

# Combined Therapy of Local and Metastatic 4T1 Breast Tumor in Mice Using SU6668, an Inhibitor of Angiogenic Receptor Tyrosine Kinases, and the Immunostimulator B7.2-IgG Fusion Protein<sup>1</sup>

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## ABSTRACT

The therapeutic efficacy of combined antiangiogenic and immune therapy was tested against weakly immunogenic and highly metastatic 4T1 breast tumor using SU6668, an angiogenesis inhibitor and recombinant murine (rm) B7.2-IgG fusion protein, an immunostimulator. SU6668 inhibits the tyrosine kinase activity of three angiogenic receptors VEGFR2 (Flk-1/KDR), PDGFR $\beta$ , and FGFR1, which play a crucial role in tumor-induced vascularization. rmb7.2-IgG is a fusion protein of the extracellular domain of B7.2, and the hinge and constant domains of murine IgG2a. Intermolecule disulfide linkages are maintained so that it forms a dimer. Our studies showed that three weekly immunizations of BALB/c mice bearing 0.5–0.8 cm 4T1 breast tumors with rmb7.2-IgG and irradiated 4T1 tumor cells (B7.2-IgG/TC) resulted in a significant inhibition of tumor growth and formation of pulmonary metastases. T-cell depletion experiments revealed that both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are required for stimulation of the antitumor and antimetastatic immune response by B7.2-IgG/TC. Treatment of mice with SU6668 substantially inhibited tumor vascularization but did not inhibit tumor infiltration by T lymphocytes or the T-cell response to rmb7.2-IgG therapy. On the contrary, tumors in mice immunized with B7.2-IgG/TC and treated with SU6668 had higher numbers of tumor infiltrating T cells than tumors of other groups. SU6668 treatments initiated either on day 3 or day 10 after inoculation of 4T1 tumor cells resulted in a significant inhibition of tumor growth. Similarly, three weekly immunizations with B7.2-IgG/TC starting either on day 7 or 12 inhibited growth of 4T1 tumors. However, the most potent antitumor effects were observed in mice treated with a combination of SU6668 and B7.2-IgG/TC. Analysis of pulmonary metastases revealed that combined therapy also had a more potent antimetastatic effect than each modality alone. These results indicate that antiangiogenic and immune therapies using SU6668 and B7.2-IgG/TC are compatible, and manifest complementary antitumor and antimetastatic effects. Combined antiangiogenic and immune therapy might represent a new strategy for cancer treatment.

## INTRODUCTION

Tumor cells have very efficient mechanisms for stimulating the new blood vessel network formation essential for tumor growth (1–3). In addition, tumor blood vessels serve as a major gateway for dissemination of tumor cells into distant anatomical locations, where they can induce a new round of neovascularization and develop into metastatic lesions. Thus, the tumor vascular system should be an important target in the battle against cancer. In recent years numerous small molecules and biological agents capable of inhibiting tumor-induced vascularization have been identified. The ability of these

agents to inhibit tumor vascularization and tumor growth has been demonstrated in numerous experimental models (4, 5). However, these effects were often incomplete and transient. Histological analysis revealed that tumor cells could survive in the vicinity of established host vessels so that on cessation of antiangiogenic therapy, tumor cells were again able to stimulate blood vessel formation, resulting in tumor reappearance (6, 7). These results suggest that antitumor strategy could benefit from attacking both tumor cells and tumor vasculature. We hypothesize that immunotherapy directed against tumor cells, combined with an antiangiogenic regimen, may provide a greater therapeutic efficacy than either single modality. Indeed, our recent studies support this hypothesis (8). We found that the antitumor effect of recombinant endostatin was higher against highly immunogenic than weakly immunogenic variants of 3LL Lewis lung carcinoma. Endostatin caused only partial inhibition of the parental 3LL tumor, whereas it induced regression in 40% of mice of the highly immunogenic variant 3LL-C75. The role of immune effector pathways in this response was demonstrated using T-cell-deficient nude mice. In those mice endostatin induced only growth inhibition but not regression of the highly immunogenic 3LL-C75 tumor (8). The conclusion that an immune response can complement the efficacy of endostatin was additionally supported by experiments in which the antitumor response was enhanced by vaccination with 3LL-C75 tumor cells. Antitumor vaccination significantly increased the efficacy of endostatin against the parental weakly immunogenic 3LL tumor. Immunizations combined with endostatin treatment resulted in regression of 3LL tumor in 50% of mice, whereas only partial inhibition of a tumor growth tumor was seen in nonimmunized mice (8). These studies demonstrate that antitumor immune response could complement the antitumor effects of endostatin therapy.

In the present study we additionally tested the therapeutic efficacy of combined antiangiogenic and immune therapy against the established 4T1 breast tumor. For these studies, the therapy included an antiangiogenic agent SU6668 and the immunostimulator rmb7.2-IgG fusion protein. SU6668 is a small molecule synthetic inhibitor of the tyrosine kinase activity of three angiogenic receptors VEGFR2<sup>3</sup> (Flk-1/KDR), PDGFR $\beta$  and FGFR1 (9). These receptors are expressed by endothelial cells and play a crucial role in tumor-induced angiogenesis (10, 11). In addition, PDGFR $\beta$  is expressed by pericytes that provide stability for newly formed vessels (12). Thus, inhibition of receptor signaling by SU6668 might interrupt tumor-induced angiogenesis, resulting in tumor cell starvation and inhibition of tumor growth. Indeed, SU6668 treatment of xenografted human tumors led to eradication or inhibition of tumor growth (9). It was also found that SU6668 therapy inhibits liver metastasis formation after intrasplenic inoculation of C26 colon tumor cells (13, 14).

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<sup>3</sup>The abbreviations used are: VEGFR, vascular endothelial growth factor receptor; rm, recombinant murine; PDGFR, platelet-derived growth factor receptor; FGFR, fibroblast growth factor receptor; TC, tumor cell; mAb, monoclonal antibody; PE, phycoerythrin; vWF, von Willebrand Factor; IL, interleukin; VEGF, vascular endothelial growth factor.

To assess the ability of antitumor immune mechanisms to complement the antitumor effects of SU6668, immunostimulation was performed using rmB7.2-IgG fusion protein. This fusion protein was purified from Chinese hamster ovary cells transfected with an expression plasmid that encoded murine B7.2 signal and extracellular domains joined to a genomic DNA segment encoding the hinge-CH2-CH3 domains of murine IgG2a. The Cys residues within the IgG hinge region were preserved such that the fusion protein forms a dimer (15). T-cell activation is a result of signaling via the T-cell receptor after its interaction with MHC I peptide. This activation also requires a costimulatory signaling via CD28-B7 pathway. CD28 molecules bind B7 with low affinity, whereas CTLA4 (CD152) has high affinity for B7. Most T cells are constitutively expressed CD28 molecules, whereas CTLA4 molecules express on activated T cells. CTLA4 after engagement with B7 molecules might deliver a down-regulatory signal (16). It was shown that treatment of tumor-bearing mice with B7.2-IgG or B7.1-IgG resulted in stimulation of the antitumor immunity and inhibition or eradication of growth of various murine tumors (15, 17, 18). These antitumor effects of soluble rmB7-IgG fusion protein may be a result of ligation of CD28 on T cells providing costimulatory signal. In addition, it was suggested that the soluble rmB7-IgG, in contrast to the cell-associated B7 molecules, may antagonize the inhibitory signal provided by the interaction of cell surface B7 and its alternate ligand CTLA4, expressed by activated T cells (15, 17, 18).

In present studies we investigated whether the therapeutic efficacy of an angiogenesis inhibitor SU6668 can be enhanced by stimulation of an antitumor immune response with rmB7.2-IgG. Combined therapy with SU6668 and rmB7.2-IgG was evaluated against the established highly metastatic 4T1 breast tumor. Our results demonstrate that combined angiogenic therapy with SU6668 and immunotherapy with rmB7.2-IgG has significantly more potent antitumor and anti-metastatic effects than either modality alone.

