Treatment of experimental breast cancer using interleukin-12 gene therapy combined with anti–vascular endothelial growth factor receptor-2 antibody

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Abstract

We have shown previously that interleukin-12 (IL-12) gene therapy induced strong antitumor effects in several syngeneic murine tumor models including 4T1 mammary adenocarcinoma. Antiangiogenic treatment with a monoclonal antibody (mAb) directed against the vascular endothelial growth factor receptor-2 (VEGFR-2) is another promising treatment approach that can cause transient suppression of tumor growth. We hypothesized that the combination of IL-12 gene therapy and anti-VEGFR-2 mAb will achieve better antitumor and antimetastatic effects against 4T1 adenocarcinoma than each treatment alone via implementation of different mechanisms. Administration of anti-VEGFR-2 mAb into BALB/c mice bearing s.c. 4T1 tumors induced significant suppression of tumor growth, as did intratumoral administration of naked IL-12 DNA. The combined treatment with anti-VEGFR-2 mAb and IL-12 DNA resulted in significantly enhanced inhibition of tumor growth as compared with each treatment alone. This combination was also effective against spontaneous lung metastases. In T-cell–deficient nude mice, both IL-12 DNA and anti-VEGFR-2 mAb were effective in suppressing tumor growth. In T-cell– and natural killer cell–deficient *scid/beige* mice, only anti-VEGFR-2 mAb was effective, suggesting that natural killer cells are involved in the antitumor effects induced by IL-12 DNA. In both types of immunodeficient mice, the combination of anti-VEGFR-2 mAb and IL-12 DNA was as effective in suppressing 4T1 tumor growth as anti-VEGFR-2 mAb alone. Antitumor effects of anti-VEGFR-2 mAb were associated with the inhibition of angiogenesis within the tumors, whereas the antiangiogenic effect of IL-12 gene therapy was not detected. Our results show a therapeutic benefit of combining IL-12 gene therapy and anti-VEGFR-2 mAb for cancer treatment. [Mol Cancer Ther 2004;3(8):969–76]

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Introduction

Although breast cancer, as other human cancers, has long been considered nonimmunogenic, it has been shown during the last several years that human breast tumors may express tumor-associated antigens such as HER-2/*neu* (1), p53 (2), and MUC-1 (3), which can be recognized by the immune system. Therefore, immunotherapeutic strategies for treatment of breast cancer have been studied in experimental settings for potential clinical application (4). Among other immunotherapeutic approaches, strategies using cytokines for inducing or augmenting the antitumor immune response have been proven effective in a variety of animal tumor models (5–7). Interleukin-12 (IL-12) has been shown to be one of the most potent antitumor cytokines in experimental models (8–12). Using a gene therapy approach for local IL-12 delivery, we have shown previously that gene gun-mediated transfer of the IL-12 cDNA expression plasmid into skin surrounding established tumors results in T-cell-mediated tumor regression (13) without systemic toxicity (14). Furthermore, a significant systemic antitumor effect of the localized IL-12 gene delivery protocol was shown against distant solid tumors (15) and spontaneous metastases (13) in immunogenic murine tumor models. However, when a weakly immunogenic 4T1 mammary adenocarcinoma was used, the effect of IL-12 gene therapy was less pronounced than that against a more immunogenic TS/A adenocarcinoma (16). Therefore, we have tested whether IL-12 cDNA immunotherapy of poorly immunogenic breast cancer could be combined with a distinct treatment modality to achieve a substantially better antitumor effect.

The progressive growth of primary neoplasms and metastases depends on the development of adequate vasculature (i.e., angiogenesis; refs. 17, 18). The expression of angiogenic growth factors in different malignancies often has a prognostic significance (19). Therefore, inhibition of angiogenesis is considered to be a promising therapeutic strategy based on the results of animal studies, which have shown that angiogenesis inhibitors can reduce metastasis and shrink established experimental tumors (18, 20–22). The vascular endothelial growth factor (VEGF) is believed to play a major role in the vascularization of human tumors (23). VEGF expression is up-regulated in tumor tissues including breast cancers (24–26) and plays an important

role in the rapid growth of dormant micrometastases (27). Inhibition of VEGF activity in tumor-bearing animals using monoclonal antibodies (mAb; refs. 18, 28–30), antisense DNA (31), or soluble VEGF receptors (32, 33) results in suppression of tumor growth or metastasis. The mAb DC101 is directed against VEGF receptor-2 (VEGFR-2) and can functionally inactivate it (34), resulting in reducing angiogenesis within the growing tumor and, consequently, inhibition of tumor growth and invasion (18). Unfortunately, in most of the studies where angiogenesis inhibitors were used, residual primary tumors or dormant metastases began to grow following the discontinuation of the therapy (35), and repeated administration of antiangiogenic drugs was required to control tumor dormancy (21). Thus, antiangiogenic therapy alone may not provide a mechanism for complete tumor eradication and prevention of tumor recurrence.

