Specific Inhibition of Cyclooxygenase 2 Restores Antitumor Reactivity by Altering the Balance of IL-10 and IL-12 Synthesis[1]

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Cyclooxygenase-2 (COX-2), the enzyme at the rate-limiting step of prostanoid production, has been found to be overexpressed in human lung cancer. To evaluate lung tumor COX-2 modulation of antitumor immunity, we studied the antitumor effect of specific genetic or pharmacological inhibition of COX-2 in a murine Lewis lung carcinoma (3LL) model. Inhibition of COX-2 led to marked lymphocytic infiltration of the tumor and reduced tumor growth. Treatment of mice with anti-PGE_2 mAb replicated the growth reduction seen in tumor-bearing mice treated with COX-2 inhibitors. COX-2 inhibition was accompanied by a significant decrement in IL-10 and a concomitant restoration of IL-12 production by APCs. Because the COX-2 metabolite PGE2 is a potent inducer of IL-10, it was hypothesized that COX-2 inhibition led to antitumor responses by down-regulating production of this potent immunosuppressive cytokine. In support of this concept, transfer of IL-10 transgenic T lymphocytes that overexpress IL-10 under control of the IL-2 promoter reversed the COX-2 inhibitor-induced antitumor response. We conclude that abrogation of COX-2 expression promotes antitumor reactivity by restoring the balance of IL-10 and IL-12 in vivo. ***The Journal of Immunology,*** **2000, 164: 361–370.**

Lung cancer is the leading cause of cancer death in the United States (1). Many tumors, including lung cancer, have the capacity to promote immune tolerance and escape host immune surveillance (2). Tumors utilize numerous pathways to inhibit immune responses including the elaboration of immune inhibitory cytokines. In addition to direct secretion of immunosuppressive cytokines, lung cancer cells may induce host cells to release immune inhibitors (3–7). We have reported previously that human lung cancer cell-derived PGE_2 can orchestrate an imbalance in the production of IL-10 and IL-12 by lymphocytes and macrophages (5). IL-10 and IL-12 are critical regulatory elements of cell-mediated antitumor immunity. Although IL-10 inhibits important aspects of cell-mediated immunity, IL-12 induces type 1 cytokine production and effective antitumor cell-mediated responses (8–12). IL-10 overproduction at the tumor site has been implicated in tumor-mediated immune suppression (13, 14). In contrast, IL-12 is critical for effective antitumor immunity (15, 16). In both tumor models and patients, the tumor-bearing state induces lymphocyte and macrophage IL-10 but inhibits macrophage IL-12 production (5, 6, 17–19). Because PGE_2 appears to be pivotal in the reciprocal regulation of IL-10 and IL-12 (20, 21), we have sought to determine the pathways responsible for its high level production at the tumor site. We find that tumor cyclooxygenase-2 (COX-2)[3] expression is a pivotal determinant of the expression of these cytokines in the tumor-bearing host.

The initiation of prostanoid synthesis from arachidonic acid involves the enzyme referred to as COX, which has also been termed PGH synthase or PG endoperoxide synthase (22). Two isoenzymes have been identified: a constitutive form (COX-1) and an inducible isoenzyme (COX-2) (23–25). COX-2 is up-regulated in response to a variety of stimuli, including growth factors and cytokines (23). Because it can lead to enhanced PGE_2 production and subsequent cytokine imbalance in vivo, tumor expression of COX-2 may be instrumental in the generation of tumor-induced abrogation of T cell-mediated antitumor responses (5). COX-2 has been implicated in the development of colon cancer and may play a role in promoting invasion, metastasis, and angiogenesis in established tumors (26–29). In addition to lung and colon carcinomas (5, 30–32), COX-2 has recently been reported to be expressed in a variety of human malignancies (33–38). We report here that specific COX-2 inhibition serves to restore the tumor-induced imbalance in IL-10 and IL-12 and promotes antitumor responses in an immunocompetent murine lung cancer model.

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[3] Abbreviations used in this paper: COX, cyclooxygenase; SSC, sodium chloride sodium citrate; EIA, enzyme immunoassay; NSCLC, non-small cell lung cancer; Lewis lung carcinoma cell line; CM, control medium.

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Materials and Methods

Reagents

Indomethacin and aspirin were obtained from Sigma (St. Louis, MO). NS-398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide], and PGE_2 were purchased from Cayman Chemicals (Ann Arbor, MI). SC-58236, anti-PGE_2 (2B5 mAb), and isotype-matched control mouse IgG1 (MOPC21) were generously provided by Searle (Skokie, IL). Recombinant mIL-1β and rat anti-mouse anti-CD40 mAb were obtained from PharMingen (San Diego, CA).

Stable transfection

A 2.3-kb *Bam*HI-*Xho*I fragment containing the open reading frame for a polypeptide of 604 amino acids of murine COX-2 was isolated and cloned into the *Bam*HI-*Xho*I site of the eukaryotic expression vector pCR 3.1 (Invitrogen, San Diego, CA). For the antisense insert, a PCR fragment was generated from the sense construct utilizing the T7 promoter as the 5′ primer binding site and positions 725–701 of the murine cDNA as the 3′ primer binding site. The PCR fragment was cloned into the pCR 3.1 TA vector. In this vector, transcription of the cDNA is controlled by the CMV promoter. This vector also contains the neomycin resistance gene that allows for selection in G418 (Life Technologies, Rockville, MD). COX-2 sense and antisense-oriented expression vectors were prepared. These expression vectors were transfected into the 3LL cell line using the superfect transfection reagents (Qiagen, Los Angeles, CA). 3LL cells were also transfected with the PCR 3.1 control vector that did not contain the COX-2 insert. After transfection, the 3LL cells were selected in 500 μg/ml of G418 for 10 days. After selection, 3LL clones expressing COX-2 sense, antisense, and control vector constructs were isolated by limited dilutions from 96-well plates. 3LL COX-2 sense and antisense clones were initially screened based on PGE_2 production. The 3LL COX-2 sense clones produced 7–9 ng/ml/10^5 cells of PGE_2, whereas the COX-2 antisense clones produced in the range of 105–285 pg/ml/10^5 cells. The clones were further characterized for COX-2 mRNA and protein by Northern and Western blot analysis, respectively. The 3LL COX-2 antisense-transfected clones expressed less COX-2 mRNA and protein than did the 3LL parental tumor cells, 3LL COX-2 sense, or 3LL control vector-transfected clones (data not shown). Northern blot analysis for the *COX-1* gene message in the 3LL COX-2 antisense, 3LL COX-2 sense, and 3LL control vector clones showed that the COX-1 message remained unaltered (data not shown).

