Abstract

Purpose: Transforming growth factor β (TGFβ) is a pleiotropic cytokine that affects tumor growth, metastasis, stroma, and immune response. We investigated the therapeutic efficacy of anti–TGFβ receptor II (TGFβ RII) antibody in controlling metastasis and tumor growth as well as enhancing antitumor immunity in preclinical tumor models.

Experimental Design: We generated neutralizing antibodies to TGFβ RII and assessed the antibody effects on cancer, stroma, and immune cells in vitro. The efficacy and mechanism of action of the antibody as monotherapy and in combination with chemotherapy in suppression of primary tumor growth and metastasis were evaluated in several tumor models.

Results: Anti–TGFβ RII antibody blocked TGFβ RII binding to TGFβ 1, 2, and 3, and attenuated the TGFβ-mediated activation of downstream Smad2 kinase, invasion of cancer cells, motility of endothelial and fibroblast cells, and induction of immunosuppressive cells. Treatment with the antibody significantly suppressed primary tumor growth and metastasis and enhanced natural killer and CTL activity in tumor-bearing mice. Immunohistochemistry analysis showed cancer cell apoptosis and massive necrosis, and increased tumor-infiltrating T effector cells and decreased tumor-infiltrating Gr-1+ myeloid cells in the antibody-treated tumors. Fluorescence-activated cell sorting analysis indicated the significant reduction of peripheral Gr-1+/CD11b+ myeloid cells in treated animals. Concomitant treatment with the cytotoxic agent cyclophosphamide resulted in a significantly increased antitumor efficacy against primary tumor growth and metastasis.

Conclusions: These preclinical data provide a foundation to support using anti–TGFβ RII antibody as a therapeutic agent for TGFβ–RII–dependent cancer with metastatic capacity. Clin Cancer Res; 16(4); 1191–205. ©2010 AACR.

Transforming growth factor β (TGFβ) is a pleiotropic cytokine that plays a pivotal role in regulating the pathogenesis of a wide variety of disorders including cancer, fibrosis, atherosclerosis and arteriosclerosis, systemic sclerosis, and inflammation (1–5). TGFβ stimulates the activation of TGFβ receptor (TGFβ R) kinase and downstream signaling cascades that initiate broad cellular and noncellular processes including survival, proliferation and differentiation, migration and motility, and deposition of extracellular matrix and induction of cytokines contributing to tumorigenesis, metastasis, angiogenesis, and inflammation. Genetic knockout of the receptor I or II and inhibition of TGFβ signaling by antagonists of TGFβ and TGFβ RII kinase have been shown to alleviate metastasis and tumor progression (6–8). However, the precise mechanism by which TGFβ exerts its complex activity in cancer pathogenesis is still not fully understood.

TGFβ has been shown to promote metastasis and tumor progression through reducing the adhesiveness and increasing the motility and proteolytic activity of cancer cells, facilitating the process of epithelial-to-mesenchymal transition (EMT), inducing angiogenesis, and recruiting Gr-1+/CD11b+ myeloid cells to tumor tissues (9–11). Gr-1+/CD11b+ myeloid cells were reported to suppress...
Translational Relevance

Therapeutic agents capable of controlling cancer growth and metastasis, and elevating weakened antitumor immunity continue to be needed. Transforming growth factor β (TGFβ) pathway promotes metastasis and tumor progression through the modulation of cancer cell invasiveness, angiogenesis, stromal activity, and immunosuppression. We show that anti–TGFβ RII antibody has significant efficacy against metastasis and tumor growth through the attenuation of TGFβ RII–mediated activity in cancer, stroma, and immune cells. Preclinical data showed that the antibody treatment resulted in significant tumor necrosis, reduction of immunosuppressive myeloid and regulatory T cells, and elevation of natural killer and CTL killing response. Co-treatment with anti–TGFβ receptor II (TGFβ RII) antibody and cytotoxic agent cyclophosphamide led to a superior antitumor efficacy against primary tumor growth and metastasis. The study has provided compelling data supporting the use of a neutralizing anti–TGFβ RII antibody as a novel therapeutic strategy for treatment of TGFβ RII–dependent tumors with metastatic potential.

immunity against tumor, promote cancer cell invasion, and induce angiogenesis and anti-vascular endothelial growth factor–resistant tumors (12–15). TGFβ also exerts immunosuppressive effects by the induction of CD4+ CD25+ regulatory T (Treg) cells and inhibition of CTL activity (16, 17). Furthermore, TGFβ plays a complex role in modulating tumor microenvironment consisting of endothelial cells, fibroblasts, immune cells, and the extracellular matrix that promote pathogenesis of cancer and metastasis (6, 18). Blockade of TGFβ signaling and TGFβ R I kinase activation by recombinant TGFβ R extracellular domain, TGFβ antisense RNAi, small-molecule receptor I kinase inhibitors, and anti–TGFβ antibody has been reported to be efficacious in the suppression of tumor growth and metastasis in animal models (19–22). These approaches were designed to attenuate TGFβ signaling–mediated activity but did not have a direct effect on TGFβ R–expressing cells to impede the receptor-mediated detrimental activity in cancer pathogenesis and immunosuppression. The binding of TGFβ ligands to TGFβ RII is a crucial step to initiate the activation of TGFβ signaling pathways (23). To date, how the blockade of TGFβ RII by a specific antagonist, i.e., a neutralizing antibody, affects tumor progression still remains elusive.

Here, we show that direct targeting of TGFβ RII by a specific neutralizing antibody has significant efficacy against primary tumor growth and metastasis through the antibody multieffects on the receptor-mediated deleterious activity in cancer, stroma, and immune cells. The high-affinity neutralizing antibodies to TGFβ RII have a strong blocking activity to TGFβ RII binding to TGFβ 1, 2, and 3, and TGFβ-mediated activation of downstream Smad2 kinase in cancer cells. We show that the antibody-mediated antitumor effect is in part attributed to the abrogation of TGFβ RII+/Gr-1+/CD11b+ myeloid and Treg cells in addition to the inhibition of autocrine stimulation of cancer cells, and enhancement of natural killer (NK) and CTL activity. These results have provided compelling data to support the use of anti–TGFβ RII antibody as a therapeutic agent for TGFβ RII–dependent cancer with metastasis capacity.

