Cyclooxygenase 2 Inhibition Promotes IFN-γ-Dependent Enhancement of Antitumor Responses[1]

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In previous studies, we demonstrated an immune suppressive network in non-small cell lung cancer that is due to overexpression of tumor cyclooxygenase 2 (COX-2). In this study, we assessed the vaccination response to tumor challenge following either pharmacological or genetic inhibition of COX-2 in a murine lung cancer model. Treatment of naive mice with the COX-2 inhibitor, SC-58236, skewed splenocytes toward a type 1 cytokine response, inducing IFN-γ, IL-12, and IFN-γ-inducible protein 10, whereas the type 2 cytokines IL-4, IL-5, and IL-10 remained unaltered. Fifty percent of mice receiving SC-58236 and an irradiated tumor cell vaccine completely rejected tumors upon challenge. Those mice that did form tumors following challenge demonstrated a reduced tumor growth. In contrast, all mice either vaccinated with irradiated tumor cells alone or receiving SC-58236 alone showed progressive tumor growth. Studies performed in CD4 and CD8 knockout mice revealed a requirement for the CD4 T lymphocyte subset for the complete rejection of tumors. To determine the role of host COX-2 expression on the vaccination responses, studies were performed in COX-2 gene knockout mice. Compared with control littermates, COX-2$^{-/-}$ mice showed a significant tumor growth reduction, whereas heterozygous COX-2$^{-/+}$ mice had an intermediate tumor growth reduction following vaccination. In vivo depletion of IFN-γ abrogated the COX-2 inhibitor-mediated enhancement of the vaccination effect. These findings provide a strong rationale for additional evaluation of the capacity of COX-2 inhibitors to enhance vaccination responses against cancer. *The Journal of Immunology,* **2005, 174: 813–819.**

Despite the identification of a repertoire of tumor Ags, hurdles persist for immune-based therapies. Tumor-induced immune suppression may be contributing to the limited efficacy of the current approaches. Many tumors, including lung cancer, have the capacity to promote immune tolerance and escape host immune surveillance (1, 2). Tumor-reactive T cells have been shown to accumulate in lung cancer tissues but fail to respond because of suppressive tumor cell-derived factors (3, 4). Tumors use numerous pathways to inhibit immune responses, including the elaboration of immune inhibitory cytokines. In addition to directly secreting immunosuppressive cytokines, lung cancer cells may induce host cells to release immune inhibitors (5–9). In previous studies, we found an immune suppressive network in non-small cell lung cancer (NSCLC)[3] that is due to overexpression of tumor cyclooxygenase 2 (COX-2) (7, 10), which is constitutively expressed in a variety of malignancies (11–14). We and others (7, 15–18) have reported that COX-2 is frequently constitutively elevated in human NSCLC. Although multiple genetic alterations are necessary for lung cancer invasion and metastasis, COX-2 may be a central element in orchestrating this process (7, 16–20). Overexpression of COX-2 is associated with apoptosis resistance (21, 22), angiogenesis (23, 24), enhanced invasion and metastasis (25–27), and decreased host immunity (7, 10, 28). This study was performed to determine the role of COX-2 inhibition in augmenting the vaccination response to tumor challenge. We report that COX-2 inhibition enhances antitumor immunity in a murine lung cancer model.

Materials and Methods

Reagents

The COX-2 inhibitor, SC-58236, was provided by Pharmacia. Abs to mouse CD40, CD3, and Ab pairs to murine IFN-γ, IL-4, IL-5, IL-10, and IL-12 along with the respective cytokine standards were obtained from BD Pharmingen. Ab pairs to murine by monokine induced IFN-γ/CXCL9, IFN-γ-inducible protein 10 (IP-10)/CXCL10, and the respective recombinant cytokine standards were purchased from R&D Systems.

Cell culture

Murine Lewis lung carcinoma cell line (here referred to as 3LL), B16 melanoma, and EL4 lymphoma cell lines are syngeneic for C57BL/6 mice and were obtained from the American Type Culture Collection. The cells were routinely cultured as monolayers in 25-cm^3 tissue culture flasks containing RPMI 1640 medium (Irvine Scientific) supplemented with 10% FBS (Gemini Biological Products), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2 mM glutamine (JRH Biosciences) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cell lines were Mycoplasma free and used up to the 10th passage before thawing frozen cells from liquid N_2.