## MATERIALS AND METHODS

**Mice.** Female, 6-week-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8–9 weeks of age. Mice were housed in a specific pathogen-free facility at the University of Pittsburgh that is accredited for animal care by the American Association of Laboratory Animal Care. Experiments were performed in accordance with the approved institutional protocol and the guidelines of the Institutional Animal Care and Use Committee.

**Tumor Cell Lines.** 4T1 breast tumor spontaneously developed in BALB/c mice. 4T1/IA<sup>d</sup>/B7.1 subline was derived from 4T1 cells cotransfected with the MHC class II *H-2A<sup>d</sup>* and *B7.1* genes (19, 20). Both lines were provided by Dr. Suzanne Ostrand-Rozenberg (University of Maryland, Baltimore, MD). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (hereafter referred to as complete medium).

**Flow Cytometry Analysis.** The analysis of MHC class I and class II molecule expression by 4T1 and 4T1/IA<sup>d</sup>/B7.1 tumor cells was performed as described previously (21). Tumor cells were incubated with anti-H-2K<sup>d</sup>, H-2D<sup>d</sup>, and H-2IA<sup>d</sup> mAb for 30 min at 4°C. After washing, cells were stained with goat antimouse IgG-PE for an additional 30 min. Cells were washed and fixed in 1% of paraformaldehyde. The expression of B7.1 molecules by tumor cells was analyzed by staining cells with anti-CD80-biotin and avidin-PE (BD PharMingen, San Diego, CA). Fluorescence signals were collected on a log scale (21).

**SU6668 and B7.2-IgG Treatment Protocols.** BALB/c mice were inoculated s.c. with  $1 \times 10^5$  of 4T1 breast tumor cells. Treatment with SU6668 (SUGEN, Inc., South San Francisco, CA) was started on day 3 or 10 after tumor cell inoculation. SU6668 dissolved in DMSO was inoculated daily s.c. at a dose of 75 mg/kg in 0.05 ml of DMSO. All of the injections were administered distant from the tumor site. Mice in other groups received injections of 0.05 ml of DMSO. Purified rmB7.2-IgG fusion protein (Wyeth/

Genetics Institute, Cambridge, MA) mixed with  $4 \times 10^6$  irradiated (15,000r) 4T1 tumor cells (hereafter referred as B7.2-IgG/TC) was given s.c. at a dose of 100  $\mu$ g in PBS. Immunizations were initiated on day 7 or 12 after tumor cell inoculation and repeated three times at weekly intervals. Tumor growth was evaluated by measurement of tumor diameters three times a week, and the tumor volume was calculated as length  $\times$  width<sup>2</sup>  $\times$  0.52. All of the data represent as mean  $\pm$  SE. Experiments were terminated when tumors reached 2 cm in diameter according to the protocol approved by Institutional Animal Care and Use Committee, University of Pittsburgh. Each group contained 7–9 mice. Organs (lungs, liver, kidney, and spleen) from sacrificed mice were removed, fixed in the Bouin's solution, and metastatic nodules were counted and their diameter was measured using dissecting microscope (22).

**In Vivo T-Cell Subset Depletion.** *In vivo* T-cell depletion was performed using ascites of anti-CD4 (GK1.5) or anti-CD8 (53–6.72) hybridomas (American Type Culture Collection, Manassas, VA). On day 5 after s.c. inoculation of  $1 \times 10^5$  4T1 cells, BALB/c mice were inoculated i.p. with 0.2 ml of ascites containing anti-CD4 or anti-CD8 mAb. Two days after inoculation of anti-CD4 or anti-CD8 mAb mice were immunized with B7.2-IgG (100  $\mu$ g) and  $4 \times 10^6$  irradiated (15,000 r) 4T1 cells. In total three immunizations were performed at weekly intervals (on days 7, 14, and 21 of tumor growth). Treatment with anti-CD4 and anti-CD8 mAbs was repeated 2 days before and 2 days after each immunization, and continued twice a week during the entire period of tumor growth. This treatment resulted in elimination of CD4 and CD8 cells as demonstrated by flow cytometry. The control group of mice was inoculated with 200  $\mu$ g of purified normal rat IgG (Sigma, St. Louis, MO).

**In Vitro Proliferative Response of Spleen Cells to B7.2-IgG Stimulation.** BALB/c mice were treated with SU6668 daily starting on day 4 after 4T1 cell inoculation. Immunizations with B7.2-IgG/TC were performed on days 7, 14, and 24. Spleens were removed when tumors reached about 1.2–1.5 cm in diameter, and spleen cell suspensions were prepared. Flat-bottomed 96-well plates were precoated with B7.2-IgG (40  $\mu$ g/ml) for 2 h at 37°C in a final volume of 0.1 ml/well (23). After washing the wells twice with Dulbecco's PBS,  $4 \times 10^5$  spleen cells were added to each well. In some wells, irradiated (15,000r) 4T1 tumor cells ( $2 \times 10^4$ ) were also added. Spleen cells were cultured for 3 days and levels of proliferation was tested by [<sup>3</sup>H]thymidine incorporation (23).

**IFN- $\gamma$  Production by Spleen Cells of Mice Treated with SU6668 and/or B7.2-IgG.** BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 cells. Daily treatments with SU6668 (75 mg/kg) were initiated on day 3 following 4T1 tumor cell inoculation. On days 7, 14, and 21, mice were inoculated with B7.2-IgG (100  $\mu$ g) mixed with  $4 \times 10^6$  irradiated 4T1 tumor cells. After 35 days of tumor growth, spleens were harvested. Spleen cells ( $4 \times 10^6$ ) and  $1 \times 10^5$  irradiated 4T1/IA<sup>d</sup>/B7.1 tumor cells were plated into 24-well plates. After 48 h of stimulation, the supernatants were collected, and IFN- $\gamma$  concentration in these supernatants was determined using IFN- $\gamma$  ELISA kit (R&D Systems, Minneapolis, MN).

**Histological Analysis of Blood Vessel Density and Tumor Infiltration by T Lymphocytes.** BALB/c mice were inoculated s.c. with 4T1 tumor cells ( $1 \times 10^5$ ) and treatment with SU6668 begun on day 4. On day 7, some of the SU6668-treated and untreated mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 26. To perform histological analysis of tumors of similar size (not more than 1.5 cm in diameter), tumors from control mice were harvested on days 19–26, and tumors from the treated mice were obtained on days 28 and 29. Four tumors per group were harvested and fixed in 2% paraformaldehyde, infused with 30% sucrose overnight, and frozen in liquid nitrogen-cooled isopentane. Five- $\mu$ m cryosections were cut and mounted on superfrost slides (Fisher, Pittsburgh, PA) and labeled as described below. Sections were washed three times in PBS containing 0.5% BSA and 0.15% glycine (pH 7.4; Buffer A). This was followed by a 30-min incubation with purified goat IgG (50  $\mu$ g/ml) at 25°C and three additional washes with Buffer A. All of the preceding steps are designed to ensure minimal nonspecific reaction to the antibodies used. Sections were incubated with anti-vWF rabbit polyclonal antibody (BD PharMingen) for 60 min and stained with antirabbit IgG-Alexa 488. The sections were then washed six times (5 min/wash) in Buffer A. To determine T-cell intratumor infiltration, sections were incubated for 60 min with anti-CD4 or CD8-biotin mAb (BD PharMingen) followed by three washes in Buffer A and 60-min incubation in Streptavidin-Alexa 488 (1  $\mu$ g/ml; Molecular Probes, Eugene, OR). Sections were then stained with Hoescht dye to label nuclei. After three washes in buffer