Materials and Methods

Reagents. All reagents and chemicals were purchased from Sigma unless otherwise noted. Human TGFβ 1, 2, and 3 were purchased from R&D Systems. Recombinant TGFβ RII Fc (TGFβ RII Fc) proteins and soluble recombinant TGFβ RII alkaline phosphatase (TGFβ RII AP) proteins were produced and purified from transfected cell culture.

Cell lines. Cell cultureware and assay plates were purchased from (BD Biosciences). Human cancer cell lines BXPC-3 and MDA-MB-231 and mouse tumor cell lines EMT6, 4T1, and CT26 were obtained from the American Type Culture Collection. MDA-MB-231 LM2-4 luciferase transfectant cells was provided by Dr. R. Kerbel (University of Toronto, Sunnybrook Health Sciences Center, Toronto, Ontario, Canada). Cells were cultured in medium with 5% to 10% FCS at 37°C in a humidified, 5% CO2 atmosphere before used for in vitro and in vivo studies.

TGFβ RII antibody generation and blocking assays. The antibodies were generated using human IgG transgenic mice (Medarex) and Lewis rats (Charles River Laboratories) through standard hybridoma technology. For blocking assay, TGFβ were coated on microplates. A serial dilution of purified antibodies was incubated with TGFβ RII AP for 1 h in TGFβ-coated plates. After wash, p-nitrophenyl phosphate substrate was added to the wells for color development. The values of absorbance at 405 nm were read on a microtiter plate reader (Molecular Devices Corp) for the quantification of TGFβ RII binding to TGFβ. IC50 of the antibodies were analyzed using the software GraphPad Prism.

Measurement of affinity of the anti–TGFβ RII antibodies. Affinities of purified anti–TGFβ RII antibodies were determined by Plasmon Resonance Technology using the Biacore instrument according to the procedures provided by the manufacturer. Kinetic analyses of the antibodies were performed by immobilizing the recombinant extracellular domain of the TGFβ RII onto the sensor surface at a low density. The association (kon) and dissociation (koff) rates were determined using the BiAevaluation 2.1 software.

Flow cytometry analysis. Aliquots of transfected cells, carcinoma cells, spleen cells, or lymph node cells were incubated with fluorescence-labeled or unlabeled primary antibodies. In some case, cells were incubated with anti–FcγII/III receptor antibody before incubation with
primary antibodies. A matched IgG isotype (Jackson ImmunoResearch) was used as a negative control. After washes, stained cells were analyzed on Guava PCA System (Guava Technologies) or fluorescence-activated cell sorting (FACS) Aria flow cytometer (BD Biosciences).

**Western blot analysis.** Semi-confluence monolayer cells were cultured overnight in serum-free medium, then treated with antibodies or isotype control IgG in the presence of 10 ng/mL TGFβ for 1 h. After washes, cell lysates were prepared and subjected to electrophoresis and Electro-Transfer to nitrocellulose membrane. Blots of phosphorylated Smad2 and Smad2 were detected using anti–phospho-Smad2 and Smad2 antibodies (Millipore Co.) and chemiluminescence system, and imaged and quantified by densitometry using the Fuji Image Analyzer.

**Cell migration and invasion assay.** In the migration assay, cells were loaded into upper chambers inserted in Collagen-coated lower chamber of Transwell plates, then incubated in serum-free medium in the presence or absence of 10 ng/mL TGFβ, a serial dilution of antibodies or isotype control IgG for 24 to 48 h. Matrigel-coated upper chambers were used for invasion assay. In myeloid cell–induced tumor cell migration assay, tumor cells and Gr-1+/CD11b+ cells purified from the spleens of tumor-bearing mice were incubated in the top chamber for 24 h. Migrated cells in the opposite side of upper chambers were fixed and stained with Hoechst solution. Stained cells were imaged and counted using a Digital Camera and software Image-Pro Plus 5.1.

**Internalization assay.** TGFβ RII+ tumor cells were incubated at 4°C or 37°C with fluorescence-labeled anti–TGFβ RII antibody in serum-free medium for 30 to 120 min. After washes, plates were placed on ice to terminate internalization. Cells were resuspended in mounting medium and applied on a microscope slide with coverslips. Image of treated cells were taken using a NIKON confocal microscope and a digital camera, and processed using an image software.

**Cytotoxic assays.** NK and CD8 T cells were isolated from spleens of treated mice using DX5 or CD8a microbeads (Miltenyi Biotech), respectively. NK and CTL activity were assessed as previously described (24). Purified NK cells were incubated with DDAO-SE–labeled target YAC-1 cells at different ratios for 4 h. CD8 T cells were tested for killing activity to DDAO-SE–labeled target EMT6 cells with the exception that the CD8 T cells were first incubated with mitomycin C–treated EMT6 cells for 5 to 7 d. After wash, cells were fixed with paraformaldehyde and permeabilized with Perm solution (BD Biosciences), and incubated with anti–cleaved caspase-3-PE and analyzed by flow cytometry. Cleaved caspase-3 expression was determined using the DDAO-SE–gated target cells in a DDAO-SE versus Caspase-3-PE dot plot.

**ELISpot assay.** NK and CD8a+ T cells were purified as above from spleens of treated tumor-bearing mice at day 9 and 14, respectively, after tumor inoculation. Purified DX5+ and CD8a+ cells were incubated with mitomycin-C–treated EMT6 cells at a ratio of 20:1 for 72 and 48 h, respectively. Spots were developed, counted, and plotted as the number of spot-forming units per 10^5 cells per instruction (BD Biosciences).

