

## Cyclooxygenase-2 Inhibition Augments the Efficacy of a Cancer Vaccine

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**Abstract** Tumor-derived cyclooxygenase-2 (COX-2) and its product, prostaglandin E<sub>2</sub>, exert strong immunoinhibitory effects that block dendritic cell function and CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation and function. We have shown previously that the addition of an oral COX-2 inhibitor to immunogene therapy using IFN-β markedly augmented therapeutic efficacy in murine tumor models. In this study, we hypothesized that COX-2 inhibition might also augment an antitumor vaccination strategy. Mice bearing tumors derived from TC1 cells, a tumor line that expresses the human papillomavirus (HPV) E7 protein, were thus vaccinated with an adenoviral vector expressing HPV E7 protein (Ad.E7). This vaccine approach effectively generated E7-specific CD8<sup>+</sup> cells and slowed the growth of small tumors but had little effect on large tumors. However, feeding mice with the COX-2 inhibitor, rofecoxib, restored the effectiveness of the vaccine against large tumors and prolonged survival. This effect was accompanied by a larger percentage of E7-specific CD8<sup>+</sup> cells in the regional draining lymph nodes and a markedly increased number of tumor-infiltrating E7-specific CD8<sup>+</sup> cells (as determined by flow cytometry) and total CD8<sup>+</sup> T cells (as determined by immunohistochemical staining). Increased immunocyte trafficking was likely mediated by the generation of a Th1-type tumor microenvironment because COX-2 inhibition increased expression levels of mRNA for IFN-γ, interleukin-12, IP-10, and MIG while lowering the expression of vascular endothelial growth factor within tumors. This study shows that the effectiveness of a cancer vaccine can be significantly improved by adding COX-2 inhibition.

As a result of the identification of an increasing number of tumor-selective antigens, vaccine strategies have been aggressively pursued to enhance antitumor immune responses (1–3). Whole tumor cells, gene-modified tumor cells, naked DNA, peptides, recombinant viral vectors, and antigen-modified dendritic cells are several of the vaccination strategies that are being evaluated. Although vaccine trials have shown occasional examples of clinical efficacy, responses have been infrequent, even in the presence of the generation of antigen-specific effector cells (1, 4).

Both the immune system and the tumor microenvironment are known to express immunosuppressive factors that reduce the effectiveness of immunotherapy (5–8). Immune regulatory pathways, mediated by molecules, such as CTLA-4 (9, 10), or immune regulatory or suppressor cells, such as CD4<sup>+</sup>/CD25<sup>+</sup> T cells (11, 12) or myeloid suppressor cells (13), dampen effective antitumor responses while, simultaneously, tumor-

stromal-derived factors, such as transforming growth factor-β (6, 14), vascular endothelial growth factor (VEGF; ref. 15), arginase (16), interleukin (IL)-10 (17), and prostaglandins (18, 19), further suppress an effective antitumor immune response. Although several studies have shown the development of antigen-specific cells in vaccinated patients, it is likely that these immune regulatory and tumor-derived factors prevent effective tumor killing and control (1, 4).

Prostaglandins derived from arachidonic acid through the cyclooxygenase (COX) enzymes have very potent immunosuppressive properties, particularly prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; refs. 18, 19). PGE<sub>2</sub> has been shown to inhibit T-cell proliferation by altering polyamine synthesis (20), inhibiting intracellular calcium release (21), and inhibiting p59 protein tyrosine kinase (22). PGE<sub>2</sub> profoundly inhibited IFN-γ and IL-2 production (23) while enhancing IL-4 and IL-10 production (24), thereby promoting a Th2 response over a Th1 antitumor response. Furthermore, PGE<sub>2</sub> has been shown to prevent the maturation and ability of dendritic cells to present antigen and to produce IL-12 and IFN-γ (24–26). In addition, PGE<sub>2</sub> has been implicated recently as promoting FOXP3 expression and CD4<sup>+</sup>/CD25<sup>+</sup> T regulatory cell activities (27).

In a murine model of malignant mesothelioma, we have shown previously that the addition of an oral COX-2 inhibitor to immunogene therapy delivered by intratumoral injection of an adenovirus expressing IFN-β markedly augmented therapeutic efficacy (28). In this article, we hypothesized that COX-2 inhibition might also augment an antitumor vaccination

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strategy. To test this hypothesis, we used a murine lung cancer cell line (TC1) that expresses a known viral tumor antigen, the human papillomavirus (HPV) E7 protein (29). We used a vaccination strategy employing an adenoviral vector expressing the E7 protein (Ad.E7) that has been shown to prevent tumor development when mice are vaccinated before tumor cell injection (30). We thus vaccinated mice bearing established flank tumors with Ad.E7 and fed them control chow or chow containing the COX-2 inhibitor rofecoxib. Our studies showed that COX-2 inhibition significantly augmented the vaccine effect. This increased efficacy was associated with increased trafficking of CD8<sup>+</sup> T cells into the tumor, increased numbers of antigen-reactive T cells, and marked up-regulation of the mRNA for cell adhesion molecules and immunostimulatory cytokines and chemokines within the tumors.

## Materials and Methods

**TC1 flank tumor model.** TC1 cells were derived from mouse lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (29) and provided by Dr. Yvonne Paterson (University of Pennsylvania, Philadelphia, PA). The cells were grown *in vitro* in RPMI, 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and 5 µg/mL penicillin/streptomycin. C57BL/6 mice (Charles River, Wilmington, MA) were shaved on their right flank and  $1 \times 10^6$  TC1 cells were injected s.c. Tumors were allowed to grow to the indicated desired volume as determined by the following formula: (long axis  $\times$  short axis  $\times$  short axis) / 5. Following treatments as outlined below, tumor growth was followed with periodic measurement of tumor volume. Mice were sacrificed when tumor volume was  $>2,000 \text{ mm}^3$  or the mice appeared lethargic or in distress. For the tumor measurement data, at least two separate experiments were done with five mice in each treatment group.

**Ad.E7 vaccination.** An adenoviral vector expressing the HPV-E7 protein under control of a cytomegalovirus promoter was provided by Hildegund Ertl (Wistar Institute, Philadelphia, PA; ref. 30) and subsequently produced in University of Pennsylvania Viral Core Facility. In some experiments, non-tumor-bearing mice were vaccinated with  $1 \times 10^9$  plaque-forming units (pfu) of Ad.E7 s.c. in one flank and boosted with the same dose 7 days later. To test the effects on tumors, when tumors achieved the desired size, mice were vaccinated s.c. in the left flank (contralateral to the tumor) with  $1 \times 10^9$  pfu of Ad.E7 vector. Seven days following the initial vaccination, mice received a booster vaccine of  $1 \times 10^9$  pfu of Ad.E7. Control animals received the same doses of a control virus (Ad.LacZ).

**Monoclonal antibodies.** The following antibodies were obtained from BD Biosciences PharMingen (San Diego, CA) and used at the indicated dilutions for flow cytometry: CD8- $\alpha$ -FITC (1:200), CD62L-phycoerythrin (PE; 1:200), CD45-PerCP (1:200), and IFN- $\gamma$ -PE (1:300).

