Soluble Type II Transforming Growth Factor-β Receptor Inhibits Established Murine Malignant Mesothelioma Tumor Growth by Augmenting Host Antitumor Immunity

Eiji Suzuki,[1] Veena Kapoor,[1] H.-Kam Cheung,[2] Leona E. Ling,[2] Peter A. DeLong,[1] Larry R. Kaiser,[1] and Steven M. Albelda[1]

[1]Thoracic Oncology Research Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania; and [2]Molecular and Cellular Biology, Biogen Idec, Cambridge, Massachusetts

ABSTRACT

Purpose: **Transforming growth factor (TGF)-β blockade has been proposed as an anticancer therapy; however, understanding which tumor patients might benefit most from such therapy is crucial. An ideal target of such inhibitory therapy might be malignant mesothelioma (MM), a highly lethal, treatment-resistant malignancy of mesothelial cells of the pleura and peritoneum that produces large amounts of TGF-β. The purpose of this study was to explore the possible therapeutic utility of TGF-β blockade on MM.**

Experimental Design: **To evaluate this hypothesis, we tested the effects of a soluble TGF-β type II receptor (sTGF-βR) that specifically inhibits TGF-β1 and TGF-β3 in three different murine MM tumor models, AB12 and AC29 (which produce large amounts of TGF-β) and AB1 (which does not produce TGF-β).**

Results: **Tumor growth of both established AB12 and AC29 tumors was inhibited by sTGF-βR. In contrast, AB1 tumors showed little response to sTGF-βR. The mechanism of these antitumor effects was evaluated and determined to be primarily dependent on immune-mediated responses because (*a*) the antitumor effects were markedly diminished in severe combined immunodeficient mice or mice depleted of $CD8^+$ T cells and (*b*) $CD8^+$ T cells isolated from spleens of mice treated with sTGF-βR showed strong antitumor cytolytic effects, whereas $CD8^+$ T cells isolated from spleens of tumor-bearing mice treated with of control IgG2a showed no antitumor cytolytic effects.**

Conclusions: **Our data suggest that TGF-β blockade of established TGF-β-secreting MM should be explored as a promising strategy to treat patients with MM and other tumors that produce TGF-β.**

INTRODUCTION

Transforming growth factor (TGF)-β is a M_r 25,000 homodimeric protein with mulitple mammalian forms [*i.e.*, TGF-β1, TGF-β2, and TGF-β3 (1, 2)], which are synthesized and secreted by various normal cells including macrophages, neutrophils, platelets, subsets of activated lymphocytes, and most transformed cells (3–6). The TGF-β isoforms play an intriguing role in tumor biology because they can function as both tumor suppressors and tumor enhancers. In normal epithelial cells, TGF-β is a potent growth inhibitor and promoter of cellular differentiation through a variety of complex signaling pathways (7). Loss of TGF-β (as seen in hemizygous Tgfb-1-null mice) or loss of function of the TGF-β receptors (especially TGF-β type II receptor) clearly enhances tumorigenicity (8). For example, somatic mutations of the TGF-β type II receptor are characteristic of colon and gastric carcinomas (9). Thus, for normal cells, TGF-β is a tumor suppressor. On the other hand, as tumors develop and progress, they almost always lose their negative growth response to TGF-β and, indeed, often produce large amounts of this cytokine. In this setting, TGF-β becomes a powerful tumor promoter due to its ability to stimulate angiogenesis, alter the stromal environment, and, importantly, cause local and systemic immunosuppression (10–13).

Despite these potentially contradictory effects, the goal of developing anticancer therapeutics based on inhibiting TGF-β has been pursued for over a decade. As proof of principal, a number of studies have clearly shown that transfection of tumor cells with antisense oligonucleotides or inhibitors of TGF-β can decrease TGF-β production and inhibit tumor growth after injection of the transfected cells (14, 15). Knockout of TGF-β responsiveness in T cells also leads to decreased tumor growth (16). Studies using soluble inhibitors of TGF-β, approaches with potential clinical utility, have also been performed. Inhibitors include antibodies (17), TGF-β–binding proteins such as decorin or soluble β-glycan (18, 19), and, most recently, soluble chimeric TGF-β type II receptor molecules that bind and sequester TGF-β1 and TGF-β3 (20–23). To date, beneficial effects have been seen in a number of mouse tumors systems (especially reduction of metastatic disease) including breast cancer (17, 18, 24), thymoma (21), hepatocellular carcinoma (15), and glioma (23).

Given the potential side effects of TGF-β blockade due to its tumor promoting and proinflammatory effects, the choices of which tumor patients might benefit most from such therapy are crucial. We postulate that an early ideal target of such inhibitory therapy would be malignant mesothelioma (MM). MM is an aggressive tumor of the serosa and pleura induced by exposure to asbestos (25). Current therapies for this tumor are only

Received 11/19/03; revised 6/3/04; accepted 6/16/04.
Grant support: National Cancer Institute grant PO1 66726.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Steven Albelda, Thoracic Oncology Research Laboratory, BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104. Phone: 215-573-9933; Fax: 215-573-4469; E-mail: albelda@mail.med.upenn.edu.

marginally effective, and mortality remains disturbingly high, with a median survival of approximately 6 to 12 months. However, there are a number of features that suggest that MM would be susceptible to the effects of TGF-β blockade, especially its immune suppressive effects.

First, significant levels of TGF-β are produced by most human MM cell lines (14, 26, 27) and murine and rat MM cell lines (14, 28). High levels of TGF-β have also been documented in patients with MM. TGF-β immunoreactivity has been observed in >90% of MM samples (29, 30). High levels of TGF-β have also been measured in pleural effusions caused by MM. Maeda *et al.* (31) measured levels of 280 pmol/L that were three to six times that seen in effusions from primary lung cancer. Using a cell-based plasminogen activator inhibitor 1 (PAI-1)-luciferase plasmid assay that measures bioactive TFG-β (32), we also observed markedly elevated levels of TGF-β (1,900 pg/mL) in the pleural fluid of MM patients that were approximately six times higher than those seen in effusions from patients with breast cancer (286 pg/mL) and non–small-cell lung cancer (293 pg/mL).[3] It is thus clear that a prominent feature of MM is TGF-β production.

Second, TGF-β has been implicated in the pathogenesis of MM in a number of studies (33). Fitzpatrick *et al.* (14) transfected a murine MM line with inducible antisense TGF-β1 and TGF-β2 plasmids and showed inhibition of anchorage-dependent growth *in vitro* and delay in tumor growth in animals after induction with zinc chloride. These findings were extended by Marzo *et al.* (34), who showed that *in vivo* administration of TGF-β2 antisense oligonucleotides delivered locally reduced tumor growth in an animal model of MM.

Third, there is evidence to suggest that MM may be amenable to immunologic therapies. In general, both human and murine MM express high levels of major histocompatibility complex (MHC) class I molecules on the cell surface. Over the past decade, we and others have demonstrated success with various immunotherapeutic approaches in mouse models of mesothelioma. For example, positive therapeutic results have been seen with interferon (IFN)-α (35), interleukin (IL)-2 (36), IL-12 (37), CD40 ligand (38), immunoliposomes (39), and adenovirally delivered IFN-β (40). A number of investigators have translated some of these animal findings into clinical trials. Several published phase I and II clinical trials have documented mesothelioma tumor responses to intrapleural infusion of IL-2, IFN-β, and IFN-γ (36, 41–44). Given this potential to respond to immunologic therapy, we therefore hypothesized that the neutralization of TGF-β could be effective for treatment of MM in terms of release from immunosuppressive environment and normalization of host immune surveillance.

To evaluate this hypothesis, we explored the effects of a soluble TGF-β type II receptor (sTGF-βR) that specifically inhibits TGF-β1 and TGF-β3 (45) in three different murine MM tumor models: AB12 and AC29 (which produce large amounts of TGF-β) and AB1 [which does not produce TGF-β (14)]. Tumor growth of both established AB12 and AC29 tumors was inhibited by sTGF-βR. In contrast, AB1 tumors showed little response to sTGF-βR. The mechanism of these findings was evaluated and determined to be primarily dependent on immune-mediated responses because (*a*) these antitumor effects were markedly diminished in severe combined immunodeficient (SCID) mice or mice depleted of $CD8^+$ T cells and (*b*) $CD8^+$ T cells isolated from spleens of mice treated with sTGF-βR showed strong antitumor cytolytic effects as assessed by the Winn assay (46). Our data suggest that TGF-β blockade of established TGF-β–secreting MM using sTGF-βR should be explored as a promising strategy to treat patients with MM.

MATERIALS AND METHODS

Animals. Pathogen-free female BALB/c and CB-17 SCID mice (6 to 8 weeks old) were purchased from Taconic Laboratories (Germantown, NY) and the Wistar Institute, respectively. Female CBA/J mice (6 to 8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA). All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. The animal use committees of the Wistar Institute and University of Pennsylvania approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

Cell Lines. Murine MM cell lines AB12, AC29, and AB1 were produced by and obtained from Dr. Bruce Robinson (14). Each of these adherent cell lines was derived independently from tumors that were induced by intaperitoneal injection of asbestos fibers into BALB/c mice (AB12 and AB1) or CBA/J mice (AC29). The ability of these lines to secrete TGF-β spontaneously in culture has been studied in detail. AB1 cells make virtually no TGF-β, whereas AB12 and AC29 cells secrete large amounts [462 and 1935 pmol/10^6 cells/24 hours, respectively (14)]. 3T3 mouse fibroblasts were obtained from American Type Culture Collection (Manassas VA). Mink lung epithelial cells (MLECs) transfected with the PAI-1 promoter driving a luciferase gene were obtained from Dr. Daniel Rifkin (New York University School of Medicine; see ref. 32). All cells were cultured and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS; Georgia Biotechnology, Atlanta, GA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. All cell lines were regularly tested and maintained negative for *Mycoplasma* spp.

