Enhanced Suppression of Melanoma Tumor Growth and Metastasis by Combined Therapy with Anti-VEGF Receptor and Anti-TYRP-1/gp75 Monoclonal Antibodies

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Abstract. *Targeted immunotherapy against tumors or angiogenesis has shown promise as an alternative approach for the treatment of malignant disease. Whether or not combining these two treatment modalities would enhance the antitumor effect was tested in mouse models of malignant melanoma. C57BL/6 mice bearing established subcutaneous B16 tumors were treated with anti-vascular endothelial growth factor receptor (anti-VEGFR) fetal liver kinase-1 (Flk-1) monoclonal antibody (mAb) DC101 and/or anti-TYRP-1/gp75 (tyrosinase-related protein-1) mAb TA99. The growth of subcutaneous B16 tumors was significantly suppressed by the mAb DC101 (63%, p<0.001) and by mAb TA99 (75%, p<0.001) treatment alone. The combined antibody (TA99+DC101) treatment resulted in a significant enhancement (93%, p<0.001) of tumor growth suppression. In a B16 pulmonary metastasis model, combined therapy with mAb DC101 and mAb TA99 resulted in a significant reduction of lung metastases compared to the control (p<0.001) and the single agent treatment groups (p<0.05). A combined modality approach that provides passive immunity to melanoma differentiation antigens as well as inhibiting tumor neovascularization may be valuable for the treatment of malignant melanoma.*

The incidence of malignant melanoma of the skin appears to be rising rapidly. An estimated 62,480 new cases are expected to be diagnosed in the United Sates this year and 8,420 patients will die of melanoma (1). Melanoma is typically characterized by rapid cell proliferation, high metastasis and dense vascularization (2-4). At the present time, there are no effective therapies for melanomas. Surgery, radiotherapy and chemotherapy only have limited effects on disease progression. Immunotherapy and antiangiogenic therapy have emerged as new therapeutic strategies for melanoma patients.

In recent years, a number of melanoma antigens recognized by the immune system have been identified (5-8). The majority of these are differentiation antigens shared by melanocytes and melanomas. TYRP-1/gp75 (tyrosinase-related protein-1) is a 75 kDa protein involved in melanin synthesis in melanosomes (9). It is synthesized in the endoplasmic reticulum, transported through the Golgi and sorted to melanosomes (10). The cytoplasmic tail of TYRP-1/gp75 constitute a retention signal sequence that targets TYRP-1/gp75 protein to melanosomes (11). Although predominantly localized within the melanosomes of melanocytes, TYRP-1/gp75 is also frequently expressed on the cell surface of melanocytes and melanomas (12). TA99 (IgG2a), a murine monoclonal antibody (mAb) specific for human and murine gp75 (13), has been shown to target and localize to subcutaneous human melanoma xenografts *in vivo* (14). *In vivo*, TA99 treatment inhibited the established subcutaneous murine B16 tumor growth in syngeneic mice (15). Murine B16 lung metastasis formation and the established metastasis in mice were inhibited by TA99 treatment (15, 16). Mice treated with TA99 often lose hair color (depigmentation), suggesting destruction of melanocytes in the skin (15, 16). Thus, gp75 is an ideal tumor target for antibody-based therapy through the activation of antibody-dependent cellular cytotoxicity (ADCC).

Melanomas are characterized by intense angiogenesis (17-20). Human melanoma has been shown to expresses angiogenic factors and receptors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and KDR/flk-1 (VEGFR) (17, 18). Angiogenesis has been correlated with progression and metastasis in melanoma (19), with VEGF considered to be one of the major inducers of tumor angiogenesis. The degree of VEGF expression

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appears to have a strong correlation with the blood vessel density in metastatic melanoma (20). Interruption of the VEGF/KDR signal pathway by neutralizing antibodies to VEGF, or with Flk-1/KDR kinase inhibitor has been shown to inhibit angiogenesis and tumor growth (21, 22). Previously, we demonstrated that mAb DC101, a blocking mAb to Flk-1, inhibited angiogenesis and growth of melanoma in an animal model (23).

Here, we investigated whether employment of immunotherapies that target tumor antigen and angiogenesis simultaneously enhances antitumor effects compared to each therapy individually. To test this hypothesis, tumor-bearing mice were treated with combined or single mAb and antitumor effects were determined.