**Flow cytometric analysis of splenocytes, lymph nodes, and tumors for E7 tetramers and intracellular IFN- $\gamma$ .** Spleens and draining lymph nodes were isolated from euthanized mice and crushed through a 70- $\mu\text{m}$  filter with RPMI plus 10% FBS (R10). Following RBC lysis, lymphocytes were washed with R10, and Fc receptors were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences PharMingen). Following one wash with PBS plus 2% FBS (staining buffer), cells were incubated for 30 minutes at 4°C with appropriate antibodies (see above) or APC-labeled H-2D<sup>b</sup> tetramers (1:500 dilution) loaded with E7 peptide (RAHYNIVTF) that were obtained from the NIAID tetramer core. After this 30-minute incubation, cell surface antibodies were added and incubated for another 30 minutes at 4°C. Samples were washed and resuspended in staining buffer or fixed in 2% paraformaldehyde. All flow cytometry was done using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). Data analysis was done using FlowJo software (Ashland, OR). Specificity of binding was confirmed by using

the ovalbumin SIINFEKL APC-labeled iTag tetramer (Beckman-Coulter Immunomics, San Diego, CA).

For fluorescence-activated cell sorting (FACS), tumors were removed from euthanized mice and minced into fine pieces in digestion buffer containing 0.1 mg/mL DNase I and 2.0 mg/mL collagenase type IV (Sigma, St. Louis, MO). Samples were incubated in digestion buffer at 37°C for 30 minutes, filtered through a 70- $\mu\text{m}$  filter, and washed twice with R10. FACS was done as described previously on  $1.5 \times 10^6$  cells.

Intracellular IFN- $\gamma$  staining was done with a fixation/permeabilization solution kit (BD Biosciences PharMingen). Briefly, cells were isolated as detailed above and  $1.5 \times 10^6$  cells were stimulated in a 96-well plate with 1 µg/mL E7 peptide (RAHYNIVTF), 50 units/mL IL-2 (Roche, Indianapolis, IN), and 1 µL/mL GolgiPlug for 5 hours at 37°C. Following several washes, cells were stained with the desired cell surface markers as detailed above, washed, fixed, and permeabilized with Cytofix/Cytoperm solution for 20 minutes at 4°C. Following two washes with PermWash, cells were stained with IFN- $\gamma$  antibody for 30 minutes at 4°C, washed, and analyzed on the Becton Dickinson FACSCalibur flow cytometer. Flow cytometry was done on two different animals in two separate experiments for a total of four samples from each treatment arm from draining lymph nodes, spleen, and tumor.

**In vivo analysis of CTL function.** Systemic CTL activity was measured *in vivo* as described previously (31). Splenocytes from normal C57BL/6 mice were washed and divided into two populations. One population was pulsed with 5 µmol/L RAHYNIVTF peptide (the HPV-E7 immunodominant peptide) for 90 minutes at 37°C, washed in PBS, and labeled with a high concentration (5 µmol/L) of CFSE (Molecular Probes, Eugene, OR). These cells serve as CTL targets. The other, control population was labeled with a low concentration of CFSE (0.5 µmol/L). A total of  $10^7$  cells of each population were mixed in 200 µL PBS and injected via tail vein into control mice or mice that had been vaccinated with Ad.E7 7 days earlier. Specific *in vivo* toxicity was determined by collecting draining lymph nodes and spleens from recipient mice 18 hours after injection and the number of cells in each target cell population was determined by flow cytometry.

**Treatment with COX-2 inhibitor.** Specific COX inhibition was achieved using the COX-2 inhibitor rofecoxib (MK-0966) obtained as a gift from Merck Frosst Canada & Co. (Kirkland, Quebec, Canada). This was incorporated into mouse chow by Test Diet (Richmond, IN) at a concentration of 0.0075%. On the day of vaccination with Ad.E7, standard mouse chow was changed to either rofecoxib chow or control chow. This chow was provided to the animals on a continuous basis until the completion of the experiment.

**Measurement of PGE<sub>2</sub> levels.** Animals were injected with TC1 tumor cells and started on COX-2 inhibitor chow or control chow (as described above) on the day of the first Ad.E7 or Ad.LacZ vaccination. Animals were boosted with Ad.E7 7 days later. Blood and tumors tissues were harvested 3 days after the booster vaccination. Blood was collected in heparinized syringes containing aspirin (10 µg/mL; Sigma). Plasma was separated immediately by centrifugation at 4°C and frozen. Tumors were sonicated for 30 seconds in 1 mL complete buffer (50 mL PBS containing one tablet of anti-protease cocktail; Roche) to which aspirin (10 µg/mL) was added. Homogenates were then spun at 3,000 rpm for 10 minutes and filtered through a 1.2- $\mu\text{m}$  syringe filter unit and frozen. Total protein in each sample was determined.

PGE<sub>2</sub> was extracted and determined by RIA according to the manufacturer's instructions using a kit from Perkin-Elmer (Boston, MA). Both interassay and intra-assay variation was  $<6\%$ .

**Immunohistochemical staining of tumors.** Animals bearing flank tumors were euthanized and the tumors were immediately placed in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA) to be stored at  $-80^\circ\text{C}$ . After sectioning, we did immunoperoxidase staining by using the avidin-biotin-peroxidase Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Monoclonal antibodies against leukocyte subsets were obtained from BD Biosciences PharMingen.

**RNA isolation and reverse transcription-PCR.** TC1 tumors from control and rofecoxib-treated mice were harvested 10 days after rofecoxib treatment and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). RNA (3  $\mu$ g) from each tumor was reverse transcribed using 0.5  $\mu$ g oligo(dT) (Promega, Madison, WI), 10 mmol/L deoxynucleotide triphosphates (Clontech, Palo Alto, CA), and 1 unit PowerScript reverse transcriptase in  $5\times$  first-strand buffer and 100 mmol/L DTT (Clontech) for 80 minutes at 42°C. Optimum primer sequences were selected after verification for gene-specific complementation using the National Center for Biotechnology Information Blast program (<http://www.ncbi.nlm.nih.gov/blast>). Primer sequences can be obtained from the authors on request. Semiquantitative analysis of gene expression was done using a Cepheid Smart Cycler following the manufacturer's protocol for SYBR Green kit supplied by Roche (Sunnyvale, CA). cDNA concentrations from each pool were normalized using  $\beta$ -actin as a control gene. Relative level of expression of each of the selected genes (fold change in rofecoxib-treated versus control) was determined. Each sample was run in triplicate and the experiment was repeated twice.

**Statistical analysis.** Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's *t* test. Comparisons with more than two groups were done using ANOVA with appropriate *post hoc* testing. Differences were considered significant when  $P < 0.05$ . Data are presented as mean  $\pm$  SE. The log-rank test was used for analysis of survival data.