TGF-β Inhibitor. The soluble recombinant murine TGF-β receptor type II-murine Fc:IgG2a chimeric protein has been described previously and was supplied by Biogen (Cambridge, MA; ref. 22). This construct binds and inhibits TGF-β1 and TGF-β3 in the 1 nmol/L range and has a half-life in mouse plasma of 14 days. Previous studies have shown biological effects at 1 (22), 2 (45), and 5 mg/kg (20). The activity of this reagent was tested in a functional TGF-β assay (32). MLECs stably transfected with a construct containing the human PAI-1 promoter fused to firefly luciferase reporter gene were seeded in 96-well plates at a density of 1.8×10^3 cells per well in DMEM containing 10% FBS. Human platelet-derived TGF-β1 protein

[3] P. DeLong, R. Carroll, A. Henry, et al., Malignant pleural effusions secondary to mesothelioma have few CD4+ CD25+ regulatory T cells but high levels of TGF-β compared to pleural effusions secondary to carcinoma, submitted for publication.

(500 pg/mL; BD Biosciences PharMingen, San Diego, CA) was added in the absence or presence of various concentrations of sTGF-βR and incubated for 16 hours at 37°C in a 5% CO_2 incubator. Cells were lysed with 1× cell lysis buffer (Analytical Luminescence; Promega, Madison, WI), and the lysates were transferred to 96-well plate. Both substrate A and substrate B (Analytical Luminescence; Promega) were added to the samples, and luciferase activity was measured using an ML1000 luminometer (Dynatech Laboratories, Inc., Alexandria, VA). Luciferase activity was reported as relative light units.

The Effect of Soluble TGF-β Type II Receptor and TGF-β Protein on AB12 and AB1 Cell Growth *In vitro*. AB12 or AB1 cells were seeded in 96-well plates at a density of 2×10^3 cells per well in 100 μL of DMEM with 10% FBS. Twenty-four hours after cell seeding, fresh media were added with various concentrations of sTGF-βR (1, 10, 100, and 1,000 ng/mL) or TGF-β1 protein (1, 10, 100, and 1,000 pg/mL) in 100 μL of DMEM with 1% FBS. After 24 and 48 hours of incubation, cancer cell viability was assessed by using the Cell Titer 96 Aqueous Non-Radioactive MTS Cell Proliferation Assay (Promega).

Immunoblot Detection of Phosphorylated and Total Smad2 Protein. To evaluate the post-receptor events in TGF-β signaling of AB12 and AB1 cells, Smad2 protein phosphorylation after TGF-β stimulation was determined. Cells in 10-cm plates were grown to 100% confluence in complete media containing 10% FBS and then serum-starved in the same media containing 0.5% FBS for 3 to 5 hours. Cells were either unstimulated or stimulated with 10 ng/mL TGF-β for varying times followed by removal of the media and two rinses with 1× PBS. The cells were then immediately frozen and stored at −80°C. To detect phosphoryated Smad2 or total Smad2 protein, the cells were lysed in 1× NuPage gel sample buffer (Invitrogen, Carlsbad, CA). Cell lysates were heated at 70°C for 10 minutes, electrophoresed on 10% NuPage Tris-Bis gels, and transferred to polyvinylidene difluoride membrane (Invitrogen). After blocking, phosphorylated Smad was detected with primary antibody rabbit anti–phospho-Smad2 (Upstate Biotechnology, Inc., Lake Placid, NY) followed by a secondary antibody, horseradish peroxidase (HRP)-conjugated donkey antirabbit $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The blot was stripped in Restore solution (Pierce Biotechnology, Inc., Rockford, IL), and total Smad2 protein was detected using a primary antibody, mouse anti-Smad2/3 (Transduction Laboratories, Lexington, KY) and a secondary antibody, HRP-conjugated secondary goat antimouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). West Femo enhanced chemiluminescence (Pierce Biotechnology, Inc.) was used to detect HRP activity.

Animal Models. BALB/c mice received injection in one flank with 1×10^6 AB12 or AB1 tumor cells. CBA/J mice received injection in one flank with 1×10^6 AC29 tumor cells. To test efficacy of sTGF-βR on "small" tumors, treatment was begun when the tumors reached a minimal volume of 150 mm^3 (approximately 10 days after tumor cell inoculation). To test efficacy of sTGF-βR on "large" tumors, treatment was begun the tumors reached a minimal volume of either 350 or 1,000 mm^3 (approximately 18 days and 25 days after tumor cell inoculation, respectively). The dose of sTGF-βR was established in pilot studies of small AB12 tumor-bearing mice treated with three doses of 0.1, 1.0, 5.0, and 10 mg/kg sTGF-βR. Doses of 1.0 mg/kg reduced AB12 tumor growth after 10 days more than doses of 0.1 mg/kg, whereas doses of 5 and 10 mg/kg had no additional effects. Thus, 1.0 mg/kg sTGF-βR per injection was used in this study. In the TGF-β blockade groups, mice received intraperitoneal administration of 1.0 mg/kg sTGF-βR, every 3 days, for six doses. Because the soluble recombinant TGF-β receptor chimeric protein was composed of the extracellular domain of the murine type II TGF-β receptor fused to the Fc portion of murine IgG2a, we used 1.0 mg/kg mouse IgG2a as control treatment, as described in previously mentioned studies (22, 47). SCID mice were also studied using the same experimental design as the BALB/c mice protocols. Tumor volumes were estimated using the formula ($\pi \times$ long axis $\times$ short axis $\times$ short axis)/6. We performed measurements of tumors twice weekly. Unless otherwise mentioned, each control or experimental group had a minimum of five mice.

Evaluation of Tumor-Infiltrating Lymphocyte Distribution by Flow Cytometry Analysis. To study tumor-infiltrating immune cells in a quantifiable way, tumors were excised at designated times and minced on ice. The dissected tumor tissues were digested by a 60-minute incubation with 400 units/mL collagenase type V (Sigma, St. Louis, MO) and 0.4 mg/mL DNase I (Roche, Basel, Switzerland) at 37°C to prepare a single cell suspension. Digested cells were stained with Allophycocyanin conjugated rat antimouse CD45 (BD Biosciences PharMingen, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated rat antimouse CD4 or CD8 (Southern Biotechnology Associates, Inc., Birmingham, AL) antibodies. Flow cytometry was performed using a DakoCytomation CyAn (DakoCytomation, Fort Collins, CO). The percentage of tumor-infiltrating leukocytes (based on the total number of digested cells) was defined. The percentage of $CD4^+$ or $CD8^+$ cells was determined as a fraction of the total $CD45^+$ cells.

Expression of H-2, I-A/E Antigens, and Fas on AB12. To evaluate whether the sTGF-βR affects cell surface expression of MHC class I ($H\text{-}2K^d$ and $H\text{-}2D^d$), MHC class II (I-A and I-E), and Fas on AB12 tumor *in vivo*, which would enhance the tumor interaction with T-cell–mediated antitumor responses, digested AB12 tumor cells (as described above) were stained with APC-conjugated rat antimouse CD45, FITC-conjugated mouse antimouse MHC class I (34-1-2S; eBioscience, San Diego, CA), phycoerythrin (PE)-conjugated rat antimouse MHC class II (M5/114.15.2; eBioscience), and PE-conjugated hamster antimouse Fas (BD Biosciences PharMingen) antibodies. Cultured AB12 cells treated with either 1,000 ng/mL control IgG2a or 1,000 ng/mL sTGF-βR for 24 hours were stained with the FITC-conjugated mouse antimouse MHC class I, PE-conjugated rat antimouse MHC class II, and PE-conjugated hamster antimouse Fas antibodies. Flow cytometry analysis was performed using a DakoCytomation CyAn.