Materials and Methods

Cell lines and animals. Spontaneous murine melanoma B16BL6 cell line was kindly provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX, USA). The expression of mouse TYRP-1/gp75 was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR), fluorescence staining and Western blotting (data not shown). Tumor cells were maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and routinely tested for Mycoplasma contamination. Six- to eight-week-old female C57BL/6 mice were purchased from Taconic Farm (Germantown, NY, USA). All experiments and procedures were performed in accordance with the United States Department of Agriculture and Human Services and NIH policies regarding the use of laboratory animals.

Antibody production. Hybridoma for mouse mAb TA99 was kindly provided by Dr. Alan Houghton (Memorial Sloan Kettering Cancer Center, New York, NY, USA). MAb TA99 was purified from mouse ascites by protein A affinity column. The Hybridoma producing mAb DC101 was generated in house as described elsewhere (24) and grown *via* continuous feed fermentation in serum-free medium. MAb DC101 was purified from serum-free medium by a protein A affinity column. Mouse IgG2a (Sigma, St. Louis, MO, USA) and rat IgG (Jackson Immuno Research Laboratories, Western Grove, PA, USA) were used as negative controls for mAb TA99 and mAb DC101, respectively. All antibodies were tested for endotoxin using the Pyrogent Plus *Limulus* amebocyte lysate kit (BioWhittaker, Walkersville, MD, USA).

Mouse tumor models. Cultured B16BL6 cells were harvested, washed and resuspended in phosphate-buffered saline (PBS) at 5×10^6 cells/ml. For the subcutaneous tumor model, 1×10^6 cells were injected subcutaneously (*s.c.*) into the right flank of syngeneic C57BL/6 mice. Once tumors reached measurable sizes, randomized groups of 10 animals then received intraperitoneal (*i.p.*) injections of mAb TA99 and/or DC101, and control Ab (mouse IgG_{2a} or rat IgG) every three days. Tumors were measured twice weekly with calipers, and their sizes calculated by the formula $[\pi/6(W1xW2xW3)]$, where W1 represents the largest tumor diameter and W3 represents the smallest tumor diameter. For the melanoma lung metastasis model, C57BL/6 mice were injected intravenously through the tail vein with 2×10^5 B16 cells in sterile saline. On the third day, mice were treated with mAb TA99 and/or mAb DC101 and control Ab (mouse IgG_{2a} or rat IgG). Twenty days after tumor injection, mice were sacrificed and the lungs were removed. Lung surface metastases were scored and counted as black nodules under a dissecting microscope.

Histology. Tumor tissues were removed from mice, fixed in 10% saline buffered formaldehyde, and embedded in paraffin. Tissues were then cut into 4-μm sections and stained with hematoxylin and eosin or the appropriate antibodies for evaluation of vessel density, tumor cell proliferation and apoptosis.

Staining of blood vessels of tumor tissue was performed as described elsewhere (20). Briefly, sections were incubated with rat anti-mouse pan endothelial cell antigen antibody (clone MECA-32, 10 μg/ml; PharMingen, San Jose, CA, USA) for 30 min at room temperature (RT), rinsed with PBS, and then incubated with biotinylated goat anti-rat IgG (1:500, Jackson Immuno Research, West Grove, PA, USA) for 30 min at RT. Finally, sections were incubated with Cy3 Streptavidin (1:500, Jackson Immuno Research) for 30 min at RT. Staining of Ki-67 antigen followed the same protocol used for vessel staining except primary anti-Ki-67 Ab (TEC3, 1:50, Dako, Carpinteria, CA, USA) was used. TUNEL staining was performed using *in situ* cell death detection kit (Roche MBP, Indianapolis, IN, USA). Sections were digested with proteinase K (20 μg/ml, Roche MBP, Indianapolis, IN, USA) for 15 min at RT, permeabilized with 0.1% sodium citrate containing 0.1% Triton® X-100 for 2 min at 4˚C, and blocked with Tris-buffered saline (TBS) for 1 h at RT. Sections were then incubated with labeling enzyme at 37˚C for 60 min. Vessel density was captured using NIH Image software (Bethesda, MD, USA) and quantified by analyzing 10 random fields per section (from three mice in each group) using Corel PhotoPaint (Fremont, CA, USA).

