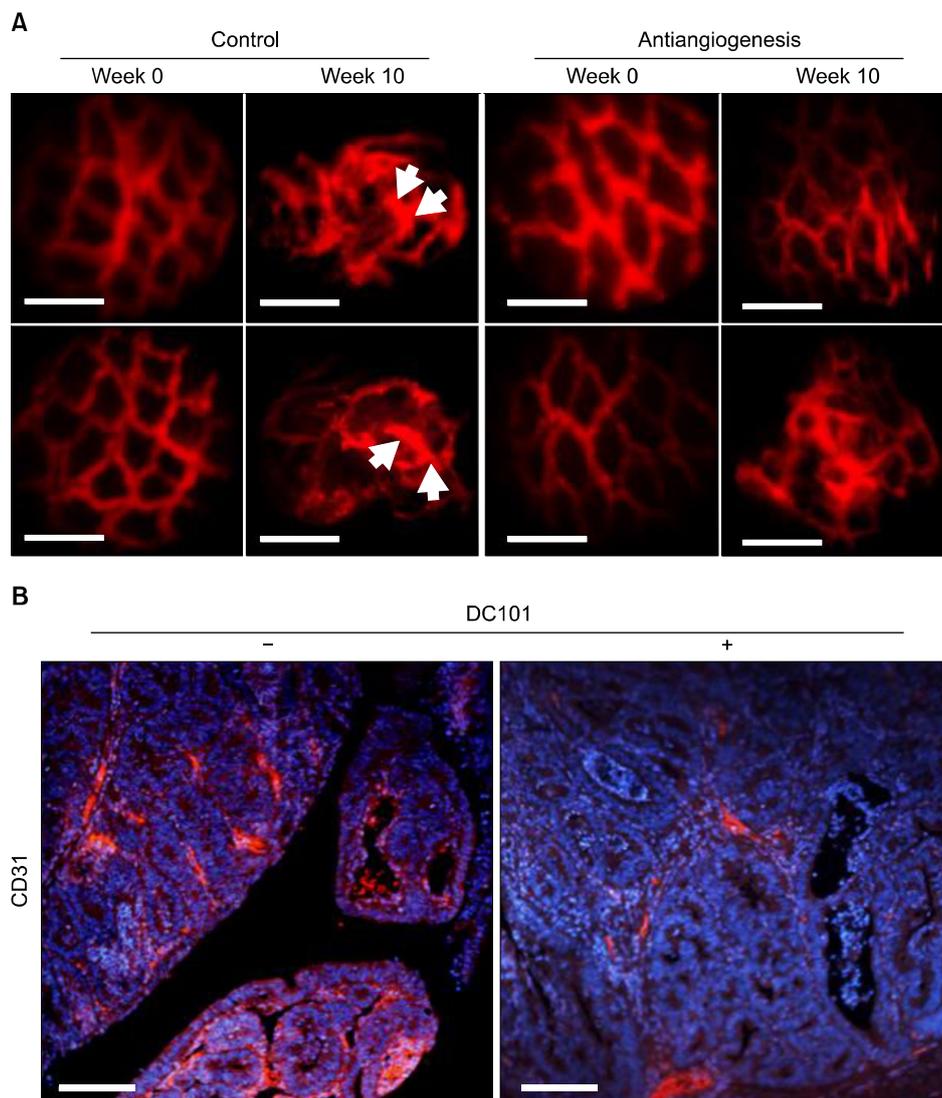


Figure 2. Normalization of early angiogenesis upon application of anti-vascular endothelial growth factor therapy. (A) Blood vessels in macroadenomas of an adenomatous polyposis coli (*Apc*) mouse before (week 0) and after (week 10) the DC101 treatment. The white arrows indicate dilated or tortuous vessels, and the yellow arrows indicate apparently normal vessels in the tumor regions. Scale bars: 100 μm . (B) Fluorescent images of the blood vessels in the macroadenomas of an *Apc* mouse with and without sunitinib treatment. Blue, 4',6-diamidino-2-phenylindole (DAPI)-stained nucleus; red, CD31-stained blood vessels. Scale bars: 200 μm .

stration of adeno-Cre made them tortured. But, the vasculature in DC101 treated mice showed the normal like shape compared with control mice (Fig. 2A, right panel of each group). The histological analysis of the adenoma tissues that were collected at week 12 showed a significantly smaller CD31-positive area—indicating a lower vascular density—in the sunitinib-treated mice when compared with the corresponding adenoma tissues in the untreated mice (Fig. 2B).