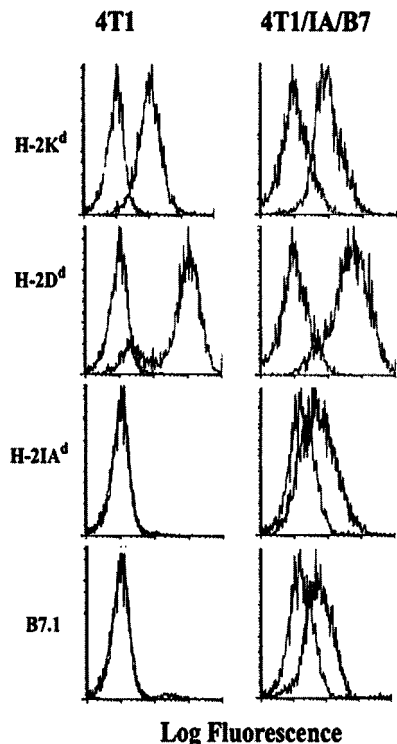


Fig. 1. Expression of B7.1 and MHC class I and class II molecules by 4T1 and 4T1/IA<sup>d</sup>/B7.1 tumor cells. 4T1 and 4T1/IA<sup>d</sup>/B7.1 tumor cells were incubated with anti-H-2K<sup>d</sup>, H-2D<sup>d</sup>, and H-2IA<sup>d</sup> mAb for 30 min at 4°C. After washing, cells were stained with goat antimouse IgG-PE for an additional 30 min. Cells were washed and fixed in 1% of paraformaldehyde. The expression of B7.1 molecules by tumor cells was analyzed by staining cells with anti-CD80-biotin and avidin-PE (BD Pharmingen). Fluorescence signals were collected on a log scale. First peak represents an isotype IgG control, the second peak shows the specific antibody binding.

they were then mounted in Gelvatol (Monsanto, St. Louis, MO) and coverslipped for subsequent observation. Images were collected using a Magnifire camera (Olympus, Melville, NY) attached to an Olympus Provis microscope. Separate images were collected for the green (Alexa 488) and blue (Hoescht dye) channels, and superposed using Adobe Photoshop (Adobe). No additional enhancement of the images was performed.

**Statistical Analysis.** Statistical analysis of the differences in tumor volume was performed using Student's *t* test. The level of significance was set at  $P < 0.05$ . Because distribution of metastatic tumors in the lungs is not normal, the data were presented as medians, and statistical analysis of the differences in metastasis formation were analyzed using the Mann-Whitney test.

## RESULTS

**Antitumor and Antimetastatic Effects of Immunostimulation with B7.2-IgG Fusion Protein.** It was shown that murine recombinant B7.2-IgG and B7.1-IgG fusion proteins have a similar ability to stimulate the antitumor immune response (15). Our studies were performed using B7.2-IgG fusion protein to stimulate the antitumor immune response against 4T1 breast tumor. 4T1 breast tumor that was originally developed in BALB/c mice is highly metastatic and weakly immunogenic (19, 20). This tumor grows very fast, usually reaching a diameter of 0.5 cm in 7 days after s.c. inoculation of  $1 \times 10^5$  4T1 cells tumors. We first evaluated the ability of B7.2-IgG fusion protein treatment to stimulate the antitumor immunity against established 4T1 breast tumor. In addition, we compared the antitumor effects induced by B7.2-IgG fusion protein mixed with irradiated 4T1 tumor cells versus vaccination with 4T1/IA<sup>d</sup>/B7.1-irradiated tumor cells. 4T1/IA<sup>d</sup>/B7.1 tumor cells were transfected with the MHC class II *H-2IA<sup>d</sup>* gene and cotransfected with the *B7.1* gene (20). Our flow cytometric

analysis revealed that 4T1 and 4T1/IA<sup>d</sup>/B7 tumor cells express relatively high levels of MHC class I H-2K<sup>d</sup> and H-2D<sup>d</sup> molecules (Fig. 1). In addition, 4T1/IA<sup>d</sup>/B7 tumor cells expressed a high level of the transfected *H-2IA<sup>d</sup>* and *B7.1* genes (Fig. 1). Thus, 4T1/IA<sup>d</sup>/B7 tumor cells could serve as antigen presenting cells that are able to present 4T1 breast tumor antigen via both MHC class I and class II molecules and provide a costimulatory signal to T cells via the B7.1-CD28 interaction (20).

BALB/c mice were inoculated s.c. with  $1 \times 10^5$  of 4T1 breast tumor cells and after 7 days mice were separated into three groups that had tumors of similar sizes (~0.5 cm in diameter). Two groups of mice were immunized s.c. with  $4 \times 10^6$  irradiated (15,000r) 4T1/IA<sup>d</sup>/B7 cells or with B7.2-IgG (100 Bg) plus irradiated  $4 \times 10^6$  4T1 tumor cells (B7.2-IgG/TC). These immunizations were repeated two additional times at a weekly interval. The results presented in Fig. 2 show that immunizations with irradiated 4T1/IA<sup>d</sup>/B7 were able to significantly inhibit the growth of established 4T1 tumors. However, treatments with B7.2-IgG/TC showed slightly higher ( $P < 0.05$ ) antitumor effects than immunizations with the genetically modified 4T1/IA<sup>d</sup>/B7 tumor cells. It is of note that the parental 4T1 tumor cells expressing only MHC class I molecules are poorly immunogenic, and immunizations of mice bearing 4T1 breast tumor with the irradiated 4T1 cells alone failed to induce the protective antitumor immune responses (data not shown). Thus, soluble B7.2-IgG fusion protein could be highly efficient in stimulation of potent antitumor immune response.

4T1 tumor cells are able to spread into different anatomical locations at the earliest stage of tumor growth (19, 20). To analyze the possible effect of antitumor vaccinations on metastasis formation, mice were killed at day 35 of tumor growth, and their lungs, liver, kidney, spleen, and lymph nodes were recovered. Visible metastases were found only in the lungs. When lungs of treated and untreated mice were examined, we found that control mice had a median of 20 metastases/lungs (range, 5–37 metastases), whereas mice immunized with B7.2-IgG/TC or with irradiated 4T1/IA<sup>d</sup>/B7 cells had a median of 5 and 4 metastases/lungs, respectively (Table 1).

These results indicate that soluble B7.2-IgG fusion protein has a

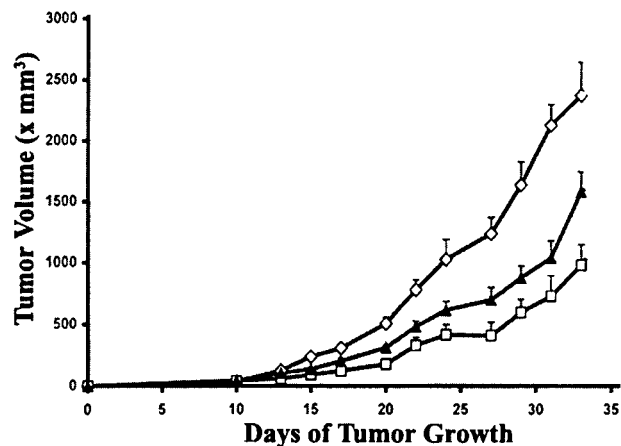


Fig. 2. Comparative antitumor effects of immunizations with soluble B7.2-IgG, irradiated 4T1 tumor cells versus 4T1 tumor cells cotransfected with the MHC class II *H-2IA<sup>d</sup>* and *B7.1* genes (4T1/IA<sup>d</sup>/B7.1). BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 7, when tumors reach ~0.5 cm in diameter, mice were inoculated s.c. with  $4 \times 10^6$  irradiated (15,000r) 4T1 tumor cells admixed with B7.2-IgG fusion protein (100  $\mu$ g;  $\square$ ). One group of mice was immunized with  $4 \times 10^6$  irradiated 4T1/IA<sup>d</sup>/B7.1 cells ( $\blacktriangle$ ). Immunizations were repeated on days 14 and 21. Data represent mean tumor volume; bars,  $\pm$  SE. Each group contained 8–9 mice. From day 22 the differences between control group and groups immunized with B7.2-IgG/TC or 4T1/IA<sup>d</sup>/B7.1 cells were significant ( $P < 0.05$ ). The differences between B7.2-IgG/TC and 4T1/IA<sup>d</sup>/B7.1 immunized groups were significant ( $P < 0.05$ ) starting day 27.