## Results

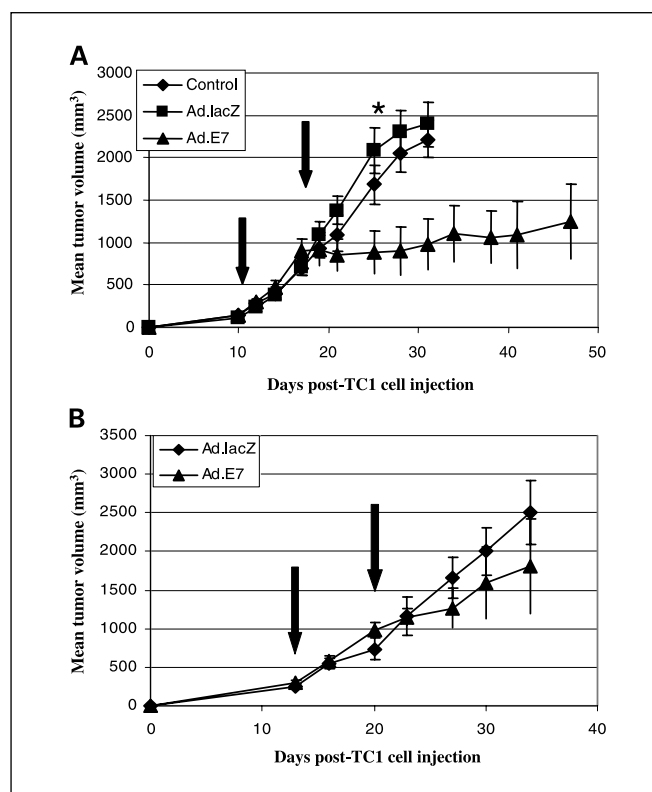
**Ad.E7 vaccination slowed the growth of small established tumors but had no effect on larger tumors.** To confirm the ability of Ad.E7 vaccination to prevent the growth of TC1 tumors, we vaccinated mice with Ad.E7 virus 14 and 7 days before injecting the contralateral flank with  $1 \times 10^6$  TC1 cells. As described by He et al. (30), vaccination before injection of TC1 cells prevented the development of any tumor compared with unvaccinated or Ad.lacZ-vaccinated mice (data not shown).

To develop a model of vaccine therapy for established tumors, we allowed tumors to grow to various sizes in the mouse flank and vaccinated in the contralateral flank at the time the tumors achieved the desired size and then boosted 7 days later. When mice with small tumors ( $\sim 100$  mm<sup>3</sup>) were vaccinated with Ad.E7, there was a significant inhibition in tumor growth shortly after the boost vaccine compared with control unvaccinated ( $P = 0.021$ ) or Ad.lacZ-vaccinated mice ( $P = 0.009$ ; Fig. 1A).

In contrast, when tumors were allowed to grow larger before vaccination ( $\sim 275$  mm<sup>3</sup>), Ad.E7 vaccination did not inhibit tumor growth when compared with Ad.lacZ-vaccinated mice ( $P = 0.373$ ; Fig. 1B).

**Ad.E7 vaccination generated functional E7-specific CD8<sup>+</sup> cells that secreted IFN- $\gamma$  and had cytotoxic T-cell activity.** To examine the specific immunologic response to Ad.E7 vaccination, spleens and draining lymph nodes were isolated from mice vaccinated 7 and 14 days previously with Ad.LacZ or Ad.E7. The lymphocytes were stained for the presence of E7-specific CD8<sup>+</sup> T cells using E7 peptide tetramer and for the production of intracellular IFN- $\gamma$  after peptide stimulation. E7 tetramer and IFN- $\gamma$  cells were expressed as a percentage of total CD8<sup>+</sup> cells. Specificity of E7 tetramer binding was confirmed by using the ovalbumin SIINFEKL tetramer as a control (data not shown).

Ad.E7 vaccination resulted in the generation of E7-specific CD8<sup>+</sup> cells at days 7 and 14 in both spleen (1.06% and 1.56%, respectively; Fig. 2A, b and d) and draining lymph node (0.61% and 0.30%, respectively; Fig. 2A, j and l) when



**Fig. 1.** Ad.E7 vaccination inhibited the growth of small tumors but was ineffective against larger tumors. **A**, tumors were allowed to grow to  $\sim 100$  mm<sup>3</sup> and vaccinated as described in Materials and Methods. Arrows, days of vaccination. Ad.E7 vaccination significantly ( $P < 0.05$ ) slowed tumor growth compared with untreated (Control) or Ad.lacZ-vaccinated animals. **B**, in contrast, Ad.E7 vaccination had no effect on the growth of larger tumors ( $\sim 275$  mm<sup>3</sup>).

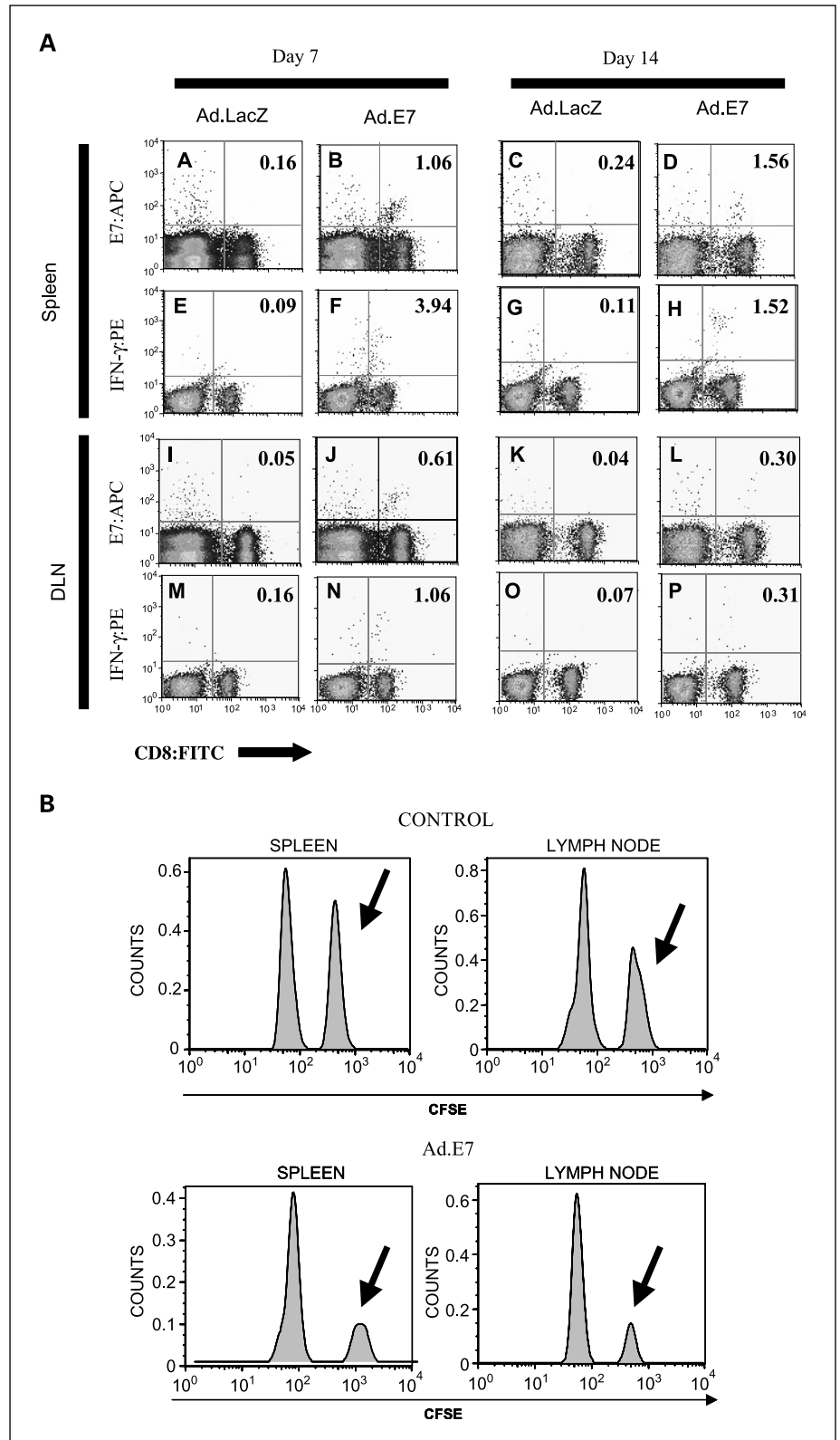
compared with scant background staining at days 7 and 14 for Ad.lacZ-vaccinated mice in both spleen (Fig. 2A, a and c) and draining lymph node (Fig. 2A, i and k). Furthermore, Ad.E7 vaccination generated functional E7-specific cells as evidenced by their production of intracellular IFN- $\gamma$  at day 7 (3.94% and 1.06%; Fig. 2A, f and h) and day 14 (1.52% and 0.31%; Fig. 2A, n and p) in the spleen and draining lymph node, respectively. Lymphocytes isolated from Ad.lacZ-vaccinated mice did not show the production of intracellular IFN- $\gamma$  when stimulated with E7 peptide in either spleen (Fig. 2A, e and g) or draining lymph node (Fig. 2A, m and o). Therefore, the vaccination strategy we employed not only generated E7-specific CD8<sup>+</sup> cells, but these cells also produced IFN- $\gamma$  when stimulated with E7 peptide.