3. Suppressed infiltration of tumor-related immune cells into the colon tumor

We then conducted an immunohistological investigation of the infiltration by tumor-related immune cells into the colon tumor upon the commencement of DC101 treatment. The numbers of F4/80+ macrophages, CD11c+ dendritic cells, and

CD335+ NK cells were similar between the untreated and treated groups, whereas the CD4+ and CD8+ cell counts were modestly lower in the treated group (Fig. 3). Considering the critical role of the CD4+ cell subpopulation in the tumorigenesis of colon cancer, the reduction of the CD4+ cell count from DC101 treatment might therefore contribute to the treatment's tumor-suppressing effect.

As cytokines secreted from immune cells can affect the survival and proliferation of colon adenomas, we first investigated the quantitative changes of IL-6 and IL-10, which have been identified as factors that enhance and suppress the survival of colon tumor cells, respectively, in control and DC101-treated animals.^{7,12} The concentrations of the two cytokines appeared to be stable in the normal regions of both the untreated and treated animals, but both IL-6 and IL-10 were reduced in the polyps of the

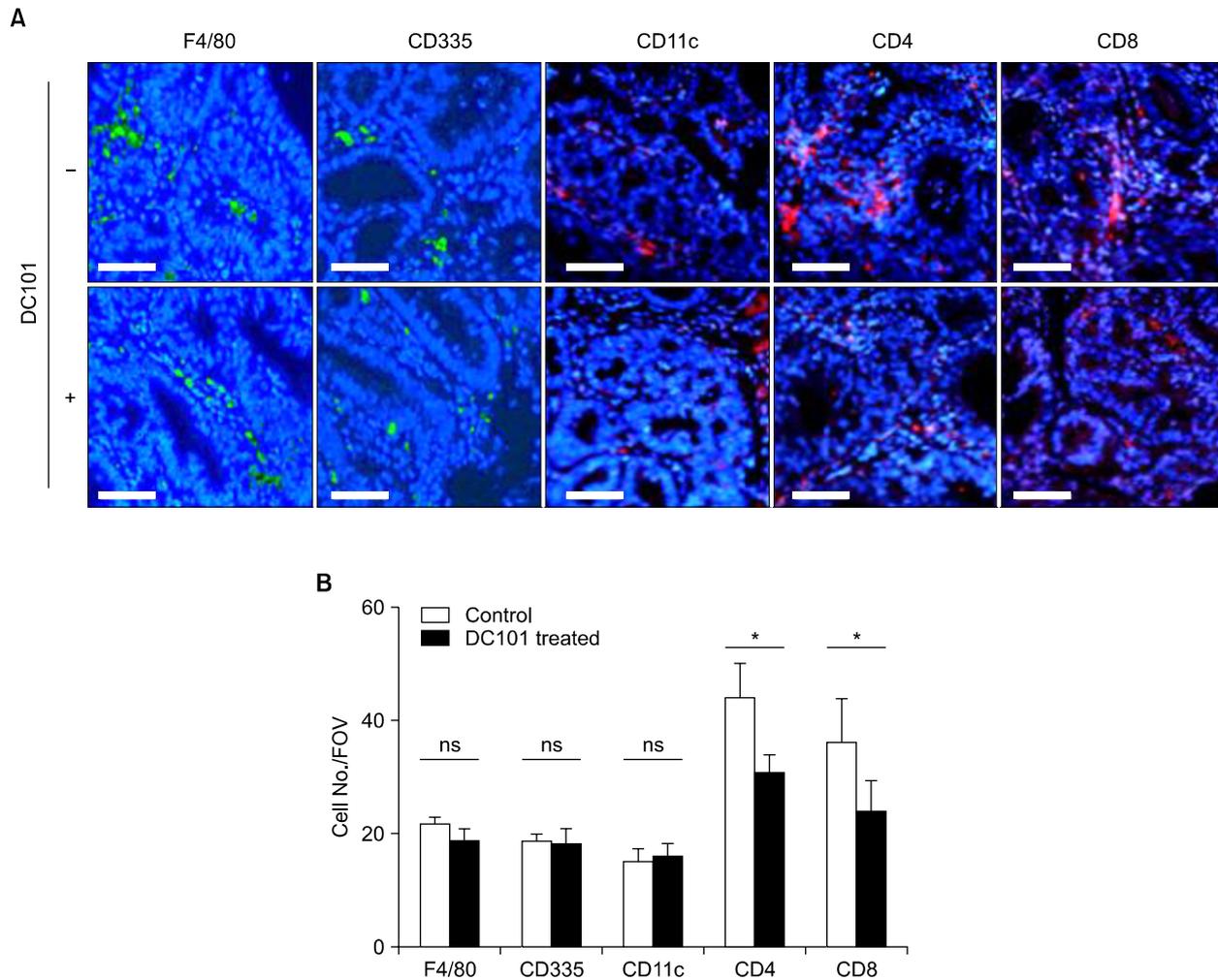


Figure 3. Reduction of infiltration by various immune cells into adenomas after DC101 treatment. (A) The mice were intraperitoneally injected with DC101 (40 mg/kg) once every 3 days for 4 weeks, and the colons were isolated and fixed with 10% formalin. For immunofluorescent staining, the tissues were blocked with 3% H₂O₂ in methanol to inactivate the endogenous peroxidases. The slides were firstly washed with phosphate-buffered