We hypothesized that the limitations of each approach (immunologic and antiangiogenic) may be overcome by the strength of the other approach. Specifically, we show here that the combination of an immunologic approach (IL-12 gene therapy) and an antiangiogenic approach (anti-VEGFR-2 mAb) can achieve better antitumor effects than each treatment given separately.

Materials and Methods

Mice

BALB/c mice were obtained from Harlan Sprague-Dawley (Madison, WI). BALB/c nude mice and CB17 *scid/beige* mice were purchased from Taconic Farms (Germantown, NY). Female mice between ages 8 and 12 weeks were used in the experiments. Housing, care, and use of mice were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (NIH publication 86-23, Bethesda, MD, 1985).

4T1 Tumor Model

The 4T1 cell line (36) was originally established from the spontaneous, moderately differentiated mammary adenocarcinoma growing in BALB/c mice and was kindly provided by F.R. Miller (Michigan Cancer Foundation, Detroit, MI). When established as primary s.c. tumors, this adenocarcinoma metastasizes primarily to the lungs (36). Tumor cell cultures were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and streptomycin/penicillin. Before being injected intradermally, tumor cells were detached from the plastic by a short incubation in trypsin-EDTA solution. Mice were shaved in the abdominal area and injected intradermally with 1×10^5 viable tumor cells in 50 μL Dulbecco's PBS. Tumor growth was monitored two to three times weekly by measuring two perpendicular tumor diameters using calipers. To determine the metastatic tumor load in the lungs, mice were sacrificed on days 24 to 28 post-4T1 tumor cell implantation and immediately injected intratracheally with India ink followed by lung fixation in Fekete's solution as described (37).

IL-12 Gene Therapy

The murine IL-12 DNA expression plasmid vector pNGVL3-mIL12 was described by us elsewhere (38). It was ~6.3 kb and constructed using a cytomegalovirus early enhancer/promoter/intron–based plasmid with a kanamycin selection gene. The p35 and p40 subunits were separated by an internal ribosomal entry site and driven by a single cytomegalovirus promoter (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI). pCMVLux, a control plasmid DNA vector (~5.6 kb) containing a luciferase gene with the cytomegalovirus promoter, was constructed as described (13). Plasmid DNA was purified in the absence of ethidium bromide or penicillin derivatives by using a commercially available column chromatography method according to manufacturer's protocol (Qiagen, Chatsworth, CA). For intratumoral injections of naked DNA, DNA (10–50 μg) was diluted in 100 μL sterile PBS and injected into the tumor using a 30 G needle every 3 to 4 days starting on day 4 post–tumor cell implantation.

DC101 mAb Therapy

DC101 mAb is a rat monoclonal IgG1 directed against murine VEGFR-2 (18). Mice received i.p. injections of DC101 mAb or rat IgG (control) at 0.8 mg per mouse every 3 days (39) starting on day 3 post–tumor cell implantation.

Depletion of T Cells and Natural Killer Cells *In vivo*

Anti-CD4 mAb and anti-CD8 mAb were produced from ascites of nude mice injected i.p. with GK1.5 and 2.43 hybridomas, respectively (both obtained from American Type Culture Collection, Manassas, VA). The mAbs, enriched for IgG by ammonium sulfate precipitation, were mixed at a dose of 300 μg each and injected i.p. into mice on day 2 after the tumor challenge and continued every 4 to 5 days. Control tumor-bearing mice received 600 μg of rat IgG (Sigma Chemical Co., St. Louis, MO) per injection. Previous experiments with this technique showed depletion of >95% of T cells in the spleen for 4 days following anti-CD4 + anti-CD8 mAb administration. Natural killer (NK) cells were depleted by injecting tumor-bearing mice i.p. with 50 μL of anti-asialo GM1 antibody (Wako, Richmond, VA) in 0.5 mL PBS. Previous experiments showed that this dose and regimen of anti-asialo GM1 antibody resulted in abrogation of NK activity detected *in vitro* against YAC-1 cells by splenocytes from treated mice.

