Advances in Brief

Tumor Cyclooxygenase 2-dependent Suppression of Dendritic Cell Function[1]

Sherven Sharma, Marina Stolina, Seok-Chul Yang, Felicita Baratelli, Jeff F. Lin, Kimberly Atianzar, Jie Luo, Li Zhu, Ying Lin, Min Huang, Mariam Dohadwala, Raj K. Batra, and Steven M. Dubinett[2]

University of California Los Angeles School of Medicine-Wadsworth Pulmonary Immunology Laboratory, Veterans Administration Greater Los Angeles Healthcare System [S. S., M. S., S-C. Y., F. B., J. F. L., K. A., J. L., L. Z., Y. L., M. H., M. D., R. K. B.]; The UCLA Lung Cancer Research Program of the Jonsson Comprehensive Cancer Center [S. M. D.]; and Division of Pulmonary and Critical Care Medicine, University of California Los Angeles School of Medicine [S. M. D.], Los Angeles, California 90073

Abstract

Dendritic cells (DCs) serve as professional antigen-presenting cells and are pivotal in the host immune response to tumor antigens. To define the pathways limiting DC function in the tumor microenvironment, we assessed the impact of tumor cyclooxygenase (COX)-2 expression on DC activities. Bone marrow-derived DCs were cultured in either tumor supernatant (TSN) or TSN from COX-2-inhibited tumors. After culture, DCs were pulsed with tumor-specific peptides, and their ability to generate antitumor immune responses was assessed following injection into established murine lung cancer. *In vitro*, DC phenotype, alloreactivity, antigen processing and presentation, and interleukin (IL)-10 and IL-12 secretion were evaluated. DCs cultured in TSN failed to generate antitumor immune responses and caused immunosuppressive effects that correlated with enhanced tumor growth. However, genetic or pharmacological inhibition of tumor COX-2 expression restored DC function and effective antitumor immune responses. Functional analyses indicated that TSN causes a decrement in DC capacity to (*a*) process and present antigens, (*b*) induce alloreactivity, and (*c*) secrete IL-12. Whereas TSN DCs showed a significant reduction in cell surface expression of CD11c, DEC-205, MHC class I antigen, MHC class II antigen, CD80, and CD86 as well as a reduction in the transporter-associated proteins, transporter associated with antigen processing 1 and 2, the changes in phenotype and function were not evident when DCs were cultured in supernatant from COX-2-inhibited tumors. We conclude that inhibition of tumor COX-2 expression or activity can prevent tumor-induced suppression of DC activities.

Introduction

COX[3] (also referred to as PG endoperoxidase or PG G/H synthase) is the rate-limiting enzyme for the production of PGs and thromboxanes from free arachidonic acid (1). The enzyme is bifunctional, with fatty acid COX (producing PGG2 from arachidonic acid) and PG hydroperoxidase activities (converting PGG2 to PGH2). Two forms of COX have been described: (*a*) COX 1, constitutively present in most cells and tissues; and (*b*) an inducible COX-2 expressed in response to cytokines, growth factors, and other stimuli (1). COX-2 is constitutively overexpressed in a variety of malignancies (2). We, as well as others, have reported that COX-2 is frequently constitutively elevated in human NSCLC (3, 4). Although multiple genetic alterations are necessary for lung cancer invasion and metastasis, mounting evidence from several studies indicates that tumor COX-2 activity has a multifaceted role in conferring the malignant and metastatic phenotypes (5, 6). Overexpression of tumor COX-2 is associated with apoptosis resistance (5), increased angiogenesis (7), decreased host immunity (3, 8), and enhanced invasion and metastasis (6, 9). In this study, we sought to determine whether tumor COX-2 expression decreases host antitumor immune responses by impacting the maturation and activity of host APCs.

Antitumor immune responses require the coordinate activities of lymphocyte effectors and professional APCs (10). DCs are professional APCs that are pivotal participants in the initiation of T-cell responses (11). DCs acquire Ag in the periphery and subsequently transport it to lymphoid organs, where they prime specific immune responses (11). The tumor microenvironment can adversely affect DC maturation and function (12). Tumor-derived cytokines that have been shown to mediate DC dysfunction include IL-10, vascular endothelial growth factor, macrophage colony stimulating factor, and IL-6 (13–15). In this study, we document a tumor COX-2-dependent suppression of DC function.

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[2] To whom requests for reprints should be addressed, at Division of Pulmonary and Critical Care Medicine, UCLA School of Medicine, 37-131 Center for Health Sciences, 10833 LeConte Avenue, Los Angeles, CA 90095-1690. Phone: (310) 794-6566; Fax: (310) 267-2789; E-mail: sdubinett@mednet.ucla.edu.

[3] The abbreviations used are: COX, cyclooxygenase; TSN, tumor supernatant; IL, interleukin; SN, supernatant; DC, dendritic cell; APC, antigen-presenting cell; PG, prostaglandin; NSCLC, non-small cell lung cancer; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody; PE, phycoerythrin; CM, culture media; EIA, enzyme immunoassay; Th, T helper; TAP, transporter associated with antigen processing; NE, nuclear extract; CE, cytoplasmic extract; MLR, mixed lymphocyte reaction.

