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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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), PDGFRβ, 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. Intermolecular 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/T) resulted in a significant inhibition of tumor growth and formation of pulmonary metastases. T-cell depletion experiments revealed that both CD4+ and CD8+ T lymphocytes are required for stimulation of the antitumor and ant metastatic immune response by B7.2-IgG/T. 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/T 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/T 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/T. Analysis of pulmonary metastases revealed that combined therapy also had a more potent antitumor effect than each modality alone. These results indicate that antiangiogenic and immune therapies using SU6668 and B7.2-IgG/T are compatible, and manifest complementary antitumor and antitumor 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 (Flk-1/KDR), PDGFRβ, and FGFR1 (9). These receptors are expressed by endothelial cells and play a crucial role in tumor-induced angiogenesis (10, 11). In addition, PDGFRβ 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 of established tumor growth (9). It was also found that SU6668 therapy inhibits liver metastasis formation after intrasplenic inoculation of C26 colon tumor cells (13, 14).
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 IgG2α. 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/IAα°B7.1 subline was derived from 4T1 cells cotransfected with the MHC class II H-2Aα and B7.1 genes (19, 20). Both lines were provided by Dr. Suzanne Ostrand-Rosenberg (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/IAα°B7.1 tumor cells was performed as described previously (21). Tumor cells were incubated with anti-H-2Kα, H-2Dα, and H-2IAα mAb for 30 min at 4°C. After washing, cells were stained with goat antimouse IgG-FITC for an additional 30 min. Cells were washed and fixed in 1% 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 × 10³ 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 was dissolved in DMSO and 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 × 10³ irradiated (15,000 r) 4T1 tumor cells (hereafter referred as B7.2-IgG/TC) was given s.c. at a dose of 100 µg 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 × width²/2. All of the data represent as mean ± 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 antibodies to anti-CD4 (GK.1) or anti-CD8 (53-6.72) hybridomas (American Type Culture Collection, Manassas, VA). On day 5 after s.c. inoculation of 1 × 10³ 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 µg) and 4 × 10⁵ 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 µg 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 µg/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 × 10⁵ spleen cells were added to each well. In some wells, irradiated (15,000r) 4T1 tumor cells (2 × 10⁵) were also added. Spleen cells were cultured for 3 days and levels of proliferation was tested by [³H]thymidine incorporation (23).

IFN-γ Production by Spleen Cells of Mice Treated with SU6668 and/or B7.2-IgG. BALB/c mice were inoculated s.c. with 1 × 10³ 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 µg) mixed with 4 × 10⁵ irradiated 4T1 tumor cells. After 35 days of tumor growth, spleens were harvested. Spleen cells (4 × 10⁵) and 1 × 10³ irradiated 4T1/IAα°B7.1 tumor cells were plated into 24-well plates. After 48 h of stimulation, the supernatants were collected, and IFN-γ concentration in the supernatants was determined using IFN-γ 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 × 10³) 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-μm 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 µg/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, solutions 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 µg/ml; Molecular Probes, Eugene, OR). Sections were then stained with Hoescht dye to label nuclei. After three washes in buffer...
signals were collected on a log scale. First peak represents an isotype IgG control, the staining cells with anti-CD80-biotin and avidin-PE (BD PharMingen). Fluorescence of paraformaldehyde. The expression of B7.1 molecules by tumor cells was analyzed with goat antimouse IgG-PE for an additional 30 min. Cells were washed and fixed in 1% B7.1 and cotransfected with the breast tumor. In addition, we compared the antitumor effects induced treatment to stimulate the antitumor immunity against established 4T1 cells tumors. We first evaluated the ability of B7.2-IgG fusion protein 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 enhance 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/A$^{49}$/B7.1-irradiated tumor cells. 4T1/A$^{49}$/B7.1 tumor cells were transfected with the MHC class II H-2IA$^{d}$ gene and cotransfected with the B7.1 gene (20). Our flow cytometric analysis revealed that 4T1 and 4T1/A$^{49}$/B7 tumor cells express relatively high levels of MHC class I H-2K$^{a}$ and H-2D$^{d}$ molecules (Fig. 1). In addition, 4T1/A$^{49}$/B7 tumor cells expressed a high level of the transfected H-2IA$^{d}$ and B7.1 genes (Fig. 1). Thus, 4T1/A$^{49}$/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/A$^{49}$/B7 cells or with B7.2-IgG (100 µg) 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 4T1IA$^{49}$/B7 were able to significantly ($P < 0.05$) 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/A$^{49}$/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/A$^{49}$/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
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 tumor activity with B7.2-IgG might be a result of interactions of B7.2.