**Proliferation assay.** Naive CD4 T cells, CD4/CD25+ Treg cells, Gr-1+/CD11b+ myeloid cells, and MHCII–APCs were isolated from spleen using cell type specific microbeads (Miltenyi Biotech). CD4 T cells were incubated in a medium with Treg cells or mitomycin C–treated myeloid cells in the presence or absence of purified MHCII + APC and 0.5 μg/mL anti–CD3e antibody (Clone 145-2C11, BD Biosciences), and a serial dilution of anti–TGFβ RII antibody or normal rat IgG for 7 d. Proliferation was assessed using the Cell Titer Glo assay (Promega).

**Treg cell induction assay.** Purified naive CD4 T cells were incubated with APCs and anti–CD3 antibody in a medium in the presence or absence of 10 ng/mL TGFβ and antibody for 7 d. Cells were then harvested for the staining of CD4+/CD25+/Foxp3+ Treg cells using fluorescence-labeled specific antibody. Stained cells were analyzed on a FACS Aria flow cytometer.

**In vivo tumor studies.** Athymic nude mice, balb/c mice (Charles River Laboratories) were used for inoculation with mouse or human carcinoma cells. For treatment of established tumor in subcutaneous models, tumors were allowed to grow to approximately 300 to 500 mm³ in size, and then mice were randomized into groups with 12 to 15 animals per group. For treatment of metastatic tumor growth in lung, mice were injected i.v. with tumor cells through the tail vein 24 h before antibody administration. Animals received i.p. administration of the anti–TGFβ RII antibody (10–40 mg/kg ×3 each week) or/and Cyclophosphamide (80 mg/kg ×1 each week). Mice in control groups received an equal volume of saline or normal IgG solution. Tumors were measured twice each week with digital calipers. Tumor volumes were calculated using the formula \(V = \frac{\pi}{6} (w_1 \times w_2 \times w_3)\), in which \(w_1\) represents the largest tumor diameter and \(w_2\) represents the smallest tumor diameter. All animal studies were conducted in accordance with the guidelines of the NIH “Guide for Care and Use of Animals” and an approved protocol reviewed by the Institutional Animal Care and Use Committee.

**Immunohistochemistry.** Paraffin-embedded tumor sections were stained with hematoxylin, dehydrated, and counterstained with Eosin. For CD8a, Gr-1, and vessel staining, sections were stained by biotin-conjugated CD8a (BioLegend), Gr-1 (AbD SeroTec), MECa-32 (Phar-mingen), or isotype control (Jackson), then incubated with horseradish peroxidase–streptavidin (1:1,000, Jackson) and 3,3′-diaminobenzidine substrate (Zymed) per instruction. Anti–Ki67 (NeoMarker), anti–pSmad2 antibody (Millipore Co.), and ApopTag Plus Peroxidase In situ Apo-osis Detection kit (CHEMICON) were used for staining of proliferative activity, p-Smad2 level, and apoptosis, respectively. Slides were cleared in xylene and coverslipped with nonaqueous mounting medium (Cytoseal). Images of stained tissue sections were taken using the Zeiss Microscope/Digital Image Camera. The percent-positive Gr-1+ cells or MECa-32–stained cells were quantitated using the ImagePro Plus software.
Fig. 1. A, anti–TGFβ RII antibodies block TGFβ 1. Blocking activity of TR1 and MT1 to TGFβ RII binding to TGFβ 1 in dose response was quantified by ELISA (points; mean; bars, SEM). Data represent one of three to four repeated experiments. B, anti–TGFβ RII antibodies block Smad2 activation. TGFβ-stimulated phosphorylation of Smad2 in human MDA-MB-231 or mouse 4T1 tumor cells was blocked by TR1 and MT1, respectively, whereas isotype control IgG had no effect. TGFβ RII Fc was used as a positive control. Total Smad2 is shown as a loading control. IC50 of TR1 and MT1 in the p-Smad2 inhibition assays was determined to be ∼5 ± 0.5 nmol/L. C, anti–TGFβ RII antibodies block tumor cell migration and invasion. TR1 and MT1 significantly inhibited TGFβ-induced migration of human MDA-MB-231 and invasion of 4T1 tumor cells as quantified in image of spots (columns, mean of multiple fields; bars, SD). Notably, TR1 treatment resulted in a significant less tumor cell migration compared with nontreated (P = 0.045, none versus TGFβ+TR1). A negative control “none” is culture without TGFβ. Each spot represents one of migrated or invaded tumor cells. Spots in multiple areas were quantified using the Image-Pro Plus software. D, anti–TGFβ RII antibodies induced internalization of TGFβ RII in tumor cells. TGFβ RII–expressing tumor cells were incubated with Alexa Fluor–labeled MT1 or TR1 in serum-free medium at 4°C or 37°C for 120 min. MT1 and TR1 induced internalization of most surface TGFβ RII in EMT6 and MDA-MB-231 tumor cells at physiologic temperature condition as shown in images taken by confocal microscope. Data represent one of two or three repeated experiments.
Statistical analysis. We determined the statistical differences for in vitro assays by Student’s t test. Tumor volume data were analyzed using repeated-measures ANOVA to determine the significant differences in tumor sizes among treatments, time points, and treatment-time interactions or t test for analysis on tumor nodules in lung between treatments. We considered values of $P < 0.05$ statistically significant and values of $P < 0.01$ highly significant.

Results

Characteristics of anti–TGFβ RII antibodies. To achieve the complete blockade of TGFβ RII-mediated activation of downstream cascades, neutralizing high-affinity antibodies specifically binding to a ligand interactive epitope of TGFβ RII were generated. Results from ELISA showed that binding specifically binding to a ligand interactive epitope of downstream cascades, neutralizing high-affinity antibody to respective human or mouse TGFβ activity of these antibodies lies on their high binding affinity to native TGFβ RIIB expressed on murine 4T1 and EMT6 carcinoma cells (Supplementary Fig. S1; Table 1). FACS data indicated that MT1 was able to bind to native TGFβ RII expressed on murine 4T1 and EMT6 carcinoma cells (Supplementary Fig. S2A), and TR1 to human MDA-MB-231 and BXPC-3 carcinoma cells (Supplementary Fig. S2B). TR1 had no cross-reactivity with mouse TGFβ RII as measured by ELISA and FACS (data not shown) and therefore was selected for in vitro studies in human xenograft tumor models to ensure that TR1 exclusively acts on human tumor cells but not on murine host cells. Anti-mouse TGFβ RII antibody MT1 with comparable binding and blocking activities similar to TR1 was used for studies on TGFβ RII-mediated functions and antitumor effects in mouse models.