Northern blot analysis

To determine the time course of *COX-1* and *COX-2* gene expression in 3LL cells, 3LL COX-2 sense clone (4SC7-3LL), and 3LL control vector clone (CV-3LL) after IL-1β stimulation, 3LL cells cultures were treated with or without mIL-1β (150 U/ml) for 1–24 h, and total RNA was isolated. Cell pellets for each of the sample time points were lysed in 4 M guanidine isothiocyanate solution by gently pipetting. A quantity of 0.2 vol of chloroform was added to the cell lysate and kept on ice for 10 min. After centrifugation at 12,000 × g for 10 min at 4°C, the upper aqueous phase was transferred to a new tube. Equal volumes of isopropanol were added to the upper aqueous phase and kept on ice for 45 min. After centrifugation at 12,000 × *g* for 10 min at 4°C, the RNA pellet was washed twice with 75% ethanol and dried at room temperature. The dried RNA pellet was dissolved in 0.1% diethyl pyrocarbonate-treated double-distilled water and adjusted to a final concentration of 1 μg/ml. Northern blotting analysis was done as previously described to detect COX message (5). Briefly, 10 μg/sample for each of the conditions was electrophoresed through a 10% denaturing formaldehyde agarose gel, and the RNA was then transferred to a Hybond nylon membrane (Amersham, Arlington Heights, IL) in 20× sodium chloride sodium citrate (SSC). The RNA was fixed to the nylon membrane by UV cross-linking (Stratagene, San Diego, CA). Both prehybridization and hybridization were performed at 68°C in rapid hybridization solution obtained from Amersham. Duplicate filters were hybridized overnight with cDNA probes for murine COX-1, COX-2, and β-actin control. The probes were labeled with a [^{32}P]dCTP using a nick translation kit (BRL, Bethesda, MD). The probes were cleaned by passage through a Pharmacia Sephadex G-50 column (Piscataway, NJ). After hybridization, the filters were washed twice for 15 min at room temperature in 2× SSC/0.1% SDS and once for 10 min at 42°C and three times at 68°C in 2× SSC/0.1% SDS. The filters were exposed to Kodak XAR-5 film overnight at −80°C, and densitometric analysis was performed.

Cell culture

Murine Lewis lung carcinoma cell line (3LL) was obtained from American Type Culture Collection (ATCC; Manassas, VA). The COX-2 antisense 3LL clone (1ASE7-3LL), the COX-2 sense clone (4SC7-3LL), and the control vector-transfected cells (CV-3LL) were utilized for these studies. The cells were routinely cultured as monolayers in 25-cm^3 tissue culture flasks containing RPMI 1640 medium (Irvine Scientific, Santa Anna, CA) supplemented with 10% FBS (Gemini Biological Products, Calabasas, CA), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2 mM glutamine (JRH Biosciences, Lenexa, KS) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cell line was *Mycoplasma* free, and cells were utilized up to the 10th passage before thawing frozen stock 3LL cells from liquid N_2. For experiments utilizing 3LL, 1ASE7-3LL, 4SC7-3LL, or CV-3LL cell supernatants, 1 × 10^5 cells/ml were cultured in 6-well plates in RPMI 1640 with or without specific COX inducers or inhibitors. For experiments utilizing aspirin, 10^5 cells/ml were incubated for 2 h in the presence of aspirin (50 μg/ml). The tumor cells were then washed twice in PBS and replated at 10^5 cells/ml. After a 24-h culture period, the tumor cell supernatants were collected and PGE_2 levels measured by enzyme immunoassay (EIA).

Splenocyte IL-10 production

Splenocytes were isolated from normal mice using a standard protocol (39). Splenocytes were cultured at a concentration of 2 × 10^6 cells/ml in tumor cell supernatants or in tumor cell supernatants from 3LL cells that were treated with COX inhibitors (aspirin, indomethacin, NS-398, SC-58236, anti-PGE_2 mAb, or control Ab). After a 72-h incubation, IL-10 production by splenocytes was assessed by ELISA. Splenic lymphocytes were isolated from mice bearing 1ASE7-3LL tumors, 4SC7-3LL tumors, CV-3LL tumors, SC-5236-treated, indomethacin-treated, and control mice bearing 14-day-old 3LL tumors. Splenic lymphocytes were cultured in control medium (CM) at a concentration of 2 × 10^6 cells/ml, and IL-10 concentrations were measured after 6 days.

APC isolation

APC were purified from total splenocyte suspension by Ab-mediated complement lysis of T and B cells. The Abs used for these depletions included TIB207 (anti-CD4), TIB150 (anti-CD8), and TIB146 (anti-B lymphocytes). Hybridoma cell lines were purchased from ATCC. After RBC depletion, splenocytes were incubated with a mixture of mAbs and rabbit complement for 60 min at 37°C. After Ab depletion, APCs were washed twice in CM. Staining for cell surface markers with Abs to CD11b, CD11c, and F480 showed that cells were >95% APC following Ab depletion. Less than 5% of cells stained positively for CD3 following Ab depletions. APC (5 × 10^6 cells/ml) from tumor-bearing mice treated with the COX-2 inhibitor SC-58236, from untreated tumor-bearing controls and control non-tumor-bearing animals were stimulated with anti-CD40 (5 μg/ml). After a 72-h culture, IL-12 levels were determined by ELISA. For macrophage purification, APC suspension was cultured in 6-well plates overnight. After a 24-h culture, non-adherent cells were removed, adherent cells were washed twice with PBS, and macrophages were removed by trypsinization. Macrophages (5 × 10^6 cells/ml) were stimulated with anti-CD40 (5 μg/ml) in CM, 3LL supernatant, 1ASE7-3LL supernatant, 4SC7-3LL supernatant, CV-3LL supernatant, in media with PGE_2, anti-PGE_2 mAb, or isotype-matched IgG control Ab, MOPC21 (10 μg/ml).

Mice

Pathogen-free-female C57BL/6 mice (8–12 wk of age) were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in the West Los Angeles Veterans Affairs Animal Research Facility. IL-10 transgenic mice were made by standard methods at University of California, Los Angeles, Transgenic Mouse Core Facility as described previously.

3LL tumor model

For tumorigenesis experiments, 5 × 10^5 3LL parent, 1ASE7-3LL, 4SC7-3LL, or CV-3LL cells were inoculated on the right suprascapular area in C57BL/6 mice, and tumor volumes and survival were monitored. Tumor growth was assessed three times per week following tumor implantation. Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula $(0.4) \times (ab^2)$, with a as the larger diameter and b as the smaller diameter. Mice were pretreated with indomethacin (10 mg/ml in drinking water) or SC-58236 (3 mg/kg 3 times per week i.p.) 1 wk before the tumor cell inoculation and for the duration of the experiment. Mice were pretreated with anti-PGE_2 mAb or control Ab 24 h before tumor inoculation and three times a week for the duration of the experiment (10 mg/kg i.p.). On day 14 after tumor inoculation, non-necrotic tumors and splenic lymphocytes were isolated from tumor-bearing mice for determination of PGE_2 and IL-10 concentrations. PGE_2 and IL-10 levels were evaluated in tumor homogenates as described previously (40).