***In vivo* Depletion of $CD4^+$ and $CD8^+$ T Cells.** To deplete specific immune effector cell subsets before and during treatments in this model, BALB/c mice received intraperitoneal injections of 200 μg of purified monoclonal antibodies purified from the anti-CD4 hybridoma GK1.5 or the anti-CD8 hybridoma 53-6.7 (obtained from the American Type Culture Collection). Injections were administered 3 days and 1 day before

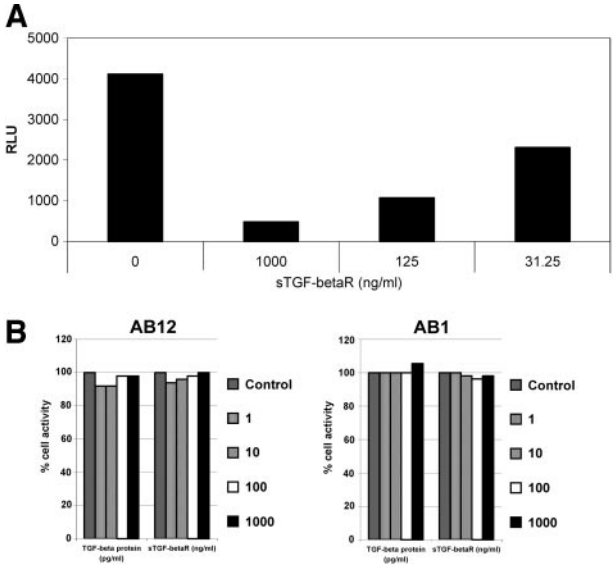

Fig. 1 Effects of TGF-β and sTGF-βR on murine mesothelioma cells. *A.* The addition of 500 pg/mL of exogenous TGF-β1 to MLECs in which a luciferase gene was driven by a TGF-β–inducible promoter (PAI-1) induced high levels of luciferase activity. Addition of 1,000, 125, and 31.25 ng/mL sTGF-βR markedly inhibited TGF-β–induced luciferase activity. *B.* AB12 or AB1 cells were seeded in 96-well plates at a density of 2×10^3 cells/well. Twenty-four hours after cell seeding, fresh media were added with various concentrations of sTGF-βR protein (1, 10, 100, and 1,000 ng/mL) or TGF-β1 protein (1, 10, 100, and 1000 pg/mL) in DMEM with 1% FBS. After 24 and 48 hours of incubation, the cancer cell viability was assessed by using the MTS assay. Neither sTGF-βR nor TGF-β1 protein had any significant effects on the growth of either of the cell lines.

inoculation with tumor cells. Thereafter, a maintenance dose of antibody was injected intraperitoneally every 7 days throughout the entire experimental period to ensure depletion of targeted cell type. $CD4^+$ and $CD8^+$ T-cell depletion was confirmed by flow cytometry of splenic suspensions at the time of tumor injection and weekly afterward (data not shown).

Evaluation of Effector Function (Winn Assay). Winn assays were performed as described previously (46). Splenocytes were isolated, and $CD8^+$ T lymphocytes were purified using the MACs system (Miltenyi Biotec, Auburn, CA). Briefly, splenocytes were reacted with anti-$CD8^+$ microbeads and run through a magnet-mounted column (Miltenyi Biotec) that allowed the $CD8^-$ cells to pass but bound the bead-labeled $CD8^+$ cells, which were then eluted. This cell population contained >90% $CD8^+$ cells by flow cytometry (data not shown). The $CD8^+$ T-lymphocyte–enriched population from normal, tumor-sensitized, or tumor-sensitized and treated mice was admixed with viable AB12 tumor cells at a ratio of 3 purified $CD8^+$ splenocytes for each tumor cell, and the mixture was inoculated into the flanks of naïve BALB/c mice. Each mixture thus contained 0.5×10^6 tumor cells and 1.5×10^6 $CD8^+$ T cells. This ratio has previously been determined to be optimal for detecting positive and negative effects (48). Tumor growth was measured after 1 week and expressed as the mean ± SE of at least five mice per group.

Statistical Analysis. Unless otherwise noticed, data comparing differences between two groups were assessed using unpaired Student's *t* test. ANOVA with *post hoc* testing was used for multiple comparisons. Differences were considered significant when P was <0.05. Statistical analysis was conducted using the StatView 5.0 for Windows program.

RESULTS

***In vitro* Studies with Soluble TGF-β Type II Receptor and TGF-β1 Protein.** Before beginning experiments in our animal models, we first evaluated the effects of our reagents using *in vitro* assays. To confirm that our sTGF-βR was able to effectively block the effects of TGF-β, we studied MLECs in which a luciferase gene was driven by a TGF-β–inducible promoter (PAI-1). As expected, the addition of 500 pg/mL of exogenous TGF-β1 induced high levels luciferase activity (4114 relative light units) in the MLECs. This activity was markedly inhibited in a dose-dependent fashion by the addition of serial concentrations of sTGF-βR (1,000, 125, and 31.25 ng/mL) to MLECs along with 500 pg/mL of exogenous TGF-β1 (Fig. 1*A*).

We next evaluated the effect of sTGF-βR and TGF-β1 protein on the growth of two of our mesothelioma tumor cell lines. AB12 and AB1 cells were grown in the presence of either 1, 10, 100, and 1,000 ng/mL sTGF-βR protein or 1, 10, 100, and 1,000 pg/mL TGF-β1 protein for 24 and 48 hours. Neither sTGF-βR nor TGF-β1 protein had any significant effects on the growth of either of the cell lines (Fig. 1*B*).

To determine whether this lack of responsiveness was due to a loss of or defect in signaling of TGF-β receptors, AB12 and AB1 lines (along with mouse 3T3 cells and MLECs as controls) were exposed to 10 ng/mL TGF-β and analyzed for the expected TGF-β receptor-dependent phosphorylation of Smad2. As shown in Fig. 2, TGF-β led to rapid phosphorylation of Smad2 in both control lines, as well as in the AB12 and AB1 tumor lines. These data indicate that the lack of effect of TGF-β on the growth of the tumor cells was not due to the loss of TGF-β

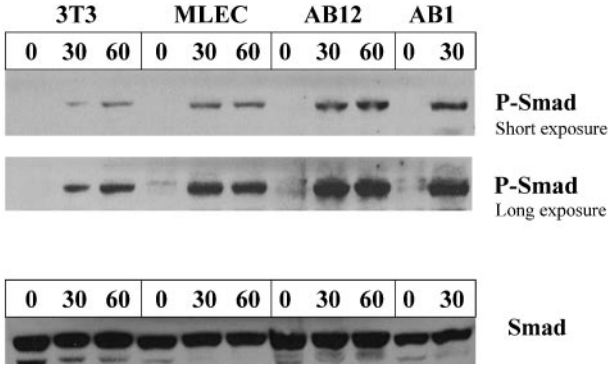

Fig. 2 TGF-β induces the phosphorylation of Smad2 in control, AB12, and AB1 cells. AB12 and AB1 mesothelioma lines (along with mouse 3T3 cells and MLECs as controls) were exposed to 10 ng/mL TGF-β1 for 30 or 60 minutes and lysed, and equal amounts of protein were analyzed by immunoblotting using a specific antibody for phosphorylated Smad2 (*top two panels*). Blots were stripped and reblotted with antibody against nonphosphorylated (total Smad2) to ensure equal loading (*bottom panel*). TGF-β1 led to rapid phosphorylation of Smad2 in both control lines, as well as in the AB12 and AB1 tumor lines.

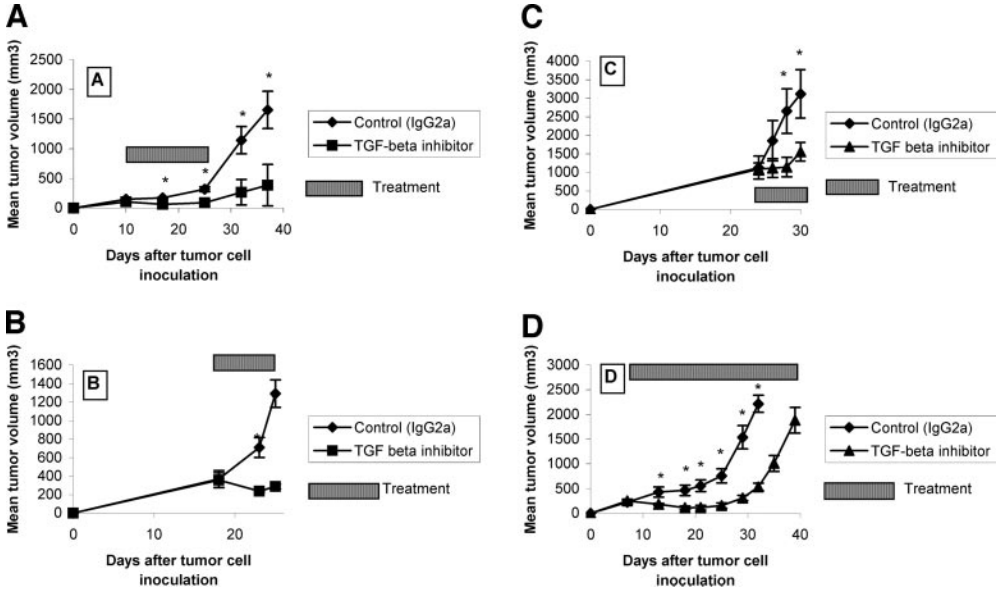

Fig. 3 sTGF-βR inhibits the growth of established TGF-β–producing AB12 tumors. *A.* Groups of mice ($n = 5$) bearing small AB12 tumors (150 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for six doses (*hatched bar*). Tumor volume was measured over time. Values are mean volume (mm^3). *Error bars* represent SE. Tumors treated with sTGF-βR were significantly smaller than tumors treated with control IgG2a on days 18, 25, 32, and 38 (∗, $P < 0.05$). The slope of tumor growth remained lower in the inhibitor-treated animals for the duration of the experiment. *B.* Groups of mice ($n = 5$) bearing large AB12 tumors (350 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for three doses (*hatched bar*). Tumors treated with sTGF-βR were significantly smaller than tumors treated with control IgG2a on days 21 and 24 (∗, $P < 0.001$). *C.* Groups of mice ($n = 5$) bearing very large AB12 tumors (1,000 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for three doses (*hatched bar*). Tumors treated with sTGF-βR were significantly smaller than tumors treated with control IgG2a on days 28 and 30 (∗, $P < 0.05$). *D.* Groups of mice ($n = 5$) bearing small AB12 tumors (150 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for a more extended period (nine doses; *hatched bar*). Tumor volume was measured over time. Values are mean volume (mm^3). *Error bars* represent SE. Tumors treated with sTGF-βR were significantly smaller than tumors treated with control IgG2a (∗, $P < 0.01$). However, even if treatment with sTGF-βR was continued throughout the experiment, the tumors were not eradicated.

receptors or defects in proximal TGF-β signaling but rather to some other abnormality of proliferative control.

