Systemic Blockade of Transforming Growth Factor-β Signaling Augments the Efficacy of Immunogene Therapy

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Abstract

Locally produced transforming growth factor-β (TGF-β) promotes tumor-induced immunosuppression and contributes to resistance to immunotherapy. This article explores the potential for increased efficacy when combining immunotherapies with TGF-β suppression using the TGF-β type 1 receptor kinase inhibitor SM16. Adenovirus expressing IFN-β (Ad.IFN-β) was injected intratumorally once in established s.c. AB12 (mesothelioma) and LKR (lung cancer) tumors or intratracheally in a Kras orthotopic lung tumor model. Mice bearing TC1 (lung cancer) tumors were vaccinated with two injections of adenovirus expressing human papillomavirus-E7 (HPV-E7; Ad.E7). SM16 was administered orally in formulated chow. Tumor growth was assessed and cytokine expression and cell populations were measured in tumors and spleens by real-time PCR and flow cytometry. SM16 potentiated the efficacy of both immunotherapies in each of the models and caused changes in the tumor microenvironment. The combination of SM16 and Ad.IFN-β increased the number of intratumoral leukocytes (including macrophages, natural killer cells, and CD8+ cells) and increased the percentage of T cells expressing the activation marker CD25. SM16 also augmented the antitumor effects of Ad.E7 in the TC1 flank tumor model. The combination did not increase HPV-E7 tetramer-positive CD8+ T cells in the spleens but did induce a marked increase in the tumors. Tumors from SM16-treated mice showed increased mRNA and protein for immunostimulatory cytokines and chemokines, as well as endothelial adhesion molecules, suggesting a mechanism for the increased intratumoral leukocyte trafficking. Blockade of the TGF-β signaling pathway augments the antitumor effects of Ad.IFN-β immun-activating or Ad.E7 vaccination therapy. The addition of TGF-β blocking agents in clinical trials of immunotherapies may increase efficacy. [Cancer Res 2008;68(24):10247–56]

Introduction

It has become increasingly apparent that cancer cells alter their adjacent microenvironment to form a permissive and supportive environment for tumor progression (1–6). Tumor-induced immunosuppression is one of the most important of these adaptations and inhibits endogenous antitumor immune responses, as well as presenting a formidable block against any immunotherapy approaches used to treat the tumor (7, 8).

Current immunotherapies (such as immunogene therapy with cytokines, tumor vaccines, and adoptive T-cell transfer) are primarily aimed at initiating or boosting the immune response to tumors and their antigens. However, the effectiveness of these therapies may be limited by the local immunosuppressive environment of the tumor. In particular, the immunosuppressive cytokine transforming growth factor-β (TGF-β) is overexpressed by tumors. Evidence suggests that TGF-β production by tumors plays a significant role in blocking immune response (9). Specifically, recent findings implicate this multifunctional cytokine in preventing T-cell infiltration into tumors, inhibition of T-cell activation/function, and mediation of T regulatory cell–induced immunosuppression (10–14). For example, evidence links clinical resistance to tumor vaccine therapy in glioblastoma patients to TGF-β expression levels (15).

The pivotal role of TGF-β in suppressing the antitumor immune response has made it a logical target for the development of antagonists to block its biological effects (9). We and others have shown that TGF-β blockades (soluble receptors/antibodies) and TGF-β receptor inhibitors have antitumor effects that, in several models, are due primarily to immunologic mechanisms (16–19). For example, we found that TGF-β blockade had antitumor effects in a murine malignant mesothelioma model and that this activity was CD8+ T-cell dependent (18, 19). In these models, TGF-β blockade resulted in the persistence of tumor-killing CD8+ T cells harvested from the spleens of the tumor-bearing animals and increased numbers of CD8+ T cells within tumors of animals treated with a soluble TGF-β receptor (18). Similar findings were observed when we used SM16, a small, orally available type I TGF-β receptor (ALK5/ALK4) kinase inhibitor that can effectively block SMAD phosphorylation within tumors (19). In our studies, and in those of Ge and colleagues (16) and Nam and colleagues (17), antitumor effects were markedly reduced in immunodeficient animals.