Anti–TGFβ RII inhibits Smad2 activation and cancer cell motility, and induces the receptor internalization in cancer cells. TGFβ stimulates the activation of TGFβ RI kinase and downstream signaling kinase Smad2 that leads to broad cellular processes, i.e., survival, proliferation, differentiation, motility, and migration (18, 25). In the kinase phosphorylation assay, anti–TGFβ RII antibodies TR1 and MT1 inhibited the TGFβ-stimulated phosphorylation of downstream signaling kinase Smad2 in human MDA-MB-231 and murine 4T1 breast carcinoma cells (Fig. 1B). TGFβ signaling has a significant role in promoting an invasive tumor phenotype and cancer cell migration and invasion that are important components of metastasis (26, 27). In tumor cell migration and invasion assays, TR1 and MT1 significantly inhibited the TGFβ-induced migration of human MDA-MB-231 breast carcinoma cells and the invasion of murine 4T1 breast carcinoma cells (Fig. 1C), respectively, whereas irrelevant control IgG had no effect. Notably, TR1 treatment resulted in a significantly less TGFβ-induced migration of tumor cells compared with that of nontreated tumor cells (Fig. 1C), implying that anti–TGFβ RII antibody inhibits the TGFβ autocrine stimulation of cancer cells. A similar antibody effect was observed in migration assays with murine EMT6 and 4T1 breast carcinoma and human BXPC-3 pancreatic cells (Supplementary Fig. S3A-C). Furthermore, we found that anti–TGFβ RII antibody had the ability to induce receptor internalization in MDA-MB-231 and EMT6 tumor cells (Fig. 1D), suggesting that the antibody-induced receptor deterioration may also contribute to the reduction of TGFβ RII-mediated downstream kinase activation and cellular activity. Interestingly, our data showed that neither TGFβ or anti–TGFβ RII antibody had significant effect on tumor cell growth in vitro (Supplementary Fig. S4). Taken together, the results suggest that the inhibitory effect of anti–TGFβ RII antibody on TGFβ-stimulated Smad2 activation and cancer cell migration and invasiveness may result in the suppression of tumor growth and metastasis in vivo.

Table 1. Binding and blocking characteristics of anti–TGFβ RII antibodies

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<th>Clone</th>
<th>Blocking activity (IC₅₀)</th>
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<td>TR1</td>
<td>0.12 nmol/L: TGFβ 1</td>
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<td>0.54 nmol/L: TGFβ 2</td>
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<td>0.19 nmol/L: TGFβ 3</td>
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<td>MT1</td>
<td>0.57 nmol/L: TGFβ 1</td>
<td>0.054 nmol/L</td>
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<td></td>
<td>0.53 nmol/L: TGFβ 2</td>
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<td>0.47 nmol/L: TGFβ 3</td>
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NOTE: The binding activity of the antibodies to TGFβ RII binding to TGFβs and the binding activity of the antibodies were measured by ELISA. IC₅₀ and ED₅₀ were determined using the GraphPad Prism software. Kinetic analyses of the antibodies were performed by Biacore instrument. KD value was determined by the calculation of dissociation (koff)/association (kon) rates using the BIAl evaluation 2.1 software. Surface binding activity of the antibodies to TGFβ RII was measured by staining the receptor-expressing tumor cells with 0.1 μg/mL TR1 or MT1 and FACS. Mean fluorescent intensity units were calculated as the mean log fluorescence multiplied by the percentage of positive population.

Abbreviation: MFIU, man fluorescent intensity unit.

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Fig. 2. A, TR1 treatment inhibited the primary tumor growth of BXPC-3 (ANOVA \( P < 0.0001 \), versus saline control) and MDA-MB-231 (ANOVA \( P < 0.02 \), versus human IgG control) in mice bearing established s.c. human xenograft tumors. The pulmonary metastatic tumor growth in TR1-treated mice was also significantly inhibited (\( P < 0.05 \)). Mice were injected i.v. with MDA-MB-231-LM2-4 luciferase–transfected metastatic tumor cells 24 h before TR1 treatment. At the indicated times, photons were captured by the IVS image system and were plotted as photons per second ×10^6. B to D, IHC analysis indicated a decreased Smad2 activation (\( P < 0.03 \); B) and proliferative activity (\( P < 0.01 \); C) in cancer cells and a significant increase in tumor necrosis (D) in TR1-treated MDA-MB-231 xenografts compared with human IgG– or saline-treated tumors. Original magnification, ×200.
Anti–TGFβ RII directly suppresses primary tumor growth and metastasis. TGFβ signaling has been shown to regulate tumorigenesis and metastasis during malignancy progression (28, 29) in part through TGFβ autocrine and paracrine stimulation of cancer cell survival and growth (30–32). We first tested if anti-human TGFβ RII antibody TR1 would have antitumor activity directly against the growth of s.c. inoculated human carcinomas and pulmonary metastasis of i.v. inoculated luciferase transfectant human tumor cells in athymic immunodeficient mice. Indeed, TR1 significantly suppressed the growth of established primary tumor xenografts of BXPC-3 by 60%,
MDA-MB-231 by 31% (Fig. 2A), and PANC-1 by 31% (data not shown). Consistent with the inhibitory activity to cancer cell migration in vitro, TR1 significantly diminished the pulmonary metastatic tumor growth of MDA-MB-231 xenografts by 81% in a tumor metastasis model (Fig. 2A). Immunohistochemistry (IHC) analysis indicated a marked decrease in Smad2 phosphorylation (Fig. 2B) and proliferative activity (Fig. 2C) in cancer cells, and a significantly increased necrosis (Fig. 2D) in TR1-treated tumors compared with the saline or human IgG–treated tumors. These in vivo results suggest that anti–TGFβ RI antibody has a direct antitumor effect on primary tumor growth and metastasis through inhibition of TGFβ-mediated autocrine and paracrine stimulation of cancer cells because TR1 does not bind to TGFβ RI expressed on host cells.