Soluble TGF-β Type II Receptor Inhibits the Growth of Small (150 mm^3) and Large (350 or 1,000 mm^3) Established AB12 Tumors. Studies were next conducted to determine the effects of sTGF-βR on the growth of established tumors derived from the TGF-β–producing mesothelioma cell line AB12. Mice ($n = 5$ mice per group) received injection in the flank with mesothelioma cells, and the tumors were allowed to grow to small size (approximately 150 mm^3, 10 days after tumor cell inoculation). At this time, 1.0 mg/kg sTGF-βR or 1.0 mg/kg IgG2A (control) was administered to mice (via the intraperitoneal route) every 3 days for a total of six doses. As shown in Fig. 3*A*, sTGF-βR inhibited the growth of small AB12 tumors significantly **($P < 0.05$)** at the conclusion of treatment and for up to 38 days. Interestingly, the slope of tumor growth remained lower in the inhibitor-treated animals for the duration of the experiment. This experiment was repeated multiple times with similar results (*i.e.*, see control data in Fig. 6*A* and *B*).

To evaluate the effects in more bulky tumors, treatment was delayed until the tumors reached a size of approximately either 350 or 1,000 mm^3, at 18 and 25 days after tumor cell inoculation, respectively. The sTGF-βR significantly decreased the growth of both 350 and 1,000 mm^3 AB12 tumors ($P < 0.001$ and $P < 0.05$, respectively; Fig. 3*B* and *C*).

Although markedly inhibited, even small tumors were not eradicated by six doses of the sTGF-βR treatment. To determine whether longer treatment would continue to maintain tumor inhibition or even augment effects, an experiment was repeated using nine doses of sTGF-βR or control IgG2a to treat small (150 mm^3) tumors. As shown in Fig. 3*D*, prolonged treatment did not eliminate tumors, and in fact, the tumors seemed to slowly escape from the growth-suppressive effects of TGF-β inhibition.

Soluble TGF-β Type II Receptor Has Significant Antitumor Effects on Another TGF-β–Producing Cell Line, AC29, but Minimal Effects on the Growth of an Established Non-TGF-β–Producing Tumor Cell Line, AB1. To evaluate whether there were antitumor effects on another TGF-β–producing line in a different mouse strain, a similar experiment was repeated. Mice bearing AC29 tumors that were approximately 250 mm^3 in size (12 days after tumor cell inoculation) were treated with six doses of sTGF-βR or control. Similar to the effects seen in the AB12 model, there were significant ($P < 0.05$) antitumor effects in AC29 tumor-bearing animals. (Fig. 4*A*). To test the effect of sTGF-βR on the growth of established tumors derived from a mesothelioma line that did not produce TGF-β, a similar experiment was performed using the AB1 tumor cell line. Tumors were treated when they reached a size of approximately 100 mm^3 in tumor volume (13 days after tumor cell inoculation). In contrast to the effects seen with AB12 or AC29 tumors, however, sTGF-βR had minimal ($P >$

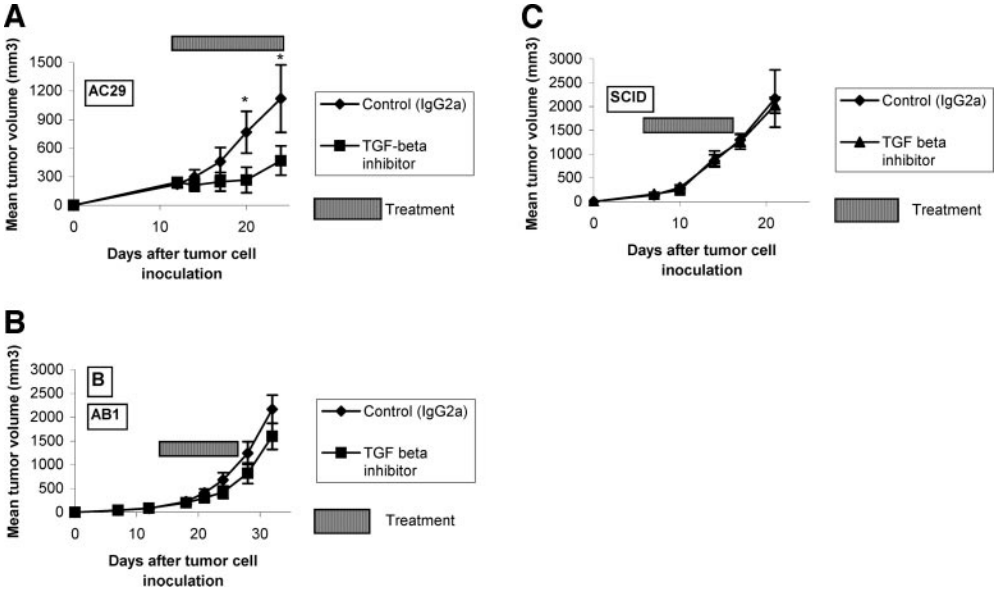

Fig. 4 Effects of sTGF-βR on mesothelioma tumor growth in three mouse models. *A.* Soluble TGF-βR inhibits the growth of another TGF-β–producing tumor (AC29). Groups of mice ($n = 5$) bearing large AC29 tumors (250 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for six doses. Because this tumor line showed large amounts of variability in size, antitumor effects were analyzed by calculating the fold change of individual tumor volumes using the following formula: fold change of individual tumor volume = individual tumor volume at each experimental time point/Individual tumor volume at beginning of treatment. The mean value of tumor volume fold change in sTGF-βR–treated mice (mean ± SE, 0.9 ± 0.2) was significantly smaller than that in control IgG2a-treated mice (mean ± SE, 3.2 ± 0.6; ∗, $P < 0.02$). *B.* Soluble TGF-βR has minimal effects on the growth of non-TGF-β–producing AB1 tumors. Groups of mice ($n = 5$) bearing small AB1 tumor (150 mm^3) were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for six doses. Tumor volume was measured over time. Values are mean volume (mm^3). *Error bars* represent SE. There were no significant differences between tumors treated with sTGF-βR and tumors treated with control IgG2a ($P > 0.05$ at all time points). *C.* Efficacy of sTGF-βR on AB12 tumors is lost in SCID mice. Immunodeficient SCID mice ($n = 5$) bearing small AB12 tumors were treated intraperitoneally with control IgG2a or sTGF-βR every 3 days for six doses. Tumor volume was measured over time. Values are mean volume (mm^3). *Error bars* represent SE. There were no significant differences between tumors treated with sTGF-βR and tumors treated with control IgG2a ($P > 0.05$ at all time points).

0.05) effects on the growth of AB1 flank tumors (Fig. 4*B*). This experiment was repeated once with similar results.

Tumor Growth Suppression by Soluble TGF-β Type II Receptor in Tumor-Bearing Mice Was Abolished in SCID Mice Models. There are a number of possible mechanisms by which TGF-β blockade might reduce tumor growth including direct antiproliferative effects on tumor cells (*i.e.*, TGF-β could be an autocrine growth factor) or by blocking angiogenesis (18, 34). However, given the lack of effect of TGF-β on the tumor cells *in vitro*, we suspected that the primary mechanism of effect was due to reduction of TGF-β–induced tumor immunosuppression. To evaluate this, studies were repeated using the AB12 cell line, but in immunodeficient SCID mice. Treatment of established tumors with sTGF-βR in SCID mice using the same protocol as described above resulted in complete loss of therapeutic effect (Fig. 4*C*). These experiments indicate that antitumor effects of sTGF-βR in this model are dependent on B or T lymphocytes and are not due to direct effects on the growth of tumor cells.

Treatment with Soluble TGF-β Type II Receptor Allows $CD8^+$ T-Cell Accumulation in the AB12 Tumors but Does Not Affect MHC Class I and II and Fas Expression on AB12 Cells. To evaluate which types of immune effector cells might be important, animals bearing AB12 tumors (350 mm^3) were treated with IgG2a (control) or sTGF-βR (treatment) at 1.0 mg/kg for three doses. Two days after the last treatment, the tumors were removed. Control-treated tumors weighed 1,000 ± 200 mg. Treated tumors were significantly ($P < 0.05$) smaller (200 mg ± 15 mg; data not shown). The tumors were then digested, and the cells were subjected to flow cytometry. The average percentage of digested cells positive for the pan-leukocyte marker CD45 was slightly higher in the treated tumors (20%) than in the control tumors (14%). However, the percentage of these leukocytes that stained positively for CD8 was twice as high in the tumors treated with sTGF-βR as in the control tumors ($P < 0.05$; Fig. 5). There were only small numbers of intratumoral $CD4^+$ T cells in either treatment group. Soluble TGF-βR treatment thus significantly increased the number of $CD8^+$ T cells within the tumor. Despite the increased number of $CD8^+$ T cells that were observed in sTGF-βR–treated AB12 tumors, treatment with sTGF-βR did not result in any increase in the MHC class I and II and Fas expression on AB12 cells *in vitro* (Table 1) or *in vivo* (data not shown).