To examine the ability of the vaccine to generate CTLs capable of lysing antigen-expressing target cells, systemic CTL activity was measured *in vivo* as described previously (31). Equal numbers of differentially 5,6-carboxy-succinimidyl-fluorescein ester (CSFE)-labeled target cells (untreated or pulsed with the RAHYNIVTF immunodominant peptide from HPV-E7) prepared from the spleens of naive C57BL/6 mice were adoptively transferred into control mice or mice vaccinated with Ad.E7. As shown in Fig. 2B, 18 hours after injection, FACS was done on spleen and lymph nodes and the number of unpulsed control cells (number of cells with moderate expression of CSFE) to pulsed target cells (number of cells with high levels of CSFE; arrows) was determined. In nonvaccinated animals, control and

target cells were found in a ratio of ~ 2:1. In contrast, the number of peptide-pulsed target cells was markedly decreased in Ad.E7-vaccinated animals resulting in a control to target ratio of 9:1. This loss of peptide-coated cells represents enhanced *in vivo* CTL activity in vaccinated animals.

**COX-2 inhibition enhanced the clinical effectiveness of Ad.E7 vaccination in animals with large tumors.** Given the lack of effect of Ad.E7 vaccination on large tumors, we therefore sought to determine if inhibition of PGE<sub>2</sub> production would enhance the effectiveness of our vaccination strategy. Our

**Fig. 2.** Ad.E7 vaccination generates E7-specific CD8<sup>+</sup> cells that functionally secrete IFN- $\gamma$  and have cytotoxic activity. **A**, FACS analysis. Spleen and draining lymph node (DLN) were isolated from vaccinated animals 7 days following initial vaccination with control (Ad.LacZ) or Ad.E7 (Day 7) and 7 days following boost vaccine (Day 14) and stained for the presence of E7-specific CD8<sup>+</sup> cells and the production of IFN- $\gamma$  using FACS. Ad.E7 vaccination generated E7-specific CD8<sup>+</sup> cells at days 7 and 14 in both spleens (*b* and *d*, respectively) and draining lymph node (*j* and *l*, respectively). When exposed to E7 peptide, these cells functionally secreted IFN- $\gamma$  at both days 7 and 14 in both spleen (*f* and *h*, respectively) and draining lymph node (*n* and *p*, respectively). All Ad.lacZ-vaccinated mice showed only scant background staining for both E7-specific CD8<sup>+</sup> cells and IFN- $\gamma$  staining at both 7 and 14 days in both spleen and draining lymph node. **B**, Ad.E7 vaccination induces an *in vivo* CTL response. Equal numbers of differentially labeled target cells prepared from the spleens of naive C57BL/6 mice representing peptide-pulsed/CFSE-high cells or control/CFSE-low cells were adoptively transferred in to control mice or mice vaccinated with Ad.E7. Eighteen hours after injection, spleen and lymph nodes were analyzed by FACS and the number of unpulsed control cells (number of cells with moderate expression of CFSE) to pulsed target cells (number of cells with high levels of CFSE; arrows) was determined. In control animals, the ratio of nonpulsed to peptide-pulsed target cells was ~ 2:1. In contrast, the number of peptide-pulsed cells was markedly decreased in vaccinated animals resulting in a control-to-target ratio of 9:1. This loss represents CTL activity.



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laboratory has shown previously that stable serum levels of the COX-2 inhibitor rofecoxib were achieved in mice after 10 days of feeding on rofecoxib chow (28). Therefore, we began rofecoxib chow on the day of the initial vaccination so that peak serum levels would be obtained 10 days after the initial vaccine and 3 days after the boost vaccine.

Combination treatment with Ad.E7 vaccination and rofecoxib was done in larger tumors (~275 mm<sup>3</sup>). As shown in Fig. 3 (similar to our previous finding), when fed control chow, Ad.E7 vaccination alone had no effect on the growth of large tumors compared with Ad.lacZ vaccination. In addition, treating mice with Ad.lacZ vaccination plus rofecoxib chow had no effect on tumor growth rate.