saline and incubated for 20 minutes in a protein-blocking solution supplemented with 4% normal bovine serum albumin; the slides were then incubated overnight at 4°C with primary antibodies against F4/80, CD11c, CD335, CD4, and CD8 (upper panel). (B) The cell count was determined from four independent images with a 20× objective field of view (FOV) before it was statistically analyzed. Scale bar, 100 μm. ns, not significant. **P* < 0.05.

treated group compared with the untreated group (Fig. 4A and 4B). The concentration of VEGF in the same tissue homogenate appeared slightly higher in the polyps of the DC101-treated animals (Fig. 4C), indicating the antagonistic function of DC101 against VEGF,¹³ although the minor concentration difference is not statistically significant.

DISCUSSION

Previous clinical studies showed that it was at the microadenoma stage that a colon tumor either remained benign or developed into a macroadenoma,^{14,15} while angiogenesis

appeared to be essential for the transformation of microadenomas into macroadenomas;¹⁶ furthermore, angiogenesis might also influence the behavior of microadenomas during tumorigenesis. In our previous study, wherein we used in vivo endoscopic imaging to analyze spontaneous colon tumors in *Apc* conditional knockout mouse models, we observed the natural progression of transient lesions that apparently mimic the microadenomas and aberrant crypt foci found in patients. Angiogenesis and abnormal vasculature gradually became apparent as these lesions grew from microadenomas to macroadenomas. After antiangiogenic treatment with DC101 or sunitinib, the vessel diameter and vascular area decreased, the