Histologic Analysis of 4T1 Tumors

Sections were deparaffinized in multiple changes of xylene and rehydrated through decreasing graded ethanols. Heat retrieval was carried out in a standard steamer in 1× target retrieval solution (DAKO, Carpinteria, CA) for 20 minutes at 95°C to 99°C followed by a 20-minute cool down at room temperature. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 10 minutes at room temperature. Sections were incubated in blocking buffer (5% bovine serum albumin, 10% normal goat serum, 0.02% Tween 20, 1× TBS) followed by incubation in biotinylated rat anti-pan endothelial cell antigen (clone MECA-32,

BD PharMingen, San Diego, CA) diluted in 0.2× blocking buffer overnight at 4°C. After successive washes in PBS, sections were incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Positive immunostaining was developed by incubation in 3,3′-diaminobenzidine (DAKO) for 5 minutes at room temperature. Sections were counterstained briefly with hematoxylin followed by dehydration, clearing in xylene, and coverslipping using a permanent mounting medium. Brightfield images of immunostained tissue were viewed on a Zeiss Axioskop (Thornwood, NY) and digitized using a Sony camera (San Diego, CA) and Scion CG-7 framegrabber (Frederick, MD). Vascularity was assessed by quantitating the number of immunopositive pixels in 10 fields at 200× per mouse, four animals per treatment group, using Corel PhotoPaint (Corel Corp., Ottawa, Ontario, Canada).

Statistical Analysis

Tumor volumes [(smaller diameter)^2 × (larger diameter)/2] and vessel density counts were analyzed using the Student's t test. $P < 0.05$ was considered statistically significant.

Results

Effect of IL-12 Gene Therapy against 4T1 Adenocarcinoma

In the first series of experiments, we evaluated the effect of IL-12 gene therapy against solid 4T1 tumors and their spontaneous metastases to determine a suboptimal dose of the plasmid to be used in combination with DC101 mAb in subsequent experiments. Different doses of IL-12 DNA (50, 25, and 12.5 μg) were injected in the tumor area. Results show a significant dose-dependent antiprimary tumor (Fig. 1A) and antimetastatic (Fig. 1B) effect of IL-12 cDNA administration ($P < 0.05$).

Effect of IL-12 Gene Therapy and DC101 mAb against 4T1 Tumor in Immunocompetent Mice

We tested whether IL-12 gene therapy combined with DC101 therapy will induce greater antitumor effect than either treatment alone. As shown in Fig. 2A, intratumoral injection of 50 μg IL-12 DNA caused significant suppression of tumor growth, as did administration of DC101 mAb. The combined treatment with DC101 mAb and IL-12 DNA was associated with more striking tumor growth inhibition than each treatment alone (Fig. 2A). When the lungs from these mice were taken and examined for metastatic growth on day 25, treatment with IL-12 cDNA alone resulted in profound antimetastatic effects; therefore, no difference could be seen when DC101 mAb treatment was combined with IL-12 gene therapy (Fig. 2C). As our goal was to determine if IL-12 DNA and DC101 mAb have an additive or synergistic antitumor effect, we decided to reduce the dose of IL-12 DNA. We thought that 10 μg IL-12 DNA, similar to 12.5 μg IL-12 DNA, would be still effective against primary tumors but would induce a weaker antimetastatic effect than 50 μg IL-12 DNA as shown in Fig. 1.