Table 1 Inhibition of spontaneous metastasis formation by immunization with the soluble B7.2-IgG fusion protein plus irradiated 4T1 tumor cells or by irradiated 4T1 cells expressing the transfected H-2IA<sup>d</sup> and B7.1 genes (4T1/IA/B7.1)

BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 7, when tumors reach ~0.5 cm in diameter, mice were inoculated s.c. with murine recombinant B7.2-IgG fusion protein (100  $\mu$ g) admixed with  $4 \times 10^6$  irradiated (15,000 r) 4T1 tumor cells (B7.2-IgG/TC) or irradiated 4T1/IA/B7.1 tumor cells. Immunizations were repeated on days 14 and 21. On day 35 of tumor growth mice were sacrificed and lung metastases were counted.

Treatment	No. of mice per group	Median no. of metastasis/lung (range)
Control	7	20 (5-37)
B7.2-IgG + TC	8	5 (2-7) <sup>a</sup>
4T1/IA/B7.1	9	4 (0-10) <sup>a</sup>

<sup>a</sup> Significantly ( $P < 0.05$ ) differs from the control group according to Mann-Whitney test.

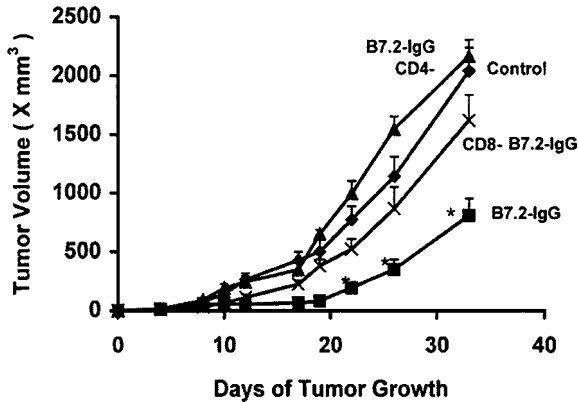


Fig. 3. Role of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the antitumor effects of B7.2-IgG/TC immunizations. BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 5 mice received i.p. injections of anti-CD4 ( $\blacktriangle$ ) or anti-CD8 ( $\times$ ) mAb. Injections of these mAbs were repeated twice a week. Control group ( $\blacklozenge$ ) of mice received 200  $\mu$ g of rat IgG. On day 7, when tumors reach ~0.5 cm in diameter, normal ( $\blacksquare$ ) or CD4<sup>-</sup>, CD8-depleted mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 21. Each group contained 7-8 mice. \*Significantly ( $P < 0.05$ ) differs from other groups; bars,  $\pm$  SE.

potent ability to stimulate antitumor and antimetastatic activity in mice with established 4T1 breast tumor.

**The Role of CD4 and CD8 in the Antitumor and Antimetastatic Effects of B7.2-IgG Therapy.** The observed stimulation of the antitumor activity with B7.2-IgG might be a result of interactions of B7.2 molecules with CD28 and/or antagonizing the inhibitory effects of CTLA4 on T lymphocytes. However, it remains unclear whether CD4, CD8, or both T lymphocytes are responsible for the antitumor effect of B7.2-IgG stimulation. To test this, we compared the antitumor effects of B7.2-IgG plus tumor cell immunizations in immunocompetent, as well as CD4<sup>-</sup> and CD8<sup>-</sup> depleted mice. BALB/c mice were inoculated with  $1 \times 10^5$  4T1 breast tumor cells. To deplete CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes, mice were inoculated i.p. with rat anti-CD4 or anti-CD8 mAb. Other groups of mice received 200  $\mu$ g of rat IgG. Antibody treatments were initiated 5 days after tumor cell inoculation and repeated 4 days later. On day 7 of tumor growth, when tumors reached ~0.5 cm in diameter, normal and T-cell-depleted tumor-bearing mice were treated with B7.2-IgG/TC. Treatments with anti-CD4 or anti-CD8 antibody were repeated 2 days after vaccination. Similar injections of antibodies were applied twice a week, 2 days before and 2 days after next immunizations. In total, three immunizations with the weekly intervals were performed, and treatment with the antibody lasted for 4 weeks. These treatments almost completely eliminated CD4 and CD8 T lymphocytes as shown by flow cytometry. Immunizations with B7.2-IgG/TC significantly ( $P < 0.05$ ) inhibited growth of 4T1 breast tumor in the immunocompetent mice (Fig. 3). Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes abrogated the

antitumor effect of B7.2-IgG/TC therapy. Tumor growth in the CD4<sup>-</sup> or CD8<sup>-</sup> depleted mice treated with B7.2-IgG/TC showed no difference from the control mice (Fig. 3). These results indicate that both CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes are important for the therapeutic activity of B7.2-IgG/TC against 4T1 breast tumor.

To evaluate the role of CD4 and CD8 lymphocytes in the antimetastatic effects of B7.2-IgG/TC therapy, all of the mice were sacrificed on day 33 of tumor growth, at the time some mice started to die. Lungs, liver, spleen, and kidney were harvested and examined under the dissecting microscope. Visible metastases were found only in the lungs. B7.2-IgG/TC immunizations significantly ( $P < 0.05$ ) reduced the number of the metastatic nodules only in the immunocompetent mice (Table 2). Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes abrogated the antimetastatic effects of B7.2-IgG/TC immunizations. These results indicate that CD4<sup>+</sup> as well as CD8<sup>+</sup> lymphocytes play a crucial role in the antitumor and antimetastatic effects of B7.2-IgG/TC immunization in BALB/c mice bearing the 4T1 breast tumor.

**Effect of SU6668 Treatment on the B7.2-IgG-induced Proliferative Response of Spleen Lymphocytes.** SU6668 is an inhibitor of tyrosine kinase activity of the angiogenic receptors VEGFR2, PDGFR $\beta$ , and FGFR1, which play a crucial role in angiogenesis (9). However, their expression and function in lymphoid cells and their precursors remains unclear. Thus, it is possible that SU6668 treatment could affect lymphocyte response to B7.2-IgG/TC immunostimulation. To investigate this, we analyzed whether SU6668 treatment *in vivo* affects spleen cells response to B7.2-IgG stimulation *in vitro*. BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. Daily treatments with SU6668 (75 mg/kg) or with its solvent DMSO (0.05 ml) were initiated 4 days later. On days 7, 14, and 24 SU6668-treated and nontreated mice were inoculated with B7.2-IgG/TC. The tumor might induce immunosuppression that is usually higher with a larger tumor. To control for these potentially confounding factors, we compared spleen cell responses in mice bearing a similar size of tumors. Spleens of control mice were harvested when tumors reach about 1.2-1.5 cm in diameter. Spleens from the treated groups of mice were removed 6 days later when their tumors had increased to comparable size. Spleens in these mice were removed 2 days after the third immunization and after 22 days of SU6668 treatments. Spleen cell suspensions were prepared, and  $4 \times 10^5$  spleen cells were plated into 96-well plate precoated with B7.2-IgG (40 mg/ml). In some wells irradiated (15,000r) 4T1 tumor cells were added ( $1 \times 10^4$ /well). [<sup>3</sup>H]Thymidine incorporation and the percentage of increase in proliferation above nonstimulated background were determined after 4 days of culture.

The results presented in Fig. 4 represent one of three experiments yielding comparable results. Spleen cells from control mice showed a relatively low response to B7.2 alone or B7.2+tumor cells. Spleen

Table 2 Abrogation of the antimetastatic effects of B7.2-IgG/TC immunization by depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes

BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 4 mice received i.p. injections of anti-CD4 or anti-CD8 mAb. Injections of these mAbs were repeated twice a week. Control group of mice received 200  $\mu$ g of rat IgG. On day 7, when tumors reach ~0.5 cm in diameter, normal or CD4<sup>-</sup>, CD8-depleted mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 21. When some mice start to die the surviving mice were killed, their lungs were harvested, and metastases were counted under dissecting microscope.