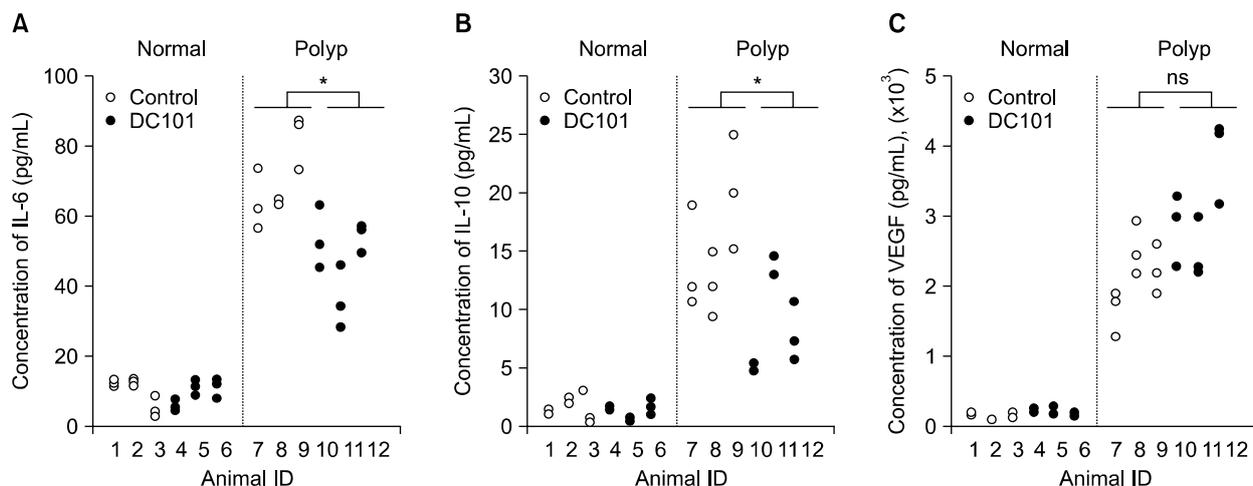


Figure 4. Changes of cytokines in tumors after DC101 treatment. Comparison of concentrations of interleukin (IL)-6 (A), IL-10 (B), and vascular endothelial growth factor (VEGF) (C) in isolated normal colon tissues and polyps. ns, not significant. * $P < 0.05$.

vessel morphology normalized, and the number of polyps declined. Unexpectedly, though, we found that the antiangiogenic therapies not only slowed the growth of transient lesions by 1 week to 3 weeks and reduced their peak size by a factor of 2 to 3, but they also delayed the timing of spontaneous regression by 4 weeks to 8 weeks, depending on the specific drug dose and genetic type; most of the untreated tumors were naturally dissolved in 10 weeks. This outcome can partly be attributed to a regeneration of the crypt that is akin to the movement of a conveyor belt.^{17,18} The cells from the crypt bottom continuously migrate up toward the luminal region, with a turnover time of 5 days in the mice, which exerts a physical pressure on the transient lesions. The antiangiogenic agents possibly hamper this crypt-regeneration process,¹⁹ and the force of vigorous differentiation might therefore be responsible for prompting the microadenoma out of the structure.

Supposing that antiangiogenic therapy does not affect the regeneration rate of the crypt, such a clearance of the tumor might be stimulated by anti-tumoral immune cells. Immune cells like the CD4+CD25+ regulatory T cells induce apoptosis by secreting cytokines that are toxic to colon tumors.^{7,8} Antiangiogenic therapy appeared to reduce the extent of the infiltration by the various immune cells into the transplanted tumors;^{20,21} therefore, it is reasonable to infer that the reduced extent of infiltration by the anti-tumoral immune cells into the microadenomas extends the survival period of the tumor cells. It is also considerably significant that antiangiogenic therapy can stimulate the activity of cancer stem-like cells, as it has been reported that the hypoxic condition caused by antiangiogenic therapy for breast cancer can intensify the aggressiveness of

tumor-initiating cells.²² Our data suggests that the decreased numbers of CD4+ and CD8+ cells and the reduced concentration of IL-6 and IL-10 in the tumor might have partially contributed to the slowdown of the natural clearance of the transient tumors after the DC101 treatment.

According to the general understanding, a tumor actively recruits the blood vessels of its host, whereby the tumor cells secrete cytokines to passively attract the vessels toward the tumor for the purpose of tumor evolution. Our data, however, contrarily suggests that early angiogenesis can be a defense mechanism for a host to clear a tumor at an early stage; our suggestion is plausible since angiogenesis can be induced (partially) by cytokines from the host's immune cells, like macrophages, as well as those from the tumor cells.²³

This research revealed new roles for angiogenesis and antiangiogenic therapy regarding the growth of aberrant crypt foci. We expect that our results will lead to the formulation of pharmacological strategies that contribute to the suppression of premalignant tumors at a very early stage. We also expect our contribution to provide a novel insight into why currently used antiangiogenic therapies can augment invasiveness and the metastatic behavior of the tumor itself in certain patients after treatment.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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