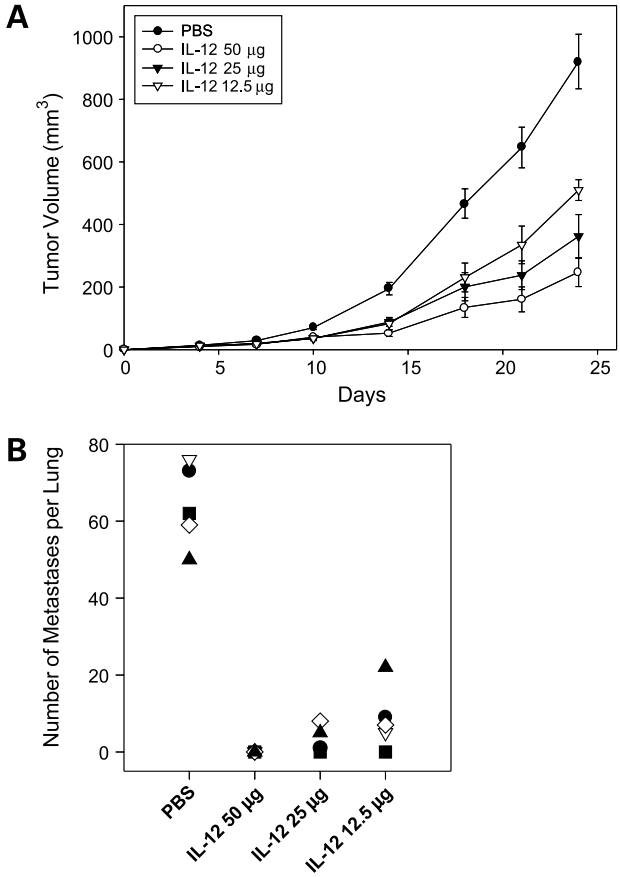

Figure 1. Dose effect of IL-12 gene therapy against 4T1 tumor. **A,** BALB/c mice were injected intradermally with 10^5 4T1 cells (day 0). IL-12 DNA or empty vector at the indicated doses were directly injected in the tumor area on days 4, 7, 10, 14, and 17 post–tumor cell implantation. *Points,* tumor volumes for five mice per group; *bars,* SE. **B,** on day 24 post–tumor cell injection, mice were sacrificed, their lungs were stained with India ink, and number of spontaneous metastases were determined.

When 10 μg IL-12 cDNA was used, the pattern remained the same. Treatment with IL-12 DNA or DC101 mAb alone caused some suppression of tumor growth. The combined treatment with DC101 mAb and IL-12 DNA resulted in significantly enhanced tumor growth inhibition as compared with each treatment alone ($P < 0.05$; Fig. 2B). In addition, there was a significant antimetastatic effect following DC101 mAb treatment as well as IL-12 DNA treatment (Fig. 2D). The combination of DC101 mAb and IL-12 DNA also resulted in an effective antimetastatic effect, although it was not statistically different from the effect of IL-12 gene therapy alone (4.5 ± 2.1 and 8.2 ± 2.1 tumor nodules per mouse, respectively). Because the low dose of IL-12 DNA (10 μg) showed synergy with DC101 mAb, we used this dose of IL-12 DNA in subsequent experiments.

Role of T Cells and NK Cells in the Antitumor Effect Induced by IL-12 DNA + DC101 mAb Therapy

In the next experiment, we asked if T cells and NK cells were required for the antitumor and antimetastatic effects

