Tumor Cyclooxygenase-2/Prostaglandin E2–Dependent Promotion of FOXP3 Expression and CD4+CD25+ T Regulatory Cell Activities in Lung Cancer

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Abstract
Cyclooxygenase (COX)-2 and its product prostaglandin (PG) E2 underlie an immunosuppressive network that is important in the pathogenesis of non–small cell lung cancer. CD4+CD25+ T regulatory (Treg) cells play an important role in maintenance of immunologic self-tolerance. CD4+CD25+ Treg cell activities increase in lung cancer and appear to play a role in suppressing antitumor immune responses. Definition of the pathways controlling Treg cell activities will enhance our understanding of limitation of the host antitumor immune pathways controlling Treg cell activities will enhance our understanding of limitation of the host antitumor immune responses. Tumor-derived COX-2/PGE2 induced expression of the Treg cell-specific transcription factor, Foxp3, and increased Treg cell activity. Assessment of E-prostanoid (EP) receptor requirements revealed that PGE2-mediated induction of Treg cell Foxp3 gene expression was significantly reduced in the absence of the EP4 receptor and ablated in the absence of the EP2 receptor expression. In vivo, COX-2 inhibition reduced Treg cell frequency and activity, attenuated Foxp3 expression in tumor-infiltrating lymphocytes, and decreased tumor burden. Transfer of Treg cells or administration of PGE2 to mice receiving COX-2 inhibitors reversed these effects. We conclude that inhibition of COX-2/PGE2 suppresses Treg cell activity and enhances antitumor responses. (Cancer Res 2005; 65(12): 5211-20)

Introduction
Cyclooxygenase (COX)-2 is constitutively overexpressed in a variety of epithelial malignancies (1). We and others have reported that COX-2 is constitutively elevated in human non–small cell lung cancer (NSCLC; refs. 2, 3). Although multiple genetic alterations are necessary for lung cancer invasion and metastasis, mounting evidence from numerous studies indicates that tumor COX-2 activity has a multifaceted role in conferring the malignant and metastatic phenotypes (4, 5). Overexpression of tumor COX-2 is associated with apoptosis resistance (6, 7), increased angiogenesis (8, 9), decreased host immunity (2, 10), and enhanced invasion and metastasis (11–13). In murine lung cancer models, we found that specific genetic or pharmacologic inhibition of COX-2 reduced tumor growth (10). In other related studies, we documented that COX-2 inhibition prevented tumor-induced suppression of dendritic cell activities (14). In this study, we sought to determine whether tumor COX-2 expression contributes to decreased host antitumor immune responses by affecting the frequency and activity of CD4+CD25+ T regulatory (Treg) cells.

Tumor-induced immune suppression has been well documented in lung cancer and other malignancies (15). Our studies have documented a COX-2-dependent immunosuppressive network in the NSCLC microenvironment. Tumor-reactive T cells accumulate in lung cancer tissues but fail to respond (16, 17), in part, because high proportions of NSCLC tumor-infiltrating lymphocytes (TIL) are Treg cells (18). Treg cells actively down-regulate the activation and expansion of self-reactive lymphocytes (19). Given that many tumor-associated antigens recognized by autologous T cells are antigenically normal self-constituents, Treg cells engaged in the maintenance of self-tolerance may impede the generation and activity of antitumor reactive T cells (20, 21). Thus, reducing the number of Treg cells or abrogating their activity within the tumor environment may induce effective tumor immunity in otherwise nonresponding hosts by activating tumor-specific and nonspecific effector cells (22–24). This is the first documentation that a tumor-induced Treg cell activity can be down-regulated by COX-2 inhibition leading to the restoration of antitumor responses.

Materials and Methods
Reagents. Dimethyl prostaglandin (PG) E2 and E-prostanoid (EP) 2/EP4 receptor agonists (Butaprost and PGE1 alcohol) were purchased from Cayman Chemical Co. (Ann Arbor, MI). FITC, phycoerythrin, tricolor-labeled anti-mouse CD3, CD4, CD25, CTLA4, and CD45RB antibodies, and isotype-matched control antibodies were purchased from PharMingen (San Diego, CA), anti-mouse CXCR3 was from Zymed Laboratories (South San Francisco, CA). Forskolin and cholera toxin were obtained from Bioworld (Plymouth Meeting, PA) and Sigma (St. Louis, MO). Joseph Portanova (Pharmacia, St. Louis, MO) provided us with the COX-2 inhibitor (SC58236), anti-PGE2 monoclonal antibody (mAb; 2B5 mAb), and isotype-matched control mouse IgG1 (MOPc21).

Mice. Pathogen-free C57BL/6 and BALB/c mice (8–12 weeks old) were obtained from Harlan (Indianapolis, IN). COX-2 knockout mice and controls on a mixed B6/129P2 background were obtained from Taconic (Germantown, NY). CC-10 TAg transgenic mice on the FVB background were bred at the West Los Angeles Veterans Affairs vivarium as described previously (25) and were generously provided by Dr. Francesco J. DeMayo (Baylor College of Medicine, Houston, TX). The EP2−/− on a 129/SvEv background and EP4−/− receptor knockout mice on a B6/D2 background were generously provided by Dr. Beverly Koller (University of North Carolina, Chapel Hill, NC). Mice were maintained in the West Los Angeles Veterans Affairs Animal Research vivarium and the institution’s animal studies review board approved all studies.

Stable transfection. A 2.3-kb cDNA fragment containing the open reading frame for a polypeptide of 604 amino acids of murine COX-2 was cloned into the HindIII-ClaI site of the retroviral vector pLNCX (Clontech, Cambridge, MA). Stable transfection was achieved using the E. coli strain DH10B and the retroviral vector pLNCX. Transfection efficiency was determined by isolation of transfected clones with puromycin-resistant colonies and expression of COX-2 protein was confirmed by Western blot analysis. Transduced cell lines were maintained in selective medium. The COX-2–transduced cell lines were used in experiments as described in the Results section.