Mice	Treatment	No. of mice	Median (range) of metastases
CD4 <sup>+</sup> /CD8 <sup>+</sup>	None	8	23 (11-63)
CD4 <sup>+</sup> /CD8 <sup>+</sup>	B7.2-IgG/TC	7	4 (0-37) <sup>a</sup>
CD4 <sup>-</sup> /CD8 <sup>+</sup>	B7.2-IgG/TC	7	45 (18-84) <sup>b</sup>
CD4 <sup>+</sup> /CD8 <sup>-</sup>	B7.2-IgG/TC	4 <sup>c</sup>	12 (2-24) <sup>b</sup>

<sup>a</sup> Significantly ( $P < 0.05$ ) differs from all other groups according to Mann-Whitney test.

<sup>b</sup> Differences between these groups and control group are not significant ( $P > 0.05$ ).

<sup>c</sup> Group contained 7 mice but 3 mice died before lungs were harvested.

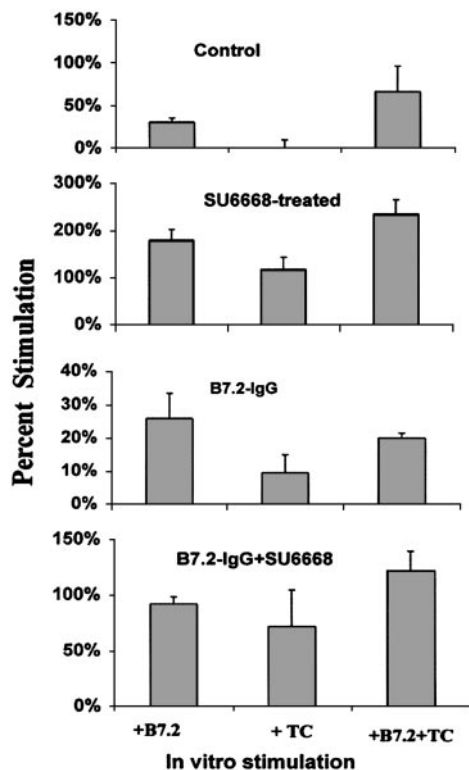


Fig. 4. Effect of SU6668 treatment on the proliferative response of spleen lymphocytes to B7.2 stimulation. BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. Starting on day 4 some of mice received daily injections of SU6668 (75 mg/kg). On days 7, 14, and 26 SU6668-treated and untreated mice were immunized with B7.2-IgG/TC. Spleens of control mice were harvested when tumor size was ~1.3 cm in diameter. Six days later, when tumors in the treated groups reached a similar size, spleens in these mice were harvested, and spleen cell suspensions were prepared. Spleens in these mice were harvested 2 days after third immunization with B7.2-IgG/TC. Spleen cells ( $4 \times 10^5$ /well) we plated into 96-well plate precoated with B7.2-IgG (40  $\mu$ g/ml). In some wells, irradiated (15,000r) 4T1 tumor cells were added ( $1 \times 10^4$  cells/well). Spleen cells were cultured 4 days and [ $^3$ H]thymidine incorporation was determined. Percentage of increase in cell proliferation was calculated as (cpm of stimulated spleen cells): (cpm of control nonstimulated spleen cells)  $\times$  100; bars,  $\pm$  SE.

cells recovered 2 days after the last *in vivo* stimulation with B7.2-IgG/TC had a higher level of *in vitro* background proliferation than spleen cells from other groups. Therefore, the additional *in vitro* stimulation with B7.2-IgG or B7.2-IgG+tumor cells resulted in a relatively small increase above this background. Significantly, however, cells from mice treated *in vivo* with SU6668 were not inhibited in their *in vitro* response to B7.2-IgG. In fact, spleen cells from SU6668-treated mice manifested the highest proliferative responses after *in vitro* B7.2-IgG or B7.2-IgG+TC stimulation. Spleen cells of mice after combined *in vivo* treatment with SU6668 and B7.2-IgG/TC also showed higher proliferative response to *in vitro* treatment with B7.2-IgG than spleen cells of control mice or mice immunized with B7.2-IgG/TC (Fig. 4). In summary, these data indicate that *in vivo* treatment of mice with SU6668 for 22 days did not inhibit spleen cell responses to B7.2-IgG and B7.2-IgG+tumor cell stimulation.

**IFN- $\gamma$  Production by Spleen Cells of Mice Treated with SU6668 and/or B7.2-IgG.** To further assess possible effects of SU6668 on the immunoreactivity, the ability of spleen cells to respond to *in vitro* restimulation and IFN- $\gamma$  production was investigated. Treatment of BALB/c mice with SU6668 was started on day 3 after s.c. inoculation of 4T1 tumor cells. Immunizations with B7.2-IgG/TC were performed on days 7, 14, and 21. After 5 weeks of tumor growth, spleens were harvested and spleen cells were resensitized *in vitro* by irradiated 4T1/IA<sup>d</sup>/B7.1 tumor cells. Two days after *in vitro* culture, the supernatants were collected, and the concentration of IFN- $\gamma$  was deter-

mined. Spleen cells of mice treated with SU6668 produced more IFN- $\gamma$  than spleen cells from control mice (Fig. 5). Spleen cells from mice treated with SU6668 or B7.2-IgG/TC produced similar amount of IFN- $\gamma$ . The highest production of IFN- $\gamma$  was found in spleen cells from mice treated with combination of SU6668 and B7.2-IgG/TC (Fig. 5). Thus, these results indicate that SU6668 treatment does not inhibit immunoreactivity of spleen cells. In fact, SU6668 complemented the immune response of spleen cells immunized with B7.2-IgG.

**Effect of SU6668 on Blood Vessel Formation and Intratumor Lymphocyte Infiltration.** BALB/c mice were inoculated s.c. with 4T1 tumor cells ( $1 \times 10^5$ ), and treatment with SU6668 (75 mg/kg) was started 4 days later. On day 7 mice were divided into four groups, and groups of SU6668-treated and untreated mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 21. To perform the histological analysis of tumor vascularization and lymphocyte infiltration, tumors of similar size (not more than 1.5 cm in diameter) were harvested. Because of the differences in tumor growth, tumors from control mice were harvested on days 19–26, and tumors from the treated mice were obtained on days 28 and 29. Four tumors per group were processed, and the histological sections stained with anti-vWF antibody. Other sections were stained with anti-CD4 or anti-CD8 mAb. The results presented in Fig. 6 showed that tumors from control and B7.2-IgG-treated mice are highly vascularized. SU6668 treatment substantially inhibited neovascularization both in the B7.2-IgG/TC-immunized and nonimmunized mice. Microscopic analysis of T-cell infiltration into tumors from control mice revealed that not more than 1–2 CD8<sup>+</sup> T lymphocytes could usually be found per high power field. In tumors from the SU6668-treated mice a similar level of CD8<sup>+</sup> lymphocytes was observed (Fig. 6). Immunizations with B7.2-IgG/TC substantially increased tumor infiltration by CD8<sup>+</sup> T cells, and some fields contained up to six CD8<sup>+</sup> lymphocytes. One would expect that reduction in tumor vascularization could result in a reduction of tumor infiltration by T lymphocytes. However, the highest infiltration of CD8<sup>+</sup> cells was found in mice treated with SU6668 and immunized with B7.2-IgG/TC. Many fields showed an infiltration by 10–25 CD8<sup>+</sup> T lymphocytes (Fig. 6).

A similar pattern of tumor infiltration by CD4<sup>+</sup> T cells was found. However, tumor infiltration by CD4<sup>+</sup> T lymphocytes was less prominent than by CD8<sup>+</sup> cells (data not shown). Thus, inhibition of

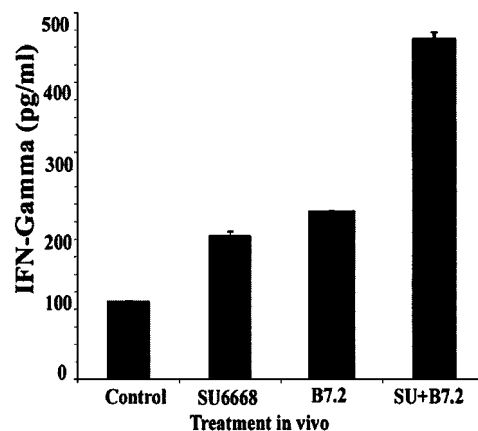


Fig. 5. IFN- $\gamma$  production by spleen cells of mice treated with SU6668 and/or B7.2-IgG/TC. BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 cells. Daily treatments with SU6668 (75 mg/kg) were initiated on day 3 following 4T1 tumor cell inoculation. On days 7, 14, and 21 mice were inoculated with B7.2-IgG (100  $\mu$ g) mixed with  $4 \times 10^6$  irradiated 4T1 tumor cells. After 35 days of tumor growth spleens were harvested. Spleen cells ( $4 \times 10^6$ ) and  $1 \times 10^5$  irradiated 4T1/IA<sup>d</sup>/B7.1 tumor cells were plated into 24-well plates. After 48 h of stimulation, the supernatants were collected, and IFN- $\gamma$  concentration in these supernatants was determined using IFN- $\gamma$  ELISA kit.