Given this augmentation of endogenous antitumor immunity, we hypothesized that combining systemic TGF-β receptor blockade with active immunotherapy would result in enhanced responses compared with either approach alone. To test this hypothesis, we combined SM16 with two immunogene therapy approaches that we characterized previously: (a) delivery of the cytokine IFN-β using an adenoviral vector (Ad.IFN-β; refs. 20, 21) and (b) vaccination using an adenoviral vector expressing a known tumor antigen [human papillomavirus-E7 protein (HPV-E7); ref. 22]. In each case, we found marked augmentation of efficacy using...
combined therapy, along with evidence of increased leukocyte infiltration, including intratumoral CD8+ T cells that were antigen specific, or showed increased expression of the activation marker CD25.

Materials and Methods

Animals. Mice were purchased from Taconic Labs or The Jackson Laboratory. Breeding pairs of Lox-Stop-Lox (LSL) KrasG12D mice (on mixed 129SvJ and C57BL/6 background) used in the orthotopic lung model were initially provided by Dr. David Tuveson of the University of Pennsylvania, Philadelphia, PA (21). The Animal Use Committee of the University of Pennsylvania approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

Cell lines. A murine malignant mesothelioma cell line, AB12, derived from an asbestos-induced tumor in a BALB/c mouse has been previously described in detail (19, 20, 23). The murine lung cancer line “LKR” was derived from an explant of a pulmonary tumor from an activated KrasG12D mutant mouse that had been induced in an F1 hybrid of 129SvJ and C57BL/6 (21). TC1 cells were derived from mouse lung epithelial cells derived from...
a C57B6 mouse and immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (24). All cell lines were injected into the appropriate syngeneic strain.

**SM16, a TGF-β receptor kinase inhibitor.** The chemical structure of SM16, a 430 molecular weight ALK4/ALK5 kinase inhibitor produced by Biogen Idec, has recently been published (25). This small molecule can be administered as formulated in mouse chow, which allows for daily oral administration (19). We have previously shown that SM16 chow at a dose of 0.45 g/kg of chow is well tolerated by the animals, results in therapeutic drug levels, and effectively blocks SMAD phosphorylation within tumor cells (19).

**TGF-RII/Fc protein.** A soluble recombinant mouse TGF-β receptor type II-murine FcIgG2a chimeric protein has been described previously (18). It binds and inhibits TGF-β1 and TGF-β3 in the 1 nmol/L range and has a half-life in mouse plasma of 14 d. Previous studies have shown biological effects at 1, 2, and 5 mg/kg.

**Animal flank tumor models.** Mice were injected on the right flank with 1 × 10^6 AB12, LKR, and TC1 tumor cells in the appropriate syngeneic host. The flank tumors were allowed to reach an average size of 200 to 250 mm^3 (approximately 14–17 d). Following treatments as outlined below, tumor growth was followed with measurement twice weekly. All experiments had at least five mice per group and were repeated at least once.

**Treatment with oral SM16 chow and intratumoral Ad.IFN-β combination.** The effect of combining oral SM16 with intratumor Ad.IFN-β therapy [10^9 plaque-forming units (pfu) of virus] was studied by treating tumor-bearing mice (AB12 and LKR). Mouse chow formulated with SM16 (0.45 g/kg chow) was given ad libitum starting when the flank tumors reached an average size of 200 to 250 mm^3 (approximately 14–17 d). One dose of 1 × 10^9 pfu of Ad.IFN-β was injected intramuscularly 6 to 7 d after SM16 treatment. The mice were followed closely and tumors were measured twice weekly.

**Treatment with oral SM16 chow and/or intratracheal Ad.IFN-β therapy in orthotopic lung cancer model.** The orthotopic lung cancer model using intratracheal Ad.IFN-β therapy has been previously described in detail (21). Briefly, to induce tumors, 100 μl of saline containing 3 × 10^6 particles of adenovirus containing Cre recombinase (Ad.Cre) were administered to all LSL.KrasG12D mice intranasally. Four groups of mice (n = 8 per group) were studied. One group got no further treatment. One group received only SM16-formulated chow starting 18 d after Ad.Cre treatment. One group received one dose of 1 × 10^9 pfu Ad.IFN-β intratracheally on day 21. The final group (combo group) got both SM16-formulated chow starting 18 d after Ad.Cre treatment and received one dose of 1 × 10^9 pfu Ad.IFN-β intratracheally on day 21. When animals appeared lethargic, had ruffled fur, or increased breathing rate, they were sacrificed.