**Anti–TGFβ RI–mediated antitumor effect is partly dependent on host immunity.** Consistent with the antitumor effect of anti–human TGFβ RI antibody in xenograft models, anti-mouse TGFβ RI antibody MT1 also significantly blocked the pulmonary metastatic tumor growth of 4T1 by 84% and of CT26 by 94% (Fig. 3A) in metastasis models of syngeneic immunocompetent mice. Furthermore, MT1 treatment inhibited the growth of established mouse 4T1 and EMT6 primary tumors by 52% and 28%, and diminished the spontaneous pulmonary metastasis of both tumor types by 89% and 84% (Fig. 3B), respectively. We found that the size of the metastatic tumor nodules was significantly smaller (approximately 0.05-0.1 mm in diameter) in the antibody-treated lungs compared with those (approximately 0.2-0.3 mm in diameter) in control IgG–treated tumors. IHC analysis revealed that MT1-treated subcutaneous tumors contained a large area of necrotic and hemorrhagic tissues with a thin outer rim of viable tumor cells (Fig. 3C). In contrast, the majority of rat IgG–treated tumor tissues comprised mostly viable tumor and stromal cells (Fig. 3C). Importantly, such significant antitumor effect was less pronounced in immunodeficient mice (data not shown). These results suggest that in addition to blocking autocrine and paracrine loop–driven tumor growth, anti–TGFβ RI antibody exerts antitumor effect in part by modulating immune responses against tumors in immunocompetent host.

**Anti–TGFβ RI enhances NK and CTL activity in immunocompetent host.** Inhibition of TGFβ signaling leads to the enhancement of NK- and CTL-mediated antitumor activity (33, 34). We assessed whether TGFβ RI blockade by MT1 increases NK and CTL activity in tumor-bearing immunocompetent mice. As predicted, NK killing activity (Fig. 4A) and tumor cell–specific killing activity of CTL (Fig. 4B) and the activity-associated IFN-γ production in NK cells and CTLs were significantly elevated in MT1-treated immunocompetent mice compared with those treated with normal rat IgG (Fig. 4C). Furthermore, we found a significant increase in tumor-infiltrating CD8 effector T cells in MT1–treated viable tumor tissue area and substantially fewer CD8 T cells localized in the periphery of rat IgG–treated tumors (Fig. 4D). These results indicate that the antitumor effects of anti–TGFβ RI antibody are in part attributed to the antibody-mediated enhancement of innate and adaptive antitumor immunity in immunocompetent host.

**Anti–TGFβ RI diminishes TGFβ RI+ Treg cells in tumor-bearing animals.** TGFβ plays an active role in the induction of CD4/Foxp3+ T cells, and Treg cells possess immunosuppressive activity to T-cell–mediated immunity against tumor cells (35, 36). We evaluated whether the blockade of TGFβ RI by MT1 has an inhibitory effect on Treg cells in tumor-bearing immunocompetent mice and found that the subset of TGFβ RI+ CD4/Foxp3 Treg cells was significantly diminished in MT1-treated tumor-bearing mice (Fig. 4E). The ablative effect on Treg cells was also observed as early as day 9 after tumor inoculation and sustained throughout treatment. Notably, anti–TGFβ RI antibody did not have an effect on the TCR engagement–induced proliferation of naive CD4 T cells in vitro and the number of peripheral CD4 as well as CD8 T cells in MT1–treated tumor-bearing mice (Supplementary Fig. S5A, B). These results indicate that anti–TGFβ RI antibody has the ability to selectively abolish TGFβ RI+ Treg cells without adverse effect on overall population of CD4 and CD8 T cells in vivo.

**Anti–TGFβ RI attenuates TGFβ induction of Treg cells and Treg cell–mediated suppression of T-cell activation and proliferation.** TGFβ is capable of converting naive T cells into Treg cells with the capacity to suppress the immune response against tumors.

Fig. 4. A, NK killing activity was significantly increased in MT1-treated tumor-bearing mice (P < 0.005; columns, mean; bars, SD). NK cells were purified from spleens at day 9 after tumor cell inoculation and were tested for killing activity against target YAC-1 cells at a ratio of 3:1 (NK versus YAC-1 cells) in a cleared caspase-3 assay. B, CTL killing activity to tumor cells was significantly increased in MT1-treated tumor-bearing mice (P < 0.005). CD8α+ cells were purified from splenocytes of treated mice at day 14 following tumor cell injection. CD8α+ cells were stimulated in vitro with mitomycin C–treated EMT6 cells for 7 d before cleaved caspase-3 assay with the target and effector cells. C, MT1 treatment significantly increased the secretion of IFN-γ in tumor cell–stimulated NK cells (P < 0.0001; left) and CD8 T cells (P < 0.005; right) as quantified by ELISpot. Columns, mean of two replicates (n = 3); bars, SD. D, the number of tumor-infiltrating CD8 T cells was significantly increased in MT1–treated viable tumor areas compared with CD8 T cells in rat IgG–treated tumors as shown by IHC analysis with anti–CD8α antibodies. The presence of CD8 T cells in tumor tissues was quantified as percentage of CD8 T cells (P < 0.005; columns, mean; bars, SEM). Original magnification, ×200. E, MT1 treatment significantly reduced the number of TGFβ RII+/Foxp3+ Treg cells (P < 0.0005; columns, mean; bars, SD). Mice bearing established EMT6 murine tumors were treated i.p. with MT1 for 24 d. Tumor-draining lymph nodes were harvested from tumor-bearing mice at day 30 after tumor inoculation for FACS with fluorescence-labeled anti–CD4, Foxp3 antibodies, and MT1.