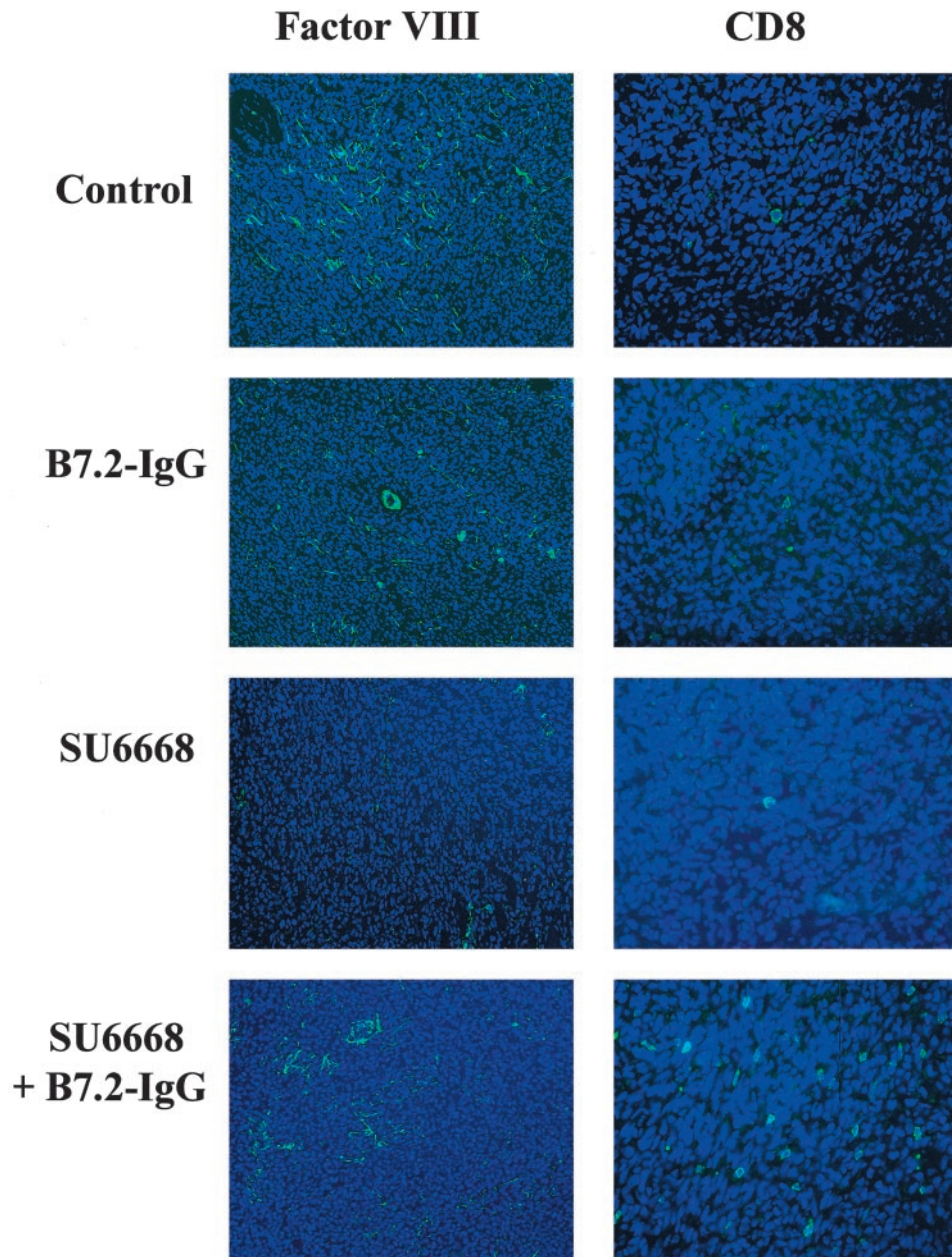


Fig. 6. Histological analysis of tumor vascularization and CD8<sup>+</sup> T-lymphocyte infiltration into tumors treated with SU6668 and/or B7.2-IgG/TC. BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. Starting on day 4, some of the mice received daily injections of SU6668 (75 mg/kg). On days 7, 14, and 21 SU6668-treated and untreated mice were immunized with B7.2-IgG/TC. To analyze tumors of similar size (1.2–1.5 cm in diameter), tumors were removed on days 16–26 from control mice and on days 28 and 29 from the treated mice. Tumors were fixed in 2% paraformaldehyde, infused with 30% sucrose overnight, and frozen. Frozen sections were stained with anti-vWF rabbit polyclonal antibody and antirabbit IgG-Alexa 488 (left panel,  $\times 400$ ). Tumor sections were also stained with anti-CD8-biotin mAb and streptavidin-Alexa 488 (right panel,  $\times 600$ ).

tumor-induced vascularization did not inhibit tumor infiltration by T cells. Rather, SU6668 treatment in combination with the antitumor immunizations resulted in augmentation of tumor infiltration by T cells.

**The Combined Antitumor Effects of Vascular Inhibitor SU6668 and B7.2-IgG/TC Immunizations.** BALB/c mice were inoculated with  $1 \times 10^5$  4T1 tumor cells, and daily treatments with SU6668 (75 mg/kg) started on day 3 after tumor cell inoculation. On days 12, 19, and 26 SU6668-treated and untreated mice were immunized with B7.2-IgG/TC. As shown in Fig. 7A, treatment with SU6668 significantly ( $P < 0.01$ ) inhibited tumor growth. Although B7.2-IgG/TC therapy started on day 12 when mice had tumors  $\sim 0.8$  cm in diameter ( $137 \pm 24$  mm<sup>3</sup>), immunization therapy stimulated an antitumor immune response that significantly ( $P < 0.05$ ) inhibited tumor growth. In mice treated with SU6668 and immunized with B7.2-IgG/TC the inhibition of tumor growth was more profound than in mice treated separately with each modality (Fig. 7A). This increased antitumor effect of combined therapy might be because initial treat-

ments with SU6668 inhibited tumor vascularization and tumor growth, and subsequent therapy with B7.2-IgG/TC was more efficient against the reduced tumor load. Indeed, after 9 days of SU6668 treatment tumor growth was inhibited, and at the time of immunization (day 12) tumor size in SU6668-treated and nontreated mice was  $92 \pm 15$  mm<sup>3</sup> and  $137 \pm 24$  mm<sup>3</sup>, respectively. This might suggest that combined SU6668 plus B7.2-IgG/TC treatment showed higher antitumor effect because immunization was applied against smaller tumors than in mice nontreated with SU6668. To exclude this possibility, we tested a different protocol so that B7.2-IgG/TC therapy was applied to mice bearing similar size tumors.