Effects of B7.2-IgG Therapy. The observed stimulation of the antitumor and antitumor metastatic effects of B7.2-IgG/TC immunizations significantly ($P < 0.05$) differs from all other groups according to Mann-Whitney test.

Table 2. Abrogation of the antitumor metastatic effects of B7.2-IgG/TC immunization by depletion of CD4$^+$ or CD8$^+$ T lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD4$^+$/CD8$^+$</th>
<th>Median (range) of metastases</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>23 (11–63)</td>
</tr>
<tr>
<td>CD8$^+$/TC</td>
<td>7</td>
<td>4 (0–37)</td>
</tr>
<tr>
<td>CD8$^+$/TC</td>
<td>4</td>
<td>12 (2–24)</td>
</tr>
</tbody>
</table>

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

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

$^c$ Group contained 7 mice but 3 mice died before lungs were harvested.

Fig. 3. Role of CD4$^+$ and CD8$^+$ T lymphocytes in the antitumor effects of B7.2-IgG/TC immunizations. BALB/c mice were inoculated s.c. with 1 × 10$^7$ 4T1 breast tumor cells. On day 5 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 μg of rat IgG. On day 7, when tumors reach −0.5 cm in diameter, normal (●), or CD4-, CD8-depleted mice were immunized with B7.2-IgG/TC. Immuizations were repeated on days 14 and 21. Each group contained 7–8 mice. *Significantly ($P < 0.05$) differs from other groups; bars, ± SE.

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

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-2 IA and B7.1 genes (4T1/IA/B7.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice per group</th>
<th>Median no. of metastasis/lung (range)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>20 (5–37)</td>
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<tr>
<td>B7.2-IgG + TC</td>
<td>8</td>
<td>5 (2–7)</td>
</tr>
<tr>
<td>4T1/IA/B7.1</td>
<td>9</td>
<td>4 (0–10)$^a$</td>
</tr>
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</table>

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

Effects 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, PDGFRβ, 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 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 × 10$^5$ spleen cells were plated into 96-well plate precoated with B7.2-IgG (40 μg/ml). In some wells irradiated (15,000r) 4T1 tumor cells were added (1 × 10$^5$/well). $[^3]$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 cell suspensions were prepared, and 4 × 10$^5$ spleen cells were plated into 96-well plate precoated with B7.2-IgG (40 μg/ml). In some wells irradiated (15,000r) 4T1 tumor cells were added (1 × 10$^5$/well). $[^3]$H[Thymidine incorporation and the percentage of increase in proliferation above nonstimulated background were determined after 4 days of culture.

Table 2. Abrogation of the antitumor metastatic effects of B7.2-IgG/TC immunization by depletion of CD4$^+$ or CD8$^+$ T lymphocytes

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

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

$^c$ Group contained 7 mice but 3 mice died before lungs were harvested.
and/or B7.2-IgG. To further assess possible effects of SU6668 on the restimulation and IFN-γ/H9253 in vitro well) we plated into 96-well plate precoated with B7.2-IgG (40/H9262 cultured 4 days and [3H]thymidine incorporation was determined. Percentage of increase in vivo spleen cells from other groups. Therefore, the additional B7.2-IgG/TC (Fig. 4). In summary, these data indicate that in vivo mice after combined treatment with SU6668 for 22 days did not inhibit spleen cell proliferation with B7.2-IgG or B7.2-IgG/T. In spleen cells from mice treated with SU6668 or B7.2-IgG/TC produced similar amount of IFN-γ. The highest production of IFN-γ 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 × 10⁵), 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⁺ T lymphocytes could usually be found per high power field. In tumors from the SU6668-treated mice a similar level of CD8⁺ lymphocytes was observed (Fig. 6). Immunizations with B7.2-IgG/TC substantially increased tumor infiltration by CD8⁺ T cells, and some fields contained up to six CD8⁺ 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⁺ cells was found in mice treated with SU6668 and immunized with B7.2-IgG/TC. Many fields showed an infiltration by 10–25 CD8⁺ T lymphocytes (Fig. 6).