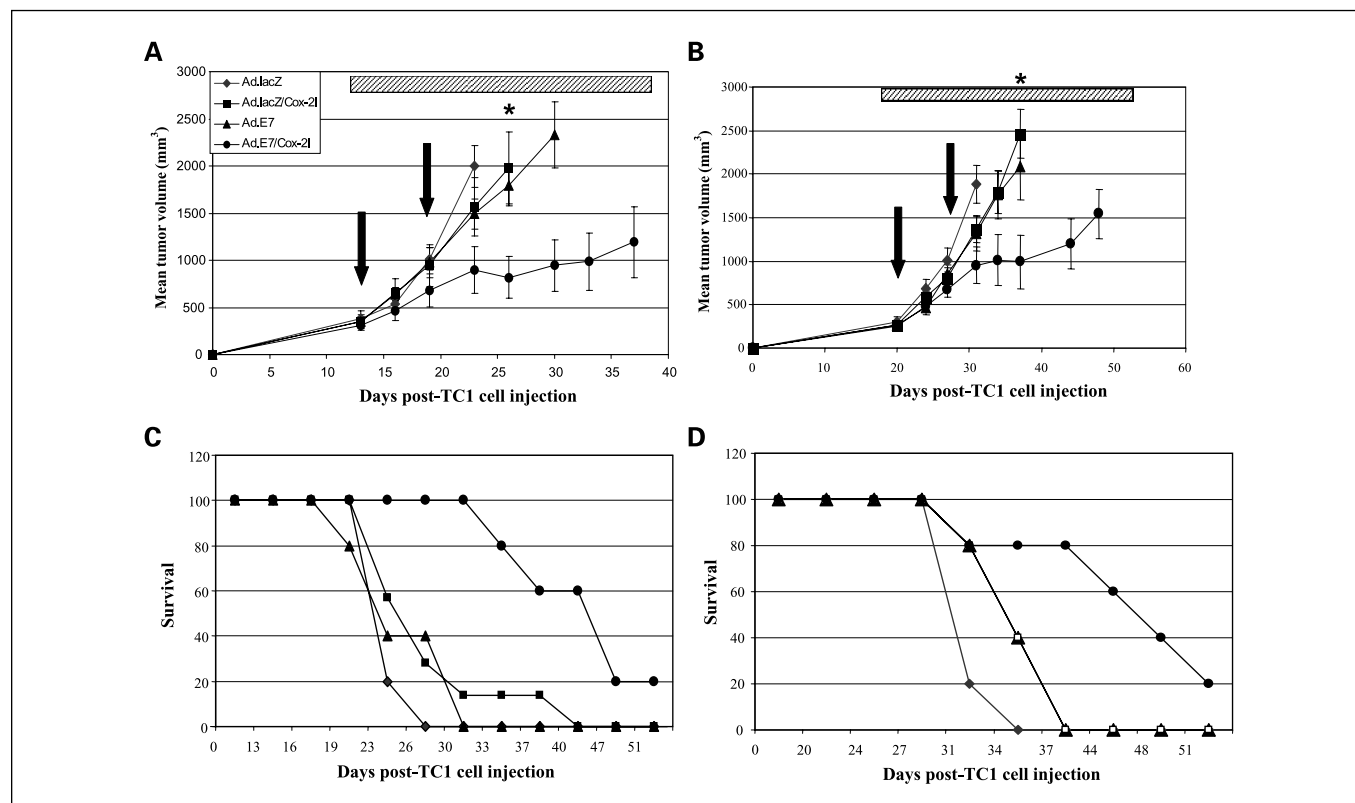
In contrast, combining Ad.E7 vaccination with rofecoxib chow resulted in significant growth inhibition compared with Ad.E7 vaccination or rofecoxib chow alone ( $P < 0.05$ ; Fig. 3A and B, two separate experiments with same conditions). The combined treatment of Ad.E7 vaccination and rofecoxib significantly improved survival ( $P < 0.008$ ) to a greater extent than any other treatment modality (Fig. 3C and D).

**Rofecoxib chow decreased serum and tumor levels of PGE<sub>2</sub>.** To confirm that the rofecoxib chow decreased PGE<sub>2</sub> levels in blood and tumor tissues, tumor-bearing animals were sacrificed after 10 days of control chow or the COX-2 inhibitor chow. Whereas plasma PGE<sub>2</sub> levels were below the limits of detection in non-tumor-bearing animals, we detected plasma levels of  $36.3 \pm 3.1$  pg/mL ( $n = 9$ ) in tumor-bearing mice.

Treatment with rofecoxib lowered this level significantly to  $21.7 \pm 2.2$  ( $P = 0.001$ ;  $n = 9$ ). PGE<sub>2</sub> levels in tumor homogenates from control animals averaged  $9.3 \pm 1.5$  ng/mg protein ( $n = 9$ ). This was significantly reduced ( $P = 0.01$ ) in rofecoxib-treated animals to  $4.1 \pm 1.2$  ng/mg protein ( $n = 9$ ). The addition of the Ad.E7 vaccine had no effect on PGE<sub>2</sub> levels in control or vaccinated animals (data not shown). In a previous study, we detected very low levels of mRNA for COX-2 in TC1 cells in culture (32), suggesting that the PGE<sub>2</sub> seen here is likely produced by tumor stromal elements (i.e., macrophages).

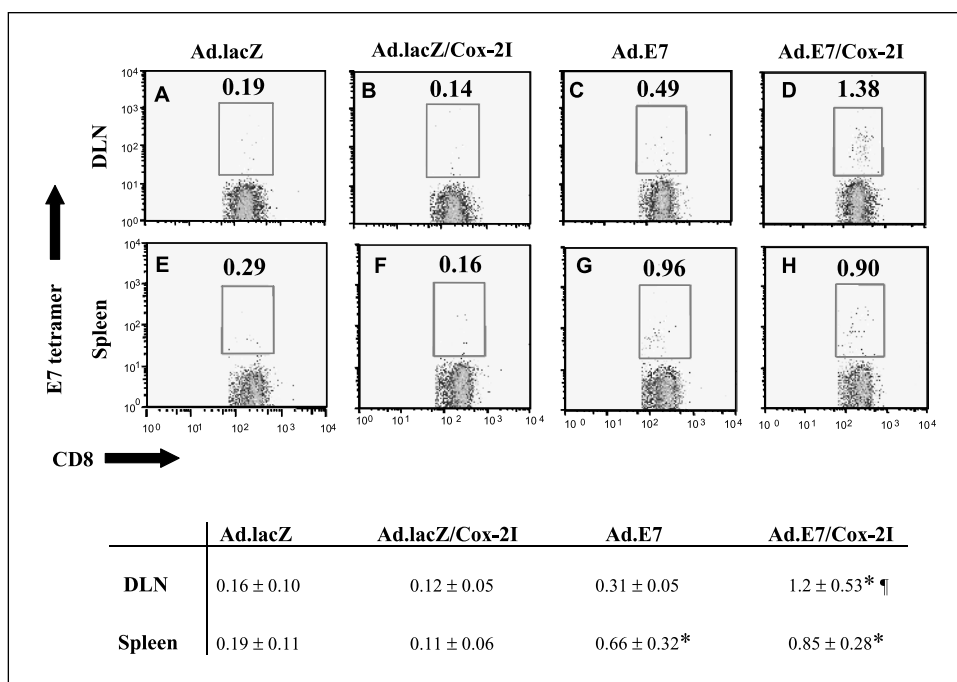
**Ad.E7 vaccination plus COX-2 inhibition enhanced the number of E7-specific CD8<sup>+</sup> cells in draining lymph nodes but not in the spleen.** We next sought to determine the mechanisms of this effect. Draining lymph nodes and spleens were isolated from tumor-bearing control and rofecoxib-treated mice at 3 and 6 days after the boost vaccine and analyzed (see Fig. 4).