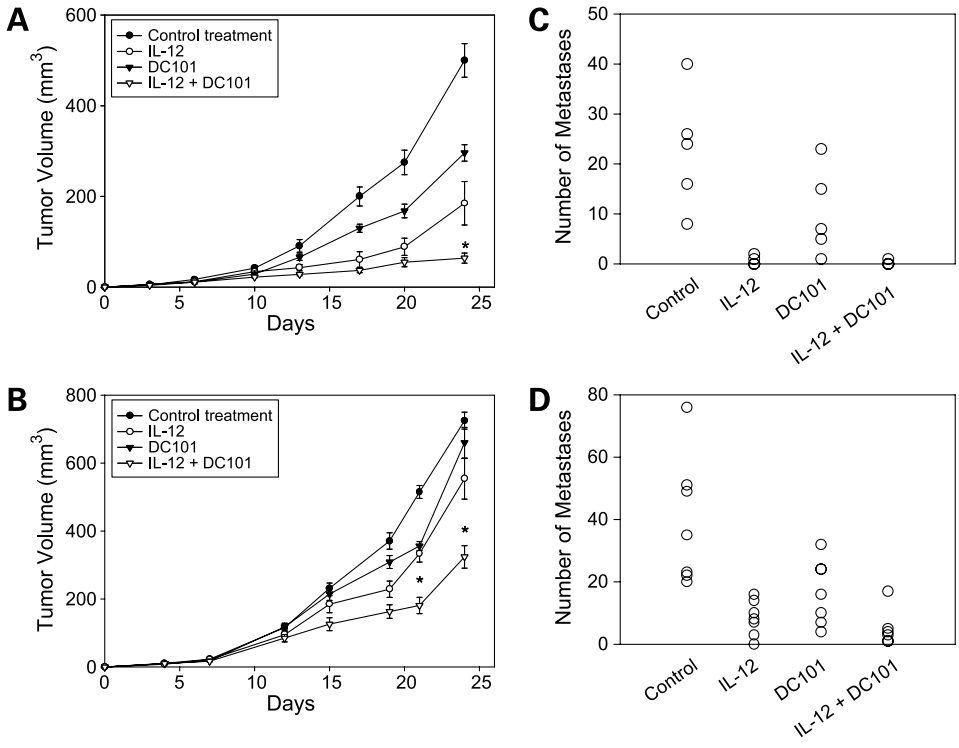

Figure 2. Antitumor effect of IL-12 gene therapy and DC101 mAb in immunocompetent mice. BALB/c mice were injected intradermally with 10^5 4T1 cells (day 0). On days 3, 6, 9, 12, 15, and 18 or 19, the mice received i.p. injections of DC101 mAb or rat IgG (*Control*) at 0.8 mg per mouse. IL-12 DNA or empty vector at 50 μg per mouse (**A** and **C**) or 10 μg per mouse (**B** and **D**) was directly injected in the tumor area on days 4, 7, 10, 13, and 17 or 18 post–tumor cell implantation. Tumor growth was serially measured. *Points,* tumor volumes for five (**A**) and seven (**B**) mice per group; *bars,* SE. *, $P < 0.05$, statistically significant differences between tumor volume values of mice treated with DC101 mAb + IL-12 DNA and mice treated with IL-12 DNA alone or DC101 mAb alone. On day 24 post–tumor cell injection, mice were sacrificed, their lungs were stained with India ink, and number of spontaneous metastases were determined (**C** and **D**). Note that in the groups receiving 50 μg IL-12 DNA alone or with DC101 mAb, three of five and four of five mice, respectively, had no metastases (**C**).

achieved by the combination of IL-12 gene therapy and DC101 mAb. BALB/c mice were depleted of T cells or NK cells by antibody administration before and throughout the treatment. The combined treatment with DC101 mAb and IL-12 DNA resulted in a similar suppression of tumor growth in control mice and in mice depleted of T cells or NK cells (Fig. 3A–C). In addition, the combined treatment with DC101 mAb and IL-12 DNA resulted in a similar antimetastatic effect in T-cell-depleted, NK cell-depleted, and nondepleted mice (Fig. 3D). Together, the results show that the antitumor effects induced by the combination of IL-12 gene therapy and DC101 mAb can be achieved in the absence of T cells and in the absence of NK cells. Next, we evaluated the relative contribution of T cells and NK cells in each individual treatment. First, we injected 4T1 tumor cells in athymic nude mice and treated them with IL-12 DNA and DC101 mAb. The results depicted in Fig. 4A show that, compared with control treatment, 10 μg IL-12 DNA induced statistically significant ($P < 0.05$) suppression of tumor growth on days 10, 14, 18, and 21 post–tumor cell implantation. DC101 mAb had a greater antitumor effect than IL-12 gene therapy, and DC101 mAb alone was as effective as the combined DC101 mAb + IL-12 treatment. When a similar experiment was done in T-cell/NK cell–deficient *scid/beige* mice, the antitumor effect of IL-12 gene therapy observed in BALB/c or nude mice was not seen, but DC101 mAb and the combination of DC101 mAb and IL-12 DNA were similarly effective (Fig. 4B). These results indicate that IL-12 requires NK cells to contribute to the antitumor effect, whereas DC101 mAb remains effective in inducing tumor growth suppression in immunodeficient mice and immunocompetent mice.

Antiangiogenic Effect of IL-12 DNA/DC101 mAb Therapy

As it is known that IL-12 can suppress tumor growth by inducing antiangiogenic effect, even in the absence of T cells (40), we investigated whether this effect of IL-12 was a contributing factor in our model. Tumor samples were collected from nude mice used in the experiment depicted in Fig. 4A on day 29 post–tumor cell implantation. The results in Fig. 5 show that DC101 treatment induced a substantial antiangiogenic effect in the tumors, whereas IL-12 DNA treatment did not. The combined treatment with DC101 mAb and IL-12 DNA was as effective in inducing the antiangiogenic effect as DC101 mAb alone (Fig. 5B).

Discussion

Invasive breast cancer is the leading cause of cancer among women and the second most fatal cancer after lung cancer (41). Two distinct therapeutic approaches, immunotherapy and antiangiogenesis therapy, are being tested clinically based on their antitumor effects in preclinical murine studies. However, each of these approaches has limitations, manifested either in low efficacy against poorly

immunogenic tumors (immunologic approach) or in inability to prevent tumor recurrence (antiangiogenic approach). These limitations can be a major obstacle for successful clinical use of these promising therapeutic strategies.

We hypothesized that a combination of these two approaches, given their different mechanisms of antitumor action, might result in the increased antitumor efficacy. Our results show that IL-12 gene therapy and anti-VEGFR-2 mAb had an additive effect against 4T1 tumors. Intratumoral injections of naked IL-12 DNA induced NK cell-dependent antitumor effects, whereas anti-VEGFR-2 mAb induced T-cell- and NK cell-independent antitumor effects. In immunocompetent mice but not in immunodeficient mice, the combination of IL-12 DNA and anti-VEGFR-2 mAb was more effective than individual therapies in inducing antitumor effects. Finally, we show that the antitumor effect of anti-VEGFR-2 mAb was associated with the inhibition of angiogenesis, whereas the antiangiogenic effect of IL-12 gene therapy was not detected in this model.