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

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-γ 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-γ 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/B7.1 tumor cells. Two days after in vitro culture, the supernatants were collected, and the concentration of IFN-γ was determined. Spleen cells of mice treated with SU6668 produced more IFN-γ 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-γ. The highest production of IFN-γ 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.
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 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, ×400). Tumor sections were also stained with anti-CD8-biotin mAb and streptavidin-Alexa 488 (right panel, ×600).

Fig. 6. Histological analysis of tumor vascularization and CD8+ 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. 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, ×400). Tumor sections were also stained with anti-CD8-biotin mAb and streptavidin-Alexa 488 (right panel, ×600).
From day 22 the differences between combined therapy and monotherapies are significant. Therapy was started with SU6668 or with B7.2-IgG (Fig. 7, immunizations. The combined antitumor effects were similar whether SU6668 treatments complement the antitumor effect of B7.2-IgG. These results indicate that significant (growth. From day 18 the differences between combined therapy and monotherapies are significant (P<0.05). 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 therapy (a). Fourth group received both SU6668 and B7.2-IgG/TC (f). 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 x 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) 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 ~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 ± 0.07 and 1.66 ± 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>
<thead>
<tr>
<th>Treatment</th>
<th>Median no. of metastases (range)</th>
<th>% metastasis-free mice</th>
<th>Mean diameter of metastasis (mm)</th>
<th>% large (&gt;3 mm) metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43 (4–107)</td>
<td>0</td>
<td>1.71 ± 0.07</td>
<td>11.4%</td>
</tr>
<tr>
<td>SU6668</td>
<td>35 (5–87)</td>
<td>0</td>
<td>1.66 ± 0.05</td>
<td>4.7%</td>
</tr>
<tr>
<td>B7.2-IgG/TC</td>
<td>3 (0–44)</td>
<td>25</td>
<td>0.89 ± 0.08</td>
<td>2.6%</td>
</tr>
<tr>
<td>Combined</td>
<td>0 (0–7)</td>
<td>50</td>
<td>1.12 ± 0.13</td>
<td>0%</td>
</tr>
</tbody>
</table>

a Significantly (P<0.05) differs from all other groups according to Mann-Whitney’s test.
b Significantly (P<0.01) differs from the control and SU6668-treated group according to Student’s test.
metastatic foci (0.089 ± 0.08 mm). In these mice only 2.6% metas-
tases 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 ± 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 sig-
nificant 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 anti-
tumor effect of B7.2-IgG treatments was found to be exclusively
mediated by CD8+ but not CD4+ cells (15).

We have found that treatment with SU6668, a small molecule
inhibitor of the angiogenic receptor tyrosine kinases VEGFR2,
PDGFRβ, 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 immnosuppressive 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 combi-
nation with SU6668 were also higher than in mice treated with
SU6668 alone strongly suggests that these two therapies are compat-
ible 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
the tumor load is relatively low. Thus, inhibition of tumor vasculari-
zation 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 den-
dritic 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 α, IL-4, IFN-) have been shown to have
antiangiogenic and antivascular effects (25, 26).

One might expect that inhibition or destruction of tumor vascular-
ization would have negative effects on tumor infiltration by T lymph-
ocytes 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

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metastatic growth it potentiated the antitumor formation of VEGF on pancreatic tumors has implied that VEGF is involved in the regulation of angiogenesis and the growth of tumors. It is possible that VEGF produced by tumors plays a role in the regulation of angiogenesis and the growth of tumors. The results indicate that the antitumor effect of VEGF is mediated by the activation of the VEGF receptor. 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 antiangiogenic immune response.

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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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