Following RBC and macrophage depletion, 6-day splenic lymphocyte culture supernatants were assessed for cytokine production. To compare 3LL, 4SC7-3LL, CV-3LL, and 1ASE7-3LL tumor formation in the lungs of C57BL/6 mice, 5×10^5 tumor cells were inoculated i.v. via a lateral tail vein. After 34 days, mice were sacrificed, and lungs were isolated and perfused by 10% formalin. Following fixation in 10% formalin for 48 h, lungs were embedded in paraffin. Sections (3–4 μm) were prepared for hematoxylin and eosin staining and histopathological examination. Tumor burden in lung sections was evaluated by microscope examination with a calibrated optical grid (a 1-cm^2 grid subdivided into 100 1.0-mm^2 squares). The total number of positive squares (with tumor occupying >50% of area) was determined for 4–6 separate high-power fields from three histologic sections as described previously (41). To evaluate IL-10-mediated immunosuppression in vivo, the splenic T lymphocytes from C57BL/6 or from IL-10 transgenic mice were isolated from spleens with Dynal beads (Dynal, Great Neck, NY) using the manufacturer's protocol. A total of 5×10^7 T lymphocytes/mouse was transferred to SC-58236-pretreated C57BL/6 mice 24 h before and 1 wk after 3LL tumor cell inoculation. Tumor volumes were assessed three times per week.

IL-10 and IL-12 ELISA

IL-10 protein concentrations from murine splenocytes cultured in CM or 3LL cell supernatants were determined by IL-10-specific ELISA as described previously (4). Briefly, 96-well Costar (Costar, Cambridge, MA) plates were coated overnight with 4 μg/ml of mouse anti-IL-10 mAb (PharMingen). The wells of the plate were blocked with 10% FBS (Gemini Biological Products) in PBS for 30 min. The plate was then incubated with the Ag for 1 h and excess Ag was washed off with PBS/Tween 20. The plate was incubated with 1 μg/ml of biotinylated mAb to IL-10 from PharMingen for 30 min, and excess Ab was washed off with PBS/Tween 20. The plates were incubated with avidin peroxidase, and after incubation in *o*-phenylenediamine substrate, the subsequent change in color was read at 490 nm with a Dynatech MR5000 spectrophotometer (Chantilly, VA). The recombinant IL-10 used in the assay as a standard was obtained from PharMingen. The sensitivity of the IL-10 ELISA was 15 pg/ml. For IL-12 measurements, an IL-12 ELISA kit was utilized (BioSource International, Camarillo, CA) and measurements were performed according to the manufacturer's instructions. The sensitivity of the IL-12 ELISA was 5 pg/ml.

PGE_2 EIA

PGE_2 concentrations were determined according to the Cayman Chemicals EIA kit protocol as described previously (5). Briefly, 96-well Costar plates were precoated overnight with 4 μg/ml of goat anti-mouse PGE_2 (BioSource International). PGE_2-acetylcholinesterase conjugate, mouse anti-PGE_2 mAb, and either standard or sample were added to each well. After an 18-h incubation at 25°C, the plate was washed five times to remove all unbound reagents. Ellman's reagent was then added to each well, and absorbance was determined at 405 nm by a Dynatech MR5000 spectrophotometer.

Statistical analysis

All in vitro results are representative of at least three independent experiments performed in triplicate. In vivo experiments were performed with at least six to eight mice per group. Differences between experimental vs control values were evaluated by Student's *t* test.

Results

Lewis lung carcinoma (3LL) cells express inducible COX-2 and abrogation of its expression down-regulates tumor PGE_2 production

Lewis lung carcinoma (3LL) is a weakly immunogenic murine lung cancer that has been shown previously to produce PGs (42). The two isoforms of COX were evaluated in 3LL. The constitutive enzyme, COX-1, has been previously found to be present in most cells and tissues, whereas the inducible isoenzyme, COX-2, is expressed in response to a variety of stimuli (23). To determine the time course of *COX-1* and *COX-2* gene expression, 3LL cells were stimulated with IL-1β for various durations, and Northern blot analysis was performed with labeled COX-1 and COX-2 cDNA probes. As shown in Fig. 1, the *COX-1* and *COX-2* genes are both expressed in 3LL cells. COX-2 mRNA was induced with maximal induction occurring 2 h after incubation with IL-1β. In contrast, COX-1 mRNA expression remained unchanged.

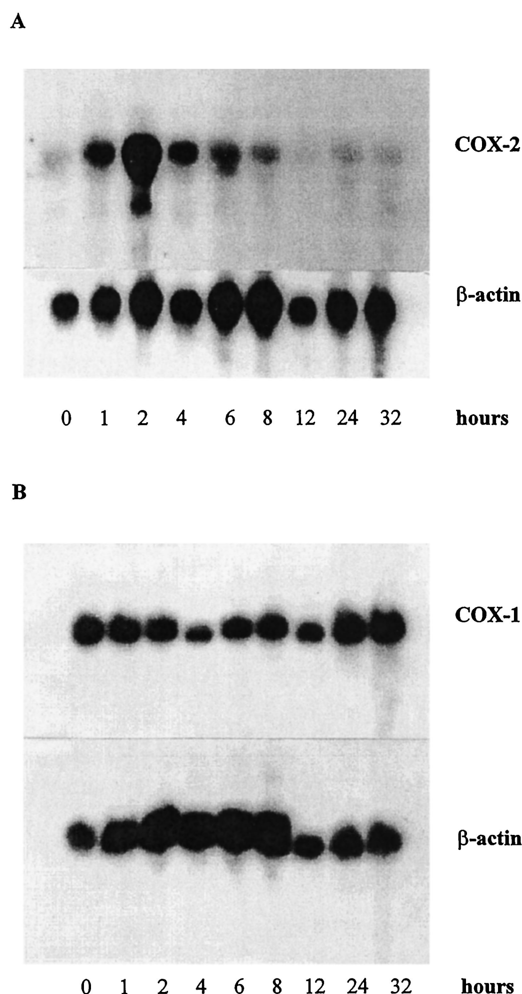

FIGURE 1. COX-2 is the inducible COX isoform in 3LL tumor cells. Following IL-1β stimulation of 3LL cells, Northern blot analysis was performed with COX-2 (*A*) and COX-1 (*B*) cDNA probes. Maximal COX-2 mRNA expression occurred 2 h after incubation with IL-1β, whereas COX-1 mRNA expression remained unchanged.

To evaluate the importance of tumor COX-2 in modifying tumor growth and host immunity, a COX-2 antisense plasmid was constructed and utilized to abrogate COX-2 production in 3LL. 4SC7-3LL and CV-3LL were utilized as controls. To assess the efficacy of the construct, the 3LL clones expressing COX-2 antisense, COX-2 sense, and control vector controls were evaluated for COX-2 mRNA expression and protein production.