Dendritic cell (DC) culture and pulsing with mut1mut2 peptides

Lymphocyte-depleted bone marrow-derived DCs from C57BL/6 mice (H-2^b) were cultured with medium containing murine GM-CSF (2 ng/ml)

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[3] Abbreviations used in this paper: NSCLC, non-small cell lung cancer; COX-2, cyclooxygenase 2; DC, dendritic cell; IP-10, IFN-γ-inducible protein 10.

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and IL-4 (20 ng/ml; R&D Systems) for 6 days as reported previously (29). Day 6 DCs were pulsed with mut1 and mut2 peptides as described previously (30). The mut1 and mut2 peptides, synthesized by Research Genetics, consist of the 52–59 aa positions of the mutated connexin 37 protein present in the 3LL cell line and have the sequence FEQNTAQP and FEQNTAQA, respectively (31).

Tumor cell irradiation

A single-cell suspension of 3LL tumor cells (2×10^6 cells/ml) was irradiated with 80 Gy of gamma irradiation in a ^{137}Cs gamma irradiator. Cells were washed three times in PBS before injection.

Mice

Pathogen-free female C57BL/6 mice (8–12 wk of age) were obtained from Harlan Sprague Dawley, and COX-$2^{-/-}$ and heterozygous COX-$2^{-/+}$ mice were obtained from Taconic Farms and maintained in the West Los Angeles Veterans Affairs Animal Research Facility. C57BL/6 CD$4^{-/-}$ and CD$8^{-/-}$ were obtained from The Jackson Laboratory. The Institutional Animal Studies Review Committee approved all studies.

Cytokine determinations

C57BL/6 naive mice received i.p. injections of SC-58236 (3 mg/kg) or diluent control on days 1, 3, and 5. On day 8, splenocytes (5×10^6/ml) were stimulated for 24 h with either anti-CD3 (1 μg/ml) for IFN-γ, IL-4, IL-5, IL-10, and IP-10 quantification or anti-CD40 mAb (5 μg/ml) for IL-12 production. The IL-12 ELISA measured both P40/P70 subunits of IL-12. ELISA quantification of cytokines was performed as previously described (32), and the plates were read with a Molecular Dynamics plate reader. C57BL/6 mice were treated with the following: 1) a single dose of 5×10^5 irradiated 3LL cells s.c.; 2) three injections of SC-58236 (0.1 mg/kg) i.p. plus one dose of 5×10^5 irradiated 3LL cells s.c.; and 3) three injections of SC-58236 (0.1 mg/kg) i.p. Fourteen days following vaccination, but without a tumor challenge, IFN-γ was determined from splenocytes after restimulation overnight with irradiated (100 Gy, ^{137}Cs gamma rays) autologous 3LL cells or syngeneic control tumors EL4 and B16 at a ratio of 10:1. Following vaccination, mouse IFN-γ-specific ELISPOT was performed with the R&D ELISPOT kit, according to the manufacturer's instructions, and spots quantified with the Immunospot Image Analyzer (Cellular Technologies) at the UCLA Immunology Core Facility.

Vaccination model

C57BL/6 mice were treated with the following: 1) a single s.c. dose of 5×10^5 irradiated 3LL cells; 2) three injections of SC-58236 (0.1 mg/kg) i.p. plus one s.c. dose of 5×10^5 irradiated 3LL cells; and 3) three i.p. injections of SC-58236 (0.1 mg/kg). To determine the role of CD4 and CD8 T cells on tumor responses, CD$4^{-/-}$ and CD$8^{-/-}$ mice were treated with three injections of SC-58236 (0.1 mg/kg) i.p. plus one s.c. dose of 5×10^5 irradiated 3LL cells.

On day 14, mice were challenged with 10^5 3LL tumor cells. In addition, C57BL/6 mice were vaccinated with peptide-pulsed DCs. A total of 10^6 mut1mut2-pulsed DCs was injected at weekly intervals s.c. for 3 wk. One week after the last vaccination, mice were challenged with 10^5 3LL tumor cells, and tumor volumes were monitored by measuring bisecting diameters as described previously (30).

Because nonsteroidal anti-inflammatory drugs have effects in addition to COX inhibition (33, 34), we assessed the involvement of COX-2 expression on vaccination effects by performing experiments in COX-2 gene knockout mice. Homozygous COX-$2^{-/-}$, heterozygous COX-$2^{-/+}$, and the control littermates were vaccinated once with 5×10^5 irradiated 3LL cells. On day 14, mice were challenged with 2.5×10^5 3LL cells.