To determine whether addition of rofecoxib to Ad.E7 vaccination increased the number of E7-specific CD8<sup>+</sup> cells systemically, spleens and draining lymph nodes were first examined by FACS. The percentage of E7-specific CD8<sup>+</sup> cells (as a percentage of total CD8<sup>+</sup> cells) seen after treatment was significantly increased ( $P < 0.05$ ) in splenocytes from Ad.E7-vaccinated mice (Fig. 4G; mean, 0.66%) and the Ad.E7/COX-2 inhibitor mice (Fig. 4H; mean, 0.85%) compared with either Ad.lacZ-vaccinated (Fig. 4E; mean, 0.19%) or combined Ad.lacZ plus rofecoxib (Fig. 4F; mean, 0.11%). However,



**Fig. 3.** COX-2 inhibition enhanced the effectiveness of Ad.E7 vaccination against large tumors. Tumors were allowed to grow to ~275 mm<sup>3</sup> and received our standard vaccination protocol in addition to chow containing rofecoxib. Arrows, days of vaccination; cross-hatched box, period of rofecoxib treatment. \*,  $P < 0.05$ , combined Ad.E7 vaccination and rofecoxib significantly inhibited the growth of tumors compared with all other treatment combinations (A and B, repeat experiments with same conditions),  $P < 0.05$ . This treatment resulted in significantly prolonged survival (C and D, survival curves for A and B, respectively). Note that the curves for Ad.LacZ and Ad.LacZ/COX-2 overlap in (D).

**Fig. 4.** Combination Ad.E7 vaccination and rofecoxib increased E7-specific CD8<sup>+</sup> cells compared with Ad.E7 vaccination alone in draining lymph node but not in the spleen. Spleen and draining lymph node were isolated from tumor-bearing, vaccinated animals (initial tumor size of 275 mm<sup>3</sup> vaccinated on days 11 and 18) 3 days following boost vaccinations and stained for the presence of E7-specific CD8<sup>+</sup> cells using FACS. Ad.E7 vaccination with or without rofecoxib showed similar levels of E7-specific CD8<sup>+</sup> cells; however, in draining lymph node, combination Ad.E7 vaccination and rofecoxib significantly increased the number of E7-specific CD8<sup>+</sup> cells compared with Ad.E7 vaccination alone. Ad.lacZ alone or in combination with rofecoxib had low levels of background staining. The table represents the mean ± SE for four mice from two separate experiments with result pooled from days 3 and 6 after boost vaccination. \*, *P* < 0.05 compared with both Ad.lacZ groups; †, *P* < 0.05 compared with both Ad.LacZ groups and Ad.E7 vaccination alone.



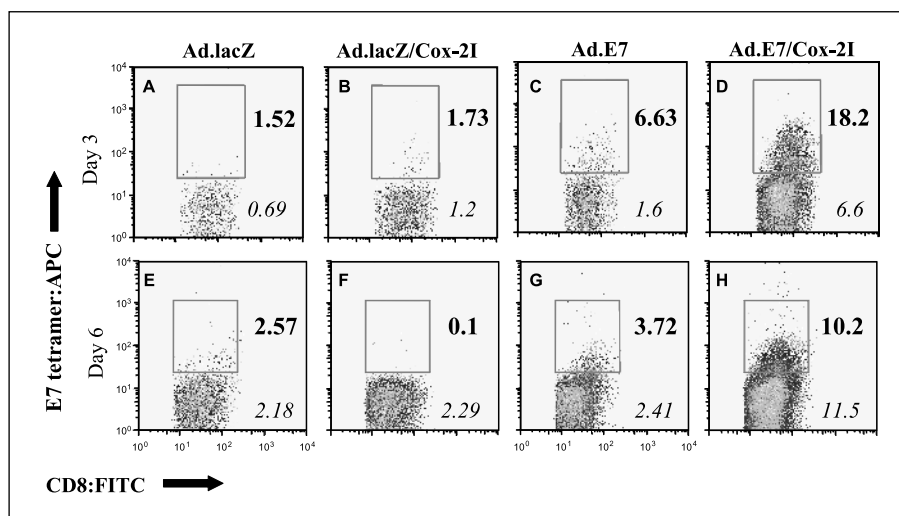
addition of rofecoxib to Ad.E7 vaccination did not increase the number of E7-specific CD8<sup>+</sup> cells in the spleen over that of Ad.E7 vaccination alone.

Lymphocytes from draining lymph node in animals treated with Ad.lacZ alone or Ad.lacZ plus rofecoxib had minimal staining for E7-specific CD8<sup>+</sup> cells (Fig. 4A; mean, 0.16% versus Fig. 4B; mean, 0.12%). The number of tetramer<sup>+</sup> cells was not significantly different in the draining lymph node (Fig. 4C; mean, 0.31%), but addition of rofecoxib to Ad.E7 vaccination significantly (*P* = 0.02) increased the number of E7-specific CD8<sup>+</sup> cells in the draining lymph node ~2.5-fold compared with Ad.E7 vaccination alone (Fig. 4C; mean, 1.2% versus Fig. 4D; mean, 0.31%). These data collectively suggested that addition of rofecoxib to Ad.E7 vaccination increased the number of E7-specific CD8<sup>+</sup> cells in the draining lymph node but not in the spleen.

*COX-2 inhibition enhanced intratumoral CD8<sup>+</sup> cells and E7-specific CD8<sup>+</sup> cells.* We also examined the number of E7-specific CD8<sup>+</sup> cells and total CD8<sup>+</sup> cells that accumulated in tumors. At days 3 and 6 after the second vaccine boost, tumors were removed, digested, and analyzed by FACS to determine the presence of E7-specific CD8<sup>+</sup> cells.

As shown in Fig. 5, following treatment initiation, combination treatment markedly increased the number of tumor-infiltrating CD8<sup>+</sup> T cells. Three days after boost vaccine, tumors showed a low number of CD8<sup>+</sup> cells in mice treated with Ad.lacZ (Fig. 5A), 0.69% of total cells counted during FACS (see italicized number in *bottom right*), Ad.lacZ plus rofecoxib (Fig. 5B; 1.2%), and Ad.E7 (Fig. 5C; 1.6%) compared with a ~4-fold increase in the number of CD8<sup>+</sup> T cells in mice treated with Ad.E7 plus rofecoxib (Fig. 5D; 6.6%). This same pattern was seen at day 6, where Ad.E7

**Fig. 5.** Combination Ad.E7 vaccination and rofecoxib increased tumor-infiltrating E7-specific CD8<sup>+</sup> cells. Initial tumors of ~275 mm<sup>3</sup> were vaccinated on days 12 and 189 and isolated 3 and 6 days following booster vaccinations with Ad.LacZ or Ad.E7, digested, and stained for the presence of CD8<sup>+</sup> T cells and E7-specific CD8<sup>+</sup> cells. Half the animals received control chow and half the animals were fed rofecoxib chow (COX-2I). **Bold-faced number on top**, percentage of the total CD8<sup>+</sup> cells that were E7 tetramer<sup>+</sup>; *italicized number at the bottom*, percentage of the total number of live cells digested from the tumor that were CD8<sup>+</sup>. Combination Ad.E7 vaccination and rofecoxib increased the number CD8<sup>+</sup> cells within the tumors ~4-fold and increased the number of E7-specific CD8<sup>+</sup> cells ~2.5-fold (D) compared with Ad.lacZ (A), Ad.E7 (C) alone, or combined Ad.lacZ/rofecoxib (B).



plus rofecoxib treated mice had a ~4-fold increase in the number of tumor-infiltrating CD8<sup>+</sup> T cells (Fig. 5H; 11.5%) compared with Ad.lacZ (Fig. 5E; 2.18%), Ad.lacZ plus rofecoxib (Fig. 5F; 2.29%), and Ad.E7 alone (Fig. 5G; 2.41%).