Based on previous studies in a variety of host cells and tumors, we anticipated that high-level PG production by 3LL would be COX-2 dependent. Initially, several 3LL COX-2 antisense, sense, and control vector-transfected clones were evaluated for PGE_2 production, COX-1 mRNA expression, COX-2 mRNA expression, and protein production as well as tumor growth in vivo. In the initial characterization of the clones, the following results were obtained for which data are not shown. The 3LL COX-2 antisense-transfected clones expressed less COX-2 mRNA and protein than did the 3LL parental tumor cells, COX-2 sense, and control vector-transfected cells. The 3LL COX-2 sense clone expressed higher levels of COX-2 mRNA than the parental or control vector-transfected 3LL cells. In the 3LL COX-2 antisense, COX-2 sense, and control vector clones, *COX-1* gene expression remained unaltered. All antisense COX-2 clones evaluated showed diminished tumor

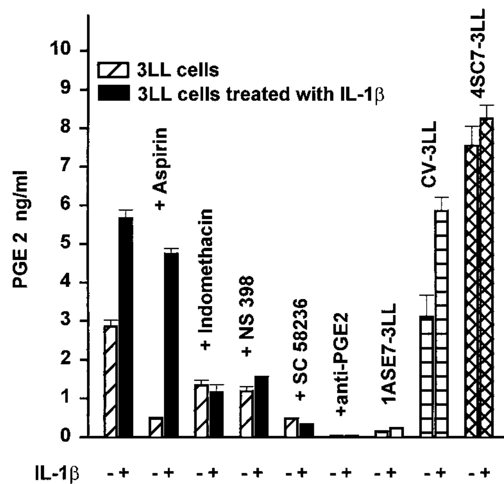

FIGURE 2. High-level PGE_2 production by 3LL cells is COX-2 dependent. Treatment of 3LL cells with COX-2 inhibitors as well as with anti-PGE_2 mAb leads to inhibition of IL-1β-stimulated PGE_2 production. The following COX inhibitors were utilized: aspirin (50 μg/ml), indomethacin (1 μg/ml), NS-398 (5 μg/ml), and SC-58236 (5 μg/ml). At these concentrations, COX inhibitors did not directly alter cell proliferation or viability (data not shown). In addition, anti-PGE_2 mAb (10 μg/ml) was utilized. A control mAb (10 μg/ml) did not alter detectable PGE_2 levels in tumor supernatants (data not shown). Results are representative of four independent experiments.

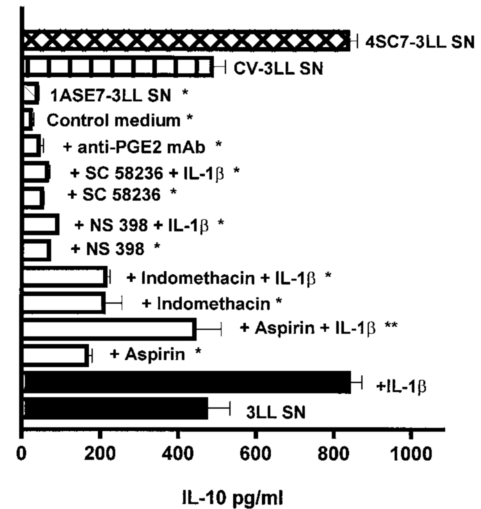

FIGURE 3. Pretreatment of 3LL cells with COX-2 inhibitors significantly decreases IL-10 production by splenocytes cultured in tumor supernatants. Splenocytes from normal C57BL/6 mice were cultured in 3LL supernatants. The tumor supernatants were initially collected after a 24-h incubation of tumors with or without COX-inhibitors and/or mIL-1β. Splenocyte IL-10 concentrations in culture supernatants (SN) were measured after a 72-h incubation of splenocytes in tumor-CM. One milliliter of each tumor supernatant was added to freshly isolated splenocytes and IL-10 production was determined by specific ELISA. Indomethacin, NS-398, SC-58236, and anti-PGE2 mAb significantly decreased both constitutive ($p < 0.05$) and IL-1β-treated tumor supernatant ($p < 0.05$) induction of IL-10 production by splenocytes. Results are representative of four independent experiments. *, $p < 0.05$ compared to 3LL SN; **, $p < 0.05$ compared to aspirin alone.

growth in vivo, whereas the in vivo growth of CV-3LL and sense clones were indistinguishable from that of the parental tumor.

To further assess the role of 3LL COX-2 expression in modulation of cytokine production and tumorigenesis, a 3LL COX-2 antisense clone, 1ASE7-3LL, that constitutively produced 90 $\pm$ 8 pg/ml/10^5 cells/24 h was selected for evaluation. The 3LL COX-2 sense clone 4SC7-3LL produced 7–9 ng/ml/10^5 cells of PGE_2 in 24 h was selected for further evaluation. The parental 3LL cell line and 3LL control vector-transfected clones (CV-3LL) constitutively produced between 2.5 and 3.5 ng/ml/10^5 cells of PGE_2 in 24 h. Stimulation of parental 3LL and CV-3LL with mIL-1β consistently led to a 2- to 3-fold increase in PGE_2 production. mIL-1β did not augment PGE_2 levels in the sense clone 4SC7-3LL (Fig. 2). In COX-2 antisense transfectants this induction of PGE_2 was abrogated completely as shown for 1ASE7-3LL in Fig. 2. Pharmacological agents that differentially block COX isoenzymes also were utilized to determine the relative contribution of each isoenzyme to high-level PGE_2 production (Fig. 2). The specific COX-2 inhibitors NS-398 and SC-58236, as well as indomethacin and aspirin, which block both isoenzymes, were utilized. Treatment of 3LL cells with indomethacin significantly decreased both constitutive and mIL-1β-stimulated PGE_2 production (Fig. 2). Aspirin was used to assess abrogation of COX-1. Aspirin has been shown to inhibit both COX-1 and COX-2 isoenzymes irreversibly by covalent modification (43). When aspirin is removed from the medium, however, newly synthesized IL-β-induced COX-2 is unaffected and therefore active (44). When 3LL cells were treated with aspirin for 2 h and then washed, mIL-1β-stimulated levels of PGE_2 production were maintained in 3LL supernatants. This suggests that following IL-1β stimulation, high-level PGE_2 production by 3LL cells is COX-2 dependent. Because specific COX-2 inhibition down-regulated the baseline, nonstimulated level of PGE_2 production as well as the IL-1β-induced levels (Fig. 2), we conclude that COX-2 also appears to contribute significantly to constitutive PGE_2 production by 3LL cells. The baseline constitutive COX-2 mRNA expression and protein production in 3LL parental cells is consistent with previous studies that have found serum to induce COX-2 (45, 46). Constitutive expression of COX-2 in normal host cells and tumor cells also has been described (47, 48). Thus, treatment of 3LL cells with mIL-1β induces COX-2 mRNA expression and this high-level COX-2 expression is responsible for increased PGE_2 levels in 3LL cells ($p < 0.05$, Figs. 1 and 2).