Flow cytometry. Antibody binding to cells was performed on cultured B16BL6 cells and freshly isolated B16BL6 cells from subcutaneous tumors. Cells were treated for 1 h on ice with either 5 μg/ml mouse IgG, or TA99 in 1% bovine serum albumin (BSA)/PBS. Cells were then washed 3x in 1% BSA/PBS and incubated for 1 h with FITC-labeled goat anti-mouse IgG (BD Pharmingen, San Jose, CA, USA). Cells were washed and analyzed by an Epics XL Flow Cytometer (Coulter, Fullerton, CA, USA).

ADCC Assay. B16BL6 cells were collected and washed with AIM V media (Invitrogen, CA, USA) and plated at a density of 10,000 cells/well in a 100 μl, in a 96-well-U bottom plate. TA99 or mouse IgG_{2a} were added at 5 μg/ml in 50 μl/well and incubated at 37˚C for 0.5 h with B16BL6 cells. Mouse spleen cells were added in a volume of 50 μl at an effector (E):target (T) ratio of 50:1. Plates were further incubated for 4 h at 37˚C. After incubation, plates were spun down at 800 ×g, and 100 μl of supernatants were gently transferred to 96-well-flat bottom plates. Lactate dehydrogenase (LDH) assay reagent was added as specified by the manufacturer (Roche) and plates were read at 490 nm. Controls in assay: target spontaneous, effector spontaneous, and target maximum (by adding 50 μl of 4% Triton®).
% Cytotoxicity = (experimental–target spontaneous–effector spontaneous) / (target maximum–target spontaneous–effector spontaneous) ×100.
% Specific lysis = (test) % cytotoxicity – (negative control) % cytotoxicity.

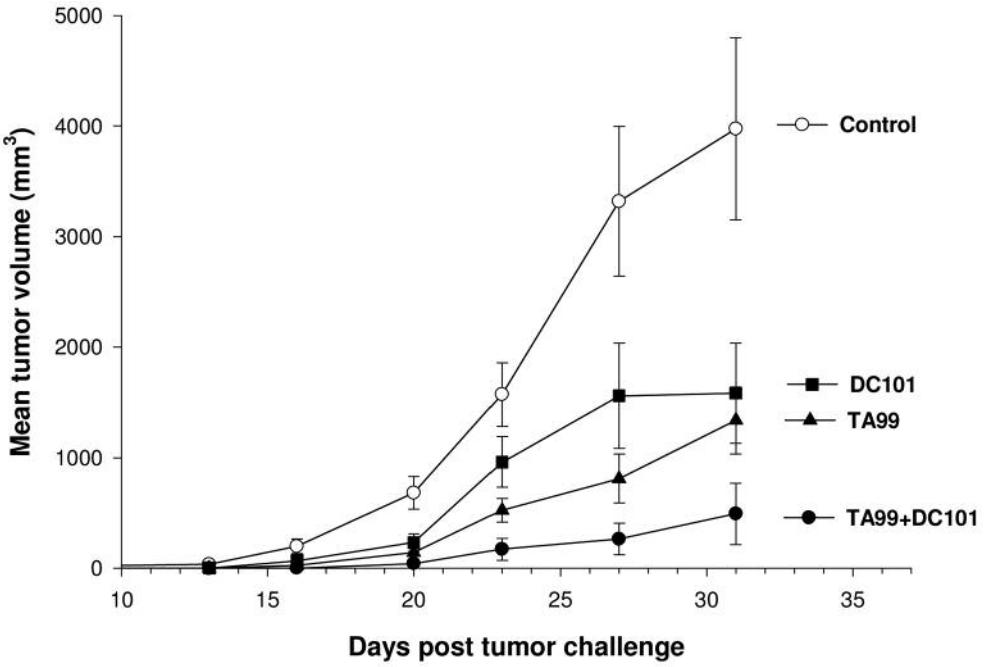

Figure 1. *Effect of DC101 and TA99 on growth of subcutaneous B16 melanoma tumor. The subcutaneous tumors were established by injecting 1×10^5 B16 cells in saline into the right flank of C57BL/6 mice. Once measurable tumors were formed, mice were randomized and received i.p. injection of 800 μg of each antibody as indicated. Injections were given every three days until the end of study. There were 10 mice in each group. Data are representative of three independent experiments Control, mouse IgG_{2a} and rat IgGAb.*

Statistical analysis. Tumor volume, lung metastases and vessel density were analyzed using Student's *t*-test. Analyses were computed using the statistical package in SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA, USA).