Tumor Growth Suppression by Soluble TGF-β Type II Receptor in Tumor-Bearing Mice Persisted after $CD4^+$ T-Cell Depletion but Was Abolished in $CD8^+$ T-Cell–Depleted Mice. To further define a functional role of $CD4^+$ and $CD8^+$ T cells on antitumor effects seen in this model, *in vivo* depletion of $CD4^+$ and $CD8^+$ T cells was performed. Mice were treated with sTGF-βR as described above, with and without depletion

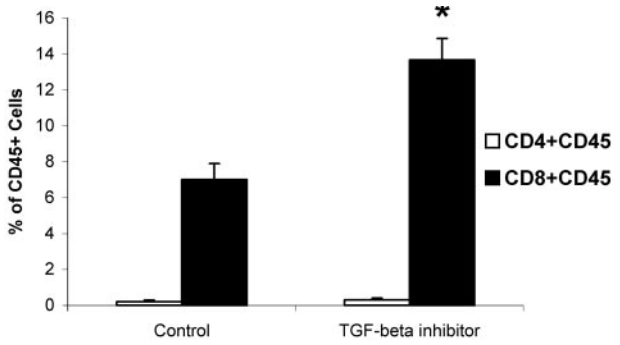

Fig. 5 $CD8^+$ T cells accumulate in AB12 tumors treated with sTGF-βR. Animals bearing AB12 tumors (350 mm^3; $n = 3$) were treated intraperitoneally with IgG2a (*Control*) or sTGF-βR at 1.0 mg/kg for three doses. Two days after the last treatment, the tumors were digested with collagenase and DNase. The cells were subjected to two-color flow cytometry analysis. CD45 and CD8 double-positive–stained cells were regarded as $CD8^+$ T cells. CD45 and CD4 double-positive–stained cells were regarded as $CD4^+$ T cells. Values are mean percentage of the $CD45^+$ cells. *Error bars* represent SE. The percentage of $CD8^+$ T cells was twice as high in the tumors treated with sTGF-βR as in the control tumors (∗, $P < 0.05$). There was minimal accumulation of $CD4^+$ T cells with or without therapy.

Table 1 Expression levels of MHC class I, MHC class II, and Fas with and without TGF-β blockade

	MHC class I	MHC class II	Fas
Control cells (IgG2a)	94.8 (5.8)	4.9 (0.8)	13.4 (1.1)
sTGF-β inhibitor-treated cells	90.7 (4.3)	4.1 (0.8)	11 (1.0)

NOTE. AB12 cells in culture were treated with control compound (IgG2a) or soluble TGF-β inhibitor. Cells were then analyzed in triplicate using fluorescence-activated cell sorting for expression levels of MHC class I, MHC class II, and Fas. Data are expressed as the percentage of cells expressing each antigen. The mean fluorescence intensity is in parentheses. There were no significant differences between treatment groups.

of the $CD4^+$ and $CD8^+$ T cells using anti-CD4 and anti-CD8 antibodies as described in Materials and Methods.

The data from the $CD4^+$ T-cell depletion study is shown in Fig. 6*A*. Depletion of $CD4^+$ T cells did not have any effect on tumor size. As described above, tumor-bearing mice treated with sTGF-βR on day 10 (Fig. 6*A*, *D10 TG*) had significantly ($P < 0.05$) smaller tumors than those treated with IgG2a (*Control*): The mean tumor volume at day 18 was 450 mm^3 in the control group *versus* 110 mm^3 in the sTGF-βR–treated group. Soluble TGF-βR inhibition thus resulted in a 77% reduction of size ($P < 0.05$). Treatment with sTGF-βR was similarly effective in the $CD4^+$ T-cell–depleted animals. The average tumor size in the sTGF-βR–treated animals after $CD4^+$ T-cell depletion was significantly smaller ($P < 0.05$; 225 *versus* 470 mm^3) than that in the $CD4^+$ T-cell–depleted mice that did not receive sTGF-βR (52% reduction in size). There was no significant difference in tumor size in the sTGF-βR–treated animals, regardless of whether $CD4^+$ T cells were depleted. These data suggest that antitumor effects by treatment with sTGF-βR were not dependent on $CD4^+$ T-cell activity.

In the $CD8^+$ T-cell depletion study (Fig. 6*B*), the mean tumor volume at day 17 was 250 mm^3 in the control group *versus* 100 mm^3 in the sTGF-βR–treated group. Soluble TGF-βR inhibition thus resulted in a 60% reduction of size ($P < 0.01$). As shown in Fig. 6*B*, $CD8^+$ T-cell depletion in non-sTGF-β–treated but tumor-bearing control mice led to markedly accelerated tumor growth (*anti-CD8 Control*). This finding is consistent with our detection of cytotoxic T lymphocyte (CTL) activity in untreated animals (see below). Tumors in $CD8^+$ T-cell–depleted mice treated with sTGF-βR (*D10 TG anti-CD8*) also grew faster than control tumors, with the tumor size

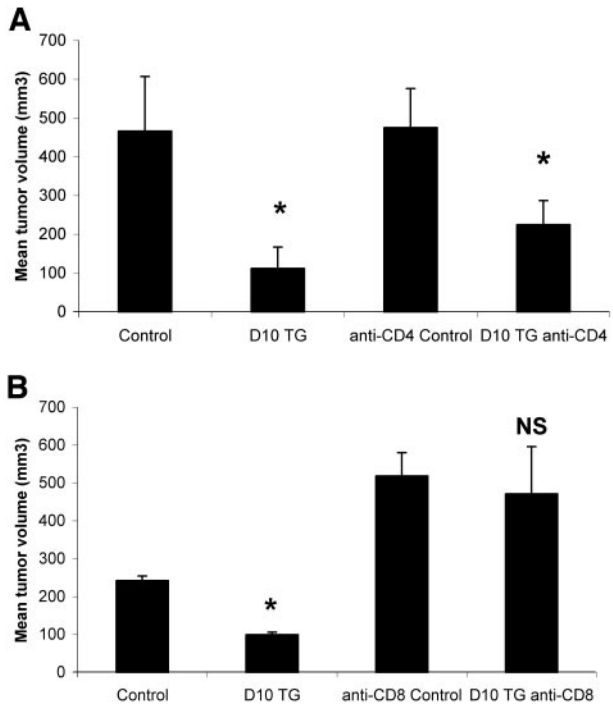

Fig. 6 Effects of $CD4^+$ or $CD8^+$ T-cell depletion on the efficacy of sTGF-βR in tumor-bearing mice. *A*, effects of $CD4^+$ T-cell depletion. Animals bearing AB12 tumors ($n = 5$) were treated intraperitoneally with IgG2a alone (*Control*), sTGF-βR at 1.0 mg/kg for six doses (*D10 TG*), IgG2a plus anti-CD4 antibody (*anti-CD4 Control*), or sTGF-βR and anti-CD4 antibody (*D10 TG anti-CD4*). Tumor size was measured after 8 days. Values are mean volume (mm^3). *Error bars* represent SE. Depletion of $CD4^+$ T cells had no effect on tumor size. Tumor-bearing mice treated with sTGF-βR on day 10 (*D10 TG*) had significantly smaller tumors than those treated with IgG2a (*Control*). $CD4^+$ T-cell–depleted mice also had significantly smaller tumors than control animals after sTGF-βR inhibition (∗, $P < 0.05$). *B*, effects of CD8+ T-cell depletion. Animals bearing AB12 tumors ($n = 5$) were treated intraperitoneally with IgG2a alone (*Control*), sTGF-βR at 1.0 mg/kg for six doses (*D10 TG*), IgG2a plus anti-CD8 antibody (*anti-CD8 Control*), or sTGF-βR and anti-CD8 antibody (*D10 TG anti-CD8*). Tumor size was measured after 7 days. Values are mean volume (mm^3). *Error bars* represent SE. Tumor-bearing mice treated with sTGF-βR on day 10 (*D10 TG*) had significantly smaller tumors than those treated with IgG2a (*Control*; ∗, $P < 0.01$). Depletion of $CD8^+$ T cells resulted in more rapid growth of tumors. However, the mean tumor volume at day 17 (510 mm^3) in the $CD8^+$ T-cell–depleted mice control group (*anti-CD8 Control*) was not statistically different (470 mm^3) in the $CD8^+$ T-cell–depleted mice treated with sTGF-βR (*D10 TG anti-CD8*; $n = 5$). Soluble TGF-βR after CD8 depletion resulted in only an 8% reduction in size. Thus, $CD8^+$ T cells are required for the TGF-β effect. *NS*, $P > 0.05$.