The experiments in T-cell-deficient nude mice (Fig. 4A) and T-cell- and NK cell-deficient *scid/beige* mice (Fig. 4B) reveal that the additive effect of IL-12 DNA and DC101 mAb, observed in immunocompetent mice (Fig. 2A and B), was abrogated. These findings imply an important role of T cells, and possibly NK cells, in the beneficial effect of the combinatorial treatment. Compared with immunocompetent mice (Fig. 2A and B), IL-12 DNA induced a reduced but statistically significant antitumor effect in nude mice and was not effective in *scid/beige* mice, whereas the antitumor efficacy of DC101 mAb was increased in both nude and *scid/beige* mice. These results conclusively show that IL-12 DNA and DC101 mAb have different antitumor mechanisms. Our results showing the additive antitumor effect of IL-12 gene therapy and DC101 mAb are consistent with recent publications demonstrating a benefit of combining immunotherapy and antiangiogenic therapy in other experimental tumor models (42–45). In a murine breast cancer model, treatment with a combination of adenovirus vectors expressing murine angiostatin and IL-12 resulted

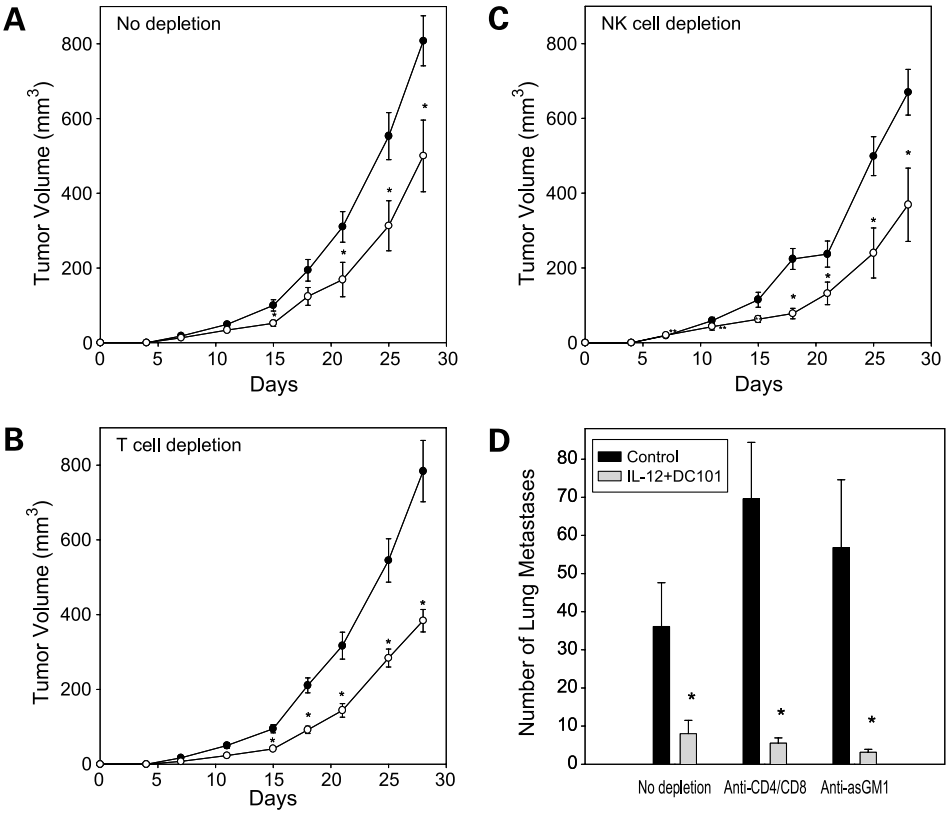

Figure 3. Combined therapy with IL-12 cDNA and DC101 is effective in mice depleted of T cells or NK cells. BALB/c mice were injected intradermally with 10^5 4T1 cells (day 0). On days 2, 7, 11, and 16, mice were injected i.p. with a combination of anti-CD4 mAb and anti-CD8 mAb (**B**) or with anti-asialo GM1 antibody (**C**) or left without antibody injection (**A**). On days 3, 6, 9, 12, 15, and 18, the mice received i.p. injections of DC101 mAb (*open circles*) or rat IgG (*solid circles*) at 0.8 mg per mouse. On days 4, 7, 10, 13, and 17 post–tumor cell implantation, the