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Palo Alto, CA). For virus production, 70% confluent 293 T cells were transfected with COX-2 sense, COX-2 antisense, or pLNCX and selected in 500 μg/mL G418 (Life Technologies, Rockville, MD). COX-2 sense and antisense clones were initially screened from 96-well plates based on PGE2 production. The COX-2 sense clones produced 7 to 9 ng PGE2/mL/10^5 cells, whereas the COX-2 antisense clones produced 105 to 285 pg PGE2/mL/10^5 cells. The parental and control vector-transduced cells produced 2.5 to 3.2 ng PGE2/mL/10^5 cells. The clones were further characterized for COX-2 mRNA and protein by Northern and Western blot analyses, respectively. The COX-2 antisense clones expressed less COX-2 mRNA and protein than did the parental tumor cells, COX-2 sense, or control vector-transduced cells (data not shown). Northern blot analyses showed that the COX-1 message remained unaltered in the parental, COX-2 sense, COX-2 antisense, and control vector-transduced cells (data not shown). In these studies, a L1C2 COX-2 antisense clone that produces 78 to 102 pg PGE2/mL/10^5 cells/24 h and a L1C2 COX-2 sense clone that produces 9 ng PGE2/mL/10^5 cells/24 h were used. In Results and Discussion, these cells are referred to as COX-2 sense and antisense clones.

Cell culture. The murine Lewis lung carcinoma (3LL, H-2b, also known as LLC, ATCC CRL-1642) from American Type Culture Collection (Manassas, VA) and the line 1 alveolar lung tumor (L1C2, H-2d) were used in these studies. B16 melanoma and EL4 lymphoma cell lines syngeneic for C57Bl/6 mice were obtained from American Type Culture Collection. The 3LL, L1C2, L1C2 COX-2 antisense, L1C2 COX-2 sense clones, and the control vector-transfected cells (CV-L1C2) were routinely cultured as monolayers in 25 cm^2 tissue culture flask at 37°C in a humidified atmosphere containing 5% CO2 in air. The culture medium contained RPMI 1640 (Irvine Scientific, Santa Anna, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), penicillin (100 units/mL), streptomycin (0.1 mg/mL), and 2 mmol/L glutamine (JRH Biosciences, Lenexa, KS). The cell lines were Mycoplasma free and used up to the 10th passage before thawing frozen cells from liquid N2.

Collection of tumor cell supernatants. Supernatants were collected from L1C2 or 3LL cells (1 x 10^6 cells/mL) following a 24-hour culture in medium culture. Supernatants were also collected from cells treated with the specific COX-2 inhibitor SC88236 (5 μmol/L), anti-PGE2 mAb (5 μg/mL), or control antibody (5 μg/mL). This concentration of anti-PGE2 mAb was chosen (5 μg/mL) because it completely neutralized PGE2 in the tumor cell supernatant (TSN) by enzyme immunoassay (EIA) measurements. For control treatment, supernatant samples from isotype-matched control antibody was used. Both L1C2 and 3LL cells constitutively produce ~3 ng/mL PGE2/24 h/10^5 cells. When treated with SC88236 (5 μmol/L) for 24 hours, the cells produce 0.5 ng PGE2/mL/24 h/10^5 cells. Addition of anti-PGE2 (5 μg/mL) to tumor cell culture decreased PGE2 below the level of detection by EIA. Addition of the isotype-matched control antibody to the tumor cell culture did not alter PGE2 concentration.

In vitro proliferation assay. Murine spleen CD4^+CD25^- and CD4^+CD25^- T cells were purified using Miltenyi beads according to the manufacturer’s instructions. Flow cytometric evaluation of Miltenyi bead purified CD4^+CD25^- T cells showed >98% of the T cells staining positive for CD4^+CD25^- but ~96% of the T cells staining positive for CD4^+CD25^. The purified CD4^+CD25^- cells revealed <1% of the cells staining positive for CD4^+CD25^-. CD4^+CD25^- T cells were treated in an increasing concentration of dimethyl PGE2 (0.65, 13, and 26 μmol/L, TSN, TSN plus anti-PGE2, TSN plus isotype-matched control antibody, TSN plus anti–transforming growth factor (TGF)-β by Western blot analysis of Foxp3. Murine spleen CD4^+CD25^- T cells were stimulated with dimethyl PGE2 (26 μmol/L) for 24 hours. Western analysis was done as described previously (26) using protein G-Sepharose purified rabbit anti-mouse Foxp3 IgG (provided by Alexander Rudensky, University of Washington, Seattle, WA) at a dilution of 1:2,000 and the Amersham Life Science (Piscataway, NJ) enhanced chemiluminescence protocol. Western blots were stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to control for loading. Densitometric analyses were done using the Perkin-Elmer Life Sciences Kodak Image Station 440 (Boston, MA).

Malignant transformation. 1.5 x 10^5 3LL tumor cells were infected s.c. in the right suprascapular area of C57Bl/6 mice. Mice bearing 5-day-old palpable tumors were treated with SC88236 (0.1-3 mg/kg) thrice weekly via i.p. injections for the duration of the experiment. COX-2^-/- mice were used for tumor models as follows: 2.0 x 10^5 3LL tumor cells were injected s.c. in the right suprascapular area of the knockout mice or age-matched controls.

For the s.c. tumor implantations, tumor volumes were monitored by measuring two bisecting diameters of each tumor with calipers thrice weekly. Tumor volume was calculated using the formula: \( V = 0.4ab^2 \), with \( a \) as the larger diameter and \( b \) as the smaller diameter.