To address the importance of IFN-γ in COX-2 inhibitor-mediated enhancement in the vaccination effect, C57BL/6 mice received three injections of SC-58236 (0.1 mg/kg) i.p. plus one s.c. dose of 5×10^5 irradiated 3LL cells. After vaccination, mice were treated three times each week with 100 μg/dose of purified monoclonal anti-IFN-γ or appropriate control Abs (rat IgG) by the i.p. route for the duration of the experiment. Anti-mouse IFN-γ monoclonal (R4-462; American Type Culture Collection) neutralizing Ab was purified by affinity chromatography from SCID mice ascites that was generated 3–4 wk after i.p. injection of 10^6 R4-462 hybridoma cells/mouse (35). Control rat IgG was obtained from Sigma-Aldrich. On day 14, mice were challenged with 5×10^5 3LL tumor cells, and tumor volumes were monitored. In response to the anti-IFN-γ Ab, there was a significant reduction of IFN-γ in vivo (data not shown).

Results

COX-2 inhibition augments splenic type 1 cytokine production

Based on our previous findings and reports in the literature (5–7, 10, 28), we hypothesized that COX-2 inhibition in vivo would skew the host response toward the type 1 cytokine phenotype. To test this, we evaluated splenic cytokine profiles that accompany COX-2 inhibition in naive, non-tumor-bearing mice. Compared with diluent controls, splenocytes from COX-2 inhibitor-treated mice had an enhanced capacity to secrete IFN-γ (1.8-fold, $p < 0.01$) and IP-10 (2.3-fold, $p < 0.01$) after anti-CD3 stimulation and IL-12 (1.5-fold, $p < 0.01$), following anti-CD40 stimulation. In contrast, production of ILs 4, 5, and 10 remained unaltered (Table I).

COX-2 inhibition enhances the antitumor vaccination response

Based on the observations documented in Table I, we postulated that COX-2 inhibition would augment vaccination efficacy and heighten the specific immune response to tumor challenge. The response to a single suboptimal dose of irradiated tumor cell vaccination (5×10^5) was enhanced in mice that received SC-58236 with four of eight mice completely rejecting the tumor challenge dose of 10^5 cells and a significant reduction in tumor growth in the animals that did form tumors on challenge (Fig. 1*A*). The same trend in tumor growth rates was evident in the groups for an additional 10 days.

COX-2 inhibition enhances 3LL antitumor vaccination response

To evaluate the effect of COX-2 inhibition on the specificity of the vaccination responses, IFN-γ ELISPOT assay was performed on splenic T cells. In ELISPOT assays, at constitutive levels without stimulation with 3LL cells, no difference was noted in the frequency of IFN-γ-producing cells among the treatment groups. However, compared with mice receiving irradiated 3LL cells or SC-58236 alone, mice treated with SC-58236 plus tumor cell vaccine had a significantly greater frequency of splenic T cells producing IFN-γ in response to restimulation in vitro with irradiated 3LL cells ($p < 0.01$) (Fig. 1*B*). Although there were minimal responses to the irrelevant control syngeneic tumor EL4, there was a significant increase in the cells producing IFN-γ in response to B16 (data not shown). However, the induction in response to the autologous tumor was significantly greater than all controls. We determined the frequency of T cells secreting IL-4, IL-5, and IL-10 following vaccination. There were no significant differences in the

Table I. *COX-2 inhibition augments splenic type 1 cytokine production*[a]

Treatment	IFN-γ	IP-10	IL-12	IL-4	IL-5	IL-10
Diluent control	4,337 ± 431	4,328 ± 430	166 ± 8	292 ± 12	80 ± 7	503 ± 11
SC-58236	7,678 ± 138*	10,138 ± 524*	243 ± 23*	320 ± 34	109 ± 27	481 ± 22

[a] C57BL/6 mice were treated for one week with three i.p. injections of SC-58236 (3 mg/kg) or diluent control. On day 8, splenocytes were stimulated for 24 h with either anti-CD3 (1 μg/ml) for IFN-γ, IP-10, IL-4, IL-5, and IL-10 quantification or anti-CD40 mAb (5 μg/ml) for IL-12 production. Splenocytes from COX-2 inhibitor-treated mice had an enhanced capacity to secrete IFN-γ and IP-10 following anti-CD3 stimulation and IL-12 following anti-CD40 stimulation compared with diluent control. IFN-γ and IP-10 are expressed as pg/ml/10^6 cells. There were no significant differences in splenocyte IL-4, IL-5, and IL-10 following COX-2 inhibitor treatment. IL-4, IL-5, IL-10, and IL-12 are expressed as ng/ml/5×10^6 cells. *, $p < 0.01$ compared with diluent control; $n = 8$ mice/group.