Materials and Methods

Reagents. PGE_2 was purchased from Cayman Chemical Co. (Ann Arbor, MI). COX-2 inhibitor (SC-58236), anti-PGE_2 mAb (2B5 mAb), and isotype-matched control mouse IgG1 (MOPc21) were provided by Joseph Portanova (Pharmacia, Peapack, NJ). Recombinant murine IL-10, GM-CSF, IL-4, purified antimouse IL-10 mAb, and rat antimouse CD40 mAb were obtained from PharMingen (San Diego, CA). FITC, PE, and tricolor-labeled antimouse CD11c, CD80 (B7-1), CD86 (B7-2), MHC class I, and MHC class II antigens were purchased from Caltag Laboratories (San Diego, CA). Rabbit antimouse IgG against TAP1 and TAP2 were provided by John J. Monaco (Howard Hughes Medical Institute, University of Cincinnati, Cincinnati, OH). PE-labeled antirabbit IgG and sera complement (rabbit HLA-ABC) were obtained from Sigma (St. Louis, MO). Purified rat antimouse DEC-205 and PE-labeled antirat IgG were purchased from Serotec (Raleigh, NC).

Stable Transfection. The 3LL COX-2 sense and antisense constructs were made as described previously (8). The 3LL COX-2 sense clones produced 7–9 ng PGE_2/ml/10^5 cells, whereas the 3LL COX-2 antisense clones produced 105–285 pg PGE_2/ml/10^5 cells. The 3LL and control vector-transfected cells produced 2.5–3.2 ng/ml/10^5 cells PGE_2. The clones were further characterized for COX-2 mRNA and protein by Northern and Western blot analysis, respectively, as described previously (8). For the studies described in this paper, we used 3LL COX-2 antisense clone 1ASE7-3LL that produces 78–102 pg PGE_2/ml/10^5 cells per 24 h and 3LL COX-2 sense clone 4SC7–3LL that produces 9 ng PGE_2/ml/10^5 cells per 24 h. In "Results and Discussion," these cells are referred as COX-2 sense and COX-2 antisense clones.

Cell Culture. Murine Lewis lung carcinoma (3LL, H-2^b, also known as LLC1, ATCC CRL-1642) cell line was obtained from American Type Culture Collection (Manassas, VA). 3LL, COX-2 antisense, COX-2 sense clone, and the control vector-transfected cells (CV-3LL) were routinely cultured as monolayers in 25-cm^3 tissue culture flasks in CM containing RPMI 1640 (Irvine Scientific, Santa Anna, CA) supplemented with 10% fetal bovine serum (FBS Gemini Bioproducts, Calabasas, CA), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 2 mM glutamine (JRH Biosciences, Lenexa, KS) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cell lines were *Mycoplasma* free, and cells were used up to the 10th passage before thawing frozen stocks of 3LL cells from liquid N_2.

Collection of TSN. 3LL, COX-2 antisense, COX-2 sense, or CV-3LL (1×10^5 cells/ml for each) was cultured in T/75 flasks in culture medium, and SNs were harvested at 24 h. SNs were also collected from 3LL cells treated with the specific COX-2 inhibitor, SC-58236 (5 μg/ml), anti-PGE_2 mAb (5 μg/ml), or control antibody (5 μg/ml). Anti-PGE_2 mAb (5 μg/ml) was chosen because this dose completely neutralized PGE_2 in the TSN as measured by EIA. For treatment of TSN with control mAb, an equivalent amount of isotype-matched control antibody was used. 3LL cells constitutively produce approximately 3 ng/ml PGE_2/24 h/10^5 cells. When treated with SC-58236 (5 μg/ml) for 24 h, the cells make 0.5 ng PGE_2/ml/24 h/10^5 cells.