The use of E7 tetramers to determine the number of E7-specific tumor-infiltrating CD8<sup>+</sup> cells (boxed area in FACS plots) revealed that, 3 days following boost vaccine, combined treatment with Ad.E7 vaccination alone markedly increased the number of E7-specific CD8<sup>+</sup> cells compared with Ad.lacZ vaccination alone or Ad.LacZ vaccination plus rofecoxib (6.63% versus 1.52% versus 1.73% of total CD8<sup>+</sup> cells, respectively; Fig. 5C versus Fig. 5A and B). However, the addition of rofecoxib to Ad.E7 vaccination dramatically increased the number of E7-specific CD8<sup>+</sup> cells ~3-fold to 18.2% of CD8<sup>+</sup> cells (Fig. 5D). A similar pattern of staining was seen at day 6 following boost vaccine with a ~2.5-fold increase in the number of E7-specific tumor-infiltrating CD8<sup>+</sup> cells in Ad.E7 plus rofecoxib (Fig. 5H; 18.2%) compared with Ad.E7 alone (Fig. 5G; 6.63%).

To confirm the FACS findings and to visualize the location of the CD8<sup>+</sup> T cells, tumors were also examined by immunohistochemistry at days 3 and 6. No increases in intratumoral macrophages or CD4<sup>+</sup> T cells were seen in any group (data not shown). Ad.lacZ-vaccinated animals had few tumor-infiltrating CD8<sup>+</sup> cells at either day 3 or 6 (Fig. 6A and E, respectively). In these large tumors, the number of infiltrating CD8<sup>+</sup> cells was not noticeably increased with either Ad.E7 vaccination alone (Fig. 6C and G) or combined Ad.lacZ vaccination with rofecoxib (Fig. 6B and F) at day 3 or 6. However, combined Ad.E7 vaccination with rofecoxib resulted in a clear increase in the number of tumor-infiltrating CD8<sup>+</sup> cells (Fig. 6D and H) and these cells were evenly distributed throughout the tumor.

**COX-2 inhibitor treatment increased intratumoral expression of Th1 cytokines.** Because the addition of rofecoxib to Ad.E7 vaccination generated a larger number of tumor-infiltrating E7-specific CD8<sup>+</sup> cells, we sought to determine if rofecoxib treatment altered the level of expression of the mRNAs of

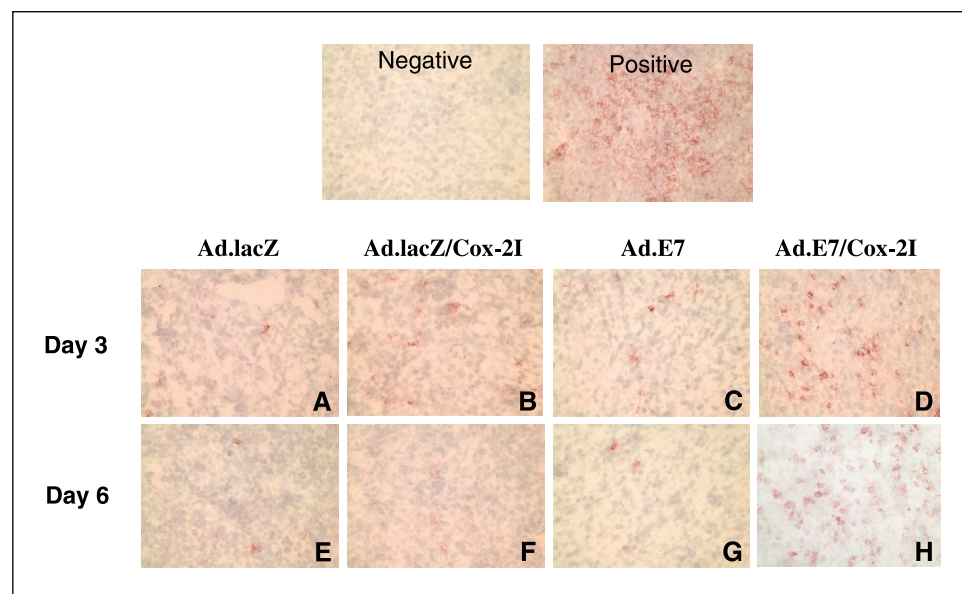
select cell adhesion molecules, cytokines, and/or chemokines within the tumors. Mice were fed control or COX-2 inhibitor chow and tumors were harvested at day 10 for RNA isolation. By real-time reverse transcription-PCR (RT-PCR; Table 1), we determined that tumors treated with rofecoxib showed marked increases (compared with mice fed control chow mice) in the expression of message levels of intercellular adhesion molecule-1 (ICAM-1; 5.9-fold), CXCL9 (MIG; 5.3-fold), CXCL10 (IP10; 3.5-fold), IL-12 (2-fold), and IFN- $\gamma$  (8.5-fold), whereas there was a relative decrease in the expression of VEGF (0.5-fold).

## Discussion

We have shown previously that oral administration of the COX-2 inhibitor rofecoxib was able to slow the growth of small murine mesothelioma and lung cancer cell tumor lines and that this inhibition was due primarily to augmentation of immunologic responses (28, 32). In addition, we found that combining COX-2 inhibition with an adenoviral vector expressing IFN- $\beta$  enhanced the efficacy of immunogene therapy (28). We did not detect increased numbers of CTLs in the spleens of treated mice using Winn assays; however, markedly increased trafficking of CD8<sup>+</sup> T cells into the tumors was noted.

These previous studies raised several interesting questions. (a) Could COX-2 inhibition be combined with other types of cancer immunotherapy, such as vaccination? (b) How was COX-2 inhibition increasing intratumoral trafficking? (c) Given the recently observed suppression of dendritic cell function by PGE<sub>2</sub> (19, 33, 34) and the activation of T-regulatory cells (27), could we also detect increased numbers of antitumor CTLs after COX-2 blockade if we had more sophisticated techniques to trace these cells?

To address these questions, we used the TC1 lung cancer cell line that expresses low levels of the known viral oncogene HPV-E7 (29). Several anti-HPV-E7 vaccine strategies have been employed to both prevent the growth of tumor cells (30) or treat established tumors (35–40). Importantly, the immunology of HPV-E7 has been well studied in C57BL/6 mice and the



**Fig. 6.** COX-2 inhibition increased the number of tumor-infiltrating CD8<sup>+</sup> cells. Tumors were removed, fixed, and stained for CD8<sup>+</sup> cells 3 and 6 days following booster vaccination with Ad.LacZ or Ad.E7. Half the animals received control chow and half the animals were fed COX-2 inhibitor chow (COX-2I). Following vaccination, Ad.lacZ-treated (A and E), Ad.lacZ/rofecoxib-treated (B and F), and Ad.E7-treated (C and G) mice all showed few intratumoral CD8<sup>+</sup> cells at days 3 and 6. In contrast, mice given the combined Ad.E7/rofecoxib treatment significantly increased the number of tumor-infiltrating CD8<sup>+</sup> cells at day 3 (D) and day 6 (H). Negative and positive controls of spleens (top) showed the specificity of CD8<sup>+</sup> staining.