**Oral SM16 treatment with Ad.E7 vaccination in the TC1 tumor model.** An E1/E3-deleted type 5 adenoviral vector expressing the HPV-E7 protein under control of a cytomegalovirus promoter (Ad.E7) has been previously described (22). To test the effects of combination treatment, animals bearing TC1 tumors (~200 mm^3 in size) were vaccinated s.c. in the left flank (contralateral to the tumor) with 1 × 10^6 pfu of Ad.E7 vector. The next day, oral chow formulated with SM16 (0.45 mg/kg chow) was started. Seven days following the initial vaccination, mice received a booster vaccine of 1 × 10^5 pfu of Ad.E7 in the left flank. Control animals received regular chow.

**TGF-RII/Fc protein with Ad.E7 vaccination in the TC1 model.** The effects of combination treatment using a different type of TGF-β inhibitor with the Ad.E7 vaccine, animals bearing large TC1 tumors (~500 mm^3 in size) were left untreated or vaccinated s.c. in the left flank (contralateral to the tumor) with 1 × 10^6 pfu of Ad.E7 vector. Five days later, i.p. injections of the TGF-RII/Fc protein (1 mg/kg every 3 for three doses) were started in one group of control and one group of vaccinated animals. The next day (7 d following the initial vaccination), vaccinated mice received a booster vaccine of 1 × 10^8 pfu of Ad.E7 in the left flank. Each group had five mice.

**Flow cytometric analysis of tumors and spleen after SM16 and Ad.E7 vaccination treatment.** In the Ad.IFN-β models, fluorescence-activated cell sorting (FACS) on tumors was performed 2 d after the dose of Ad.IFN-β. In the vaccination model, spleens were harvested for FACS 7 d after the second Ad.E7 vaccination (previously determined to be the time of optimal response) and tumors were harvested for FACS 2 d after the second Ad.E7 vaccination (a time when the tumor size was similar among groups). Splenocytes and tumor cells were studied by FACS analysis as previously described (22). The allopurinol-targeted H-3Hdeterase (1,200 dilution) loaded with E7 peptide (RAHYNIVTF) was obtained from the National Institute of Allergy and Infectious Diseases tetramer core. The fluorescently labeled antibodies were all purchased from BD Biosciences. Flow cytometry was done using a Becton Dickinson FACSCalibur flow cytometer. Data analysis was done using FlowJo software.

**RNA isolation and real-time reverse transcription-PCR.** To evaluate changes in the tumor microenvironment induced by SM16, mice with tumors (~200 mm^3) were treated with SM16 chow or control chow (n = 5 in each group). Tumors were removed after 5 d and flash frozen, and the RNA from each tumor was isolated. For both treatment conditions, a pool of RNA was created by adding the same amount of RNA from each of the five tumors within the group. cDNA was made from each pool, RNA levels were normalized to β-actin levels, and quantification of tumor mRNA levels was performed as previously described (26). Relative levels of expression of each of the selected genes (fold change in SM16 treated versus control) were determined. Each sample was run in quadruplicate and the experiment was repeated at least once.

**Intratumoral cytokine assays.** Mice were treated as above. Tumors were measured at 5 d after treatment, sonicated for 30 s, spun at 3,000 rpm for 10 min, and filtered through a 1.2-μm syringe filter unit. Total protein in each individual sample (n = 5 in each group) was determined. Mouse cytokine expression for each sample was measured using a multiplex Luminex bead assay system as previously described (26).

**Statistical analyses.** For the reverse transcription-PCR (RT-PCR) and protein experiments comparing differences between two groups, we used unpaired Student’s t tests. For FACS studies and flank tumor studies comparing more than two groups, we used ANOVA with appropriate post hoc testing. For the modified survival study (Fig. 1D), we used Kaplan-Meier survival curves and analyzed with the Mantel-Cox log-rank test. Differences were considered significant when P < 0.05. Data are presented as mean ± SE.

**Results**

SM16 augments immunotherapies in multiple tumor models. The effect of simultaneous TGF-β receptor blockade and immune activation was examined by combining SM16 treatment with two immunogene therapy models.