A representative FACS plots from each group with an average of at least three animals are shown (left) and quantified (bars on the right). F, MT1 inhibited TGFβ–mediated induction of Treg cells; columns, mean (n = 4); bars, SD. Purified naive CD4+ cells were stimulated with anti–CD3 antibodies and purified APCs in the presence or absence of TGFβRI and MT1. Cells were then harvested for staining of CD4+/CD25+/Foxp3+ Treg cells. Stained cells were analyzed by FACS. G, MT1 reversed Treg cell–mediated suppression of naive T-cell proliferation; columns, mean (n = 3); bars, SD. Purified CD4+/CD25+ Treg cells were cocultured with purified CD4+CD25– naïve T cells in the presence of anti–CD3 antibody and APCs. Following 7 d of coculture, cells were tested for proliferation using the Cell Titer Glo assay. Data represent one of two repeated experiments.

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response (37, 38), and Treg cells have a role in suppression of antitumor immunity (39, 40). Our in vitro data showed that anti–TGFβ RII antibody significantly blocked the TGFβ-mediated induction of Treg cells (Fig. 4F). More importantly, anti–TGFβ RII antibody significantly blocked the Treg cell–mediated inhibition of naïve T-cell proliferation stimulated by anti-CD3 antibody and MHC class II–positive antigen-presenting cells (APC; Fig. 4G). These in vitro results are consistent with the in vivo result of the inhibition of Treg cells in anti-TGFβ RII antibody–treated tumor-bearing mice in this study. Thus, the antitumor effect of anti–TGFβ RII antibody is in part attributed to the blockade of TGFβ induction of Treg cells and Treg-mediated suppression of T-cell response in addition to the antibody-mediated depletion of TGFβ RII+ Treg cells in vivo.

**Anti–TGFβ RII abrogates tumor-derived TGFβ RII+/Gr-1+/CD11b+ myeloid cells.** Gr-1+/CD11b+ myeloid cells have a seminal role in promoting metastasis and angiogenesis in tumor stroma and in mediating immunosuppression during tumor progression (11, 13, 15). To determine whether the antitumor activity of anti–TGFβ RII antibody MT1 is attributable to the direct inhibition of myeloid cells in tumor-bearing mice, we evaluated the alteration of Gr-1+/CD11b+ population and the subset of TGFβ RII+ myeloid cells in a major organ, i.e., spleen. Gr-1+ myeloid cells at different time points during the course of treatment. We found that there was <5% of Gr-1+/CD11b+ myeloid cell present in the spleens of normal mice, whereas the number of the peripheral myeloid cells was significantly increased to over 40% in spleens of tumor-bearing mice at day 30 of tumor cell inoculation (Fig. 5A). However, MT1 treatment markedly reduced myeloid cell population (Fig. 5B) and remarkably eliminated the subset of peripheral TGFβ RII+/Gr-1+/CD11b+ myeloid cells in tumor-bearing mice (Fig. 5C). Similar effects were also observed as early as at day 14 and 21 after tumor inoculation (data not shown). The increased infiltration of myeloid cells in tumors has been observed by other investigators in IHC analysis using the anti–Gr-1 antibody (11). In this study, we found a significant decrease in Gr-1+ myeloid cell infiltration in MT1-treated viable tumor tissue area compared with those in the same area of tumors treated with control IgG (Fig. 5D). The results suggest that attenuation and reduction of TGFβ RII+ myeloid cells prevents the tumor-mediated expansion and recruitment of myeloid cells in peripheral blood circulation and tumor tissues.

**Anti–TGFβ RII blocks myeloid cell–mediated tumor cell migration and T-cell suppression.** Gr-1+/CD11b+ myeloid cells have been reported to promote tumor cell invasiveness by secreting matrix metalloproteinases in vitro and in vivo (11). Whether TGFβ RII is functionally expressed in Gr-1+/CD11b+ myeloid cells and whether TGFβ RII+ myeloid cells directly augment cancer cell invasiveness remain unclear. We found that the blockade of TGFβ RII by MT1 significantly inhibited tumor cell migration induced by tumor-derived Gr-1+/CD11b+ myeloid cells in vitro (Fig. 5E). The result suggests that Gr-1+/CD11b+ myeloid cells have the capacity of directly mediating cancer cell migration through TGFβ signaling activation, and blockade of TGFβ RII+ myeloid cell–mediated migration of tumor cells may partly contribute to the inhibition of metastasis by the antibody in vivo.

One study suggested that the blockade of immunosuppressive Gr-1+/CD11b+ myeloid cell accumulation in tumor-bearing mice might facilitate a more effective immune-enhancing therapy in the treatment of advanced tumors (41). We tested the effect of MT1 on the suppressive activity of tumor-derived myeloid cells to T-cell response and found that MT1 significantly blocked the inhibitory activity of tumor-derived myeloid cells to the activation and proliferation of naïve T cells stimulated by anti-CD3 antibody and MHC-II+ APC (Fig. 5F). These results suggest that the blockade of TGFβ RII function in myeloid cells by the neutralizing antibody prevents the inhibitory effect of tumor-derived myeloid cells on T-cell response, thereby facilitating T-cell–mediated antitumor immunity.

**The anti–TGFβ RII plus cyclophosphamide regimen has a superior antitumor efficacy.** Cyclophosphamide has been reported to be a potent cytotoxic agent with the capacity of suppressing hematopoietic and myeloid progenitor cells as well as myeloid cells (42). In this regard, we reasoned that cotreatment with anti–TGFβ RII antibody MT1 and cyclophosphamide might have a more potent antitumor effect on tumor growth and metastasis. Remarkably, combination treatment with anti–TGFβ RII antibody MT1 and cyclophosphamide resulted in a significantly increased efficacy against primary tumor growth (Fig. 6A) and spontaneous pulmonary metastasis (Fig. 6B) in EMT6 tumor-bearing mice compared with MT1 or cyclophosphamide monotherapy. IHC analysis indicated a significantly higher level of apoptosis in MT1-treated tumors and more pronounced apoptosis in MT1 and cyclophosphamide-treated tumors (Fig. 6C). The increased antitumor effect of the combination treatment is likely due to the antibody-mediated ablation of TGFβ RII+ myeloid cells and the recruitment of myeloid cells, as well as cyclophosphamide effect on cancer cells and myeloid cell function because cyclophosphamide had no effect on the total numbers of Gr-1+/CD11b+ myeloid cells in spleens of treated tumor-bearing mice (Fig. 6D). These results suggest that combination treatment with anti–TGFβ RII antibody and myeloid cell suppressive chemotherapy may have an advantage in antitumor intervention.