same mice received IL-12 DNA (*open circles*) or control vector (*solid circles*) directly injected in the tumor area at 10 μg per mouse. Tumor growth was serially measured. *Points,* tumor volumes for eight mice per group; *bars,* SE. **D,** the combined treatment with DC101 mAb and IL-12 DNA resulted in a similar suppression of tumor growth in control mice and mice depleted of T cells or NK cells. *, $P < 0.05$. On day 28 post–tumor cell injection, mice were sacrificed, their lungs were stained with India ink, and number of spontaneous metastases were determined. The combined treatment with DC101 mAb and IL-12 DNA resulted in a similar antimetastatic effect in T-cell–depleted, NK cell-depleted, and nondepleted mice.

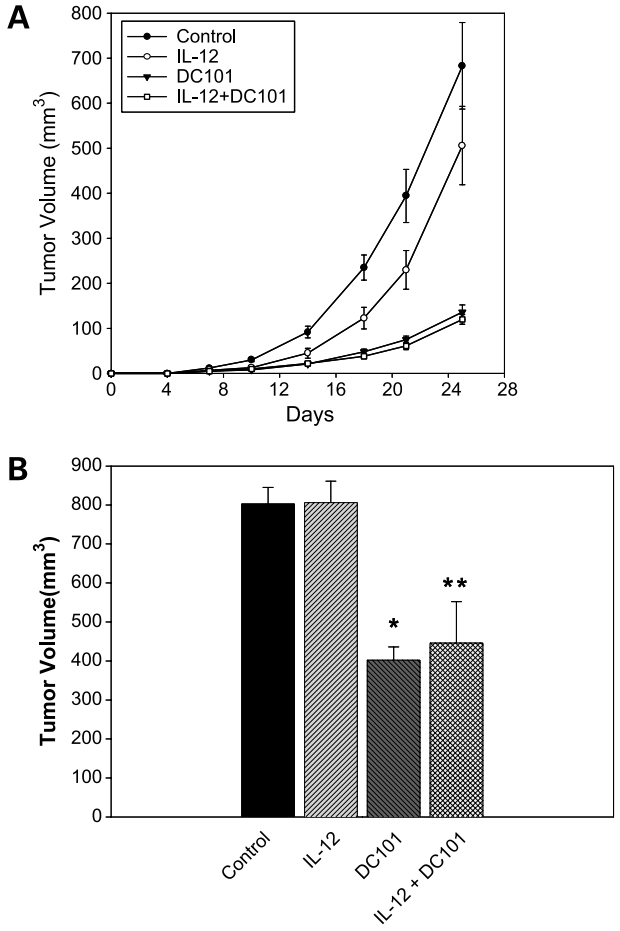

Figure 4. Antitumor effect of IL-12 gene therapy and DC101 mAb in nude mice and *scid/beige* mice. BALB/c nude mice (**A**) and CB17 *scid/beige* mice (**B**) were injected intradermally with 1×10^5 4T1 cells (day 0). IL-12 DNA (10 μg) or empty vector was given on days 4, 8, 11, 14, 18, 22, and 26 (**A**) or days 4, 7, 10, 13, and 17 (**B**). DC101 mAb (0.8 mg) or rat IgG was given on days 3, 6, 9, 12, 15, 18, 21, and 25 (**A**) or days 3, 6, 9, 12, 15, and 18 (**B**). Tumors were serially measured. *Points,* tumor volumes for five mice per group at different time points; *bars,* SE (**A**). *Columns,* tumor volumes for four mice per group on day 26 post–tumor cell implantation (**B**); *bars,* SE. *, $P < 0.001$, **, $P < 0.025$ as compared with control.

in a greater antitumor effect as compared with each treatment alone (45). In this article, the antitumor activity of IL-12 gene therapy was associated with both immune activation and antiangiogenesis.