First, the combination of SM16 with Ad.IFN-β was tested in two independent tumor models known to respond to Ad.IFN-β (20, 21). Mice bearing large AB12 (malignant mesothelioma) or LKR (lung cancer) flank tumors (approximately 200–250 mm^3) began daily oral administration of SM16 or were given control chow. Seven days later, one dose of Ad.IFN-β was administrated intratumorally. As shown in Fig. 1A, treatment of AB12 tumors with either SM16 alone or Ad.IFN-β was only minimally effective in inhibiting the growth of these large tumors. In contrast, treatment with the combination of SM16 and Ad.IFN-β resulted in marked shrinkage of the tumors, with tumors being significantly smaller (P < 0.05) than that of control or single treatments at all time points measured. Impressively, combination therapy led to complete tumor regression in three of five animals versus no complete regressions in any other group. In the LKR model (Fig. 1B), Ad.IFN-β alone temporarily slowed the growth of large tumors, whereas SM16 treatment actually decreased the size of the tumors and induced one complete regression. However, combination therapy was even more effective (P < 0.05 compared with control and each treatment alone), rapidly shrinking all tumors and eventually inducing complete remissions in four of the five tumors.
Figure 2. Effect of SM16 on Ad.E7 immunogene vaccine model. A, mice (n = 5 for each group) bearing large TC1 tumors (~200 mm³) were treated in one of four ways: (a) one group was left untreated (diamonds; control), (b) one group (SM16) was placed on the TGF-β1 receptor kinase inhibitor chow for the duration of the experiment (see arrows; triangles, SM16), (c) one group (Ad.E7) was injected with 2 × 10⁶ pfu of Ad.E7 s.c. in the contralateral flank, with a second dose given 7 d later (see arrows; circles, Ad.E7), and (d) one group (combo) received the two doses Ad.E7 plus SM16 chow (beginning the day after the first dose of Ad.E7; see arrows; squares). Tumor volumes were measured every 3 d. The tumors from mice in the combo group were statistically smaller than control tumors (P < 0.01) and tumors from the SM16 alone and Ad.E7 alone treatment groups. *, P < 0.05. B, mice were treated as above, except, instead of using SM16 chow, TGF-β1 was blocked using i.p. injections of 1 mg/kg TGF-βRII/Fc protein every 3 d for three doses (hatched column). The tumors from mice in the combo group were statistically smaller than control tumors and tumors from the TGF-β1 inhibitor and Ad.E7 alone treatment groups. *, P < 0.05.

We also performed a modified survival study using mice that conditionally express an oncogenic KrasG12D allele. LSL KrasG12D-positive mice were intratracheally injected with Ad.Cre. Eighteen days later (at a time of detectable tumor burden [21]), mice were started on either SM16-containing chow or regular chow. Three days later (day 21), one dose of 1 × 10⁹ Ad.IFN-β was administered intratracheally. As shown in Fig. 1C, the median survival time for control mice was 46 days, with all mice sacrificed by 52 days. SM16-treated mice and Ad.IFN-β-treated mice had small but significantly prolonged median survivals (P < 0.05) compared with the control, but there was no difference between the SM16 treatment and Ad.IFN-β treatment groups. However, in the group that received both SM16 and Ad.IFN-β treatment, median survival was doubled compared with control (82 days). This increase was significant compared with all the other groups (P < 0.01).

Next, we examined whether TGF-β receptor blockade potentiates a tumor antigen vaccine approach (Fig. 2A). As previously observed (22), treatment of established TC1 tumors with two doses of Ad.E7 alone led to significant (P < 0.05) slowing of tumor growth compared with control but did not induce tumor regression. Treatment with oral SM16 led to a similar significant (P < 0.05) degree of tumor slowing. There were no significant differences in tumor size between SM16 and Ad.E7 groups. In contrast, treatment with the combination of Ad.E7 and SM16 led to clear tumor regression. The tumors in the combination group were statistically smaller than control tumors (P < 0.01) and tumors from the SM16 alone and Ad.E7 alone treatment groups (P < 0.05). An independent replicate of this study is shown in Supplementary Fig. S1.

To confirm that this augmentation was not due to an “off-target” effect of the TGF-β receptor blocker, we conducted a similar study (in very large TC1 tumors) using Ad.E7 in combination with sTGF-βRII/Fc, a soluble TGF-β receptor fusion protein that binds TGF-β1 and TGF-β3, functioning like a neutralizing antibody (18). As shown in Fig. 2B, the size of tumors in the combination group was significantly smaller than those in the single treatment groups.

SM16 alters the tumor microenvironment by increasing immunostimulatory cytokines and ICAM-1. To assess the effect of SM16 alone on the tumor microenvironment, the effects of 5 days of SM16 treatment on a variety of cytokine and chemokine mRNA levels within the tumors were measured by quantitative PCR. As shown in Table 1A, SM16 induced significant increases in the mRNA levels of several inflammatory cytokines and chemokines, including tumor necrosis factor-α (TNF-α), IFN-γ, interleukin (IL)-1β, IL-6, IP10 (CXC1L10), MIG (CXC1L9), and MCP-1 (CCL2). Significant changes were also seen in message levels of key enzymes that affect arginine metabolism (which affects T-cell activation): arginase was down-regulated and inducible nitric oxide synthase (iNOS) was up-regulated.