3LL-derived PGE_2 enhances splenocyte IL-10 production and inhibits macrophage IL-12 production

Previous studies indicate that the tumor-bearing state is often characterized by an increase in IL-10 production but diminished capacity to produce IL-12 (6, 18). One possibility is that tumor or host cell-derived PGE_2 is the cause of both augmentation of IL-10 and the simultaneous decrement in IL-12 production (5). To study the effect of 3LL tumor cell-derived PGE_2 on splenocyte production of IL-10, splenocytes from normal C57BL/6 mice were cultured in 3LL supernatants. The supernatants were collected after a 24-h incubation with various combinations of mIL-1β, COX inhibitors, anti-PGE_2, or control mAb. Splenocyte-derived IL-10 concentrations were measured after a 72-h incubation of splenocytes in tumor-conditioned medium. Whereas supernatants themselves did not contain detectable concentrations of IL-10 (data not shown), tumor supernatants from 3LL, CV-3LL, and 4SC7-3LL caused an increase in splenocyte IL-10 production (Fig. 3). Neither COX inhibitors nor IL-1β itself had a direct effect on constitutive splenocyte IL-10 production (data not shown). 3LL COX-2 expression and PGE_2 production were predominantly responsible for enhanced splenocyte IL-10 production. This was evidenced by the fact that the increase in splenocyte-derived IL-10 production was significantly inhibited by treatment of tumor cells with COX-2

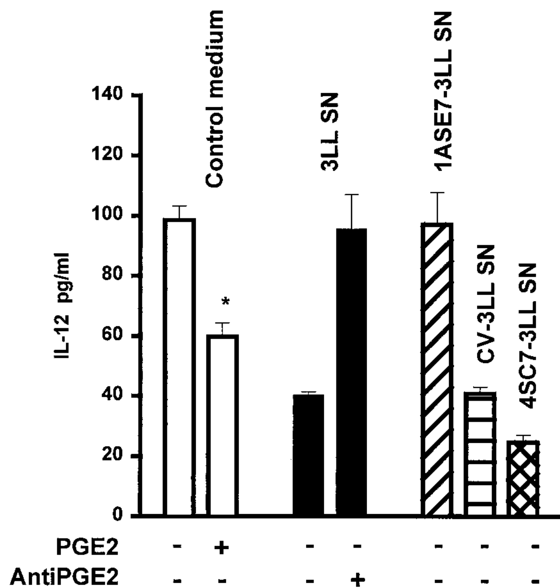

FIGURE 4. Tumor-derived PGE_2 reduces macrophage IL-12 production in vitro. One milliliter of medium or tumor supernatant was added to freshly isolated C57BL/6 splenic macrophages. Macrophages were cultured in either CM, 3LL supernatant (SN), CM with PGE_2 (5 μg/ml), 3LL SN with anti-PGE_2 mAb (10 μg/ml), control mAb (10 μg/ml), 1ASE7-3LL cell SN, 4SC7-3LL SN, or CV-3LL SN. After a 72-h incubation, IL-12 production by macrophages was determined by specific ELISA. Macrophages cultured in PGE_2-containing medium, 3LL, 4SC7-3LL, or CV-3LL SN evidenced significantly decreased IL-12 production ($p < 0.05$). Ab-mediated neutralization of PGE_2 in 3LL SN or culture in 1ASE7-3LL SN restored IL-12 production. Results are representative of three independent experiments.

inhibitors, including indomethacin, NS-398, or SC-58236 (Fig. 3, $p < 0.05$). COX-2 antisense-transfected tumor supernatants also had a decreased capacity to induce IL-10 as shown for 1ASE7-3LL in Fig. 3. The abrogation of splenocyte IL-10 induction by specific anti-PGE_2 mAb, but not control Ab, confirmed that PGE_2 was the COX metabolite in the tumor supernatant responsible for IL-10 induction. In experiments that evaluated the production of splenic lymphocyte and macrophage IL-10 in response to tumor supernatants, we found that lymphocytes made the greatest contribution to IL-10 synthesis (data not shown).

To investigate the effect of tumor-derived PGE_2 on macrophage IL-12 production, splenic macrophages were cultured in the following conditions: CM, 3LL supernatant, supernatant from 1ASE7-3LL cells, supernatant from 4SC7-3LL, supernatant from CV-3LL, CM with PGE_2 (5 μg/ml), anti-PGE_2 (10 μg/ml), or control mAb (10 μg/ml). Splenic macrophages cultured in 3LL, 4SC7-3LL, and CV-3LL supernatants showed a significant decrement in IL-12 production (Fig. 4). A similar decrement in macrophage-derived IL-12 was demonstrated when PGE_2 was added to macrophages in CM. Ab-mediated neutralization of PGE_2 in 3LL supernatant caused a restoration of macrophage IL-12 production to levels observed when macrophages were cultured in CM alone. Similarly, macrophage IL-12 production in 1ASE7-3LL supernatant was comparable to levels in CM. These findings indicate a tumor COX-2-dependent regulation of IL-10 and IL-12 in host lymphocytes and macrophages. Furthermore, PGE_2 appears to be the COX-2 metabolite responsible for increased lymphocyte-derived IL-10 and decreased macrophage IL-12 production in vitro.

COX-2 inhibition in vivo decreases tumorigenesis with concomitant reduction in IL-10 and restoration of IL-12 production

To determine the effect of tumor COX-2 expression on tumorigenesis in vivo, we studied tumor burden in the lungs of C57BL/6 mice after i.v. injection of either parental 3LL cells, CV-3LL, 4SC7-3LL, or 1ASE7-3LL transfectants. Compared with mice bearing parental 3LL, CV-3LL, or 4SC7-3LL, mice injected with 1ASE7-3LL-transfected cells showed a significant reduction in tumor burden 34 days after tumor inoculation (Fig. 5*A*, $p < 0.01$). Histologic evaluation of lung sections revealed that 1ASE7-3LL cells formed discrete tumor nodules that were extensively infiltrated with lymphocytes (Fig. 5, *D* and *E*). In contrast, parental 3LL, CV-3LL, or 4SC7-3LL injection led to formation of significantly larger tumor nodules that evidenced homogeneous tumor fields without inflammatory infiltration (Fig. 5, *B* and *C*).

To assess the importance of COX-2 inhibition on restoration of antitumor reactivity at a s.c. site, we evaluated the antitumor efficacy of both specific COX-1 and COX-2 inhibitors. Indomethacin-, SC-58236-, and anti-PGE_2-treated tumor-bearing mice showed significantly reduced tumor growth compared with 3LL tumor volumes in untreated mice. Control Ab did not alter 3LL tumor growth. The in vivo growth patterns of parental 3LL, 4SC7-3LL, and CV-3LL transfectants were indistinguishable (Fig. 6). Whereas the proliferation of 1ASE7-3LL in vitro was indistinguishable from that of the parental 3LL line (data not shown), the 1ASE7-3LL showed a significant reduction in tumorigenesis in vivo that was comparable to mice bearing parental 3LL tumors receiving COX-2 inhibitors or anti-PGE_2 mAb (Fig. 6). Mice bearing 1ASE7-3LL tumors and those treated with anti-PGE_2 mAb or COX-2 inhibitors had prolonged survival (median 50 days) compared with control tumor-bearing mice or mice treated with control Ab (median 30 days, $p < 0.05$; data not shown).