Results

To test the combined effects of TA99 and DC101 *in vivo*, mice (n=10) bearing established subcutaneous tumor were treated with each antibody individually or in combination. Growth of B16 tumors was significantly inhibited by mAb DC101 (63%, $p<0.001$) and mAb TA99 (75%, $p<0.001$) compared to the IgG antibody-treated group (Figure 1). Combined antibody treatment resulted in a significant enhancement (93%, $p<0.001$) of tumor growth inhibition, but there was no significant difference in efficacy between single agent TA99 and DC101 (Figure 1). By the end of the study (day 32), five mice treated with the DC101/TA99 combination underwent tumor regression. No tumor regressions were observed in single agent TA99- or DC101-treated mice.

The enhanced tumor inhibition with these agents was also observed in the B16 pulmonary metastasis model (Figure 2). The number of lung surface metastases were reduced with TA99 ($p=0.00001$) and DC101 ($p=0.004$). Mice treated with TA99 plus DC101 at the same dose level had significantly fewer metastases when comparing different treatment groups.

Table I. *Summary of results from B16 melanoma lung metastasis model*

Treatment group	Mean no. of metastases	Std. error	*P*-value*
Control Ab	238	10	-
DC101	96	10	0.0041
TA99	49	9	0.00001^1, 0.038^2
DC101+TA99	11	7	0.0004^1, 0.016^2, 0.033^3

*Compared to [1]control antibody, [2]DC101, [3]TA99.

The results of the B16 lung metastasis study are summarized in Table I. TA99 treatment significantly reduced lung metastasis more than DC101 treatment ($p=0.038$). TA99 plus DC101 treatment further significantly enhanced inhibition of metastasis compared to DC101 ($p=0.016$) and TA99 ($p=0.033$) alone. Furthermore, the suppression of metastasis was dose dependent on TA99 and DC101 (Figure 3). Each antibody at 50 μg was sufficient to inhibit B16 metastasis in comparison with control antibodies ($p=0.002$), mouse IgG2a plus rat IgG. The 800 mg/injection dose had the highest metastasis inhibitory effect (96% inhibition, $p<0.001$), and the results were consistent with those shown in Table I.