Although protein measurements of tissue cytokines are subject to several limitations (such as uncontrolled proteolysis and variation in efficiency of protein extraction), we also made homogenates of AB12 tumors and analyzed them using a Luminex bead assay. As shown in Table 1B, values measured from the SM16-treated versus control tumors showed significant increases in several cytokines and chemokines, including Rantes, MCP-1, MIP1α, and IL-6, although there was not perfect correspondence with the RT-PCR data.

In addition, the message level of the cell adhesion molecule ICAM-1 increased by 1.5- to 2.6-fold. To determine if this increase reflected augmented protein expression on tumor endothelial cells, FACS analysis was performed on pooled samples of tumors in which intratumoral endothelial cells were identified as the CD45⁺ / CD31⁺ cells. As shown in Fig. 3D, the mean fluorescence intensity (MFI) of ICAM-1 on endothelial cell from control tumors versus SM16-treated tumors increased from 69.5 to 108.9 on AB12 tumors, 34.2 to 66.0 in LKR tumors, and 24.8 to 50.2 on TC1 tumors. These studies were repeated in an independent experiment with very similar results.

SM16 increases the percentage and the functional status of CD8⁺ cells in tumors from all three models. To assess the effect of SM16 on the presence of CD8⁺ T cells and other leukocytes in tumors, mice were treated for 1 week with SM16 or control chow, and tumors were harvested and subjected to FACS. This time point was chosen because after 1 week of treatment, the tumors have responded to SM16 but are still large enough for analysis. Figure 4 and Supplementary Table S1 summarize these data.

In all three cell lines, treatment with SM16 (compare “control” with “SM16” in each panel of Fig. 4) resulted in significant (P < 0.05) increases in the percentage of total cells in the tumor of CD45⁺ and CD8⁺ cells. In AB12 and LKR cells, significant (P < 0.05) increases in CD11b⁺ cells were also seen. In AB12 cells, the number of natural killer (NK) cells increased significantly (P < 0.05).

To assess the activation state of these cells, we also used FACS to investigate the expression of CD25 [a T-cell activation...
marker (27, 28) in CD8+ T cells from AB12 or LKR tumors treated with SM16 versus control. Representative examples for AB12 tumors using dot plots are shown in Fig. 3B. Data from all five tumors in each group were compared using the MFI of CD25 on CD8+ T cells. In control-treated AB12 tumors, MFI was 4.0 ± 0.5. However, SM16-treated tumors showed a significant 2-fold increase in CD25 expression compared with control tumors (MFI of 7.9 ± 0.8; P < 0.05). In LKR tumors, expression of CD25 was higher on the CD8+ T cells at baseline (MFI, 15.2 ± 0.4), but again, SM16 treatment significantly increased expression of CD25 (MFI of 25.8 ± 4.1; P < 0.05).

Combination of SM16 with immunotherapies significantly increases tumor-associated CD8+ cells and myeloid cells more than single-agent therapy. We next tested the hypothesis that changes in tumor-associated immune cells correlated with the enhanced efficacy seen with the combination treatments in Figs. 1 and 2. The leukocyte cell populations were thus measured as a percentage of total cells from tumors using FACS 2 days after Ad.IFN-β in the AB12 or LKR models (which was 1 week following initiation of SM16 therapy in the SM16 alone or combination treatment regimens) or 2 days after the second dose of Ad.E7 in the TC1 model. At this time point, the tumors were large enough for

Table 1. PCR and protein analysis of tumor from control and SM16-treated tumors

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<td>Fold change</td>
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| B. Protein measurements of AB12 tissue homogenates