To determine the capacity of COX-2 inhibition to decrease IL-10 and increase IL-12 in vivo, non-necrotic tumor nodules and spleens from tumor-bearing mice were harvested 14 days after tumor inoculation. PGE_2 and IL-10 levels were evaluated in tumor homogenates (Table I) and splenocytes (Table II). Tumor nodules from 3LL-, CV-3LL-, and 4SC7-3LL-bearing mice produced significantly more PGE_2 and IL-10 than did tumor nodules from anti-PGE_2 mAb-, indomethacin-, or SC-58236-treated mice (Table I). The 1ASE7-3LL tumors also evidenced reduced PGE_2 and IL-10 production within tumor tissues, and mice bearing these tumors had significant reductions in IL-10 and PGE_2 within the spleen compared with tumor-bearing controls ($p < 0.05$, Tables I and II). Pharmacological inhibition of COX in vivo also reduced IL-10 and PGE_2 production by splenic lymphocytes compared with untreated tumor-bearing mice (Table II).

To assess COX-2-dependent modulation of IL-12 production by APC, splenic APC were isolated from 1ASE7-3LL, 4SC7-3LL, and CV-3LL tumor-bearing mice, mice bearing parental 3LL tumor after treatment with the COX-2 inhibitor SC-58236, or untreated tumor-bearing controls. APC were stimulated with anti-CD40 (5 μg/ml) and IL-12 production was determined by ELISA (Table III). APC from both SC-58236-treated and 1ASE7-3LL tumor-bearing mice produced significantly more IL-12 than APC from untreated tumor-bearing controls ($p < 0.05$).

The data presented above indicate that tumor COX-2 expression leads to PGE_2-dependent overproduction of IL-10 by host lymphocytes that could be responsible for enhanced tumor growth (19, 49). The following portion of the study was performed to deter-

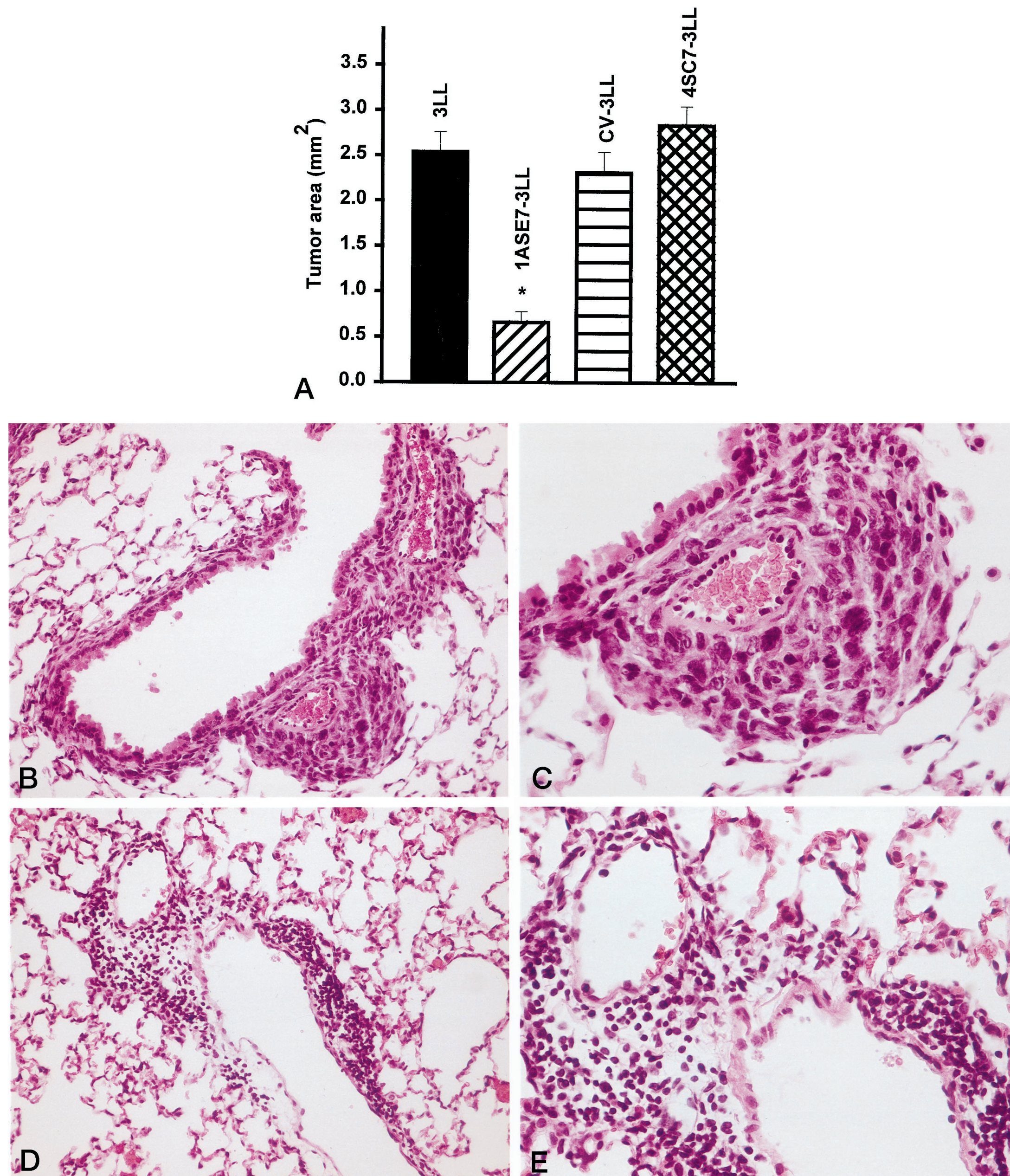

FIGURE 5. COX-2 antisense-transfected 3LL tumors show decreased tumorigenesis in the lungs of C57BL6 mice. A total of 5×10^5 parental 3LL tumor cells, 1ASE7-3LL, 4SC7-3LL, or CV-3LL transfectants were injected i.v. via a lateral tail vein in C57BL/6 mice. After 34 days, lungs were isolated, perfused, and fixed in 10% formalin and stained by hematoxylin and eosin. *A*, Tumor area was assessed by light microscopic examination with a calibrated optical grid (a 1-cm^2 grid subdivided into 100 1.0-mm^2 squares). Total number of positive squares (with tumor occupying >50% of area) was determined for 4–6 separate high-power (× 20 objective) fields from three histologic sections. Compared with mice receiving parental 3LL tumor cells, mice bearing 1ASE7-3LL showed significantly less tumor burden within the lungs ($p < 0.01$). Control vector and COX-2 sense-transfected tumors had an in vivo growth pattern that was similar to parental tumor (data not shown). *D* and *E*, Histologic evaluation of lung sections revealed that 1ASE7-3LL formed discrete tumor nodules that were extensively infiltrated with lymphocytes. *B* and *C*, In contrast, parental 3LL injection led to formation of significantly larger tumor nodules that evidenced homogeneous tumor fields without significant inflammatory infiltration. Control vector and COX-2 sense lung tumor nodules also evidenced homogeneous tumor fields without significant inflammatory infiltration (data not shown). Magnification: *B* and *D*, ×160; *C* and *D*, ×320.