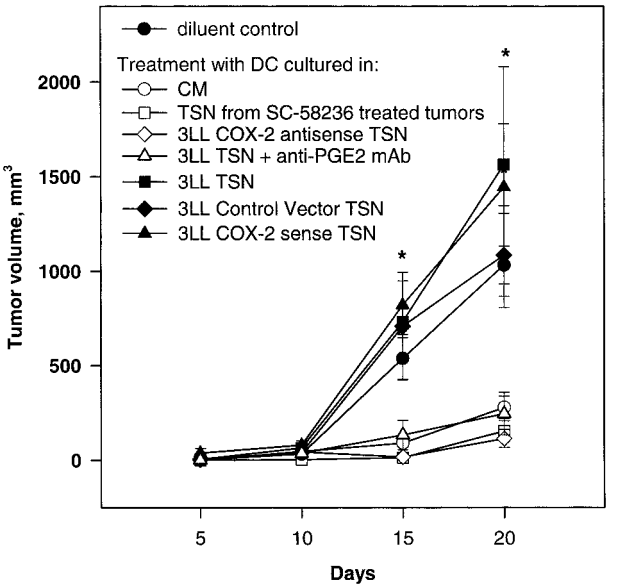

Fig. 1 DCs cultured in TSN do not mediate antitumor responses *in vivo*. Bone marrow-derived DCs were cultured in either TSN or TSN from COX-2-inhibited tumors. After culture, DCs were pulsed with tumor-specific peptides, and their ability to generate antitumor immune responses was assessed after injection into established murine lung cancer. DCs cultured in CM and pulsed with mut1/mut2 peptides led to a significant, consistent reduction in tumor growth. DCs cultured in 3LL, COX-2 sense, and control vector-transfected 3LL TSN did not have antitumor effects and led to enhanced tumor growth compared with CM ($P < 0.01$). Administration of DCs cultured in TSN from COX-2-inhibited cells or 3LL TSN treated with anti-PGE_2 mAb led to reduced tumor growth that was identical to that demonstrated with DCs cultured in control medium ($P < 0.01$ compared with DCs cultured in 3LL TSN). DCs propagated in 3LL TSN treated with control antibody showed no change in tumor volumes (data not shown).

Treatment of 3LL cells with anti-PGE_2 (5 μg/ml) decreased PGE_2 below the level of detection by EIA.

Mice. Pathogen-free C57Bl/6 and BALB/c mice (8–12 weeks of age) were obtained from Harlan (Indianapolis, IN) and maintained in the West Los Angeles VA Animal Research vivarium. All studies were approved by the institution's animal studies review board.

PGE_2 EIA. PGE_2 concentrations were determined according to the Cayman Chemical Co. (PGE_2 EIA kit protocol). Absorbance was determined at 405 nm by Molecular Devices Microplate Reader (Sunnyvale, CA).

Isolation and *in Vitro* Propagation of DCs. For all of the experiments, DCs were isolated and treated with TSNs as described here. Lymphocyte depleted bone marrow-derived DCs from C57Bl/6 mice (H-2^b) were cultured with medium containing murine GM-CSF (2 ng/ml) and IL-4 (20 ng/ml; R&D Systems, Minneapolis, MN) for 8 days as reported previously (16). To model the tumor microenvironment, DCs were continuously cultured in the presence of TSN, with medium changed every other day. DCs were cultured in murine GM-CSF and IL-4 with TSN from 3LL cells, COX-2 antisense clone, COX-2 sense clone, control vector clone, and SN from 3LL cells treated with COX-2 inhibitor (5 μg/ml). DCs were also cultured in