**Table 1.** COX-2 inhibition increased the expression of Th1 cytokines and various cell adhesions molecules

|               | Experiment 1 | Experiment 2 |
|---------------|--------------|--------------|
| ICAM-1        | 6.6          | 5.1          |
| VCAM-1        | 1.8          | 2.0          |
| TNF- $\alpha$ | 1.4          | 2.0          |
| IFN- $\gamma$ | 7.3          | 9.6          |
| IL-12         | 2.2          | 1.8          |
| IP-10         | 4.0          | 3.0          |
| MIG           | 4.8          | 5.7          |
| IL-10         | 1.5          | 1.6          |
| Arginase      | 0.5          | 1.4          |
| VEGF          | 0.4          | 0.6          |

NOTE: Tumors were harvested from mice 10 days following initiation of control or rofecoxib chow for mRNA isolation. Real-time RT-PCR was done in triplicate for each sample. Values were normalized to  $\beta$ -actin expression and the fold change in each inflammatory mediator was determined (level in tumor from mouse fed rofecoxib chow / level in tumor from mouse fed normal chow). Two independent experiments were done.

immunodominant epitope of the protein (RAHYNIVTF) is known, allowing the use of MHC class I tetramers and intracellular cytokine stimulation to follow immune responses (38–40). We took advantage of a previously characterized adenoviral vaccine expressing HPV-E7 (30) and (a) confirmed that the vector was effective in prevention studies, (b) showed that this vaccine was able to generate significant numbers of HPV-E7 tetramer<sup>+</sup> CD8<sup>+</sup> T cells in the spleen and draining lymph nodes that had CTL activity (Fig. 2), and (c) showed that two doses of vaccine could slow the growth of small tumors (Fig. 1A). Like most vaccines, however, Ad.E7 was ineffective against larger, established tumors (Fig. 1B). This size-dependent phenomenon is likely because (a) larger tumors suppress effective immune responses due to their greater production of immune suppressing factors and (b) the relative proportions of tumor cells to effector cells is increased with larger tumors, thereby preventing effective cytolysis to control tumor growth.

The most important finding of this study was that although rofecoxib alone and Ad.E7 alone had minimal therapeutic effects on the growth of larger tumors the combination was able to significantly augment efficacy and led to a marked slowing of tumor growth (Fig. 3). This finding is consistent with reports from two recent studies. In one study, a cell-based vaccine was shown to be more effective against tumor challenge when combined with either COX-2 inhibition or COX-2 knockout, and with elimination of COX-2 activity, there is a significant increase in IFN- $\gamma$  production (41). In addition, the combination of a poxvirus-based vaccine against human carcinoembryonic antigen with a COX-2 inhibitor augmented antitumor immunity and survival in a transgenic mouse model of colon cancer (42).

We believe that this observed antitumor effect was due to a combination of factors. Like our previous study (28), we observed a markedly increased number of CD8<sup>+</sup> T cells within the tumors treated with combination therapy compared with single therapies as determined by both immu-

nostaining (Fig. 6) and flow cytometry (Fig. 5). Even more importantly, we observed up to 3-fold higher numbers of antigen-specific E7 tetramer<sup>+</sup> cells within tumors (Fig. 5). This observation likely explains most of the enhanced efficacy we observed. The reason for this increased trafficking of tumor cells is not known for certain; however, real-time RT-PCR studies showed that COX-2 inhibition markedly changed the cytokine/chemokine balance within the tumors (Table 1). Tumors in the rofecoxib-treated mice had marked elevations in message levels for the endothelial cell adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) and the Th1 type cytokines/chemokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), INF- $\gamma$ , IL-12, IP-10 (CXCL10), and MIG (CXCL9). On the other hand, the message for the angiogenic and immunosuppressive molecule VEGF was suppressed by ~50%. These findings regarding an up-regulation of Th1 cytokines and chemokines are similar to those described recently by Sharma et al. (27) and others in lung cancer models treated with a COX-2 inhibitor (27, 43, 44); however, unlike these investigators, we did not observe down-regulation of IL-10 in our model. Although we did not directly measure protein levels of these mediators in this study, such measurements were made by Sharma et al. and confirmed a close correlation between the PCR and protein data (27). We thus postulate that COX-2 inhibition altered the tumor microenvironment allowing enhanced chemoattraction and trafficking of T cells into the tumors. It is also possible that this more stimulatory environment in the COX-2 inhibitor-treated animals along with a reported decrease in T regulatory cells (27) prevented the well-described inactivation of T cells within tumors, thereby allowing activation and proliferation of the CD8<sup>+</sup> T cells that have infiltrated the tumor, but we did not test this hypothesis directly. There may also have been indirect effects. Initial enhanced tumor cell killing in the combination group might lead to decreased tumor cell-mediated immunosuppression and augmented efficacy of tumor CTL.

A second potential way that COX-2 inhibition could augment immune responses would be to enhance antigen presentation and T-cell generation. To examine this question, we quantified the numbers of E7-specific CD8<sup>+</sup> T cells in the spleens and tumor draining lymph nodes of mice in each treatment regimen. As shown in Fig. 4, Ad.E7 vaccination increased the number of E7 tetramer<sup>+</sup> cells in the spleens of treated mice; rofecoxib alone did not increase this number. However, at both days 3 and 6, the numbers of E7 tetramer<sup>+</sup> cells were two to three times higher in the tumor draining lymph node of those mice treated with Ad.E7 plus rofecoxib compared with those animals treated with only Ad.E7. These data suggest that COX-2 inhibition might also be increasing dendritic cell activation and trafficking to the draining lymph node allowing for greater E7-specific CD8<sup>+</sup> cell generation at that lymphoid location.

Although vaccination strategies to activate the immune system for the prevention of infectious disease have been a great medical advance, application of vaccine strategies for the prevention and treatment of cancer has not encountered the same degree of success. As strategies to enhance antitumor T cells and their function have improved, it is becoming increasingly clear that tumor-related immunosuppression plays a major role in limiting therapeutic responses. This study and



the study by others (19, 27, 33, 34) suggest that blockade of PGE<sub>2</sub> produced by tumor cells and/or tumor-associated macrophages can limit a significant portion of this immunosuppressive response.

In conclusion, we have shown the clinical effectiveness of adding a COX-2 inhibitor to a vaccination strategy and that this effect is mediated by an alteration of the immunosuppressive tumor microenvironment leading to increased production and infiltration of antigen-specific CD8<sup>+</sup> cells into draining lymph node and tumors. We propose that this treatment approach

could be combined with current modalities of immunotherapy in a broad range of cancer patients to help control micro-metastatic disease and primary tumor burden.

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