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NOTE: (A) Tumors (n = 5 for each treatment group) from control and SM16-treated animals were treated for 5 d and then harvested, digested, and had RNA extracted. Equal amounts of RNA from each tumor in group were pooled and subjected to real-time RT-PCR analysis (TC1, AB12, and LKR). RNA was normalized using β-actin levels. The fold change of each molecule, using the expression level in untreated controls as the denominator, was calculated. Each assay was run in at least quadruplicate. (B) Tumors (n = 5 for each treatment group) from control and SM16-treated animals were treated for 5 d and then harvested, digested, and had protein extracted. Cytokine and chemokine levels for each tumor sample from each group were determined. Data are expressed as a ratio of SM16-treated to control levels. t tests were used to compare groups. Abbreviations: NS, not significant; ND, not determined.
of all tumor cells with 16.8% of tumor cells represented by CD8+ populations of tumor-associated immune cells. The overall combination of SM16 and Ad.E7 markedly increased three most dramatic changes seen in the vaccinemodel, where the untreated tumors or the tumors treated with single therapies. The tumors treated with combination therapy when compared with percentage of CD25-expressing CD4+ T cells was not increased and were not significantly changed by the treatments. The present only as a very low percentage of total tumor cells (<0.7%) from vehicle-treated mice. In all three models, CD4+ cells were, respectively, 5.7-, 56-, and 2-fold higher than in the tumors and NK+ cells were generally significantly increased (Fig. 4; Supplementary Table S1). Antigen-specific T cells in the Ad.E7 model. The TC1/Ad.E7 model provided the opportunity to measure the effects of each treatment on T cells directed at the immunodominant tumor antigen using MHC class I tetramers loaded with the appropriate HPV-E7 peptide.

As shown in Fig. 5A (averages of five mice per group) and Fig. 5B (representative examples), in control tumor-bearing animals, an average of 1.4% of the CD8+ cells in the spleen was tetramer positive. This represents a relatively weak endogenous antitumor response. Treatment with SM16 alone did not significantly increase this percentage (1.7%). As we have previously reported (22), Ad.E7 vaccination significantly (P < 0.05) increased the percentage of tetramer-positive CD8+ T cells to 5.3% (a 3.8-fold increase compared with control). The tetramer-positive percentage in the combination group (6.3%) was significantly (P < 0.05) increased (4.5-fold) compared with the control and SM16 group but was not statistically different from the Ad.E7 alone treatment group. Thus, neither SM16 nor the combination treatment caused a systemic enhancement in antitumor T cells greater than Ad.E7 alone.

Figure 5C (also see Supplementary Table S1 and Fig. 4F) shows that the percentage of CD8+ cells within individual tumors was markedly increased by combination therapy. After a Ficoll gradient spin, CD8+ cells from individual tumors were pooled and analyzed for expression of the tetramer. As shown in Fig. 5D, the percentage of intratumoral CD8+ T cells that were tetramer positive at baseline and after SM16 treatment alone was ~10%. As might be expected, Ad.E7 therapy increased this percentage to 16.9%. However, in the combination group, the percentage of tetramer-positive cells rose significantly to >50%. Thus, in this model, the combination of SM16 and Ad.E7 seems to markedly increase the localization of antitumor CD8+ T cells to the tumor microenvironment rather than enhance the systemic population of tumor-directed CD8+ T cells.

Discussion

The studies presented here using the ALK5/ALK4 inhibitor SM16 in three different syngeneic tumor models (AB12 mesothelioma, LKR, and TC1 lung carcinomas), in flank and orthotopic models, and with two different immune-activating therapies (Ad.E7 and Ad.IFN-β) support the utility of combining inhibition of TGF-β signaling with immune-activating therapies. These results show that TGF-β receptor blockade via SM16 can augment a variety of adenovirus-based, immune-activating therapies in a spectrum of tumors. Similar results using a soluble TGF-β receptor (Fig. 2B)
suggest that our findings are applicable to other TGF-β blocking strategies.

Analysis of the mechanism of action in each model highlights the similarities and differences between the response of these tumor models to these therapies. A common finding was an increase in intratumoral CD8+ T cells, which was augmented significantly in the combination treatments (Fig. 4). This effect is consistent with the previously described mechanisms of action for SM16, Ad.IFN-β, and Ad.E7 (18–20). However, the increase in tumor-associated NK (NK1+) and macrophages (CD11b+) cells with SM16 alone, in both the AB12 and LKR models, identifies a novel response to TGF-β inhibition. Interestingly, tumor-associated CD11b+ cells were not increased by SM16 in the TC1 model, suggesting that inherently different immunosuppressive mechanisms may be in play in these