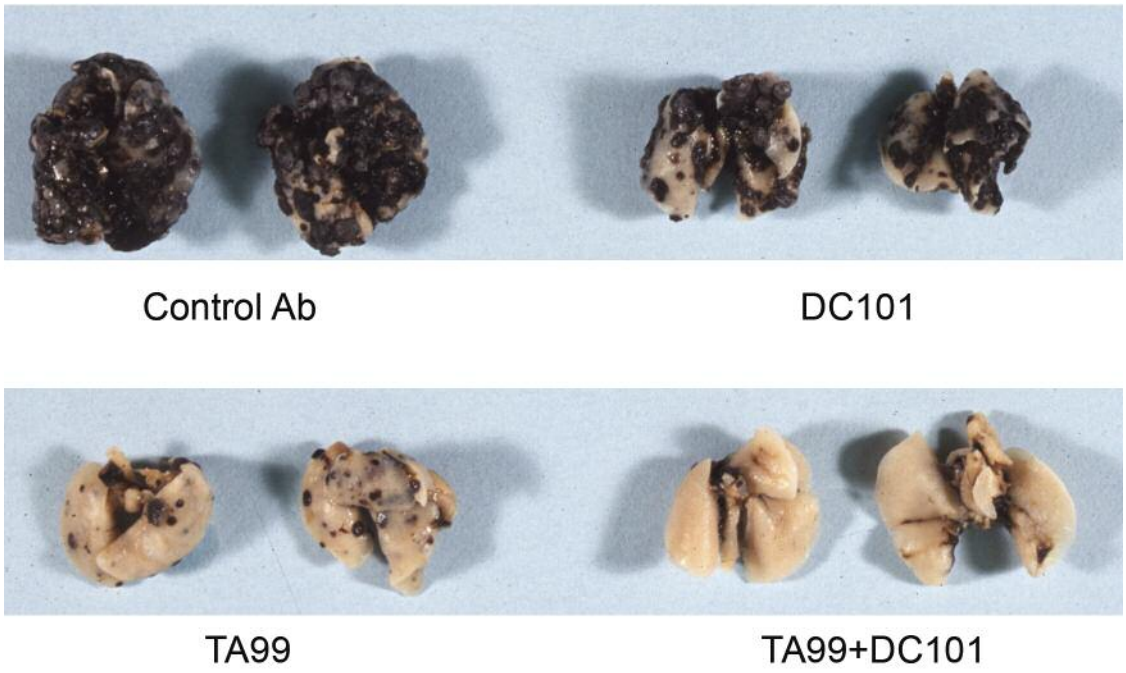

Figure 2. *DC101 and TA99 combined treatment enhances suppression of melanoma metastases. Syngeneic C57BL/6 mice (n=10) were injected intravenously through the tail vein with 2×10^5 B16BL6 cells. On day three, mice were treated with DC101, or TA99, or DC101 with TA99, or control antibodies every three days. Twenty days after tumor injection, mice were sacrificed and surface lung metastases were scored. The picture shows the images of lungs from different groups at the end of study.*

TYRP-1/gp75 is an abundant melanosomal protein. It is difficult to detect the cell surface gp75 expression in melanoma cell lines. It has been reported that surface TYRP-1/gp75 expression on tumor cell lines is increased *in vivo* (12). To confirm this observation, we isolated fresh B16BL6 cells from subcutaneous tumors and compared the surface TYRP-1/gp75 expression in the cultured B16BL6 cells with that of freshly isolated cells (Figure 4A). Expression of TYRP-1/gp75 was not detected on the surface of cultured B16BL6 cells. On the other hand, TYRP-1/gp75 on cell surface can be easily detected in freshly isolated cells from tumor. This may explain why TA99 specifically localized to melanoma *in vivo*. Effector cell activation by TA99 was evaluated by ADCC assay (Figure 4B). ADCC activity induced by TA99 correlated with TYRP-1/gp75 surface expression: 100% of fresh tumor cells were specifically lysed at an E:T ratio of 50:1, while ADCC against the cultured cells was barely detectable.

We have previously shown that DC101 inhibited angiogenesis in Matrigel and human xenografts (20). In this study, we evaluated the antiangiogenic effect of DC101 in B16 tumor (Figure 5). Endothelial cells were stained with anti-endothelial antibody and the vessel density was quantified by image analysis of stained sections. Vessel density was significantly reduced by 70%

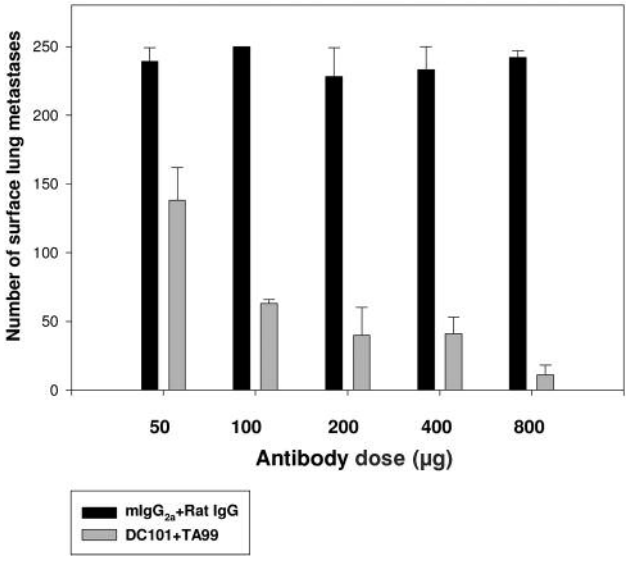

Figure 3. *Dose-dependent inhibition of melanoma pulmonary metastases by DC101 and TA99 therapy. Syngeneic C57BL/6 mice were injected intravenously through the tail vein with 2×10^5 B16BL6 cells. On day three, mice were given equal doses of rat IgG and mouse IgG2a, or DC101 and TA99 as indicated in the figure every three days. Twenty days after tumor injection, mice were sacrificed and surface lung metastases were scored. *Significantly different at $p<0.05$. Data are representative of two independent experiments.*