To determine the antitumor effects of COX-2 inhibition in a model with pulmonary specific tumor growth, CC-10 TAg transgenic mice wherein the adenocarcinomas develop in an organ-specific manner were used. In these transgenic mice, the SV40 large T antigen is expressed under control of the murine Clara cell-specific promoter, CC-10 (27). Mice expressing the transgene develop diffuse bilateral bronchoalveolar cell carcinoma and have an average life span of 4 months. The COX-2 inhibitor SC88236 (3 mg/kg) or the diluent were given i.p. in 6-week-old transgenic mice thrice weekly for 12 weeks. At 4 months, mice were sacrificed and lungs were isolated for quantification of tumor surface area. Tumor burden was assessed by microscopic examination of H&E-stained sections as described previously (25). Ten mice from each group were not sacrificed so that survival could be assessed.

To determine the role of Treg cells on COX-2 inhibition-mediated tumor reduction, CD4^+CD25^- Treg cells were purified from mouse spleens of non-tumor-bearing mice using Miltenyi beads. Treg cells were stimulated in vitro overnight with anti-CD3 (1 μg/mL) and PGE2 (26 μmol/L) before 450 nm with the Molecular Dynamics plate reader (Sunnyvale, CA). The experiments were repeated thrice.

Total RNA preparation, cDNA synthesis, and real-time PCR for Foxp3. Foxp3 was quantified by real-time PCR. Briefly, murine spleen CD4^+CD25^- and CD4^+CD25^- T cells were purified and cultured (5 x 10^5 cells/mL) for 24 hours in medium containing PGE2 (0, 6.5, and 26 μmol/L), line 1 alveolar carcinoma (L1C2), or 3LL TSN, TSN plus 5 μg/mL anti-PGE2, TSN plus isotype-matched control antibody, TSN from tumor cells treated with SC88236 (5 μmol/L), EP2 agonist (5 μmol/L), and EP4 agonist (5 μmol/L).

To determine if PGE2 treatment enhances spleen cell Foxp3 gene expression in vivo, mice were treated with diluent or dimethyl PGE2 (2.5 mg/kg/dose) for 1 week and spleen cells (10^7 cells) were quantified for Foxp3 gene expression.

For quantitative real-time PCR (QPCR) analysis, RNA was isolated using the Qiagen kit (Valencia, CA). The cDNA was prepared with a kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Foxp3 gene expressions was quantified using the SYBR Green quantitative PCR kit in the iCycler (Bio-Rad, Hercules, CA) and corrected with β-actin housekeeping control. Amplifications were done in a total volume of 20 μL for 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target to β-actin and then comparing this normalized value with the normalized expression in a reference control sample to calculate a fold change value. The primers for the amplons spanned intron/exon boundaries to minimize amplification of genomic DNA. Primer sequences were as follows: β-actin 5'-CCACAGCTGAGGAAGAAATC-3' and 5'-TCTCCAAGGAAGAGAGGAT-3' and Foxp3 5'-CCAGAGAACACGGACGACACCTT-3' and 5'-TTCTCACCACGGACGGCCTTG-3'. Western blot analysis of Foxp3. Murine spleen CD4^+CD25^- T cells were stimulated with dimethyl PGE2 (26 μmol/L) for 24 hours. Western analysis was done as described previously (26) using protein G-Sepharose purified rabbit anti-mouse Foxp3 IgG (provided by Alexander Rudensky, University of Washington, Seattle, WA) at a dilution of 1:2,000 and the Amersham Life Science (Piscataway, NJ) enhanced chemiluminescence protocol. Western blots were stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to control for loading. Densitometric analyses were done using the Perkin-Elmer Life Sciences Kodak Image Station 440 (Boston, MA).
transferring to COX-2 inhibitor–treated mice or COX-2 knockout mice bearing 5-day established tumors. Treg cells (4 × 10^5) were transferred on days 5 and 12. The COX-2 inhibitor was given starting on day 5 for the duration of the experiment.

Neutralizing antibody-mediated blockade of PGE2 was used as follows. Mice were pretreated with anti-PGE2 mAb or control antibody 24 hours before tumor inoculation and then thrice weekly for the duration of the experiment (10 mg/kg ip). To determine if PGE2 could reverse the COX-2 inhibitor–dependent reduction in tumor growth, dimethyl PGE2 (5 mg/kg) was given with the COX-2 inhibitor (3 mg/kg) to mice bearing 5-day established tumors thrice weekly for the duration of the experiment.

To evaluate CD4+CD25+ regulatory cells. The capacity of tumor-derived products to modulate CD4+CD25+ T-cell Foxp3 gene expression was assessed by QPCR analyses. Compared with Treg cells cultured in culture medium, TSN induced the tumor cell Foxp3 gene expression by 5-fold (P < 0.01). Due to constitutively elevated COX-2 expression, the tumor environment is a rich source of PGE2 (29). Neutralizing antibody-mediated-mediated blockade of PGE2 or TSN from COX-2 inhibitor–treated tumor cells abrogated tumor-induced Treg cell Foxp3 gene expression (P < 0.01). Control antibody did not significantly alter the Treg Foxp3 gene expression (data not shown). PGE2 increased CD4+CD25+ T-cell Foxp3 gene expression in a dose-dependent manner (5-9 fold; P < 0.01; Fig. 1B). As determined by Western blot analysis, PGE2 mediated an increase in Treg cell Foxp3 protein. Densitometric analysis revealed a 20-fold increase in Foxp3 protein in PGE2-treated CD4+CD25+ cells compared with diluent-treated control (P < 0.01; Fig. 1C).


To determine if TSN could induce spleen Treg cells completely abrogated tumor-induced Foxp3 induction in the COX-2 inhibitor–treated T-cell population. Control antibody did not significantly alter the TSN-mediated increase in CD4+CD25+ T-cell Foxp3 induction. Consistent with these findings, in a dose-dependent manner, PGE2 induced Foxp3 by 1.2- to 6-fold in the CD4+CD25+ T-cell population (Fig. 1F).