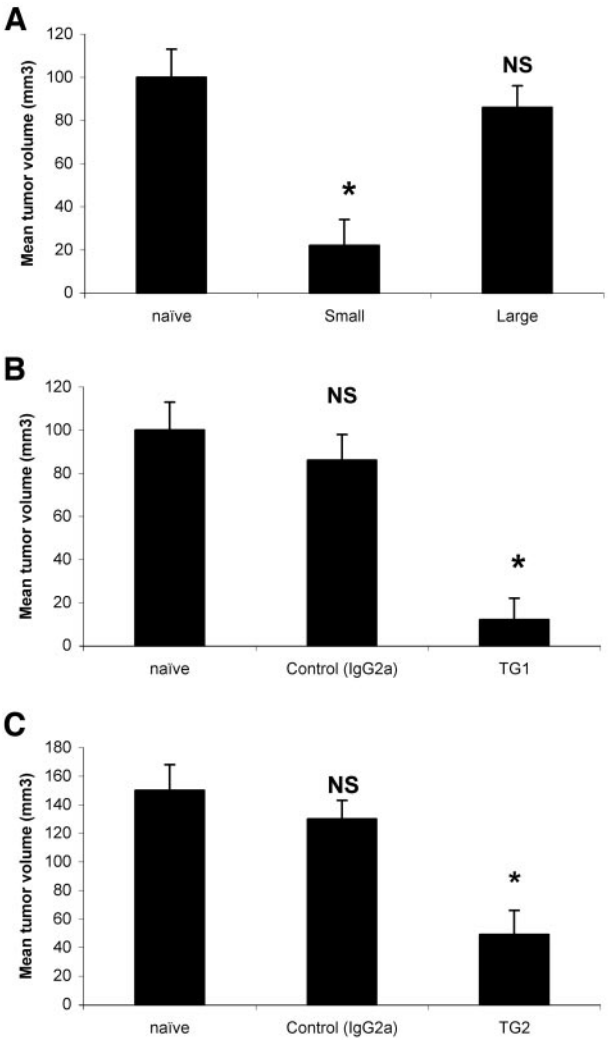

Fig. 7 Antitumor $CD8^+$ T cell activity assessed by Winn assays in treated and nontreated animals. *A*, spontaneous CTL activity in the splenocytes of AB12 tumor-bearing mice. Winn assays were performed. $CD8^+$ T cells were isolated from the spleens of non–tumor-bearing animals or from animals bearing small (100 mm^3) or larger (350 mm^3) tumors. These splenocytes were mixed with fresh AB12 tumor cells in a ratio of 3 $CD8^+$ T cells to 1 tumor cell, and the mixtures were injected into the flanks of naive BALB/c mice ($n = 5$). Tumors were then allowed to grow for 1 week. Tumor cells mixed with $CD8^+$ T cells from non–tumor-bearing animals grew to a size of approximately 100 mm^3. Tumor cells mixed with $CD8^+$ T cells from animals bearing small tumors (*Small*) grew to only 30 mm^3, a 68% inhibition of the growth ($*$ $P < 0.01$). However, the mixture of AB12 cells and $CD8^+$ T cells from animals bearing large tumors (*Large*) grew to the same size as the mixture of AB12 cells and $CD8^+$ T cells from naive animals. Values are mean volume (mm^3). *Error bars* represent SE. These data indicate that CTL activity was present in the spleens of animals with small tumors but that this activity was lost as the tumors increased in size. *B.* Soluble TGF-βR treatment affects CTL activity in small tumors. Animals with established small tumors (150 mm^3) were treated with control IgG2a (control) or sTGF-βR (*TG1*) for four doses. At this time, tumors from control animals were about 350 mm^3 in size, whereas treated tumors stayed at 150 mm^3 in size. Winn assays were performed as described above using $CD8^+$ T cells derived from splenocytes from each treatment group. Tumor cells mixed with $CD8^+$ T cells from naive animals grew to an average size of 100 mm^3 ($n = 5$), and tumor cells mixed with $CD8^+$ T cells from animals treated with IgG2a-bearing tumors grew to only slightly less than that in the $CD8^+$ T-cell–depleted mice controls treated with IgG2a (*anti-CD8 Control*). The mean tumor volume at day 17 was 510 mm^3 in the $CD8^+$ T-cell–depleted mice control group *versus* 470 mm^3 in the $CD8^+$ T-cell–depleted mice treated with sTGF-βR. The effect of sTGF-βR after CD8 depletion thus resulted in only an 8% reduction in size ($P > 0.05$). These data show that sTGF-βR exerts the majority of its antitumor effects through $CD8^+$ T-cell–dependent mechanisms.

Soluble TGF-β Type II Receptor Treatment Affects the Activity of Cytotoxic T Lymphocytes. To confirm the importance of $CD8^+$ CTLs in our observations, we assayed for splenic CTLs using an *in vivo* tumor neutralization assay (Winn assay). This assay allows for assessment of antitumor activity in an *in vivo* situation and without the need for *ex vivo* stimulation.

We first established the activity level and time course of antitumor CTLs in untreated animals. $CD8^+$ T cells were isolated from the spleens of non–tumor-bearing animals or from animals bearing small (100 mm^3) or large (350 mm^3) tumors. These splenocytes were then mixed with fresh AB12 tumor cells in a ratio of 3 $CD8^+$ T cells to 1 tumor cell, and the mixtures were injected into the flanks of naive BALB/c mice. This ratio was established from preliminary dose titration studies establishing that both positive and negative effects could be seen at this ratio. Tumors were allowed to grow for 1 week.

Splenocytes from non–tumor-bearing animals showed no antitumor cytolytic effects; injection of tumor cells alone or tumor cells mixed with $CD8^+$ T cells from naive animals grew to a size of approximately 100 mm^3 in 1 week (data not shown). As shown in Fig. 7*A*, tumor cells mixed with $CD8^+$ T cells from animals bearing small tumors (*Small*) at a lymphocyte to tumor ratio of 3:1 grew to only 30 mm^3. Compared with tumors mixed with naive $CD8^+$ T cells (*naïve*), this represented a 68% decrease of the growth ($P < 0.01$). In contrast, this antitumor cytolytic activity was lost in $CD8^+$ T cells from animals bearing large tumors (*Large*), in which tumors grew to the same size as did tumor cells mixed with naive $CD8^+$ T cells. Thus, in animals bearing small tumors, some spontaneous antitumor $CD8^+$ T-cell activity is induced by the AB12 tumor cells. This

the same size. However, the tumor volumes of mixtures of AB12 cells and $CD8^+$ T cells from animals treated with sTGF-βR ($n = 5$) was significantly smaller than the tumor volume of mixture of AB12 cells and $CD8^+$ T cells from naive animals (*naïve*; $*$, $P < 0.01$), indicating CTL activity. Values are mean volume (mm^3). *Error bars* represent SE. *C.* Soluble TGF-βR treatment affects CTLs activity in large tumors. Animals with established large tumors (300 mm^3) were treated with control IgG2a (control) or sTGF-βR (*TG1*) for four doses. At this time, tumors from control animals were about 1,200 mm^3 in size, whereas treated tumors stayed at 300 mm^3 in size. Winn assays were performed as described above using $CD8^+$ T cells derived from splenocytes from each treatment group. Tumor cells mixed with $CD8^+$ T cells from naive animals grew to an average size of 150 mm^3 (*naïve*; $n = 5$). Tumor cells mixed with $CD8^+$ T cells from animals treated with IgG2a grew to same size (control) as tumor volume of AB12 cells and naive $CD8^+$ T cells mixture (*naïve*; $n = 5$). The tumor volume of AB12 cells and $CD8^+$ T cells from animals treated with sTGF-βR (*TG2*; $n = 5$) was significantly smaller than the tumor volume of AB12 cells and $CD8^+$ T cells from naive animals (*naïve*; $* P < 0.05$), indicating CTL activity. Values are mean volume (mm^3). *Error bars* represent SE. *NS*, $P > 0.05$.

is consistent with the blockade data above (Fig. 6*B*) showing that depletion of $CD8^+$ T cells leads to faster tumor growth. Although these CTLs probably function to slow the initial growth of tumor cells, they are not able to eradicate the tumors, and interestingly, as the tumor increases in size, this $CD8^+$ T-cell activity is lost.

Given this baseline, we next studied the effect of sTGF-βR treatment on $CD8^+$ T-cell activity. We first determined whether the CTL activity that was spontaneously induced in animals bearing small AB12 tumors could be preserved by sTGF-βR treatment. Animals with established small tumors (150 mm^3) were treated with control IgG2a or sTGF-βR for four doses. After this treatment, the tumors grew to a size of about 350 mm^3 in the control animals, whereas they were reduced to about 100 mm^3 in treated animals. Splenocytes were isolated, and $CD8^+$ T cells were purified from these animals, as well as from a non–tumor-bearing naive animal. As shown in Fig. 7*B*, tumor cells mixed with $CD8^+$ T cells from naive animals grew to an average size of 100 mm^3. As above, $CD8^+$ T cells from animals treated with IgG2a (*Control*) bearing tumors of about 350 mm^3 had no antitumor cytolytic activity. However, $CD8^+$ T cells from animals bearing the small tumors treated with sTGF-βR (*TG1*), showed *persistent* antitumor cytolytic activity. The growth of tumor cells was inhibited by 90% ($P < 0.01$) as compared with the tumor volume of the naive group. These data indicate that sTGF-βR could prevent loss of CTL activity that was associated with tumor growth.

Despite the fact that we could not detect splenic CTL activity in large AB12 tumors (350 mm^3), treatment of animals bearing these large-sized tumors was effective. We next asked whether TGF-β blockade in large AB12 tumors could actually restore CTL activity. Animals with established large tumors (350 mm^3) were treated with control IgG2a or sTGF-βR for four doses. At this time, tumor size in the control animals was about 1,200 mm^3. Treated tumors remained stable in size at about 350 mm^3. Splenocytes were isolated, and $CD8^+$ T cells were purified from these animals, as well as from a non–tumor-bearing naive animal. The $CD8^+$ T cells were mixed with fresh AB12 cells and injected into the flanks of naive animals, where tumor growth was assessed. As shown in Fig. 7*C*, tumor cells mixed with $CD8^+$ T cells from naive animals grew to an average size of 150 mm^3. As above, $CD8^+$ T cells from animals bearing tumors treated with IgG2a (*Control*) had no antitumor cytolytic activity. However, $CD8^+$ T cells from animals bearing large tumors treated with sTGF-βR (*TG2*) showed significant antitumor cytolytic activity. The growth of tumor cells was inhibited by 67% ($P < 0.05$) as compared with the tumor volume of naive animals. These data indicate that sTGF-βR could not only prevent the loss of CTL but was also able to regenerate CTL activity after it had been lost.