BALB/c mice were inoculated with  $1 \times 10^5$  4T1 breast tumor cells. Immunization with B7.2-IgG/TC was applied first at day 7. At this time point tumor size was  $81 \pm 14$  mm<sup>3</sup>. Daily treatment with SU6668 (75 mg/kg) started 3 days after first immunization (day 10). Immunizations were repeated on days 14 and 21 (Fig. 7B). Separate treatments with SU6668 and B7.2-IgG significantly ( $P < 0.01$ ) inhibited tumor growth. However, combined treatment with SU6668

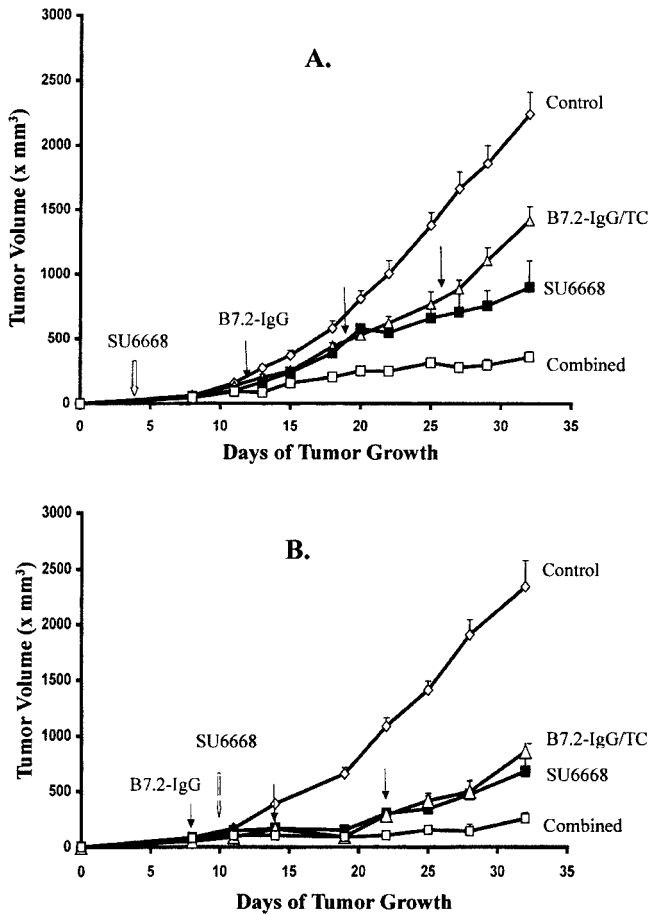


Fig. 7. The antitumor effects of combined SU6668 and B7.2-IgG/TC therapy. A, BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. Starting on day 4, some of the mice received daily injections of SU6668 (75 mg/kg). On days 12, 19, and 26 SU6668-treated and untreated mice were immunized with B7.2-IgG/TC (see arrows). Control group of mice ( $\diamond$ ) received daily injections of the solvent (DMSO). Second group was treated with SU6668 (75 mg/kg) solved in DMSO ( $\blacksquare$ ). Third group was immunized with B7.2-IgG/TC ( $\blacktriangle$ ). Fourth group received both SU6668 and B7.2-IgG/TC ( $\square$ ). Starting day 20 all applied therapies showed significant ( $P < 0.05$ ) inhibition of tumor growth. From day 18 the differences between combined therapy and monotherapies are significant ( $P < 0.05$ ). From day 29 the differences between SU6668 and B7.2-IgG/TC monotherapies are significant ( $P < 0.05$ ). B, BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 7 some of the mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 21 (see arrows). Treatment with SU6668 (75 mg/kg) was started on day 10 (see large arrow) and was repeated daily. From day 19 the differences between control and treated groups are significant ( $P < 0.05$ ). From day 22 the differences between combined therapy and monotherapies are significant ( $P < 0.05$ ); bars, SE.

and B7.2-IgG/TC had significantly ( $P < 0.05$ ) higher therapeutic effect than either therapy alone (Fig. 7B). These results indicate that SU6668 treatments complement the antitumor effect of B7.2-IgG immunizations. The combined antitumor effects were similar whether therapy was started with SU6668 or with B7.2-IgG (Fig. 7, A and B).

This complementation is based on the possibility that the immunization with B7.2-IgG/TC induces immune-mediated tumor cell destruction that is additionally augmented by treatment with SU6668, leading to inhibition of tumor vascularization resulting in tumor cell starvation and death.

**Effect of SU6668 and B7.2-IgG Therapy on Metastasis Formation.** The 4T1 breast tumor is highly metastatic, and metastatic cells can be found in various organs and tissues at the time when 4T1 tumors were as small as  $\sim 4$  mm in diameter (19, 20). Therefore, it was of interest to test the effect of SU6668 and B7.2-IgG therapy not only on local tumor growth but also on the formation of distant metastases. To test this, we analyzed metastatic growth in mice treated with SU6668 and B7.2-IgG/TC. When control mice in the experiment described above began dying (see Fig. 7B), all of the remaining mice were sacrificed and their organs harvested. Visible metastases were found only in the lungs. All of the lungs of the control mice contained numerous metastatic nodules (median number of metastases was 43; Table 3). SU6668 treatments did not significantly reduce the number of metastases (35 per lungs). In contrast, B7.2-IgG/TC immunizations significantly reduced metastasis formation (3 metastases per lung) with 25% of mice showing no visible metastases. The most profound reduction in metastasis formation was found in mice treated in a combination of SU6668 and B7.2-IgG/TC. In 50% of mice (4 of 8) lungs had no visible metastases, and lungs of 3 other mice had only 1 metastatic nodule (Table 3). These results indicate that therapy with B7.2-IgG/TC in combination with SU6668 had more profound inhibitory effects on 4T1 breast tumor metastasis formation than each therapy applied separately.

Surprisingly, repeat experiments showed that SU6668 as a single modality substantially inhibited local tumor growth but not development of spontaneous lung metastases in the same mice. One would expect that SU6668 therapy should inhibit metastatic tumor vascularization and growth of the pulmonary metastases. So, at least, this should be reflected in reduction of their size. However, measurement of the individual metastatic foci showed no reduction in their diameter compared with the control group (Table 3). Indeed, the mean diameter of lung metastases in the control and SU6668-treated mice was  $1.71 \pm 0.07$  and  $1.66 \pm 0.05$  mm, respectively. Thus, the vast majority of metastatic foci was below 2 mm in diameter. It is believed that tumors can grow up to 2–3 mm in diameter without development of their own tumor vascular system (24). Thus, this could explain why SU6668 treatment did not affect metastatic growth. However, some metastatic nodules in the lungs of the control and SU6668-treated mice were larger than 3 mm in diameter. To determine whether SU6668 affected development of large metastases, we calculated the proportion of large ( $>3$  mm) metastases in these groups. In the control mice 33 metastases among 288 (11.4%) were  $>3$  mm in diameter. In the SU6668-treated mice only 18 among 386 metastases (4.7%) were  $>3$  mm (Table 3). Immunizations with B7.2-IgG/TC significantly ( $P < 0.05$ ) reduced not only the number but also the diameter of

Table 3 The antimetastatic effect of combined SU6668 and B7.2-IgG/TC therapy

BALB/c mice were inoculated s.c. with  $1 \times 10^5$  4T1 breast tumor cells. On day 7 some of mice were immunized with B7.2-IgG/TC. Immunizations were repeated on days 14 and 21. Treatment with SU6668 (75 mg/kg) started on day 10 and was repeated daily. When tumors in control mice reached 2 cm in diameter (see Fig. 7B) mice were sacrificed, and lung metastases were counted and diameter of each metastatic nodule was measured under the dissecting microscope. The diameter of metastasis in each group is presented as mean  $\pm$  SE. Each group contained 8–9 mice.

Treatment	Median no. of metastases (range)	% metastasis-free mice	Mean diameter of metastasis (mm)	% large ( $>3$ mm) metastases
Control	43 (4–107)	0	$1.71 \pm 0.07$	11.4%
SU6668	35 (5–87)	0	$1.66 \pm 0.05$	4.7%
B7.2-IgG/TC	3 (0–44) <sup>a</sup>	25	$0.89 \pm 0.08^b$	2.6%
Combined	0 (0–7) <sup>a</sup>	50	$1.12 \pm 0.13^b$	0%

<sup>a</sup> Significantly ( $P < 0.05$ ) differs from all other groups according to Mann-Whitney's test.