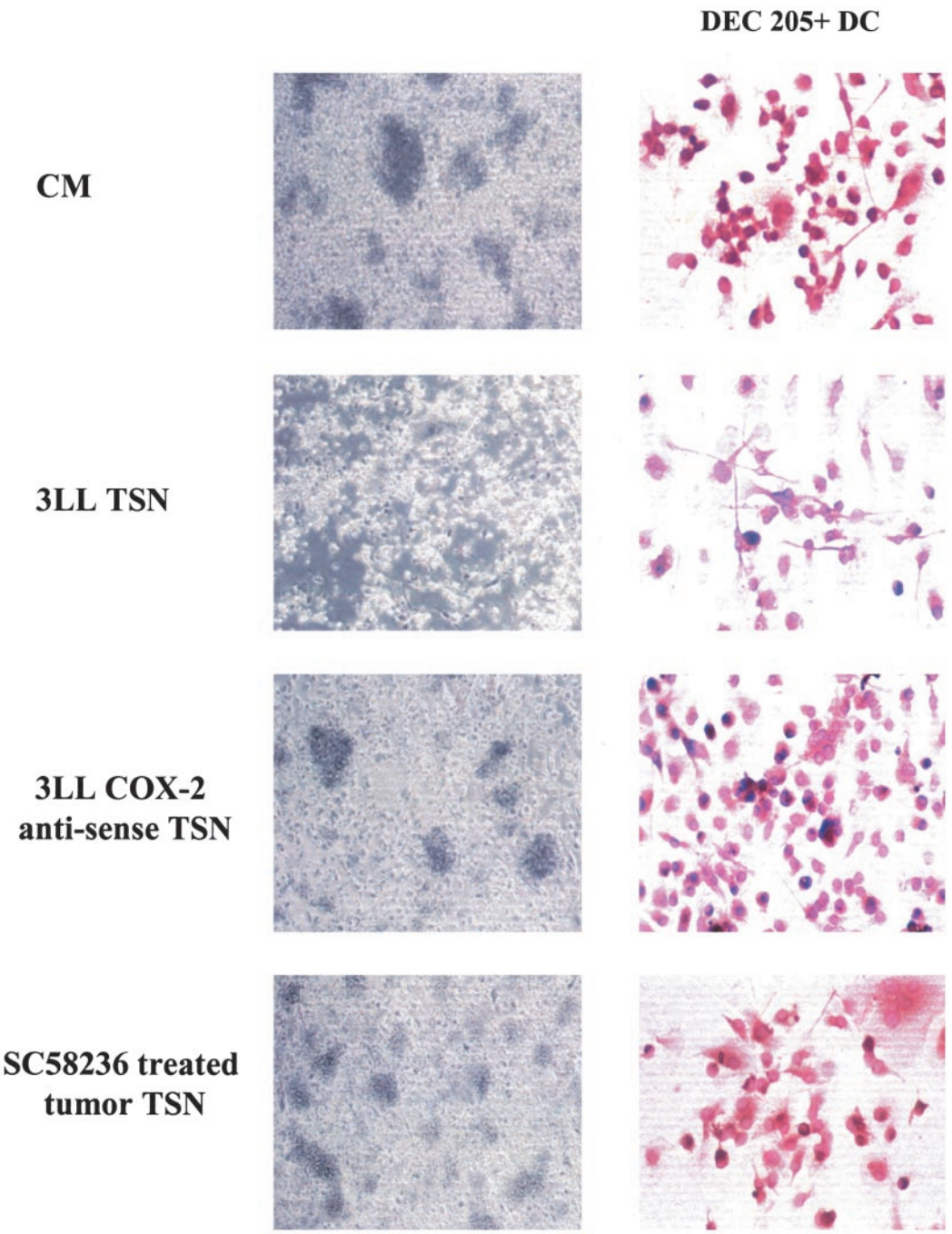

Fig. 2 Tumor-derived COX-2 decreases DC DEC-205 expression. DCs were assessed for DEC-205 staining after culture in TSN or TSN from COX-2-inhibited tumors. DC clustering and DC cell surface staining for DEC-205 were markedly decreased in the presence of 3LL SN. DCs cultured in SN from 3LL COX-2 sense and control vector-transduced constructs had morphology and DEC-205 staining patterns that were indistinguishable from DCs cultured in 3LL SN (data not shown). DCs cultured in TSN from COX-2-inhibited cells showed augmentation in the number of proliferating clusters as well as the number of cells staining positively for DEC-205.

medium containing PGE_2 (5 ng/ml) and in TSN treated with control or anti-PGE_2 mAb (5 μg/ml).

3LL Tumor Model. The mut1 and mut2 peptides, synthesized by Research Genetics (Huntsville, AL), consist of the 52–59 amino acid positions of the mutated connexin 37 protein present in the 3LL cell line and have the sequence FEQNTAQP and FEQNTAQA, respectively (17). Day 8 DCs were pulsed with mut1 and mut2 peptides as described previously (16). 3LL tumor cells (5×10^5) were inoculated by s.c. injection in the right suprascapular area of C57Bl/6 mice. The mut1/mut2-

Table 1 Tumor-mediated modulation of DC cell surface phenotype is dependent on COX-2 activity and PGE_2 production

Control mAb did not modify TSN-induced alterations in DC phenotype (data not shown). Results are representative of three independent experiments.

	CD11C		CD80		CD86		MHC I		MHC II		TAP1		TAP2	
DC growth conditions	%	MCF	%	MCF	%	MCF	%	MCF	%	MCF	%	MCF	%	MCF
CM	97	1457	23	144	57	816	94	480	79	134	79	500	78	275
3LL TSN	40[a]	515[a]	5[a]	37[a]	33[a]	402[a]	36[a]	248[a]	35[a]	194	38[a]	422	28[a]	277
TSN from SC58236-treated 3LL tumors	85[b]	717[b]	31[b]	127[b]	71[b]	866[b]	89[b]	856[b]	90[b]	770[b]	73[b]	252	75[b]	282
3LL COX-2 antisense TSN	77[b]	460	10[b]	58	64[b]	309	82[b]	216	42[b]	156	82[b]	190[a]	53[b]	179
3LL COX-2 sense TSN	25[a]	283	7[a]	21	26[a]	377	56[a]	518	31[a]	208	71	56[a]	86	88[a]
3LL control vector TSN	46[a]	483	3[a]	124	28[a]	702	86	121[a]	34[a]	296[a]	40	77[a]	24[a]	88[a]
CM + PGE_2	62[a]	716	14[a]	58	37[a]	480[a]	83[a]	303[a]	50[a]	89[a]	41	334	32	240
3LL TSN + anti-PGE_2 mAb	63[b]	869[b]	12[b]	53[b]	45[b]	468	62[b]	930[b]	53[b]	236[b]	40	370	65[b]	351[b]

[a] $P < 0.05$ compared with DCs propagated in CM.
[b] $P < 0.01$ compared with DCs propagated in 3LL TSN.

pulsed DCs propagated in the TSNs were evaluated for antitumor reactivity *in vivo*. Five day established tumors were treated with 10^6 peptide-loaded DCs by intratumoral injection at weekly intervals for 3 weeks as described previously (16). Tumor growth was assessed 3 times/week by measuring two bisecting diameters of each tumor with calipers as described previously (16).

Flow Cytometry. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the University of California Los Angeles Jonsson Cancer Center Flow Cytometry Core Facility. For flow cytometric evaluation, two or three fluorochromes (PE, FITC, and tricolor) were used to gate on the CD11c (PharMingen) bright populations of bone marrow-derived DCs in the evaluation of MHC class I antigen, MHC class II antigen, CD80, CD86, DEC-205, TAP1, and TAP2. Cells were identified as DCs by gating on forward and side scatter profiles. A total of 15,000 gated events were collected and analyzed using CellQuest software (Becton Dickinson). For TAP and DEC-205 markers, intracytoplasmic staining of DCs was performed. DCs were first stained with cell surface CD11c FITC and then permeabilized using Cytofix/Cytoperm solution (PharMingen) according to the manufacturer's instructions. The permeabilized DCs were stained for DEC-205 or TAP and analyzed by flow cytometry.