**Results**

**Prostaglandin E2 enhances the suppressive activity of CD4+CD25+ regulatory cells.** TSN-treated Treg cells were evaluated for their capacity to inhibit anti-CD3-stimulated T-cell proliferation. Exposure to TSN significantly increased Treg cell inhibitory activity in a COX-2/PGE2-dependent manner. Compared with control, TSN-treated Treg cells showed a 2.6-fold increase in inhibitory activity (Fig. 1A). Neutralizing antibody-mediated blockade of PGE2 or TSN from COX-2 inhibitor–treated tumor cells completely abrogated the augmentation of inhibitory activity induced by TSN. Neutralizing antibody-mediated blockade of TGF-β partially reversed the augmentation of inhibitory activity (P < 0.05). The control antibody did not significantly alter the TSN-induced inhibitory effect (data not shown). Compared with control untreated CD4+CD25+ cells, PGE2 significantly increased the suppressive capacity of Treg cells in a dose-dependent manner (1.5- to 3-fold; P < 0.01; Fig. 1A). In contrast, CD4+CD25− cells did not inhibit proliferation (data not shown).

**Tumor-derived prostaglandin E2 induces FOXP3 in CD4+CD25+ regulatory cells.** The capacity of tumor-derived products to modulate CD4+CD25+ T-cell Foxp3 gene expression was assessed by QPCR analyses. Compared with Treg cells cultured in culture medium, TSN induced the tumor cell Foxp3 gene expression by 5-fold (P < 0.01). Due to constitutively elevated COX-2 expression, the tumor environment is a rich source of PGE2 (29). Neutralizing antibody-mediated-mediated blockade of PGE2 or TSN from COX-2 inhibitor–treated tumor cells abrogated tumor-induced Treg cell Foxp3 gene expression (P < 0.01). Control antibody did not significantly alter the Treg Foxp3 gene expression (data not shown). PGE2 increased CD4+CD25+ T-cell Foxp3 gene expression in a dose-dependent manner (5-9 fold; P < 0.01; Fig. 1B). As determined by Western blot analysis, PGE2 mediated an increase in Treg cell Foxp3 protein. Densitometric analysis revealed a 20-fold increase in Foxp3 protein in PGE2-treated CD4+CD25+ cells compared with diluent-treated control (P < 0.01; Fig. 1C).


The EP2/EP4 receptor agonist 11-deoxy-PGE1 and the selective EP2 receptor agonist Butaprost induced Foxp3 gene expression by 25- and 16-fold, respectively. Consistent with these findings, forskolin, a pharmacologic activator of adenyly cyclase, and cholera toxin, which activates the Gαs subunit of G proteins, thus mimicking Gαs-coupled receptor signaling (EP2 and EP4), also induced Foxp3 by 16- and 14-fold, respectively (Fig. 1D). To further delineate the EP receptor mediating PGE2 increase in Treg cell Foxp3 gene expression, we used EP2 and EP4 knockout mice. Although the absence of EP4 receptor expression by Treg cells significantly reduced PGE2-mediated induction of Treg cell Foxp3 gene expression, the absence of the EP2 receptor expression by Treg cells ablated this induction (Fig. 1E).

**Prostaglandin E2 induces FOXP3 in CD4+CD25+ T cells.** To determine if TSN could induce Foxp3 in CD4+CD25+ T cells, this population was cultured in TSN for 3 days. Compared with diluent-treated control, a 1.7-fold induction in CD4+CD25+ Foxp3 expression was documented. Neutralizing antibody-mediated blockade of PGE2 and TSN from COX-2 inhibitor–treated Tumor cells completely abrogated tumor-induced Foxp3 induction in the COX-2 inhibitor–treated T-cell population. Control antibody did not significantly alter the TSN-mediated increase in CD4+CD25+ T-cell Foxp3 induction. Consistent with these findings, in a dose-dependent manner, PGE2 induced Foxp3 by 1.2- to 6-fold in the CD4+CD25+ T-cell population (Fig. 1F).
of COX-2/PGE2 inhibition on Treg cell activity and tumor burden in murine lung cancer models. COX-2 inhibition significantly reduced the CD4⁺CD25⁺ T-cell population by 60% at the tumor site (P < 0.01). Consistent with these findings, genetic inhibition of tumor COX-2 reduced CD4⁺CD25⁺ T cells at the tumor site by 30% (P < 0.05; Fig. 2A). Because CXCR3⁺ T cells can amplify antitumor responses (30), we quantified the frequency of this cell population at the tumor site. In contrast to Treg cells, COX-2 inhibition increased CXCR3⁺ T at the tumor site by 10% (data not shown). COX-2 inhibition decreased tumor-induced TIL Foxp3 gene expression by 60% (P < 0.01) at a time point when tumor volumes were equivalent in both groups of mice. Antibody-mediated neutralization of PGE2 in vivo reduced Treg cells by 30% and TIL Foxp3 expression by 50% (P < 0.05; Fig. 2A and B).

The COX-2 inhibitor–mediated decrease in Foxp3 gene expression was evident systemically. Compared with naive controls, CD4⁺CD25⁺ Treg from spleens of tumor-bearing mice had a 26-fold induction in Foxp3 gene expression (P < 0.01). COX-2 inhibitor treatment decreased the tumor-induced Treg Foxp3 expression by 42% (P < 0.05; Fig. 2C).

To determine the effect of COX-2 inhibition on Treg cell activity, we evaluated the ability of Treg cells to inhibit anti-CD3-stimulated proliferation in vitro. Compared with non-tumor-bearing controls, diluent-treated tumor-bearing mice showed a 3.5-fold increase in the Treg cell inhibitory activity. COX-2 inhibitor treatment
completely abrogated the tumor-induced T cell inhibitory activity (P < 0.05; Fig. 2D).