**Discussion**

Studies based on genetic modification and the use of TGFβ signaling inhibitors have provided considerable knowledge for understanding the mechanisms of TGFβ signaling–modulated pathogenesis in tumor, metastasis, angiogenesis, and immunosuppression (4, 5, 18). In this study, we have shown that the blockade of TGFβ RII by a neutralizing anti–TGFβ RII antibody effectively impedes the TGFβ-stimulated activation of downstream Smad2 kinase in cancer cells, and cancer cell invasiveness and migration in vitro. The results of in vivo studies have shown...
Fig. 5. A, the number of Gr-1+/CD11b+ cells were significantly increased in rat IgG-treated EMT6 tumor-bearing mice as determined and shown by FACS as percentage (43.1%, \(P < 0.0005\)) compared with those (2.8%) in normal mice and quantified as bars (\(n = 4\); columns, mean, bars, SD). B, the number of Gr-1+/CD11b+ myeloid cells from spleens of MT1-treated tumor-bearing mice were reduced (\(P < 0.05\)) as shown in percentages (16.1%) versus rat IgG-treated mice (32.5%). C, MT1 diminished TGF\(\beta\) RII+ myeloid cells in spleens of treated tumor-bearing mice (\(P < 0.0001\)) as shown in percentages (3.9%) versus those in rat IgG-treated mice (32.5%). D, IHC analysis with anti–Gr-1 antibody indicated that myeloid cells were significantly decreased in MT1-treated viable tumor areas (left). Right, the percentage of Gr-1+ myeloid cells were quantified (\(P < 0.01\); columns, mean, bars, SEM). Original magnification, \(\times 200\). E, MT1 significantly inhibited the Gr-1+/CD11b+ myeloid cell–induced migration of EMT6 tumor cells compared with that of nontreated tumor cells (\(P < 0.01\)). The myeloid cells were isolated from spleens of tumor-bearing mice. Each spot represented one of migrated tumor cells. Spots were quantified (columns, mean of multiple fields; bars, SD) using the Image-Pro Plus software. F, MT1 significantly blocked the tumor-derived myeloid cell–mediated inhibition of naïve T-cell proliferation (\(P < 0.05\)). Naïve CD4+/CD25- T cells and myeloid cells were cocultured and treated with MT1 in the presence of anti–CD3 antibody and APCs. Myeloid cell culture alone was set as a negative control. Data represent one of two repeated experiments.
that the anti-TGFβ RII antibody has the ability to suppress metastasis and tumor growth through the inhibition of Smad2 activation and proliferative activity as well as invasiveness in cancer cells. We have also found that the anti-tumor efficacy is attributed to the antibody-mediated abrogation of myeloid cells and immunosuppressive Treg cells as well as enhancement of NK and CTL activity.

Our data suggest that the antibody-mediated abrogation of Gr-1+/CD11b+ population, particularly for the subset of TGFβ RII+ myeloid cells, and the inhibition of myeloid
cell-suppressive activity to T-cell activation and myeloid cell–mediated tumor cell migration have a significant contribution to the overall antitumor efficacy. The reduction and inhibition of TGFβ RII+/Gr-1+/CD11b+ myeloid cells is likely due to the antibody-mediated inhibition of the recruitment and functionality of myeloid progenitor cells and the depletion of TGFβ RII+ myeloid cells. This hypothesis is supported by the finding of a study showing no difference in the number of myeloid cells in tumor-bearing mice treated with anti–TGFβ antibody, which had no direct effect on TGFβ R in cells (22). Furthermore, combination treatment with anti–TGFβ RII antibody and myeloid cell–suppressive cytotoxic agent cyclophosphamide significantly increased efficacy against primary tumor growth and pulmonary metastasis in tumor-bearing mice. These findings suggest that the antibody-mediated reduction and inhibition of Gr-1+/CD11b+ myeloid cells and the depletion of the subset of tumor-derived TGFβ RII+ myeloid cells in combination with myeloid cell–suppressive agents may represent a more effective therapeutic strategy for the treatment of cancer and metastasis.

Studies have shown that Treg cells mediate the suppression of NK and CTL activity and TGFβ3 inhibits the function of cytolytic factors of perforin, granzyme A, and granzyme B in immune effector cells (17, 33, 43, 44). We found that anti–TGFβ RII treatment prevented the TGFβ3-mediated induction and inhibitory activity of Treg cells and abrogated TGFβ3-RII+/Foxp3+ Treg cells, as well as increased NK and CTL killing activity and Th1 activity–associated production of IFN-γ in peripheral NK and CD8 T cells in tumor-bearing immunocompetent mice. Importantly, anti–TGFβ RII treatment resulted in a significant increase in tumor-infiltrating CD8 effector T cells (Fig. 4D) in viable tumor tissues. The elevation of tumor-infiltrating lymphocytes in tumors is likely due to the anti–TGFβ RII antibody–mediated blockade of Treg cell suppression of activation and proliferation of T cells, or/and the antibody-mediated mobilization of tumor-infiltrating lymphocytes in tumor-bearing mice.