Immunohistochemistry. DCs were cultured for 8 days in Lab-TeK Chamber Slide System (Nalgene Nunc Int., Naperville, IL). The cells were fixed in acetone and dried and immersed in 1.5% hydrogen peroxide/methanol mixture for 15 min. Immune staining was performed with rat antimouse DEC-205 (Serotec) in a dilution of 1:25 at room temperature for 0.5 h. For detection, biotinylated secondary antibodies and avidin-biotin peroxidase complex solution were used from the Vectastain Quick Elite Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. 3,3′-Diaminobenzidine treatment was performed with a 3,3′-diaminobenzidine kit from Vector Laboratories. Cells were counterstained with hematoxylin.

Mixed Lymphocyte Reaction. DCs were evaluated for mixed lymphocyte reactions with BALB/c ($H\text{-}2^d$) T lymphocytes as the responder cells. DCs were cocultured with BALB/c splenic T cells in CM at varying DC:T cell ratios for 5 days as described previously (16).

Western Blot Analysis of RelB. The Western analysis was performed as described previously (18) using anti-RelB and anti-actin antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA) and the Amersham Life Science enhanced chemiluminescence protocol. Densitometric analysis was performed using the Perkin-Elmer Life Sciences Kodak Image Station 440 (Boston, MA).

IL-10 and IL-12 ELISA. IL-10 and IL-12 protein concentrations were quantified after 72 h from 2×10^6 cells/ml DCs by ELISA as described previously (8) using IL-10 antibody pairs (PharMingen) and an IL-12 kit (Biosource International, Camarillo, CA).

***In Vitro* Antigen Presentation Assay Using the B3Z Hybridoma Cells.** To evaluate the capacity of DCs to process and present antigens, *in vitro* DCs were incubated for 24 h with 1 mg/ml chicken ovalbumin. DCs were incubated at different cell numbers with the T-cell hybridoma B3Z (2×10^5 cells) for 24 h. The β-galactosidase reporter T-cell hybridoma (B3Z) recognizes the K^b class I molecule and an ova peptide, SL8 (SIINFEKL). B3Z was generously provided by N. Shastri (Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA). Activated B3Z cells were detected by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining (19) and quantified by light microscopy.

Statistical Analysis. All *in vitro* results are representative of at least three experiments performed in triplicate. Differences between experimental and control values were evaluated by Student's *t* test.

A total of 10–12 mice/group were used for the *in vivo* experiments.

Results and Discussion

The central importance of functional APCs in the immune response against cancer was well defined in studies by Huang *et al*. (10). These investigations revealed that even highly immunogenic tumors require host APCs for antigen presentation. Thus, optimal function of APCs is a critical factor for effective anti-tumor control (12). However, suppressed DC function has been reported in tumor-bearing models and patients (20–22).

We have shown previously that bone marrow-derived DCs pulsed with the 3LL-specific peptide antigens can reduce tumor