Accompanying the decrease in Treg cell frequency and activity, COX-2 inhibition (SC58236 dose, 0.5-3 mg/kg) led to a decrease in tumor growth rates (Fig. 3A; P < 0.01 compared with diluent-treated control). Consistent with their importance in promoting tumor growth, transfer of CD4+CD25+ T cells significantly reversed the COX-2 inhibition-mediated antitumor responses. In vivo neutralization with antibody-mediated blockade of PGE2 significantly reduced the tumor growth rate. Conversely, PGE2 administration partially, but significantly, reversed the COX-2 inhibitor–mediated tumor reduction (Fig. 3B). Nonsteroidal anti-inflammatory drugs (NSAID) may affect targets other than COX-2 isozymes. Therefore, tumor growth was evaluated in COX-2 knockout mice. Consistent with the studies of Williams et al. (31), in comparison with age-matched controls, COX-2 knockout mice showed reduced tumor growth, similar to the results shown with COX-2 inhibitor treatment (Fig. 3C).

To determine the effect of COX-2 inhibition on Treg cells in a spontaneous lung cancer model, we used CC-10 SV40 TAg transgenic mice in which the adenocarcinomas develop in an organ-specific manner. Consistent with the findings in the s.c. tumor model, COX-2 inhibition decreased the frequency of Treg cells by 50% and Foxp3 gene expression by 60% in CC-10 SV40 TAg transgenic mice (Fig. 2B). Accompanying the decrease in Treg cells, there was reduced tumor burden in COX-2 inhibitor–treated CC-10 mice compared with the diluent-treated control group. In addition to marked reduction in tumor burden, histologic examination revealed areas of distinct lymphocyte infiltration in remaining tumor (Fig. 4A-E). Survival was prolonged in the SC58236 treatment group compared with diluent-treated controls (P < 0.001; Fig. 4F).

Based on previous reports indicating that tumor progression can be modified by host cytokine profiles (32, 33) and that COX-2 expression maybe an important determinant of cytokine expression, we measured the cytokine production from tumor sites following COX-2 inhibition. We evaluated tumor homogenates for the presence of TGF-β, PGE2, IL-10, IFN-γ, IL-12, MIG/CXCL9, IP-10/CXCL10, and GM-CSF. COX-2 inhibitor–treated mice showed a significant induction in type 1 cytokines but a decrease in immunosuppressive mediators. Compared with the diluent-treated group, mice treated with COX-2 inhibitor had significant reductions in TGF-β (1.5-fold; P < 0.05), PGE2 (2.5-fold; P < 0.05), and IL-10 (2-fold; P < 0.05) but an increase in IFN-γ (8-fold; P < 0.001), IL-12 (2-fold; P < 0.05), MIG/CXCL9 (2.4-fold; P < 0.01), IP-10/CXCL10 (7-fold; P < 0.05), and GM-CSF (6.5-fold; P < 0.001; Fig. 5A and B). A similar cytokine profile was observed in lung homogenates of CC-10 mice treated with the COX-2 inhibitor (data not shown).

Compared with diluent-treated controls, mice treated with SC58236 showed an enhanced tumor-specific T cell release of IFN-γ (Fig. 5C).

**Discussion**

Recent studies document the importance of COX-2 expression in human lung cancer (2, 34, 35). COX-2 overexpression underlies an immunosuppressive network in NSCLC. Progression of a premalignant lesion to the metastatic phenotype is associated with markedly higher COX-2 expression. This is also evident when lung cancer lymph node metastases are compared with primary adenocarcinomas (3). Accordingly, Khuri et al. (34) found that tumor COX-2 overexpression seems to portend a shorter survival among patients with early-stage NSCLC. We reported recently that COX-2

Figure 1. A, PGE2 enhances the suppressive activity of CD4+CD25+ Treg cells. Treg cells were evaluated for their capacity to inhibit anti-CD3-stimulated T-cell proliferation. Murine CD4+CD25+ and CD4+CD25− T cells (2 × 10^5) were treated with dimethyl PGE2 (0, 6.5, 13, and 26 μM/L), TSN, TSN + anti-PGE2, TSN + isotype-matched control antibody, TSN + anti-TGF-β1, or TSN from tumor cells treated with COX-2 inhibitor. *, P < 0.01. Neutralizing antibody–mediated blockade of TGF-β1 partially reversed the augmentation of inhibitory activity. **, P < 0.05. The absence of the EP4 and EP2 receptors on Treg cells inhibited the PGE2-dependent induction of Treg cell gene expression. *, P < 0.01. Lane 1, diluent-treated control; P < 0.01. Lane 2, PGE2-treated CD4+CD25+; lane 3, PGE2-treated CD4+CD25−. B, EP2 and EP4 receptor agonists induce Foxp3 expression. Murine splenic CD4+CD25+ T cells (5 × 10^6/mL) were cultured for 24 hours with EP receptor agonists (5 μM/L). Foxp3 gene expression was quantified using the SYBR Green quantitative PCR kit in the iCycler and corrected with 18S gene expression. **, P < 0.01. Foxp3 gene expression requires EP4 and EP2 receptor expression. Murine splenic CD4+CD25+ T cells (5 × 10^6/mL) were cultured for 24 hours with PGE2 (5 μM/L) and neutralizing antibody-mediated blockade of PGE2 in TSN or TSN collected from COX-2 inhibitor–treated tumor cells. Bars, SE. Representative of three independent experiments.

Figure 2. SC58236 reverses the COX-2 inhibition-mediated antitumor responses. A, SC58236 significantly decreased TGF-β1 protein expression in IL-10–treated tumor cells. *, P < 0.01. Lane 1, IL-10–treated control; Lane 2, IL-10–treated SC58236; lane 3, IL-10–treated PGE2. Lane 4, IL-10–treated PGE2 + SC58236. B, SC58236 decreases IL-12 protein expression in IL-10–treated tumor cells. *, P < 0.01. Lane 1, IL-10–treated control; Lane 2, IL-10–treated SC58236; lane 3, IL-10–treated PGE2. Lane 4, IL-10–treated PGE2 + SC58236. Bars, SE. Representative of three independent experiments.