DISCUSSION

The data from this study demonstrate that sTGF-βR was effective in inhibiting the growth of two established experimental murine MMs that produced high levels of TFG-β (AB12 and AC29) but did not suppress the growth of a murine mesothelioma that produced low levels of TGF-β (AB1). Strong inhibitory effects were seen in both small (100 mm^3) and large (350 and 1,000 mm^3) AB12 tumors. These data suggest that blockade of TGF-β secreted by the tumor itself (rather than associated inflammatory cells) is most important in causing tumor growth inhibition, but it is possible that other types of tumors that might induce stronger stromal cell TGF-β production might also be susceptible to sTGF-βR inhibition.

Given the multiple ways in which TGF-β can exert its biological effects, it was of interest to explore the mechanisms responsible for our observations. We initially determined whether TGF-β served as an autocrine growth factor or growth inhibitor for MM cells. Marzo *et al.* (34) showed that application of TGF-β2 antisense oligonucleotides inhibited the *in vitro* proliferation of a murine mesothelioma cell line (AC29). This scenario seems unlikely in our experiments because the AB12 mesothelioma cells were resistant to any growth-stimulatory or -inhibitory effects of added TGF-β or sTGF-βR in culture (Fig. 1). Loss of growth responsiveness to TGF-β is extremely common in tumor cells (49). Interestingly, this failure of TGF-β to inhibit cell growth was not due to a loss of TGF-β receptors or defects in the early portions of TGF-β signaling. In both tumor lines tested, TGF-β induced strong phosphorylation of the proximal downstream signal-transducing molecule, Smad2 (see Fig. 2). This is not surprising because it has been reported that loss of TGF-β or Smad functions accounts for a relatively small proportion of cases in which tumor cells become resistant to TGF-β–mediated cytostasis or apoptosis. More often, this inhibition occurs at a level downstream of the core TGF-β–signaling pathway (9). We also saw no effects of TGF-β blockade on MHC class I, MHC class II, or Fas expression in tumor cells in culture (Table 1) or *in vivo*. This lack of direct antitumor activity was confirmed by *in vivo* experiments showing loss of all inhibitory activity of the sTGF-βR in immunodeficient SCID mice. These SCID mice experiments also make the direct inhibition of TGF-β–induced angiogenesis or direct alterations in peritumoral stroma unlikely explanations for our results, although it is formally possible that growth factors or angiogenic factors produced by B or T cells that are induced by TGF-β play a role in tumor development.

Instead, our SCID mice data suggested that the immunologic effects of TGF-β blockade were key, implicating B or T lymphocytes. Very few $CD4^+$ T cells were identified within the tumor (Fig. 5), and depletion studies showed no loss of efficacy of sTGF-βR after depletion of these cells. A number of our experiments suggested that $CD8^+$ T cells were of central importance. In the AB12 tumor system, the animals initially appear to have a suppressive but not curative immune response against the tumor. This was demonstrated by the initial slow growth of the tumor and the presence of $CD8^+$ T cells with antitumor cytolytic activity as measured in the Winn assay (Fig. 7*A*). The functional importance of these CTLs is consistent with the fact that the AB12 tumors grow at a much faster rate in SCID mice (Fig. 4*C*) and in animals depleted of $CD8^+$ T cells (Fig. 6*B*). As the tumor gets larger, however, this endogenous CTL activity is lost completely from the spleen, perhaps explaining the more rapid growth of the tumors at this time.

Soluble TGF-βR treatment of small established tumors was associated with the infiltration of the tumor with $CD8^+$ T cells (Fig. 5) and the continued activity of CTLs (Fig. 7*B*). At a time when untreated animals had very little antitumor activity in their

splenic $CD8^+$ T cells, $CD8^+$ T cells from the sTGF-βR–treated animals almost completely inhibited tumor growth in a Winn assay (Fig. 7*B*). The $CD8^+$ T-cell depletion studies confirm the functional importance of these lymphocytes in the sTGF-βR effect. In intact mice, sTGF-βR inhibited tumor growth by 60%, whereas after $CD8^+$ T-cell depletion, sTGF-βR inhibited tumor growth by only 8% (Fig. 5). Although we showed minimal CTL activity in control animals bearing large AB12 tumors, strong CTL activity by Winn assay was observed in animals bearing large tumors that were treated with sTGF-βR (Fig. 7*C*).

In this study, we grew mesothelioma tumors in the flanks of animals rather than in the pleural or peritoneal spaces. Although we have not previously seen major differences in therapeutic responses to agents such as adenoviral vector encoding the IFN-β gene (Ad.IFNβ) between these orthotopic and nonorthotopic locations,[4] there are some differences in the immunologic environment, and this issue is currently being studied in our lab.

It is interesting to speculate how these data might apply to the use of sTGF-βR as TGF-β inhibitors in cancer therapy. First, given the fact that lack of TGF-β in "knockout mice" has been associated with an inflammatory phenotype (reviewed in Ruzek *et al.*, ref. 51), there are concerns about possible side effects. At least two studies have suggested that this may not be a serious problem with the use of such inhibitors. Yang *et al.* (50) showed that lifetime exposure to a soluble TGF-β anatagonist protected mice against metastasis without adverse side effects. Ruzek *et al.* (51) showed minimal effects on immune parameters after chronic anti–TGF-β monoclonal antibody administration to normal mice. Although the ability of TGF-β blockers to induce inflammatory side effects may not be limiting, the use of such inhibitors in a chemopreventive mode should probably be undertaken with caution. Inhibition of nontransformed epithelial cell growth is an important property of TGF-β, and a number of animal models have shown that blockade of TGF-β can enhance carcinogenesis (8, 52, 53).

On the other hand, our data and that of others support the potential use of sTGF-βR as TGF-β inhibitors for treatment of some malignancies. Our experiments suggest that the therapy may be most effective in tumors that make large amounts of TGF-β and that generate some level of endogenous antitumor immune response. Certainly, MM is the prototype of such a tumor; however, loss of TGF-β responsiveness and elevated secretion of TGF-β have been observed in many other tumors including brain tumors, head and neck tumors, lung cancer, breast cancer, colon cancer, pancreatic cancer, prostate cancer, ovarian cancer, multiple myeloma, and melanoma (49). Additional studies will be needed to test the sensitivity of these tumors to TGF-β blockade.

Although TGF-β blockade markedly slowed the growth of AB12 and AC29 tumors, complete regressions were not observed (see Fig. 4*D*). We do not currently know the reason for this limitation; however, there are a number of possible explanations. One potential limitation of the sTGF-βR used in these studies is that is binds only the TGF-β1 and TGF-β3 isoforms. Because semiquantitive polymerase chain reaction analysis of RNA from human and murine mesothelioma cell lines showed high levels of expression of TGF-β1 and TGF-β2 (14), this lack of ability to block the TGF-β2 isoform may account for some residual TGF-β activity and tumor persistence. It is also possible that production of other potential immunosuppressive factors, such as IL-10 and prostaglandin E_2, increase as the tumors progress and override the effects of TGF-β blockade. Our previous observations that AB12 tumor growth was markedly suppressed in mice fed with the cyclooxygenase (COX)-2 inhibitor rofecoxib (48) clearly implicate COX-2 as a potentially important immunosuppressive factor. Future studies using multiple approaches (*i.e.*, TGF-β blockade plus COX-2 inhibition) will test this hypothesis.

Indeed, the observation that one of the key mechanisms for efficacy appeared to be due to enhancement of endogenous antitumor immune effects suggests combination therapy with other anticancer enhancing agents may be more effective than TGF-β blockade alone. For example, McEarchern *et al.* (54) showed that cells transfected with murine IFN-γ and antisense TGF-β were markedly more immunogenic than cells transduced with a single gene. Using a dendritic cell vaccine against a mouse mammary tumor, Kobie *et al.* (55) found that neutralization of TGF-β using an antibody enhanced the ability of the vaccines to inhibit established tumors. As our ability to generate potent antitumor CTLs improves, it will become increasing important to block endogenous tumor immunosuppression that prevents these CTLs from entering tumors or from being inactivated once they reach the tumor site.

Given that blockade of TGF-β may be useful in anticancer therapy, it is worth considering the optimal way to accomplish this. Antisense oligonucleotide and interfering RNA technologies may be useful, but they are probably not yet clinically effective enough. Although intravenous administration would be necessary, clinically effective monoclonal antibodies (*i.e.*, anti-CD20 antibodies) and genetically engineered chimeric soluble receptors (*i.e.*, soluble tumor necrosis factor α receptors) have been produced and used successfully in the clinic. Thus, clinical trials using humanized sTGF-βR would appear to be feasible.

In summary, this study provides data to show that blockade of TGF-β using sTGF-βR can markedly slow the growth of small and large established mesothelioma tumors via its ability to enhance endogenous antitumor immune effects. Given the lack of effective treatments for mesothelioma and many other solid tumors, TGF-β blockade, used alone or in combination with other therapies, may be a useful addition to our antitumor therapeutic armamentarium.

REFERENCES

1. Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-beta: biological function and chemical structure. Science (Wash DC) 1986;233:532–4.