The antitumor mechanisms of IL-12 against some poorly immunogenic tumors have been attributed to the antiangiogenic effects of IL-12 (40, 46), which may involve NK cells (47), IFNγ (48), and IFNγ-induced secondary mediators such as the chemokine IP-10 (48). We have recently published that 4T1 is a poorly immunogenic tumor, and IL-12 gene therapy induced the antimetastatic effects against that tumor, which were T cell independent but involved NK cells and IFNγ (16). The results of this study with *scid/beige* mice, showing the lack of tumor inhibition by IL-12 (Fig. 4B), confirm that NK cells are important for the antitumor effect of IL-12 and suggest that IL-12 may activate NK cells to directly kill tumor cells as we reported previously (16). However, our inability to detect the antiangiogenic effect of IL-12 at the late stage of tumor growth (Fig. 5) does not preclude the possibility that IL-12 gene therapy was acting through this mechanism in the early stages of the treatment possibly via NK cell-mediated cytotoxicity of endothelial cells (47). In most published articles on the antiangiogenic effects of IL-12, this effect was observed within 1 week after the treatment (48, 49), whereas, in our study, the tumor samples were analyzed

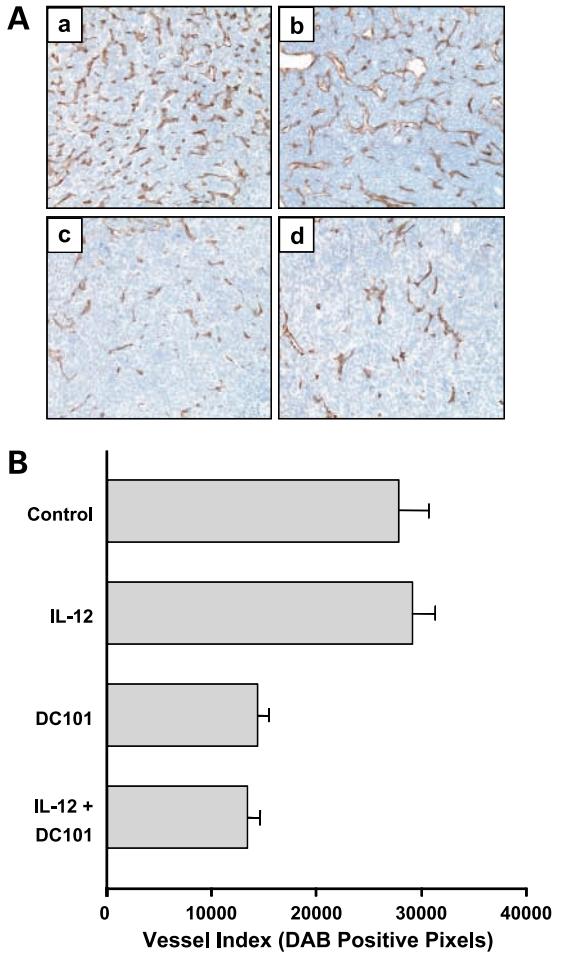

Figure 5. Effect of DC101 mAb and IL-12 cDNA on angiogenesis. 4T1 tumor cells (10^5) were injected intradermally in nude mice (day 0). On days 3, 6, 9, 12, 15, 18, 21, and 25, mice were injected i.p. with 0.8 mg DC101 mAb or rat IgG. On days 4, 8, 11, 14, 18, 22, and 26, mice were injected intratumorally with 10 μg IL-12 DNA or empty vector. On day 29, four mice from each group were sacrificed. **A,** tumor samples from control (*a*), IL-12 (*b*), DC101 (*c*), or IL-12 + DC101 (*d*) groups were removed, fixed, and processed for anti-pan endothelial cell antigen (MECA-32) immunohistochemistry (200×). **B,** vascularity was assessed by quantitating the number of immunopositive pixels in 10 fields per mouse, four animals per treatment group (200×). *Columns,* mean of 10 fields per mouse, four mice per group; *bars,* SE.

3 weeks after the initiation of the treatment. We were not able to histologically analyze the angiogenesis in the tumors within 1 week after the treatment because of the tumor size limitation.

In contrast to IL-12, the antiangiogenic effect of DC101 mAb could be observed in the tumors 3 weeks following the initiation of the therapy (39) and was readily detected in our study (Fig. 5). The antitumor activity of DC101 mAb directed against VEGFR-2 was associated with reduced tumor-induced neovascularity, enhanced apoptosis in tumor cells and endothelial cells, and reduced production of endothelial cell matrix metalloproteinase type 9 (50). Our data confirm studies showing antitumor and antimetastatic effects of DC101 mAb (39, 50–52) and additionally show that a combination of DC101 mAb and IL-12 gene therapy can result in a greater antitumor effect than each treatment alone. Our results show that these two treatments have different antitumor mechanisms: DC101 mAb reduces angiogenesis in the tumor by a NK cell-independent mechanism, whereas IL-12 gene therapy induces tumor growth suppression, which requires NK cells. We suggest that the results of this study support the development of a clinical strategy testing the combination of IL-12 and antiangiogenic therapies for metastatic breast cancer and possibly other malignancies.

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