Figure 3. COX-2 inhibitor–mediated tumor reduction (Fig. 3B) in comparison with age-matched controls, COX-2 knockout mice. Consistent with the studies of Williams et al. (31), in comparison with age-matched controls, COX-2 knockout mice showed reduced tumor growth, similar to the results shown with COX-2 inhibitor treatment (Fig. 3C).

Figure 4. COX-2 Inhibition Reduces Treg Activities in Lung Cancer

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tumor-bearing mice had a marked induction in tumor-bearing and SC58236-treated tumor-bearing mice for quantification of Five days following tumor inoculation, mice were treated with diluent or SC58236 (3 mg/kg thrice weekly). Murine splenocytes were isolated from naive, diluent-treated ()), or L1C2-COX-2 antisense (COX-2 AS) evaluated. L1C2, L1C2-COX-2 sense (COX-2 S

Figure 2. A, COX-2/PGE2 inhibition reduces CD4+CD25+ Treg cells in vivo. The effect of COX-2/PGE2 inhibition on the frequency of CD4+CD25+ Treg cells was evaluated. L1C2, L1C2-COX-2 sense (COX-2 S), or L1C2-COX-2 antisense (COX-2 AS) cells were inoculated (1.5 × 10^7 tumor cells) in BALB/c mice. Five days following tumor inoculation, L1C2 tumor-bearing mice were treated with diluent or SC58236 (3 mg/kg thrice weekly). Mice were pretreated with anti-PGE2 mAb or control antibody 24 hours before tumor inoculation and then thrice weekly. One week after treatment, TILs were purified from tumor cell digests using Percoll gradient and the leukocyte population was stained for CD4 and CD25; 10,000 gated events were collected and analyzed by flow cytometry using the CellQuest software. Compared with diluent-treated control, systemic COX-2 inhibition led to a significant decrease in the percentage of CD4 T cells expressing CD25 at the tumor site. *, P < 0.01. Similarly, genetic inhibition with COX-2 antisense constructs significantly reduced tumor cells in the TILs. *, P < 0.05, compared with diluent and COX-2 sense. In vivo neutralization with antibody-mediated blockade of PGE2 reduced Treg cells. *, P < 0.05. In contrast, control antibody did not significantly alter the frequency of Treg cells in the TILs of the diluent treatment group. Representative of three independent experiments (n = 8 mice per group). B, COX-2/PGE2 inhibition reduces TILs Foxp3 gene expression in vivo. The effect of COX-2/PGE2 inhibition on TILs Foxp3 gene expression was evaluated. 3LL tumor cells (1.5 × 10^7) were inoculated in C57BL/6 mice. Five days following tumor inoculation, mice were treated with diluent or SC58236 (3 mg/kg thrice weekly). For groups receiving antibodies, mice were pretreated with anti-PGE2 mAb or control antibody 24 hours before tumor inoculation and then thrice weekly. The CC-10 TAg transgenic mice were treated at 6 weeks old with diluent or SC58236 (3 mg/kg thrice weekly). TILs were evaluated for Foxp3 gene expression by QPCR. Compared with naïve controls, splenocytes from tumor-bearing mice were inoculated (1.5 × 10^7) were purified from spleens and 3LL tumor cells (1.5 × 10^7) were inoculated in C57BL/6 mice. Five days following tumor inoculation, mice were treated with diluent or SC82B36 (3 mg/kg thrice weekly). Murine splenocytes were isolated from naïve, diluent-treated tumor-bearing and SC82B36-treated tumor-bearing mice for quantification of Foxp3 gene expression by QPCR. Compared with naïve controls, splenocytes from tumor-bearing mice had a marked induction in Foxp3 gene expression. *, P < 0.01. The COX-2 inhibitor treatment significantly decreased the tumor-induced splenocyte Foxp3 expression. *, P < 0.01 compared with diluent-treated tumor-bearing mice. Columns, mean for eight mice per group; bars, SE. C, COX-2 inhibition decreases tumor-induced spleen Treg Foxp3 expression in vivo 3LL tumor cells (1.5 × 10^7) were inoculated in C57BL/6 mice. Five days following tumor inoculation, mice were treated with diluent or SC82B36 (3 mg/kg thrice weekly). Murine splenocytes were isolated from naïve, diluent-treated tumor-bearing and SC82B36-treated tumor-bearing mice for quantification of Foxp3 gene expression by QPCR. Compared with naïve controls, splenocytes from tumor-bearing mice had a marked induction in Foxp3 gene expression. *, P < 0.01. The COX-2 inhibitor treatment significantly decreased the tumor-induced splenocyte Foxp3 expression. *, P < 0.01 compared with diluent-treated tumor-bearing mice. Columns, mean for eight mice per group; bars, SE. D, COX-2 inhibition in tumor-bearing mice decreases the suppressive activity of CD4+CD25+ Treg cells. 3LL tumor cells (1.5 × 10^5) were inoculated in C57BL/6 mice. Five days following tumor inoculation, mice were treated with diluent or SC82B36 (3 mg/kg thrice weekly). One week following treatment, Treg cells were evaluated for their capacity to inhibit anti-CD3-stimulated T-cell proliferation. CD4+CD25+ and CD4+CD25- cells (2 × 10^5) were purified from spleens and 3 × 10^5 cells were added to plate-bound, anti-CD3-coated plates (1 μg/mL) and soluble anti-CD28 (1 μg/mL) containing 5 × 10^4 spleen T cells in quadruplet wells per condition in 96-well plates for 72 hours. Compared with non-tumor-bearing controls, diluent-treated tumor-bearing mice had an increase in the Treg cell inhibitory activity on effector T-cell proliferation. *, P < 0.01. COX-2 inhibitor treatment completely abrogated the tumor-induced T-cell inhibitory activity. *, P < 0.05. CD4+CD25+ cells did not inhibit the proliferation of splenic T cells (data not shown). Columns, mean for eight mice per group; bars, SE.

expression is responsible for CD44-dependent tumor invasion (11), chemokine-dependent angiogenesis (8), and survivin-mediated apoptosis resistance in NSCLC (7, 36). Thus, in addition to suppressing immunity, tumor COX-2 expression has been found to promote angiogenesis, increase tumor resistance to apoptosis, and enhance tumor invasiveness and metastasis (6, 8, 11, 12, 36–38).