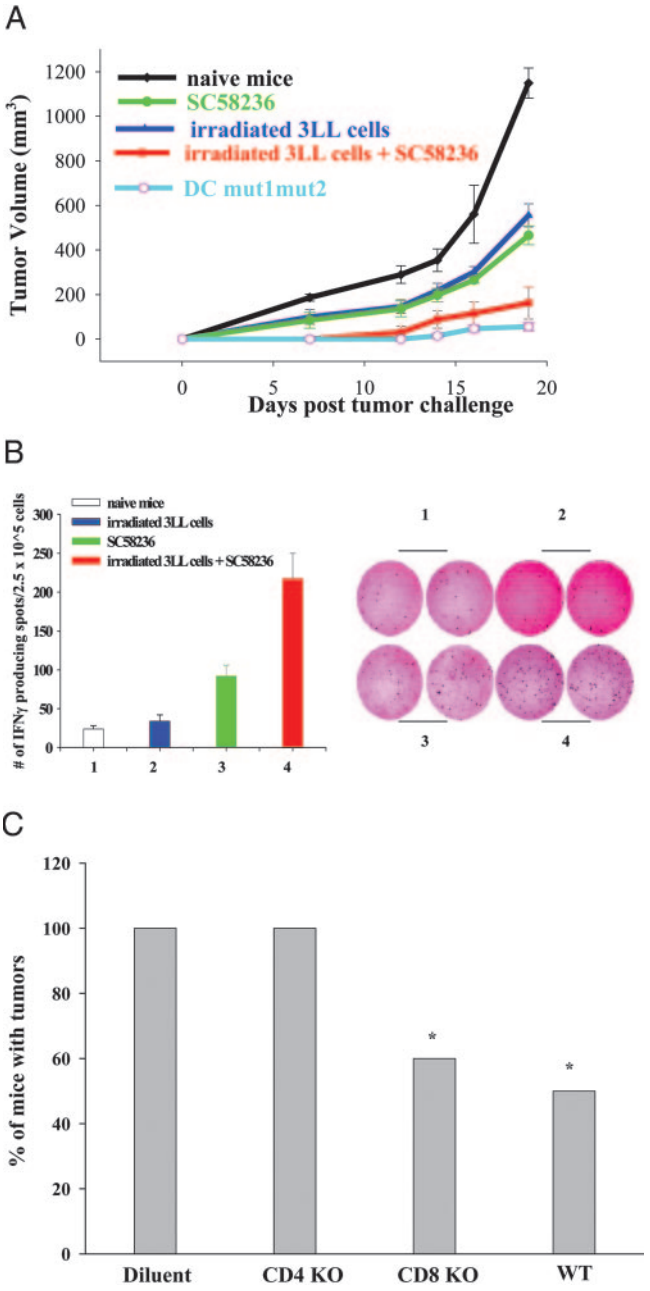

FIGURE 1. *A* and *B*, C57BL/6 mice were treated with the following: 1) a single dose of 5×10^5 irradiated 3LL cells s.c.; 2) three injections of SC-58236 (0.1 mg/kg) i.p. plus one dose of 5×10^5 irradiated 3LL cells s.c.; and 3) three injections of SC-58236 (0.1 mg/kg) i.p. On day 14, mice were challenged with a s.c. dose of 10^5 3LL tumor cells for tumor volume determinations, or splenocytes from mice without a tumor challenge were restimulated overnight with irradiated 3LL cells and syngeneic control tumors at a ratio of 10:1 for ELISPOT assay. *A*, COX-2 inhibition enhances the vaccination response to tumor challenge. Although when administered individually, the COX-2 inhibitor or irradiated 3LL tumor cells led to reduced tumor growth on rechallenge, and all mice subsequently succumbed to progressive tumor. In contrast, the vaccination response was enhanced significantly in mice that received both irradiated 3LL and COX-2 inhibitor. The response in mice vaccinated with irradiated 3LL and COX-2 inhibitor was comparable to mice immunized with mut1mut2 peptide-pulsed DC (10^6); $n = 8$ mice/group ($p < 0.05$ irradiated 3LL plus COX-2 inhibitor group compared with irradiated 3LL cells or SC-58236 groups). In contrast to the irradiated 3LL alone or SC-58236 alone groups that did not completely reject tumors on challenge, four of eight mice in the irradiated 3LL plus SC-58236 group completely rejected the tumor challenge, and the remaining mice had a reduced tumor growth rate. *B*, COX-2 inhibition enhances the vaccination response to 3LL cells. Mouse IFN-γ-specific ELISPOT was performed with the R&D ELISPOT kit and spots quantified with an Immunospot Image Analyzer (Cellular Technologies) at the UCLA Immunology Core Facility. The data for ELISPOT wells in *B* are representative spots of tumor-specific splenic T lymphocytes secreting IFN-γ. Representative data of one mouse per group from a total of eight mice per treatment group are presented. In accord with the tumor challenge data, splenocytes from mice vaccinated with irradiated 3LL cells plus SC-58236 had a significantly greater frequency of cells releasing IFN-γ when restimulated with irradiated 3LL cells ($p < 0.01$); $n = 8$ mice/group. *C*, Six- to 8-wk-old CD4 knockout (KO) and CD8 KO mice on C57BL/6 background were treated with three injections of SC58236 (0.1 mg/kg) i.p. plus one s.c. dose of 5×10^5 irradiated 3LL cells. Control C57BL/6 mice received s.c. diluent saline injections. On day 14, mice were challenged with 10^5 3LL tumor cells. Studies performed in CD4 and CD8 KO mice reveal a requirement for CD4 T lymphocyte subset for complete rejection of tumors (*, $p < 0.01$ compared with diluent control and CD4 KO mice; $n = 10$ mice/group).

frequency of T cells secreting IL-4 and IL-5 in response to 3LL cells among the various treatments. However, compared with the naive control and other treatment groups, there was a 2-fold decrease in the frequency of T cells secreting IL-10 for the SC-58236 plus irradiated tumor cell treatment group (data not shown).

Consistent with the ELISPOT data, mice treated with SC-58236 combined with tumor cell vaccine also showed an enhanced 3LL tumor-specific ($p < 0.01$) and B16-nonspecific ($p < 0.05$) release of IFN-γ (Table II).

CD4 T cell requirement for COX-2 inhibitor-mediated enhancement of antitumor vaccination response

To determine the effector T cell requisite for the COX-2-mediated enhancement of the vaccination effects, studies were performed in CD4 and CD8 knockout mice. These studies revealed a requirement for the CD4 T lymphocyte subset for the complete rejection of the tumors following SC-58236 and irradiated tumor cell vaccine administration. Compared with $CD4^{-/-}$ mice, where all animals succumbed to tumors, 40% of the $CD8^{-/-}$ mice and 50% of the wild-type C57BL/6 mice were protected from a tumor challenge ($p < 0.01$) (Fig. 1*C*).

Ab-mediated neutralization of IFN-γ in vivo reverses the antitumor vaccination response

To determine the importance of IFN-γ in COX-2 inhibitor-mediated enhancement of the vaccination effect, IFN-γ was neutralized with anti-IFN-γ Ab in mice treated with COX-2 inhibitor plus vaccine. Neutralization of IFN-γ abrogated the COX-2 inhibitor-mediated enhancement of the vaccination effect (Fig. 2*A*) and reduced the frequency of tumor-specific T cells producing IFN-γ (Fig. 2*B*) ($p < 0.01$ between the control Ab and anti-IFN-γ-treated group).