Figure 4. FACS analysis of leukocytes within treated tumors. A and B, mice (n = 5 per group) were treated with control chow, SM16, one dose of Ad.IFN-β, or the combination (Combo) of SM16 and Ad.IFN-β. AB12 tumors (A) or LKR tumors (B) were removed from all groups 2 d after Ad.IFN-β had been administered (i.e., day 22 in Fig. 1), digested, and subjected to FACS. Columns, mean for each group; bars, SE. Left, the percentage of CD45+ and CD11b+ cells of total tumor cells; right, the percentage of CD8+ T cells and NK1.1+ cells of total tumor cells. *, P < 0.05, between control versus SM16 group; **, P < 0.05, between combo group versus control, SM16, and Ad.IFN-β groups; †, P < 0.05, between combo group versus control and Ad.IFN-β groups. C, TC1 tumor-bearing mice were treated with control chow, SM16, two doses of Ad.E7, or the combination (Combo) of SM16 and Ad.E7. Tumors were removed from all groups 2 d after Ad.E7 had been administered (i.e., day 24 in Fig. 2A), digested, and subjected to FACS. Left, percentage of CD45+ and CD11b+ cells of total tumor cells; right, percentage of CD8+ T cells of total tumor cells. *, P < 0.05, between control versus SM16 group; †, P < 0.05, between control versus SM16 group; †, P < 0.05, between combination versus control, SM16, and Ad.E7; ‡, P < 0.05, between combination versus control and SM16.
different models. However, a further enhancement of CD11b+ and NK1+ populations by the combination treatments above the single-agent treatments was a common feature in all three tumor models. These data support the utility of the combination treatments in models where different immunosuppressive mechanisms may be operative.

Another novel finding was the ability of TGF-β receptor blockade alone to cause significant alterations in the tumor microenvironment favoring T-cell activation and the Th1 phenotype, as well as T-cell and leukocyte infiltration. SM16 treatment induced the expression of the message levels for cytokines that are chemoattractive for T cells and NK cells (29). An increase in tumor endothelial ICAM-1 expression and the enhanced expression of message levels for TNF-α, IFN-γ, and IL-1β, cytokines that stimulate adhesion molecule expression, are also consistent with a process that would increase T-cell and leukocyte infiltration (30). It should be noted that our RT-PCR results did not show perfect correlation with intratumoral cytokine measurements; however, this could be due to difficulties in sample proteolysis and inefficient extraction from tumor specimens.

Figure 5. FACS analysis of leukocyte populations in spleens and tumors in the Ad.E7/TC1 vaccine model. A and B, tetramer staining of splenocytes. Spleens of animals from each group (n = 5; control, Ad.E7, SM16, and combination of SM16 and Ad.E7) were harvested 7 d after the second vaccination with Ad.E7 and subjected to FACS using MHC class I tetramers loaded with the immunodominant HPV-E7 peptide. A, average percentage of tetramer-positive CD8+ T cells within the spleens. Columns, mean; bars, SE. *, P < 0.05, compared with control. B, representative FACS tracing from each group. The numbers in each box are the percentage of tetramer-positive CD8+ T cells. C, FACS staining of tumors. TC1 tumors from each treatment group were digested and subjected to FACS 2 d after the second dose of Ad.E7, a time when the tumors in the combination group were rapidly shrinking, but still large enough to analyze. C, representative FACS tracings of side scatter versus CD8 expression. The number in each panel is the percentage of CD8+ cells of the total tumor population (Fig. 4F shows the averaged data from each group). D, cells from three tumors of each treatment group were pooled, run through a Ficoll gradient, and subjected to FACS using MHC class I tetramers loaded with the HPV-E7 peptide. The numbers in the upper boxes show the percentage of tetramer-positive/CD8+ cells and the numbers in the lower boxes show the percentage of tetramer-negative/CD8+ cells. This study was repeated with similar results.
The SM16-induced increase in the percentage of activated CD8⁺ T cells (CD25⁺), the increased Th1 cytokines, and the decreased arginase message levels also suggest that TGF-β blockade promotes a tumor environment promoting the activation of tumor-associated T cells. This is consistent with previous reports showing that TGF-β induces macrophage arginase levels (31) and is inhibitory to T-cell activity via inhibition of IFN-γ and perforins (12, 13). In the TC1 model, Ad.E7 treatment alone increased tetramer-positive T cells in both the spleen and tumors, whereas SM16 had no effect on this T-cell population in either location on its own. However, the combination of SM16 and Ad.E7 induced a further increase in tumor-associated, tetramer-positive cell above that induced by Ad.E7, with no significant change in splenic tetramer-positive cells. These results suggest that TGF-β receptor blockade does not increase the overall number of tetramer-positive T cells but does increase either the trafficking or persistence in the tumor of this antitumor T-cell population. These findings suggest that TGF-β is a key proximal immunosuppressive modulator of the tumor microenvironment that can inhibit T-cell and leukocyte trafficking, function, or persistence in tumors.