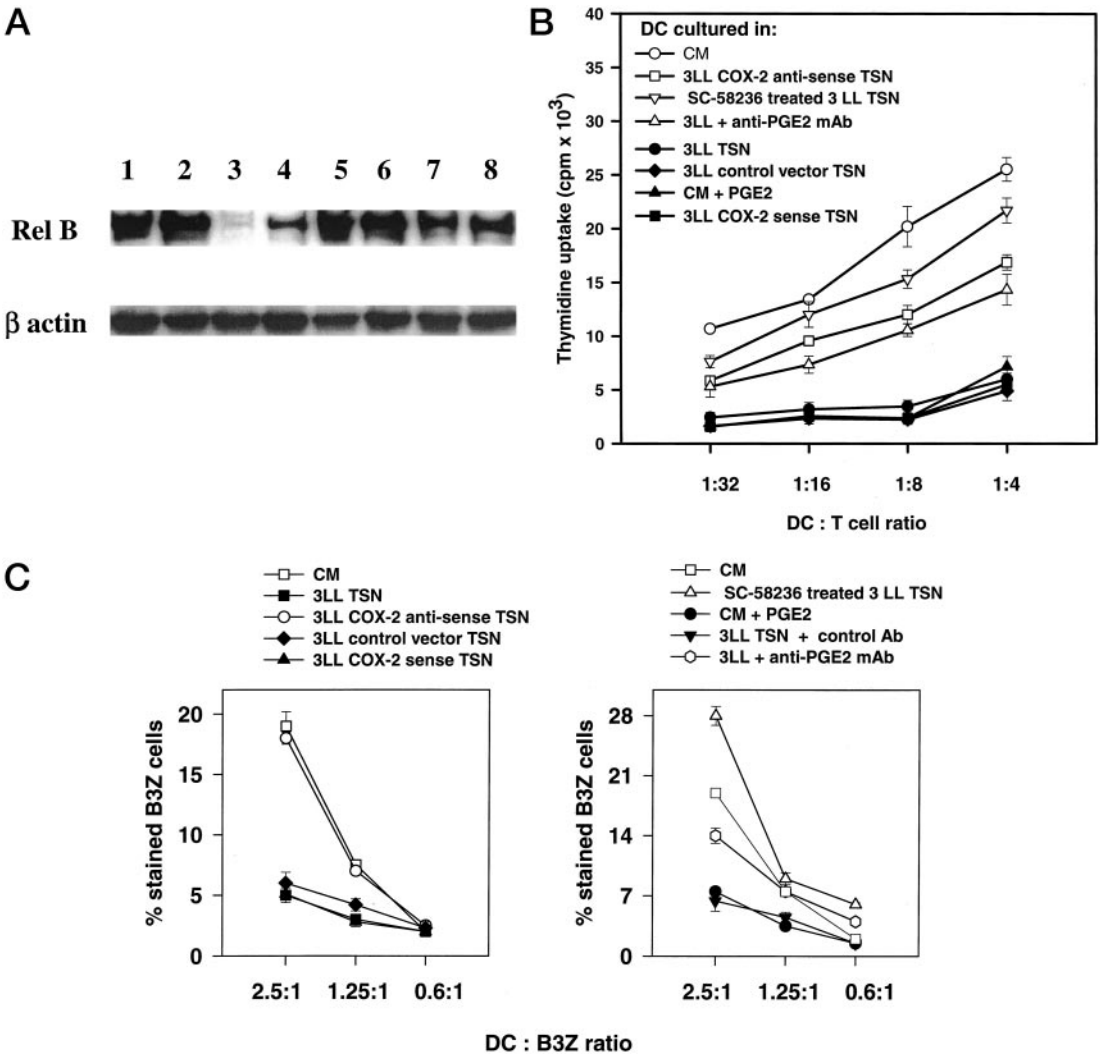

Fig. 3 *A*, TSN-treated DCs have decreased nuclear and cytoplasmic RelB protein. DCs were cultured in TSN or TSN from COX-2-inhibited tumors, and nuclear and cytoplasmic RelB were evaluated by Western blots. Densitometric analysis showed that compared with DCs cultured in CM, DCs cultured in TSN had low levels of RelB in the nucleus and cytoplasm. The RelB in the nucleus and cytoplasm in DCs cultured in 3LL COX-2 sense TSN was indistinguishable from that in DCs cultured in 3LL TSN (data not shown). The levels of RelB in the cytoplasm and nucleus were maintained at control levels after culture of DCs in SN from COX-2-inhibited 3LL cells. Results are representative of three experiments. *Lane 1*, CM NE; *Lane 2*, CM CE; *Lane 3*, 3LL TSN NE; *Lane 4*, 3LL TSN CE; *Lane 5*, 3LL COX-2 antisense TSN NE; *Lane 6*, 3LL COX-2 antisense TSN CE; *Lane 7*, SC-58236 treated 3LL NE; *Lane 8*, SC-58236-treated 3LL CE. *B*, 3LL COX-2-dependent decrease in allogeneic activity. The alloreactive stimulatory capacity of DCs cultured in TSN or TSN from COX-2-inhibited tumors was assessed by MLR. DCs propagated in TSN from 3LL, CV-3LL, or COX-2 sense SN had marked reductions in their capacity to promote MLR with allogeneic BALB/c responder T cells ($P < 0.01$ compared with DCs in CM). The reduced MLR activity of DCs observed in TSN could be replicated by adding PGE_2 (5 ng/ml) to the CM. DCs cultured in TSN from COX-2-inhibited 3LL or SN from 3LL cells treated with anti-PGE_2 mAb all showed a significant increase in alloreactivity when compared with DCs in 3LL SN ($P < 0.01$ compared with DCs in 3LL SN). 3LL SN treated with the control antibody did not affect the MLR. Results are representative of three experiments. *C*, tumor-derived PGE_2 reduces the capacity of DCs to process and present antigens to the T-cell hybridoma B3Z. DCs were cultured in TSN or TSN from COX-2-inhibited tumors, and their capacity to process and present ovalbumin was assessed using the ova-specific T-cell hybridoma B3Z. DCs cultured in 3LL, COX-2 sense, and control vector-transfected 3LL TSN had reduced capacity to process and present ovalbumin compared with DCs in CM ($P < 0.01$). This reduction could be reproduced after culturing DCs in CM containing PGE_2. Compared with DCs in 3LL TSN, DC antigen processing and presenting capacity was reversed after culture in TSN from COX-2-inhibited 3LL or 3LL TSN treated with anti-PGE_2 mAb ($P < 0.01$). Treatment of 3LL SN with the control antibody did not affect DC capacity to process and present antigen. Results are representative of three experiments.

volume and extend survival in mice after intratumoral therapy (16). In contrast, in the current study, DCs propagated in TSN and pulsed with specific tumor peptide antigens did not reduce tumor volume but in fact promoted tumor growth compared with DCs in CM ($P < 0.01$; Fig. 1). Because tumor COX-2 expression can impact anti-tumor immunity (8), we hypothesized that the immunosuppressive effect on DCs could be partly due to COX-2-dependent soluble products in the TSN. To test

this hypothesis, we evaluated the genetic and pharmacological inhibition of murine lung cancer COX-2 expression *in vitro* for its subsequent impact on DC anti-tumor activity *in vivo.* The anti-tumor efficacy of DCs was maintained after culture in SN from COX-2-inhibited tumor cells ($P < 0.01$ compared with DCs in 3LL TSN; Fig. 1). Because the capacity of DC trafficking is an important parameter of maturation, intratumoral injection of DCs may bypass this physiological step. Future studies will be required to determine whether TSN-exposed DCs have the capacity to traffic *in vivo*.