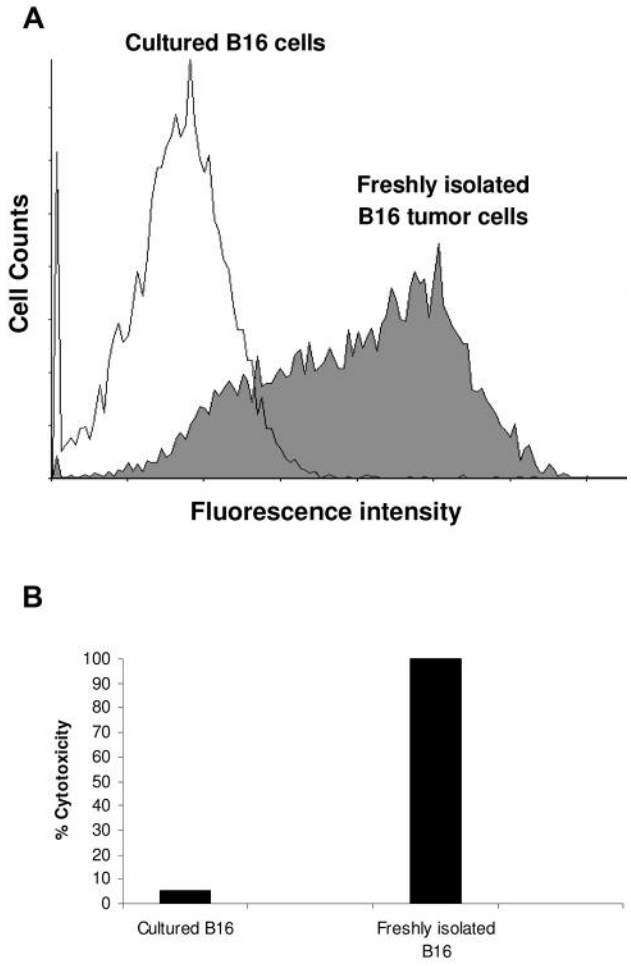

Figure 4. *TA99-induced ADCC against melanoma cells correlates with TYRP-1/gp75 surface expression. A, Comparison of surface TYRP-1/gp75 expression between cultured and freshly isolated B16BL6 cells. B, TA99-induced ADCC activity against cultured and freshly isolated B16BL6 cells.*

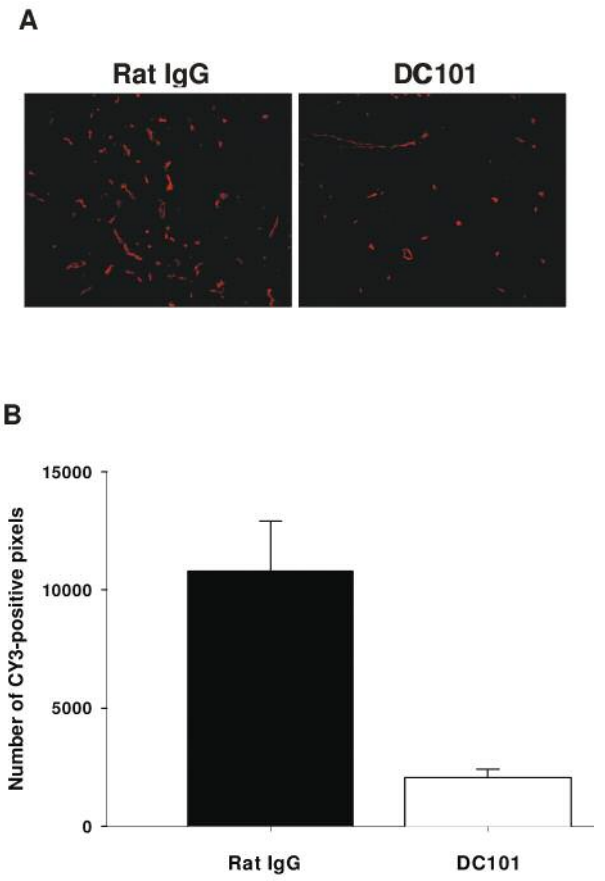

Figure 5. *DC101 treatment reduces vessel density in melanoma tumor. B16BL6 tumors from mice treated with rat IgG or DC101 were resected after 10 day of antibody treatment. Samples were fixed, paraffin-embedded and sectioned at 4 μm. Vessels were stained with biotinylated anti-mouse Meca32 antibody and followed by fluorescent-streptavidin. Microvessel density was quantitated by image analysis of stained section as described in the Materials and Methods section.*

by DC101 treatment compared to controls (p=0.001). TA99 had no direct effect on endothelial cells and vessel density (data not shown).