We have emphasized the role of TGF-β on immune cells; however, interpretation of our studies needs to take into account the complexity of the role of TGF-β in tumor biology (1, 2, 8) because multiple cells within a tumor make, activate, and respond to TGF-β. For example, using genetic models of mammary carcinogenesis in mice that result in selective loss of TGF-β signaling in tumor cells, investigators have observed marked changes in the secretion of specific chemokines by tumor cells that seem to then alter the tumor-associated myeloid cell populations and the tumor microenvironment (32, 33). As another example, loss of TGF-β receptor expression in lung cancer cells has been associated with increased invasiveness and increased production of the chemokine CCL5 (34). Our experiments use a "global" inhibitor that would presumably block TGF-β signaling in stromal cells, leukocytes, and in the tumor cells themselves (cell autonomous effects). It is currently unclear how important the blockade of each cell type might be. Additional effects of TGF-β on tumor biology could also be involved in antitumor effects, such as a recent report suggesting that TGF-β could subvert the immune system in directly promoting tumor growth through IL-17 (35).

One issue that should be considered with any type of inhibitor compound is that of specificity. As recently published (25), the activity of SM16 [similar to other such inhibitors such as SD-093 or SD208 (16)] is primarily directed against ALK5 (Kᵢ, 10 nmol/L) and ALK4 (Kᵢ, 1.5 nmol/L), although there is some moderate off-target activity to Raf (IC₅₀, 1 μmol/L) and p38/SAPKα (IC₅₀, 0.8 μmol/L). To try to address the question of off-target effects, we performed a study with the Ad.E7 vaccine in combination with a completely different class of TGF-β blocking agent, a soluble type I receptor (18), and showed very similar effects as we saw with SM16 (Fig 2B).

Previous studies have shown increased immunogenicity and antitumor responses when TGF-β inhibition (mediated by antisense oligonucleotides or dominant-negative receptors) is targeted to tumor cells and immune cell types, which are then used as vaccines or in adoptive transfer (36–40). Some of these approaches are moving into clinical trials (41–44). There are only a limited number of reports where systemic inhibitors of TGF-β have been combined with immunotherapy in the intact animal, however. Some success in a rat model of glioma has been achieved by combining intracranial injection of antisense TGF-β-i oligonucleotides with an irradiated tumor cell line (plus IFN-γ; ref. 45). Induction of anti-TGF-β antibodies by injection of plasmid DNA encoding a Xenopus TGF-β2 gene increased the therapeutic efficacy of a tyrosinase-related protein-2 plasmid DNA vaccine (46). Perhaps most relevant to this study is a report by Kobie and colleagues (47) showing that administration of an antibody against TGF-β enhanced the ability of an intratumorally injected dendritic cell vaccine to inhibit the growth of established mouse breast cancer cells.

These aforementioned studies, as well as others, have revealed the immunosuppressive effects of TGF-β in blocking systemic generation or function of antitumor T cells (16, 17) and mediating T regulatory cell activity (48). However, the experiments presented here identify additional mechanisms directed at immune and inflammatory cell infiltration, function, or persistence that may play an important role when combining TGF-β inhibition with immune-promoting therapies. These local intratumoral effects of TGF-β blockade may be extremely important because many cancer patients progress despite exhibiting relatively high percentages of circulating antitumor T cells (i.e., in melanoma) or showing the presence of T cells surrounding tumor tissue (49), supporting the idea that T cells must be able to move into the tumor, survive there, and effectively exert their direct and indirect (macrophage and NK cell activation) antitumor activities to be effective (50). Consistent with this idea, we have recently shown that after adoptive T-cell transfer, the number of cytotoxic T cells with tumors is enhanced after blockade of TGF-β receptor function (51).

Individual immune-activating therapies, such as tumor and dendritic cell vaccines, adoptive immune cell transfer, and adenovirus-based therapies (including Ad.IFN-β), are now being tested as single agents in clinical trials (52–54). The work presented here, and that of others in the field, suggests that a greater potential for efficacy may be achieved by combining these therapies with treatments targeting key modulators of immunosuppression, such as TGF-β, to promote an “immune-friendly” tumor microenvironment.

Disclosure of Potential Conflicts of Interest

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