2. Massague J. The TGF-beta family of growth and differentiation factors. Cell 1987;49:437–8.

3. Van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, Roberts AB. Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. J Biol Chem 1988; 263:7741–6.

[4] E. Suzuki and S. Albelda; Unpublished observations.

4. Yamamoto H, Hirayama M, Genyea C, Kaplan J. TGF-beta mediates natural suppressor activity of IL-2-activated lymphocytes. J Immunol 1994;152:3842–7.

5. Noble PW, Henson PM, Lucas C, et al. Transforming growth factor-beta primes macrophages to express inflammatory gene products in response to particulate stimuli by an autocrine/paracrine mechanism. J Immunol 1993;151:979–89.

6. Derynck R, Jarrett JA, Chen EY, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature (Lond) 1985;316:701–5.

7. Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. Nat Genet 2001;29:117–29.

8. Akhurst RJ. TGF-β antagonists: why suppress a tumor suppressor? J Clin Investig 2002;109:1533–6.

9. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev 1996;7: 93–102.

10. Ranges GE, Figari IS, Espevik T, Palladino MA Jr. Inhibition of cytotoxic T cell development by transforming growth factor β and reversal by recombinant tumor necrosis factor. J Exp Med 1987;166: 991–8.

11. Tada T, Ohzeki S, Utsumi K, et al. Transforming growth factor-β-induced inhibition of T cell function: susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. J Immunol 1991;146:1077–82.

12. de Visser KE, Kast WM. Effects of TGF-β on the immune system: implications for cancer immunotherapy. Leukemia (Baltimore) 1999; 13:1188–99.

13. Gorelik L, Flavell RA. Transforming growth factor-β in T-cell biology. Nat Rev Immunol 2002;2:46–53.

14. Fitzpatrick DR, Bielefeldt-Ohman H, Himbeck RP, et al. Transforming growth factor-beta: antisense RNA-mediated inhibition affects anchorage-independent growth, tumorigenicity and tumor-infiltrating T-cells in malignant mesothelioma. Growth Factors 1994;11:29–44.

15. Maggard M, Meng L, Ke B, et al. Antisense TGF-beta2 immunotherapy for hepatocellular carcinoma: treatment in a rat tumor model. Ann Surg Oncol 2001;8:32–7.

16. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-β signaling in T cells. Nat Med 2001;7:1118–22.

17. Arteaga CL, Hurd SD, Winnier AR, et al. Anti-transforming growth factor (TGF)-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity: implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. J Clin Investig 1993;92:2569–76.

18. Bandyopadhyay A, López-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. Cancer Res 2002;62:4690–5.

19. Stander M, Naumann U, Dumitrescu L, et al. Decorin gene transfer-mediated suppression of TGF-beta synthesis abrogates experimental malignant glioma growth in vivo. Gene Ther 1998;5:1187–94.

20. George J, Roulot D, Koteliansky VE, Bissell DM. In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis. Proc Natl Acad Sci USA 1999;96:12719–24.

21. Won J, Kim H, Park EJ, et al. Tumorigenicity of mouse thymoma is suppressed by soluble type II transforming growth factor β receptor therapy. Cancer Res 1999;59:1273–7.

22. Zheng H, Wang J, Koteliansky VE, Gotwals PJ, Hauer-Jensen M. Recombinant soluble transforming growth factor β type II receptor ameliorates radiation enteropathy in mice. Gastroenterology 2000;119: 1286–96.

23. Witham TF, Villa L, Yang T, et al. Expression of a soluble transforming growth factor-beta (TGFbeta) receptor reduces tumorigenicity by regulating natural killer (NK) cell activity against 9L gliosarcoma in vivo. J Neuro-oncol 2003;64:63–9.

24. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastasis. J Clin Investig 2002;109:1551–9.

25. Sterman DH, Kaiser LR, Albelda SM. Advances in the treatment of malignant pleural mesothelioma. Chest 1999;116:504–20.

26. Gerwin BI, Lechner JF, Reddel RR, et al. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res 1987;47:6180–4.

27. Lauber B, Leuthold M, Schmitter D, et al. An autocrine mitogenic activity produced by a pleural human mesothelioma cell line. Int J Cancer 1992;50:943–50.

28. Kuwahara M, Takeda M, Takeuchi Y, et al. Transforming growth factor β production by spontaneous malignant mesothelioma cell lines derived from Fisher 344 rats. Virchows Archiv 2001;438:492–7.

29. Kumar-Singh S, Weyler J, Martin MJH, Vermeulen PB, Van Marck E. Angiogenic cytokines in mesothelioma: a study of VEGF, FGF-1 and -2, and TFGβ expression. J Pathol 1999;189:72–8.

30. Jagirdar J, Lee TC, Reibman J, et al. Immunohistochemical localization of transforming growth factor beta isoforms in asbestos-related diseases. Environ Health Perspect 1997;105:1197–203.

31. Maeda J, Ueki N, Ohkawa T, et al. Transforming growth factor-beta 1 (TGF-beta 1)- and beta 2-like activities in malignant pleural effusions caused by malignant mesothelioma or primary lung cancer. Clin Exp Immunol 1994;98:319–22.

32. Abe M, Harpel JG, Metz CN, et al. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 1994; 216:276–84.

33. Fitzpatrick DR, Peroni DJ, Bielefeldt-Ohman H. The role of growth factors and cytokines in the tumorigenesis and immunobiology of malignant mesothelioma. Am J Respir Cell Mol Biol 1995; 12:455–60.

34. Marzo AL, Fitzpatrick DR, Robinson BWS, Scott B. Antisense oligonucleotides specific for transforming growth factor β2 inhibit the growth of malignant mesothelioma both *in vitro* and *in vivo*. Cancer Res 1997;57:3200–7.

35. Bielefeldt-Ohmann H, Fitzpatrick DR, Marzo AL, et al. Potential for interferon-alpha-based therapy in mesothelioma: assessment in a murine model. J Interferon Cytokine Res 1995;15:213–23.

36. Robinson B, Bowman R, Manning LS, Musk AW, Van Hazel G. Interleukin-2 and lymphokine-activated killer cells in malignant mesothelioma. Eur Respir Rev 1993;3:220–2.

37. Caminschi I, Venetsanakos E, Leong C, et al. Cytokine gene therapy of mesothelioma. Immune and antitumor effects of transfected interleukin-12. Am J Respir Cell Mol Biol 1999;21:347–56.

38. Nowak AK, Robinson BWS, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. Cancer Res 2003;63:4490–6.

39. Lanuti M, Rudginsky S, Force S, et al. Cationic lipid:bacterial DNA complexes elicit anti-tumor effects and adaptive immunity in murine intraperitoneal tumor models. Cancer Res 2000;60:2955–63.

40. Odaka M, Sterman D, Wiewrodt R, et al. Eradication of intraperitoneal and distant tumor by adenovirus-mediated interferon-beta gene therapy due to induction of systemic immunity. Cancer Res 2001;61: 6201–12.

41. Christmas TI, Manning LS, Garlepp MJ, Musk AW, Robinson BW. Effect of interferon-alpha 2a on malignant mesothelioma. J Interferon Res 1993;13:9–12.

42. Astoul P, Viallat JR, Laurent JC, Brandely M, Boutin C. Intrapleural recombinant IL-2 in passive immunotherapy for malignant pleural effusion. Chest 1993;103:209–13.

43. Boutin C, Nussbaum E, Monnet I, et al. Intrapleural treatment with recombinant gamma-interferon in early stage malignant pleural mesothelioma. Cancer (Phila) 1994;74:2460–7.

44. Goey SH, Eggermont AM, Punt CJ, et al. Intrapleural administration of interleukin 2 in pleural mesothelioma: a phase I-II study. Br J Cancer 1995;72:1283–8.

45. Smith JD, Bryant SR, Couper LL, et al. Soluble transforming growth factor-β type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. Circ Res 1999;84:1212–22.

46. Winn HJ. In vivo methods for the assessment of antibody-mediated tumor immunity. J Natl Cancer Inst Monogr 1972;35:13–8.

47. Lammerts E, Roswall P, Sundberg C, et al. Interference with TGF-beta1 and -beta3 in tumor stroma lowers tumor interstitial fluid pressure independently of growth in experimental carcinoma. Int J Cancer 2002;102:453–62.

48. DeLong P, Tanaka T, Kruklitis R, et al. Use of cyclooxygenase-2 inhibition to enhance the efficacy of immunotherapy. Cancer Res 2003; 63:7845–52.

49. Pasche B. Role of transforming growth factor beta in cancer. J Cell Physiol 2001;186:153–68.

50. Yang Y-A, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects. J Clin Investig 2002;109:1607–15.

51. Ruzek MC, Hawes M, Pratt B, et al. Minimal effects on immune parameters following chronic anti-TGF-β monoclonal antibody administration to normal mice. Immunopharmacol Immunotoxicol 2003;25: 235–57.

52. Bottinger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM. Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. Cancer Res 1997;57: 5564–70.

53. Amendt C, Schirmacher P, Weber H, Blessing M. Expression of a dominant negative type II TGF-β receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. Oncogene 1998;17:25–34.

54. McEarchern JA, Besselsen DG, Akporiaye ET. Interferon gamma and antisense transforming growth factor beta transgenes synergize to enhance the immunogenicity of a murine mammary carcinoma. Cancer Immunol Immunother 1999;48:63–70.

55. Kobie JJ, Wu RS, Kurt RA, et al. Transforming growth factor β inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res 2003;63:1860–4.