Histological and immunochemical staining of tumor removed from antibody-treated mice demonstrated differences in necrosis, cell proliferation and apoptosis (Figure 6). Tumors taken from DC101-, TA99- and TA99/DC101-treated mice showed extensive areas of necrosis and fibrosis. Tumors were confined in small areas by necrotic cells and fibrils, especially in tumors from DC101/TA99-treated mice (Figure 6D). TA99 and DC101 treatment reduced the number of proliferating tumor cells dramatically (Figure 6E-H) as measured by the presence of Ki-67 nuclear antigen. In addition, extensive apoptosis was observed in tumors from DC101- and/or TA99-treated mice (Figure 6I-L).

Discussion

The anti-TYRP-1/gp75 antibody TA99 and the anti-flk-1 antibody DC101 inhibit tumor growth by different mechanisms. We hypothesized that the combination of both antibodies would enhance the antitumor effect compared with single antibody treatment. The results from subcutaneous and metastatic models have confirmed our hypothesis. In these models, TA99 was more effective than DC101 at the same dose level. We used 800 μg/injection as a maximal dose, since it is the lowest dose of DC101 necessary to achieve maximum antitumor effects in our previously reported *s.c.* tumor model (23). It is important to note that the ratio of monoclonal antibodies used may affect their antitumor activity. In this study, we used a 1:1 ratio for TA99 and DC101, which may not be the optimal ratio in clinical testing.

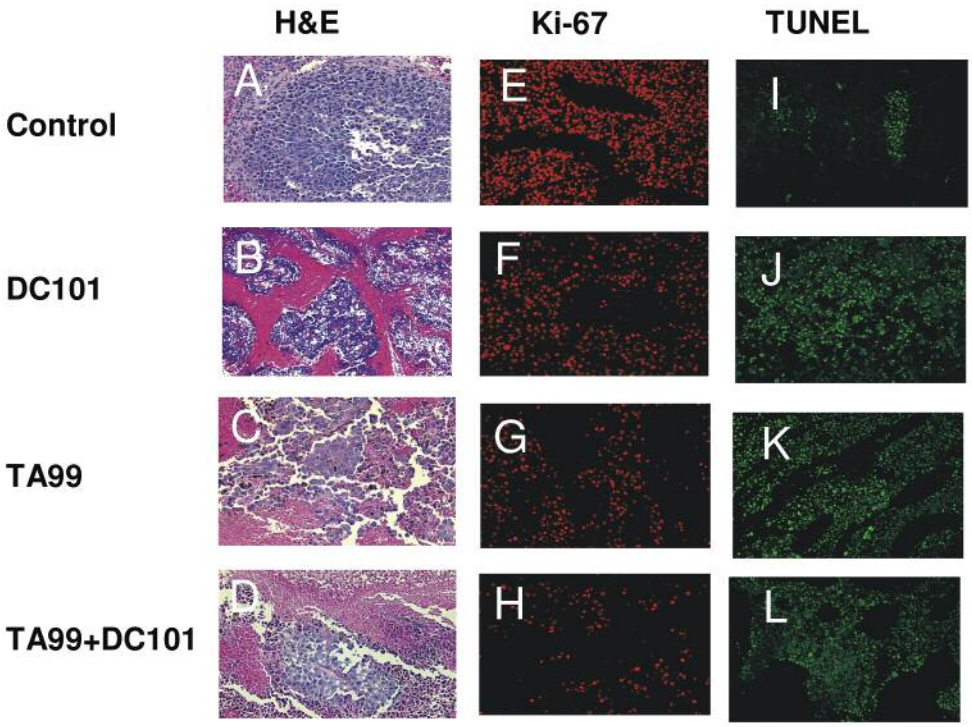

Figure 6. *DC101 and TA99 mAb treatment of B16BL6 melanoma increased tumor cell apoptosis, reduced tumor cell proliferation and increased tumor necrosis. Subcutaneous B16 tumors after 10 days of antibody therapy were resected, fixed, embedded and sectioned. Slides were then stained for Ki-67 antigen and apoptotic nuclei. Shown here are representative sections from the control the IgG (A, E, I), DC101 (B, F, J), TA99 (C, G, K) and DC101/TA99 groups (D, H, L). A-D, H&E sections; E-H, sections were stained with antibodies to Ki-67 antigen in proliferating cells; I-L, sections were examined for apoptosis using TUNEL assay that labels apoptotic nuclei with a fluorescent marker. Magnification ×200.*