mine whether lymphocyte overproduction of IL-10 could reverse the COX-2 inhibitor-mediated antitumor effect. To determine the role of lymphocyte-derived IL-10 on tumor progression in vivo, splenic T lymphocytes isolated from IL-10 transgenic mice that overproduce IL-10 under control of the IL-2 promoter were transferred to SC-58236-pretreated mice 24 h before and 7 days after 3LL tumor injection. As a control, splenic T lymphocytes isolated from normal C57BL/6 mice were transferred i.v. to 3LL tumor-bearing mice treated with SC-58236. Mice receiving the IL-10 transgenic lymphocytes evidenced a reversal of the antitumor effects of COX-2 inhibition (Fig. 7). In contrast, those mice that received transfer of equivalent numbers of normal C57BL/6 lymphocytes showed no modification in the COX-2 inhibitor-mediated antitumor response. Thus, the antitumor response in this model appears to be due to a COX-2 inhibitor-induced down-regulation of the immunosuppressive effect of lymphocyte-derived IL-10.

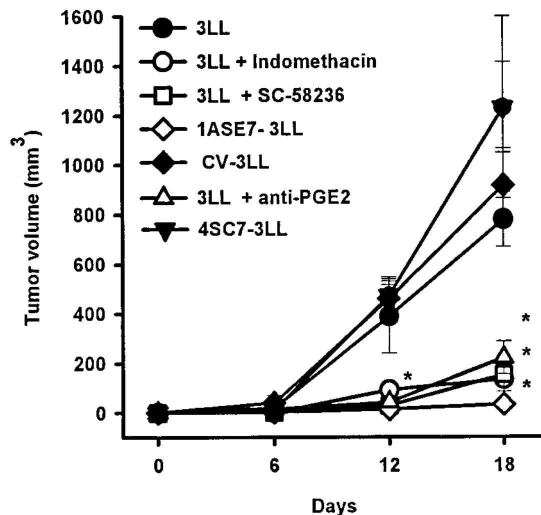

FIGURE 6. COX-2 inhibition reduces s.c. 3LL tumor growth in vivo. A total of 5×10^5 3LL cells were injected s.c. in the right flank of C57BL/6 mice (10–15/group). Mice were treated with anti-PGE_2, indomethacin, or SC-58236 as described in *Materials and Methods*. Treatment of tumor-bearing mice with COX-2 inhibitors significantly decreased tumorigenicity. The COX-2 control sense and control vector-transfected 3LL cells had the same growth rate in vivo as did the parental 3LL cells. In contrast, the 1ASE7-3LL transfectants had a significantly reduced growth rate that was comparable to the kinetics of tumor growth for COX-2 inhibitor or anti-PGE_2-treated mice. *, $p < 0.05$ compared to untreated tumor-bearing mice.

Table II. *COX-2 inhibition in vivo reduces PGE_2 and IL-10 production by splenic lymphocytes*[a]

Lymphocytes from	IL-10 Production (pg/ml/10^6 cells)	PGE_2 Production (pg/ml/10^6 cells)
Control non-tumor bearer	<15*	58 ± 23*
Untreated 3LL bearer	347.2 ± 12.4	515 ± 56
CV-3LL	322.6 ± 15.7	540 ± 43
4SC7-3LL	364.0 ± 22.3	583 ± 49
1ASE7-3LL	160.0 ± 14.0*	220 ± 5*
SC-58236-treated 3LL bearer	32.6 ± 4.3*	221 ± 67*
Indomethacin-treated 3LL bearer	<15*	183 ± 21*

[a] On day 14 after tumor inoculation, non-necrotic tumors and splenic lymphocytes were isolated from tumor-bearing mice for determination of PGE_2 and IL-10 concentrations. PGE_2 and IL-10 levels were evaluated in fresh tumor homogenates and in lymphocyte supernatants after 6 days in culture.

*, $p < 0.05$ compared to untreated tumor-bearing mice.

Discussion

To determine how inhibition of COX-2 expression effects antitumor immunity in vivo, we studied the effect of both pharmacological and specific genetic inhibition of COX-2 expression on lymphocyte and APC cytokine production in a murine model. In this study we postulated that COX-2 inhibition would 1) lead to reduced tumor growth and 2) result in restoration of IL-10/IL-12 balance in vivo.

COX-2 isoenzyme expression is significantly increased in human lung cancers compared with normal, non-cancerous lung tissues (5, 32). Our previous studies documented an immunosuppressive network in human non-small cell lung cancer (NSCLC) that is due to overexpression of tumor-derived COX-2 (5). We report here that 3LL cells express COX-2 at low levels constitutively, and this isoenzyme is induced following stimulation with mIL-1β in vitro. In contrast, COX-1 mRNA is expressed constitutively and expression remains unchanged following IL-1β stimulation. The increase in COX-2 mRNA in 3LL cells correlated with increases in COX-2 protein and PGE_2 synthesis. High-level PGE_2 production by 3LL cells after stimulation with IL-1β was documented to be COX-2 dependent as evidenced by genetic or pharmacologic inhibition of COX-2 in 3LL cells in vitro. Thus, the capacity for COX-2 induction in 3LL was similar to that previously described in human lung cancer and suggested that these tumor cells would be suitable for assessment in vivo (5).

In vivo inhibition of COX-2 with either indomethacin or SC-58236 led to reduction in tumor growth and prolonged survival. Because drugs that inhibit COX may have effects in addition to COX inhibition (50–52), we also assessed the involvement of COX-2 expression in tumorigenesis in experiments performed with 3LL cells genetically modified with COX-2 antisense or control constructs. Although the 3LL COX-2 sense or control vector-transfected tumors had growth rates comparable to the parental 3LL cells, the COX-2 antisense-transfected 3LL revealed a significant reduction in tumorigenesis that was comparable to tumors in mice receiving COX-2 inhibitors. Histologic evaluation of regressing tumors following COX-2 inhibition revealed marked lymphocytic infiltration. The histologic appearance suggested that an immune-mediated pathway was operative in mediating tumor reduction. To assess this possibility, we evaluated modulation of cytokine balance that accompanied COX-2 inhibition in vivo.

Previous studies suggest that populations of T cells in the tumor-bearing host may develop suppressor activities through the induc-

Table I. *COX-2 inhibition in vivo reduces PGE_2 and IL-10 production by tumor tissues*[a]

Tumors from	IL-10 Production (pg/ml/g of tumor tissue)	PGE_2 Production (ng/ml/g of tumor tissue)
Untreated mice (3LL)	734 ± 74	27.2 ± 1.9
CV-3LL	702 ± 57	29.0 ± 2.3
4SC7-3LL	928 ± 106	48.7 ± 4.2*
1ASE7-3LL	184 ± 27*	10.9 ± 2.1*
SC-58236-treated	398 ± 38*	16.8 ± 0.7*
Indomethacin-treated	268 ± 60*	12.3 ± 1.1*

[a] On day 14 after tumor inoculation, non-necrotic tumors and splenic lymphocytes were isolated from tumor-bearing mice for determination of PGE_2 and IL-10 concentrations. PGE_2 and IL-10 levels were evaluated in fresh tumor homogenates and in lymphocyte supernatants after 6 days in culture.