Although tumor COX-2 expression mediates immunosuppression (10, 14), the specific molecular and cellular pathways in the complex COX-2-dependent immunosuppressive network are now being unraveled that link the IKK/nuclear factor-κB pathways in tumor-associated macrophages as well as in preneoplastic lesions (39–42). In addition to tumor-associated macrophages contributing to the immunosuppressive milieu, tumor-reactive T cells accumulate in lung cancer tissues but fail to respond (16, 17). In fact, a high proportion of NSCLC TILs are CD4+CD25+ Treg cells that exert inhibition of autologous T-cell proliferation (18). Several studies
have reported increased CD4+CD25+ Treg cells in peripheral blood lymphocytes and TILs in various malignancies (43–46). In murine models, depletion of CD4+CD25+ T cells significantly augments the efficacy of cancer vaccination (22–24), implying that these cells suppress immune responses against cancer cells. Thus, the pathways controlling Treg cell activities may be important for the understanding of antitumor host immune responses in lung cancer.

Recent efforts to identify specific molecular markers for Treg cells resulted in the identification of Foxp3, a forkhead transcription factor family member encoded on the X chromosome (47). This is the most specific marker for Treg cells and is specifically expressed in CD4+CD25+ T cells in the thymus and the periphery (48). In addition, forced expression of the Foxp3 gene can convert murine naïve T cells to Treg cells that phenotypically and functionally resemble naturally occurring CD4+CD25+ Treg cells (47–49). Furthermore, inoculations of CD4+CD25+ T cells prepared functionally resemble naturally occurring CD4+CD25+ Treg cells (48). In addition, forced expression of the Foxp3 gene can convert murine naïve T cells to Treg cells that phenotypically and functionally resemble naturally occurring CD4+CD25+ Treg cells (47–49). Furthermore, inoculations of CD4+CD25+ T cells prepared from normal mice can prevent autoimmune disease in Foxp3-deficient mice (49). Collectively, these findings indicate that Foxp3 is a critical control gene for the development and function of natural CD4+CD25+ Treg cells. Consistent with this concept, Rudensky et al. have shown that CD4+CD25+ T cells from Foxp3-deficient mice lack regulatory activity (49).

Although Foxp3 expression seems to play a key role in Treg cell-lineage commitment, it is not clear what signals regulate Foxp3. Because Treg cell activity is increased in the NSCLC microenvironment, we postulated that TSN would induce Treg cell Foxp3 gene expression. Because of constitutively elevated COX-2 expression, the tumor environment is a rich source of PGE2. High concentrations of PGE2 in the tumor environment promote tumor cell survival by inhibiting apoptosis (7), inducing tumor cell proliferation (50), increasing tumor progression and migration (11, 12), and inhibiting T-cell-mediated antitumor responses (10, 14, 29).

We determined the role of tumor COX-2 expression and PGE2 in TSN on Treg cell Foxp3 expression. Foxp3 was chosen to monitor Treg cells because CD25 is not specific for these cells. TSN-induced Treg cell Foxp3 in a COX-2/PGE2-dependent manner. In addition, PGE2 receptor agonists suggested that the increase in Foxp3 in Treg cells was mediated via the EP2/EP4 receptor pathways. Consistent with these findings, PGE2-induced Treg cell Foxp3 gene expression in vivo. Furthermore, PGE2-mediated induction of Foxp3 in Treg cells was functionally significant; in a dose-dependent manner, PGE2 augmented the suppressive capacity of CD4+CD25+ cells as shown by their ability to limit CD3-stimulated splenic T-cell proliferation. The results of these studies suggest that PGE2 modulates Treg cell activity by inducing Foxp3. In addition, TSN induced Foxp3 in CD4+CD25+ T cells after 72 hours in a COX-2/PGE2-dependent manner. The duration of PGE2 exposure may be important for inducing CD4+CD25+ Foxp3 expression; although not evident at

![Figure 3](https://www.aacrjournals.org/doi/10.1158/0008-5472.CAN-05-0322)
24 hours, Foxp3 gene expression was noted after 72 hours of PGE2 exposure. The data in Fig. 1F suggest that PGE2 treatment of mice in vivo may also induce Foxp3 in the CD4+CD25− population in Fig. 1G.

Based on the in vitro results, we tested the effect of COX-2/PGE2 inhibition on Treg cell activity in established murine lung cancer models. COX-2/PGE2 inhibition reduced the Treg cell frequency and activity, attenuated Foxp3 expression in TILs, and ultimately

Figure 4. A, COX-2 inhibition mediates potent antitumor responses in CC-10 SV40 TAg transgenic mice. The COX-2 inhibitor SC58236 (3 mg/kg) and diluent control were given i.p. in 6-week-old transgenic mice thrice weekly for 12 weeks. At 4 months, when the control mice started to succumb because of progressive pulmonary tumor growth, mice in all of the treatment groups were sacrificed and their lungs were isolated and embedded in paraffin. H&E staining of paraffin-embedded lung tumor sections from control-treated mice evidenced large tumor masses throughout both lungs without detectable lymphocytic infiltration (A and C). In contrast, the systemic COX-2 inhibition group evidenced extensive lymphocytic infiltration with marked reduction in tumor burden (B and D). Arrows, tumor (1) and infiltrating mononuclear cells (2). Magnification, ×320 (A and B) and ×320 (C and D). E, significant reduction in tumor burden following systemic COX-2 inhibition. P < 0.01. There was reduced tumor burden in systemic COX-2 inhibitor–treated CC-10 mice compared with the diluent-treated control group, F, survival was prolonged in the SC58236 treatment group. P < 0.001. Points, mean for 10 mice per group; bars, SE. Survival plots are from three independent experiments.