TGFβ3 was reported to be a vital cytokine that mediates endothelial cell functions and vascular endothelial growth factor-A secretion by tumor cells, thereby promoting tumor angiogenesis (45, 46). We found that anti–TGFβ RII antibody significantly inhibited the TGFβ3-induced production of vascular endothelial growth factor-A by MDA-MB-231 human breast tumor cells and 4T1 mouse breast tumor cells in vitro (Supplementary Fig. S6A), and the blocked TGFβ3-stimulated motility of TGFβ RII+ endothelial cells in wound healing assay (Supplementary Fig. S6B), suggesting that anti–TGFβ RII antibody may have antiangiogenic effect in vivo. IHC analysis showed a significant inhibition of vascularization in TR1-tREATED MDA-MB-231 xenograft (Supplementary Fig. S6C) and 4T1 tumors (data not shown) but no significant difference in the number and density of vessels in anti–TGFβ RII and control antibody–treated tumors in EMT6 tumor model (data not shown). These results suggest that the TGFβ3 RII inhibition–mediated anti-angiogenic effect may depend upon the degrees of angiogenesis dependency of a given tumor. The mechanism underlying anti–TGFβ RII effects on tumor angiogenesis remains to be further investigated.

Studies have shown that TGFβ3 plays a crucial role in the modulation of the differentiation and motility of tumor stromal cells, i.e., cancer-associated fibroblasts and myofibroblasts that promote cancer cell invasion and metastasis (47, 48). We found that anti–TGFβ RII antibody significantly blocked the TGFβ3-mediated migration of fibroblasts (Supplementary Fig. S6D). Thus, the effect of anti–TGFβ RII antibody on cancer cell invasion and metastasis may be in part attributed to the blockade of the TGFβ3-mediated functionality of cancer-associated fibroblasts in vivo. Additional studies are required for further determining the role of TGFβ3 RII in regulating the functions of cancer-associated fibroblast and myofibroblasts during cancer progression.

Collectively, it is conceivable that the antitumor efficacy of anti–TGFβ RII antibody in controlling tumor growth and metastasis is primarily attributed to the multifaceted effects of the antibody on cancer cells, immune effector, and immunosuppressive cells as well as stroma cells including myeloid and angiogenic cells and fibroblasts. In fact, anti-mouse TGFβ3 RII antibody treatment led to significantly increased apoptosis in tumors in syngeneic tumor model (Fig. 6C). Conversely, anti-human TGFβ RII antibody that has no effect on host stroma and immune cells induced less significant apoptosis in the xenograft model. Thus, the anti–TGFβ RII antibody–mediated inhibition of cancer cell growth and invasiveness and the abolition of stroma cells, i.e., myeloid and Treg cells, as well as the enhancement of NK and CTL activity are plausibly the major mechanisms of action of the antibody in the suppression of metastasis and tumor progression.

Fig. 6. A, MT1 treatment significantly suppressed s.c. primary tumor growth (P < 0.05; columns, mean; bars, SEM). Antitumor efficacy against primary tumor growth was significantly increased in combination treatment with MT1 and cyclophosphamide (CTX) compared with monotherapy (P < 0.0005, MT1 versus MT1 + cyclophosphamide; P < 0.001, cyclophosphamide versus MT1 + cyclophosphamide). B, lungs were collected from treated mice at the end of study. Metastatic tumor nodules were quantified for incidence of pulmonary metastasis (n = 12; columns, mean; bars, SD). MT1 and cyclophosphamide treatment significantly suppressed spontaneous pulmonary metastasis (P < 0.00001; rat IgG versus MT1; P < 0.00001, rat IgG versus cyclophosphamide). Combination treatment with MT1 and cyclophosphamide led to a nearly complete suppression of pulmonary metastasis compared with monotherapy (P < 0.000005, rat IgG versus MT1 + cyclophosphamide; P < 0.005, MT1 versus MT1 + cyclophosphamide; P < 0.0005, cyclophosphamide versus MT1 + cyclophosphamide). C, MT1 significantly induced apoptosis in treated tumors (P < 0.001, MT1 versus rat IgG). The level of apoptosis was more pronounced in MT1 and cyclophosphamide-treated tumors (P < 0.05, MT1 + cyclophosphamide versus MT1). D, splenocytes were collected from treated mice at the end of study. Gr-1+/CD11b+ myeloid cells were quantified by FACS (n = 4; columns, mean; bars, SD). MT1 treatment significantly suppressed the numbers of Gr-1+/DC11b+ myeloid cells (P < 0.005), but cyclophosphamide had no effect on myeloid cells (P > 0.5). Combination treatment with MT1 and cyclophosphamide resulted in a similar effect on numbers of Gr-1+/DC11b+ myeloid cells (P < 0.005).

No statistic significance between MT1 and combination treatment (P = 0.25).
growth. Owing to the direct action on TGFβ-dependent cancer and tumor-associated cells and the potential capacity of inducing antibody-dependent cell cytotoxicity and complement-dependent cytotoxicity response, anti–TGFβ RII antibody is likely superior to other means, i.e., anti–TGFβ ligand antibodies and TGFβ RI kinase inhibitors in control of TGFβ and its cognate receptor–mediated deleterious activity in tumor growth and metastasis. Of note, anti–TGFβ RII antibody has no effect on the proliferation of naïve CD4 T cells stimulated by anti-CD3 antibody and APC in vitro (Supplementary Fig. S5A), and the number of overall peripheral CD4 and CD8 T cells in tumor-bearing mice (Supplementary Fig. S5B). Mice treated with anti–TGFβ RII antibody did not have body weight loss and were generally healthier than nontreated tumor-bearing mice. These data suggest that anti–TGFβ RII antibody has a favorable safety profile and limited toxicity to the host.

In summary, the results in this study have shown that the neutralizing anti–TGFβ RII antibody is capable of selectively attenuating TGFβ-mediated deleterious activity in tumor growth, metastasis, and immunosuppression, and enhancing antitumor immunity. These preclinical data have provided compelling rationale to support the use of a neutralizing anti–TGFβ RII antibody as a novel therapeutic strategy for treatment of TGFβ RII–dependent tumors with metastatic capacity.

Disclosure of Potential Conflicts of Interest
All authors are full time employees of ImClone Systems.

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References


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