<sup>b</sup> Significantly ( $P < 0.01$ ) differs from the control and SU6668-treated group according to Student's test.

metastatic foci ( $0.089 \pm 0.08$  mm). In these mice only 2.6% metastases had diameter  $>3$  mm. Combined treatment with SU6668 and immunization with B7.2-IgG/TC caused additional reduction in the number of metastases. Although their diameter was significantly ( $P < 0.05$ ) smaller than in control mice (mean diameter was  $1.12 \pm 0.13$  mm) their size was similar to those found in mice treated with B7.2-IgG/TC alone (Table 3). In these mice in total only 10 metastatic foci were found and none of them were  $>3$  mm.

Thus, antiangiogenic therapy with SU6668 in combination with immunostimulation with B7.2-IgG/TC had more potent antitumor and antimetastatic effects than each modality used alone.

## DISCUSSION

Our data show that treatment of mice bearing established 4T1 breast tumors with soluble B7.2-IgG fusion protein results in a significant inhibition of tumor growth and metastasis formation. T-cell depletion experiments indicate that the therapeutic effects of B7.2-IgG/TC immunizations require both CD4 and CD8 cells. In previous experiments with the highly immunogenic MethA sarcoma the antitumor effect of B7.2-IgG treatments was found to be exclusively mediated by CD8<sup>+</sup> but not CD4<sup>+</sup> cells (15).

We have found that treatment with SU6668, a small molecule inhibitor of the angiogenic receptor tyrosine kinases VEGFR2, PDGFR $\beta$ , and FGFR1, significantly inhibits vascularization and growth of 4T1 breast tumors. Our data indicate that SU6668 did not inhibit the ability of spleen cells to respond to B7.2-IgG stimulation. Rather, spleen cells from SU6668-treated mice showed a higher proliferative response to B7.2 stimulation than spleen cells from control mice. In addition, SU6668 treatment did not affect generation of dendritic cells from bone marrow and the ability of spleen cells to respond to IL-2 and generate lymphokine activated killer cells and CTLs (data not shown). If SU6668 was immunosuppressive it would be expected that the antitumor effects of B7.2-IgG in SU6668-treated mice would be lower than in nontreated mice. In contrast, mice treated with SU6668 followed by treatment with B7.2-IgG/TC manifested a higher level of tumor inhibition than mice treated with B7.2-IgG/TC alone. The fact that the antitumor effects of B7.2-IgG/TC in combination with SU6668 were also higher than in mice treated with SU6668 alone strongly suggests that these two therapies are compatible and manifest a complementary therapeutic effect.

The increased antitumor activity of combined therapy with SU6668 and B7.2-IgG/TC could be based on two independent mechanisms directed against: (a) a tumor vascular system that might lead to indirect tumor cell death via nutrient deprivation; and (b) tumor cells, resulting in direct tumor cell destruction by immune mechanisms. In addition, these two therapies could interact and complement each other. The reduction of tumor mass as a result of an inhibition of tumor vascularization by SU6668 might increase the efficacy of the antitumor immune response stimulated by B7.2-IgG. The efficacy of immunotherapy appears directly correlated with tumor size, with immunological eradication of tumor cells being most efficient when tumor load is relatively low. Thus, inhibition of tumor vascularization and reduction of tumor mass should enhance the success rates of combined therapy. In addition to its debulking effect, SU6668 might promote tumor cell apoptosis as a result of tumor cell starvation. These apoptotic cells can be phagocytized by macrophages or dendritic cells and, thus, additional stimulate specific T-cell responses. Conversely, immunotherapy could increase the antivascular effects of antiangiogenic agents. The immune response is associated with the production of various cytokines and chemokines, some of which (IL-12, tumor necrosis factor  $\alpha$ , IL-4, IFN- $\gamma$ ) have been shown to have antiangiogenic and antivascular effects (25, 26).

One might expect that inhibition or destruction of tumor vascularization would have negative effects on tumor infiltration by T lymphocytes and, hence, tumor cell destruction. It should be noted that SU6668 treatment inhibited but did not completely destroy tumor vasculature. The histological analysis of tumors revealed that SU6668 treatment substantially inhibited formation of new sprouting blood vessels in immunized and nonimmunized mice, but numerous blood vessels could still be found. The remaining blood vessels supply nutrients that are required for tumor cell survival and proliferation. Thus, T cells might extravasate from the same blood vessels that feed tumor cells. Indeed, histological analysis revealed the presence of CD8<sup>+</sup> T lymphocytes in tumors of mice treated with SU6668. The number of infiltrated CD8<sup>+</sup> T cells substantially increased in mice immunized with B7.2-IgG/TC. However, mice treated with SU6668 immunizations with B7.2-IgG/TC resulted in a more profound tumor infiltration by CD8<sup>+</sup> cells. The question is how might reduction in tumor vessels result in an increase in tumor infiltration by T cells? Several explanations could be offered to this paradox. First, SU6668 might induce apoptosis of endothelial cells by blocking the survival signal delivered by VEGF or fibroblast growth factor. Increase in endothelial death might increase blood vessel leakage and tumor infiltration by lymphocytes. However, in this case an increase in lymphocyte infiltration would be expected in tumors from nonimmunized mice treated with SU6668. That was not the case. Secondly, the observed increase in tumor infiltration by T cells in mice treated in combination with SU6668 and B7.2-IgG/TC might be because of an increase in their intratumor proliferation. Indeed, reduction in blood supply was shown to increase tumor cell death. Phagocytosis of dead tumor cells by macrophages or dendritic cells might increase antigen presentation and stimulate intratumor proliferation of T lymphocytes, resulting in an increase in numbers of tumor-infiltrated lymphocytes. These lymphocytes could kill tumor cells and work in concert with SU6668 in their inhibition of tumor growth.

It is of note that although SU6668 treatment was highly efficient in inhibition of primary 4T1 tumor growth it did not affect metastasis formation and growth. Indeed, the number and size of the metastatic nodules in control and SU6668-treated mice were similar. The inability of SU6668 to affect metastatic growth in this model could be explained by taking into consideration that: (a) lungs are highly vascularized; and (b) it is believed that tumors can grow up to 2–3 mm in diameter without development of their own vascular system (24). The spontaneous metastases developed in the lungs of control mice bearing 4T1 breast tumors were  $<2$  mm in diameter ( $1.71 \pm 0.07$  mm) and probably did not develop their own vascular system. Therefore, SU6668 treatment showed no significant inhibition in the number and size of pulmonary metastasis in these mice. These results do not exclude the possibility that SU6668 can inhibit metastatic growth but illustrate the limitation of models in which metastases are growing in the presence of the primary tumor for a limited time frame. Under this condition metastatic nodules are small and are unable to form the tumor-induced vascular system that could be a target for SU6668. The antimetastatic effects of SU6668 should be tested against experimental or postoperative tumor metastases that have the opportunity to grow longer, form their own vasculature, and develop into large metastatic tumors. The failure of SU6668 to affect development and growth of small metastatic nodules exemplifies the limitation of antiangiogenic therapy in that it is unable to completely eliminate tumor cells that are supported by normal tissue vasculature. These residual tumor cells can be eradicated by immune mechanisms. Indeed, in mice immunized with B7.2-IgG/TC, immune lymphocytes were able to completely eradicate or inhibit growth of lung metastases as reflected in reduction in number and size of the metastatic nodules. It is of note that although SU6668 as a single modality did not inhibit



metastatic growth it potentiated the antimetastatic effect of B7.2-IgG/TC immunization. Some evidence suggests that VEGF produced by tumors has immunosuppressive effects (27, 28). It is possible that SU6668 by blocking the VEGFR on the hematopoietic cells and their progenitors might abrogate the immunosuppressive effect of VEGF and, thus, increase the efficacy of the B7.2-IgG-induced antitumor and antimetastatic immune response.

In summary, our data indicate that therapies using the angiogenesis inhibitor SU6668 and the immunostimulator B7.2-IgG fusion protein are compatible, and induce a complementary antitumor and antimetastatic effect. Combined antiangiogenic and immune therapy might represent a new strategy for cancer treatment.

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## Combined Therapy of Local and Metastatic 4T1 Breast Tumor in Mice Using SU6668, an Inhibitor of Angiogenic Receptor Tyrosine Kinases, and the Immunostimulator B7.2-IgG Fusion Protein

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