Figure 5. A-C, COX-2 inhibition induces a type 1 cytokine profile, T-cell-specific responses, and a decline in the immunosuppressive molecules. Five-day-old established tumors were treated with diluent or SC58236 (3 mg/kg thrice weekly) for 2 weeks. Nonnecrotic tumors were homogenized and evaluated for the presence of GM-CSF, IFN-γ, MIG/CXCL9, IP-10/CXCL10, IL-12, and TGF-β by ELISA and PGE2 by EIA. The cytokine and PGE2 measurements were normalized to total protein determined in the homogenates. For T-cell-specific IFN-γ release, splenic T lymphocytes were restimulated overnight with irradiated (100 Gy, Cs 137 γ-rays) autologous 3LL cells or syngeneic control tumors EL4 and B16 at a ratio of 10:1 and IFN-γ was quantified by ELISA. Compared with diluent-treated controls, mice treated with SC58236 had significant increase in GM-CSF, IFN-γ, MIG/CXCL9, IP-10/CXCL10, and IL-12 (A) but a decrease in the immunosuppressive molecules TGF-β, IL-10, and PGE2 (B). Results are expressed as pg/mg protein, *, P < 0.01. C, compared with diluent-treated controls, mice treated with SC58236 showed an enhanced 3LL tumor-specific T-cell release of IFN-γ. Results are expressed as pg/mL/106 cells. P < 0.001. Columns, mean for eight mice per group; bars, SE.
decreased tumor burden. The COX-2-dependent antitumor responses were due in part to a decrease in Treg cell frequency and activity as shown by the fact that transfer of CD4+CD25+ Treg cells significantly reversed these effects. In contrast, transfer of CD4+CD25+ did not affect tumor growth rates.

The biological basis for the benefit of NSAIDs in cancer has not been fully clarified. In addition, depending on the particular agent used and its dosage, the NSAIDs have both COX-2-dependent and COX-2-independent effects. Hence, we tested the effects of genetic inhibition of COX-2 on Treg cells.

Consistent with data obtained with COX-2 inhibitors, genetic inhibition of tumor COX-2 also reduced the frequency and activity of CD4+CD25+ T cells. We tested the effect of COX-2 inhibitors on tumor growth in SCID mice. Although tumor growth reduction was seen in SCID beige mice (data not shown), the COX-2 inhibitor–dependent reduction in growth rate was more pronounced in immunocompetent mice. Furthermore, immunocompetent tumor-bearing mice treated with SC58236 showed an enhanced tumor-specific T-cell release of IFN-γ. This suggests the importance of a functional immune system for the full manifestation of COX-2 inhibitor–mediated antitumor responses.

Concomitantly, a decrease in Treg cells led to a reciprocal increase in CXCR3+ T cells, restoration of type 1 cytokine, and antiangiogenic chemokines (MIG/CXCL9 and IP-10/CXCL10) at the tumor site. Apart from a decrease in TGF-β, PGE2, and IL-10, the tumor sites of COX-2 inhibitor–treated mice revealed significant increases in IFN-γ, IL-12, IP-10/CXCL10, MIG/CXCL9, and GM-CSF. In addition, in our in vitro data show that PGE2 can induce FoxP3 in CD4+CD25+ cells. Hence, a decrease in PGE2 at the tumor sites may reduce the frequency of Treg cells by decreasing the conversion of CD4+CD25+ to the CD25+ phenotype. The importance of PGE2 in inducing the regulatory phenotype was evident as PGE2 administration in vivo partially, yet significantly, reversed the COX-2 inhibitor–mediated decrease in tumor reduction. These findings suggest that although PGE2 is important other COX-2-dependent metabolites may also influence this pathway.

It is important to note that COX-2 inhibitor treatment decreased TGF-β at the tumor site and that neutralizing TGF-β in TN5 partially reversed the augmentation of Treg inhibitory activity. Recent studies suggest that TGF-β converts CD4+CD25+ T cells into Foxp3-expressing CD4+CD25+ Treg (51). In that study, the Foxp3 induction was dependent on the levels of TGF-β, suggesting a causal influence of TGF-β. TGF-β has been shown to regulate in vivo expansion of Foxp3-expressing CD4+CD25+ Treg cells (52). Hence, a decrease in TGF-β at the tumor site may decrease the conversion of CD4+CD25+ T cells into Foxp3-expressing Treg as well as decreasing the expansion of Foxp3-expressing CD4+CD25+ Treg cells. Further studies will be required to determine the role of TGF-β in the PGE2-dependent stimulation of Foxp3 and Treg cells.

In the current studies, we evaluated both genetic inhibition of COX-2 and COX-2 knockout mice as well as COX-2 pharmacologic inhibitors to define COX-2-dependent events. Our current findings are the first demonstration of tumor COX-2/PGE2-dependent modulation of the Treg cell activity in lung cancer. The tumor-induced effect on Treg cell activity is reversible when tumor COX-2 expression is inhibited genetically or pharmacologically. These findings lend further support to the suggestion that tumor COX-2 pathways may be important targets for chemoprevention as well as genetic or pharmacologic therapy in lung cancer. Additional studies are required to determine whether cancer clinical trials that use COX-2 inhibition reduce Treg cell function.

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