TYRP-1/gp75 is the most abundant melanosomal protein in melanoma and melanocytes (25). It is synthesized in the endoplasmic reticulum, transported through the Golgi and sorted to melanosomes (10). The *C*-terminal of TYRP-1/gp75 constitutes a retention signal sequence that target gp75 protein to melanosomes (11). It is difficult to detect gp75 surface expression for most human melanoma cells *in vitro*. For reasons not yet clear, gp75 cell surface expression may be increased in the *in vivo* environment (Figure 4). TA99 can localize to melanoma tumors *in vivo* (14), suggesting that gp75 can be expressed on the cell surface *in vivo*. The melanin release pathway may explain why gp75 reaches the cell surface (26). When melanin is accumulated to a certain level within melanosomes, melanosomal membranes fuse with cell membranes and the luminal side of melanosomes becomes the cell surface side, leading to the release of melanin into the extracellular space. During this process, gp75, which is normally located in the luminal region of melanosomes, is displayed on the cell surface.

Antibodies employ several mechanisms in their antitumor activities. (i) They trigger the activation of the complement system; (ii) they activate NK cells or macrophages through the Fc receptors; (iii) they block growth signals; and (iv) they induce apoptosis of target cells. Unlike growth factor receptors, gp75 does not contain an intracellular signal-transducing motif (11). Anti-gp75 antibodies alone have no inhibitory effects on melanoma growth *in vitro*. Incubation of the gp75-positive melanoma cells with TA99 up to 72 h *in vitro* did not change cell morphology or growth compared to cells incubated with control human IgG (data not shown), suggesting that the activation of immune effectors may be the antitumor mechanism of TA99 *in vivo*. Depletion of NK cells or knock-out Fc receptor in mice reduced TA99-induced antitumor activity (15, 16). In our study reported here, TA99 alone demonstrated antitumor activity in subcutaneous and metastatic models, which is in agreement with previous reports (12, 15).

VEGF plays a critical role in angiogenesis (21). It serves as a potent and specific mitogen for endothelial cells and enhances vascular permeability. DC101 blocks the VEGF growth signal of endothelial cells and inhibits new blood vessel formation (23, 24). It is unknown if other immune cells are activated by DC101 once it binds to endothelial cells. In our experience, DC101 is more effective at the early stages of tumor development. Once a tumor reaches a certain size, its antitumor effects are reduced. It is possible that other mechanism(s) exist by which tumor endothelial cells can proliferate even when flk1 is blocked or tumor growth is less dependent on new blood

vessel formation, such as the report of melanoma cells forming tunnel-like structure to support growth (27).

Although, DC101 and TA99 have different antitumor mechanisms, the combined antitumor effects may not be a simple additive effect. DC101, which disrupts endothelial cells within tumors, could enhance TA99-mediated tumor destruction by facilitating penetration of TA99 and immune effector cells into the tumor. On the other hand, TA99 induced tumor destruction may lead to reduced tumor-derived angiogenic factors and therefore less new blood vessel formation in tumors. TA99 could also recruit immune effector cells such as NK cells to tumor sites. Cytokines secreted by NK cells, such as INFγ, could further inhibit angiogenesis (28).

Tumors employ multiple mechanisms for their uncontrolled proliferation, invasion, angiogenesis and metastasis. It is therefore logical to envision that a combination of approaches that target different mechanisms will be more effective at inhibiting tumor growth or destroying tumors than a single agent approach. Similar to our results, enhanced antitumor activities by targeting both tumor and angiogenesis have been reported recently. Tonra *et al*. reported that the combination of anti-VEGFR blocking antibody (DC101) and anti-EGFR (C-225) therapy inhibited pancreatic and colon cancer growth more than single agent therapy (29). Shaheen *et al*. also reported the enhanced growth inhibition of colon cancer carcinomatosis by antibodies to vascular endothelial cells and EGFR (30).

At the present time, there are numerous immunotherapy and antiangiogenic clinical trials underway. These trials are targeting tumor antigens, or endothelial cells, or both. We believe that our combination therapeutic approach has provided a rationale for the design of effective clinical trials in the treatment of melanoma.

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