*, $p < 0.05$ compared to untreated tumor-bearing mice.

Table III. *COX-2 inhibition in vivo augments anti-CD40-stimulated IL-12 production by APC*[a]

Mice	IL-12 Production (pg/ml/72 h/5 × 10^6 cells)
Control non-tumor bearer	209.3 ± 7.6*
Untreated 3LL bearer	104.2 ± 12.2
CV-3LL	106.6 ± 9.8
4SC7-3LL	94.0 ± 10.0
1ASE7-3LL	178.8 ± 9.5*
SC-58236-treated 3LL bearer	233.8 ± 16.3*

[a] Splenic APC were isolated from 1ASE7-3LL tumor-bearing mice, mice bearing parental 3LL tumor after treatment with the COX-2 inhibitor SC-58236, or untreated tumor-bearing controls. APC were stimulated with anti-CD40 (5 μg/ml) and IL-12 production was determined by ELISA.

*, $p < 0.05$ compared to untreated tumor-bearing mice.

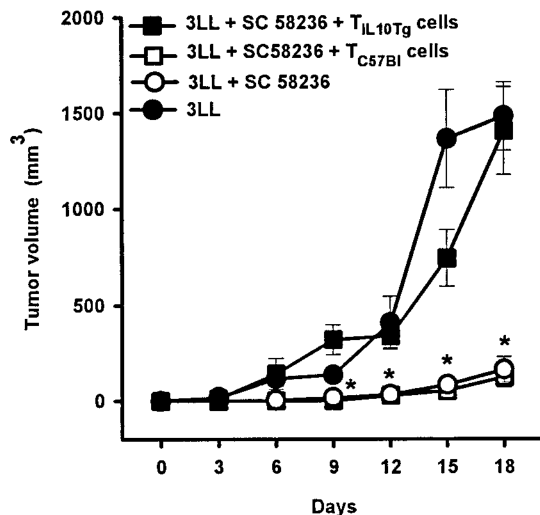

FIGURE 7. T lymphocytes from IL-10 transgenic mice reverse the antitumor effect of COX-2 inhibition. T lymphocytes from C57BL/6 mice or from IL-10 transgenic mice were isolated from spleens using Dynal beads. A total of 5×10^7 splenic T lymphocytes/mouse were transferred to SC-58236-pretreated mice 24 h before and 8 days after 3LL tumor cell inoculation. Tumor volume was assessed three times per week. *, $p < 0.01$.

tion of *IL-10* gene expression (4, 19). In accord with these studies documenting the importance of lymphocyte-derived IL-10 in the generation of tumor-induced tolerance, we speculated that the unregulated overproduction of PGE_2 by the tumor could be a central element in the immunosuppressive network because it leads to enhanced lymphocyte and macrophage IL-10 production.

IL-10 has the capacity to inhibit antitumor responses through several pathways. IL-10 has been documented to limit type 1 cytokine production (53, 54), Ag presentation (8, 55), and Ag-specific T cell proliferation (12). Pretreatment of tumor targets with IL-10 renders the tumor cells more resistant to CTL-mediated lysis (56, 57). IL-10 may also limit the access of functional APC to the tumor site (14). We have previously found that production of IL-10 by cutaneous carcinomas provides a mechanism for evasion of the local T cell immune response (13). We also found that transgenic mice overexpressing IL-10 under the control of the IL-2 promoter were unable to limit the growth of immunogenic tumors (49). Administration of blocking IL-10 mAbs restored in vivo antitumor responses in these transgenic mice. These findings support the suggestion that enhanced lymphocyte-derived IL-10 production antagonizes antitumor immunity (19). We have previously reported that NSCLC-derived PGE_2 increases the transcriptional rate of IL-10 mRNA in PBL (4). We now report that COX-2 inhibition is an effective intervention to decrease the induction of IL-10 in the tumor-bearing host. The significance of the COX-2 inhibitor-induced decline in IL-10 production is demonstrated by transfer of IL-10 overproducing lymphocytes to tumor-bearing mice; transfer of these transgenic lymphocytes to normal mice overcomes the COX-2 inhibitor-induced antitumor response.

In addition to the modulation of IL-10 levels, we find that COX-2 inhibitor therapy results in up-regulation of IL-12. Macrophage IL-12 has previously been shown to be down-regulated in tumor-bearing mice (18), and administration of this cytokine has been shown to have antitumor effects (58–62). Elevated levels of IL-12 may promote increased type 1 cytokine release and thus enhance cell-mediated antitumor immune responses. In addition, through its induction of IFN-γ, IL-12 may serve to limit angiogenesis (63, 64).

However, other nonimmune-mediated mechanisms also may be operative. For example, overexpression of COX-2 can cause tumor cells to resist apoptosis (26, 65). In addition, COX-2 expression in colon tumor lines enhances tumor invasiveness (26), increases metastatic potential (27), and promotes angiogenesis (28). A recent study in gastric carcinomas found that COX-2 overexpression significantly correlated with tumor invasion into lymphatic vessels and metastasis to lymph nodes (66). In our current studies, although the proliferation of 1ASE7-3LL in vitro was comparable to that of the parental cell line, these COX2 antisense transfectants showed a marked decrease in tumorigenicity in vivo. Furthermore, mice receiving IL-10 transgenic lymphocytes evidenced a reversal of the antitumor effects of COX-2 inhibition. Thus, in addition to promoting a malignant phenotype, our findings suggest that COX-2 also plays an important role in antagonizing host immune reactivity against malignant cells.

Despite therapeutic efforts, lung cancer remains the major cause of cancer-related death in the United States (67). Although immunologic-based therapies have shown some success for other malignancies, lung cancer has been largely unresponsive (68). The lung tumor environment promotes immunosuppressive networks and the development of tolerance (5, 69). Our current findings identify tumor COX-2 expression as a critical element in the development of immunosuppression and documents that in vivo abrogation of this enzyme causes significant tumor reduction. Tumor-derived PGs play an important role in augmenting production of inhibitory cytokines such as IL-10 (4, 21) while suppressing endogenous production of cytokines including IL-12 that are necessary for effective host cell-mediated antitumor immune responses (20, 70). New therapies are needed for NSCLC (68), and our current findings suggest potential new avenues for therapeutic intervention. COX-2 is a focal point of immune-mediated PG production and thus a potential early point of intervention in attempts to restore effective cell-mediated immune responses in the tumor microenvironment. Our current findings add to our understanding of the complex interaction between pulmonary tumor-derived PG and the cytokine network at the tumor site. Tumor COX-2 expression may be an important therapeutic target for pharmacological or gene therapy intervention in NSCLC.

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