PGE_2 is an important COX-2-dependent regulator of cell-mediated immunity and serves as a potent inducer of the Th2 immune response (23). PGE_2 acts at several levels to mediate these effects. PGE_2 acts to limit IFN-γ production directly and inhibits the effects of IL-12 receptor expression and responsiveness (24). In addition, by selectively inducing IL-12p40, in the absence of p35 production, PGE_2 also acts to inhibit the bioactive effects of the IL-12p70 heterodimer (25). PGE_2 mediates these effects via distinct prostaglandin E receptors that function in limitation of T-cell proliferation as well as antigen presentation (26). Because PGE_2 has previously been shown to affect lymphocyte and APC function (8, 23), we determined whether PGE_2 present in the TSNs had a role in down-regulating DC-mediated antitumor immune responses. Neutralizing antibody-mediated blockade of PGE_2 *in vitro* blocked the immunosuppressive effect of the TSN and yielded fully functional DCs with potent *in vivo* activities (Fig. 1). Western blot analysis revealed that the DCs expressed detectable levels of the prostaglandin E receptor subtypes 2, 3, and 4 (data not shown).

Based on the *in vivo* results, we performed *in vitro* assays to define the impact of tumor COX-2 expression and PGE_2 production on DC phenotype and function. We found that DC phenotype (Fig. 2 and Table 1), cytoplasmic and nuclear RelB protein levels (Fig. 3*A*), alloreactivity (Fig. 3*B*), antigen presentation capacity (Fig. 3*C*), and production of IL-12 and IL-10 (Fig. 4, *A* and *B*) are markedly altered by tumor COX-2 expression. Consistent with limited *in vivo* antitumor capacity, DCs cultured in TSN showed a decreased immune stimulatory molecule expression that correlated with marked limitation in DC functional activities. Recognition of target cells by CD8+ CTL requires the presence of cell surface peptide epitopes in the context of MHC class I molecules (27). In the majority of cases, these epitopes are generated from endogenously expressed proteins by breakdown in the cytosol via a large multicatalytic protease complex, the proteasome (28). Thereafter, the resultant peptides are moved from the cytosol into the endoplasmic reticulum by the TAP, a heterodimeric complex composed of TAP1 and TAP2 subunits (29). TAP therefore forms a major link between antigen generation and presentation. Indeed, TAP inactivation reduces both MHC class I loading and the surface expression of antigen-loaded complexes (30). We assessed DC capacity to process and present the ovalbumin antigen in an *in vitro* assay using the B3Z T-cell hybridoma that recognizes the ova peptide SIINFEKL (19). Consistent with the reduction in the number of TSN-cultured DCs expressing TAP, these DCs had a reduced capacity to process and present the ovalbumin antigen (Fig. 3*C*). Tumor COX-2 inhibition resulted in maintenance of DC TAP and Ag presenting properties. Additional

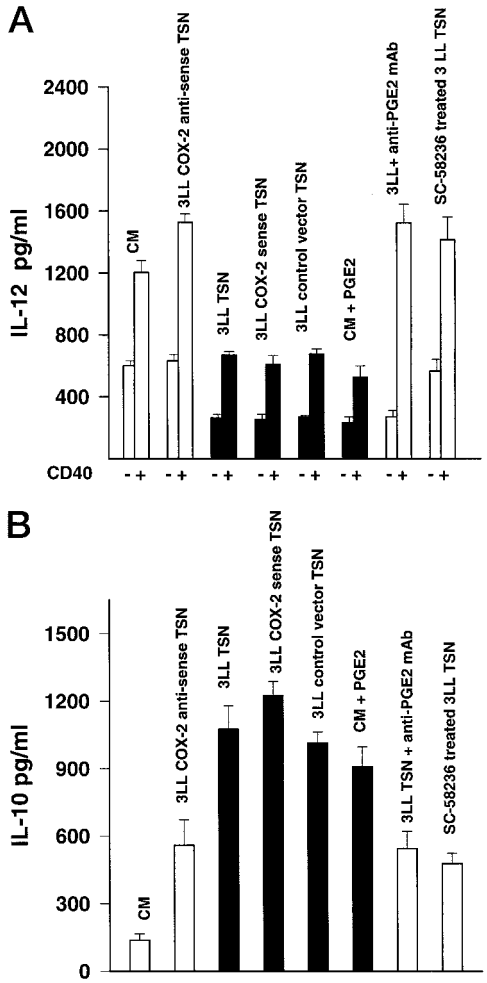

Fig. 4 *A* and *B*, 3LL COX-2 metabolites inhibit IL-12 and enhance DC IL-10 production. DCs were cultured in TSN or TSN from COX-2-inhibited tumors for 72 h. IL-12 and IL-10 secreted in the culture SNs were quantified by ELISA. DCs cultured in TSN showed a marked decrease in IL-12 and a concomitant increase in IL-10 production in comparison with DCs in culture medium ($P < 0.01$). The alteration of IL-12 and IL-10 production by DCs in TSN could be replicated by adding PGE_2 to the DC culture medium in place of TSN. Increased DC IL-10 and decreased IL-12 production in TSN were prevented after culture in SN from COX-2-inhibited 3LL or in 3LL SN treated with anti-PGE_2 mAb ($P < 0.01$). CD40 stimulation of DCs cultured in CM led to a consistent up-regulation of IL-12 production. In contrast, CD40-stimulated DCs in TSN showed a limited capacity for IL-12 induction. However, IL-12 production was maintained at levels comparable to CD40-stimulated DCs in culture medium after culture in SN from COX-2-inhibited 3LL or 3LL SN treated with anti-PGE_2 mAb. Results are representative of three experiments.

experiments are warranted to determine the mechanisms leading to restoration of DC antigen presentation *in vitro*.

DC maturation has been associated with RelB activation (31). Failure to develop functional antigen presenting activity in the tumor environment is accompanied by decreased RelB translocation (32). TSN-cultured DCs demonstrated a COX-2-dependent reduction of both cytoplasmic and nuclear RelB (Fig. 3*A*). The unanticipated finding of decreased cytoplasmic RelB is

not yet fully explained and may represent an additional component of the pathway whereby COX-2-expressing tumors suppress antigen presentation. Additional studies are warranted to determine whether the decrease in RelB caused by the TSN is due to a decrease in the synthesis of RelB and/or an increase in the rate of RelB degradation. The decrease in RelB also coincided with down-regulation of DC cell surface molecules and activity. Whereas PGE_2 caused a significant reduction in DC surface immune stimulatory molecules, anti-PGE_2-treated TSN partially blocked the alteration in DC phenotype, suggesting that other tumor-derived factors such as vascular endothelial growth factor may also be responsible for these effects. The alterations in DC phenotype in TSN cannot be attributed to granulocyte colony-stimulating factor, IL-6, or IL-10 because 3LL cells do not produce detectable levels of these cytokines (data not shown).

Our previous studies indicated that COX-2 inhibition reversed the tumor-induced augmentation in lymphocyte and macrophage-derived IL-10 and suppression of macrophage IL-12 production (8). In the current study, we focused specifically on the role of tumor COX-2 expression in regulating DC production of these cytokines. Consistent with the reduction in antitumor capacity of TSN-cultured DCs, there was also a marked decrement in IL-12 production that could be replicated by adding PGE_2 to the CM (Fig. 4*A*). Our results are in agreement with studies showing that PGE_2-promoted maturation results in DCs that produce only low amounts of IL-12 and bias the development of naïve cells toward the production of Th2 cytokines (33). In apparent contrast to our findings are those indicating that PGE_2 enhances tumor necrosis factor α-induced DC maturation (34, 35). However, these studies focus on specific culture conditions for human monocyte-derived DCs that, for example, include cytokines in addition to PGE_2 such as tumor necrosis factor α, IL-1β, and IL-6. Our findings are consistent with recent studies documenting a role for paracrine or autocrine PGE_2 release impacting IL-12 production and functional activities in murine bone marrow-derived DCs (36). Collectively, these findings suggest that the tumor COX-2 metabolite PGE_2 is an important modulator of DC IL-12 secretion and can limit the initiation of Th1 responses required for antitumor immunity.

In contrast to IL-12, IL-10 production by TSN-cultured DCs was significantly enhanced in a PGE_2-dependent manner (Fig. 4*B*). This is in agreement with previous findings that have shown PGE_2 to be a potent inducer of macrophage and lymphocyte IL-10 secretion (3, 23). DC production of IL-10 has been demonstrated to limit the maturation of monocyte-derived DCs and their capacity to initiate Th1 responses (37). IL-10 inhibits IL-12 production in immature DCs, but, in addition, it prevents DC development when present at early maturational stages, inducing tolerogenic DCs (35). IL-10-exposed DCs have a Th2-driving function that may contribute to their immunosuppressive activity *in vivo* (38).

Recent studies document the importance of COX-2 expression in human lung cancer (3, 39, 40). 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 (4). Accordingly, Khuri *et al.* (39) found that tumor COX-2 overexpression appears to portend a shorter survival among patients with early-stage NSCLC. We recently reported that COX-2 expression is responsible for CD44-dependent NSCLC invasion (6). 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 (5, 6, 41). Our current findings are the first demonstration of tumor COX-2-dependent suppression of host DC maturation and function. These deleterious effects are reversible when tumor COX-2 expression is either genetically or pharmacologically inhibited. These findings lend further support to the suggestion that tumor COX-2 maybe an important target for chemoprevention as well as genetic or pharmacological therapy in lung cancer. Additional studies are required to determine whether cancer clinical trials that use COX-2 inhibition will lead to heightened DC function.

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