COX-2 knockout mice have enhanced antitumor vaccination response

Because drugs that inhibit COX may have effects in addition to COX inhibition (36–38), we assessed the involvement of COX-2 expression by performing the vaccination experiments in COX-2 knockout mice. Compared with nonvaccinated wild-type and $COX\text{-}2^{+/-}$ heterozygotes, $COX\text{-}2^{-/-}$ knockout mice without vaccination had a reduced tumor growth rate (Fig. 3*A*, ▲, $p < 0.05$). The reduction in tumor growth rate was further enhanced in $COX\text{-}2^{-/-}$ knockout mice receiving a suboptimal dose of irradiated tumor cells. $COX\text{-}2^{-/-}$ mice vaccinated with a suboptimal dose of irradiated tumor cells had a significant reduction in tumor growth

Table II. *COX-2 inhibition enhances vaccine immune response to autologous 3LL and nonspecific response to B16 tumor cells*[a]

Treatment Groups	Constitutive	3LL	B16	EL4
Naive	80 ± 1	156 ± 16	160 ± 1	100 ± 2
Irradiated 3LL	80 ± 2	**680 ± 4**	**280 ± 4**	100 ± 3
SC-58236	80 ± 2	300 ± 5	100 ± 3	120 ± 2
SC-58236 + irradiated 3LL	90 ± 4	**2010 ± 15**∗	**440 ± 6**†	110 ± 4

[a] C57BL/6 mice were treated with the following: 1) one s.c. dose of 5×10^5 irradiated 3LL cells; 2) three i.p. injections of SC-58236 (0.1 mg/kg) plus one s.c. dose of 5×10^5 irradiated 3LL cells; and 3) three i.p. injections of SC-58236 (0.1 mg/kg). On day 14, splenocytes were restimulated overnight with irradiated 3LL cells, B16, or EL4 at a ratio of 10:1, and IFN-γ was quantified in the culture medium. Mice treated with SC-58236 plus irradiated 3LL cells showed an enhanced 3LL tumor-specific and -nonspecific B16 secretion of IFN-γ. Results are expressed as pg/ml/10^6 cells; ∗, $p < 0.01$ and †, $p < 0.05$ compared with irradiated 3LL alone or SC-58236 alone; $n = 8$ mice/group.

compared with vaccinated wild-type and heterozygous COX-$2^{-/+}$ mice (Fig 3*A*, ∗, $p < 0.05$). Compared with vaccinated homozygous COX-$2^{-/-}$ knockout mice, heterozygous COX-$2^{-/+}$ vaccinated mice had an intermediate reduction in tumor growth (Fig. 3*A*). Compared with wild-type and COX-$2^{+/-}$ heterozygous mice, COX-$2^{-/-}$ knockout vaccinated mice had a greater frequency of IFN-γ-producing, tumor-specific T cells in vitro (Fig. 3*B*). There were minimal responses to EL4 but a slight increase in IFN-γ in response to B16 tumors (data not shown).

Discussion

Although various immune-based approaches have been attempted for the treatment of lung cancer, none has proven to be reliably effective. In contrast, immune approaches have proven more successful in melanoma and renal cell carcinoma (39), leading to the impression that thoracic malignancies are nonimmunogenic and will not be amenable to immunological interventions. However, in groundbreaking studies, Boon and colleagues (40, 41) found that protective immunity could be generated against nonimmunogenic murine tumors. These studies suggest that a tumor's apparent lack of immunogenicity is indicative of a failure to elicit an effective host response rather than a lack of tumor Ag expression (42). Accordingly, a new paradigm emerged that focused on generating antitumor responses by therapeutic vaccination (43, 44). In this setting, vaccination refers to an intervention that unmasks tumor Ags, leading to the generation of specific host-immune responses against the tumor.

Although tumor Ags have been identified, several questions remain to be addressed before effective immune-based therapies can be realized. The active immune suppression induced by the tumor is a problem of paramount importance, which has been well documented in lung cancer and other malignancies (45). Tumor-reactive T cells have been shown to accumulate in lung cancer tissues but fail to respond because of suppressive tumor cell-derived factors (3, 4). Moreover, tumor cells may also direct surrounding inflammatory cells to release suppressive cytokines in the tumor milieu (5, 6). We previously found that tumor COX-2 expression regulated host expression of ILs 10 and 12 in NSCLC in a PGE_2-dependent manner (10).

Based on our previous findings and reports in the literature (5–7, 10, 28), we hypothesized that COX-2 inhibition would skew the host response toward the type 1 cytokine phenotype. To test this, we evaluated splenic cytokine profiles that accompany COX-2 inhibition in naive, non-tumor-bearing mice. Compared with diluent controls, splenocytes from COX-2 inhibitor-treated mice had an enhanced capacity to secrete IFN-γ and IP-10 after anti-CD3 stimulation and IL-12 following anti-CD40 stimulation. Based on this observation, we postulated that COX-2 inhibition would augment vaccination efficacy and heighten the specific immune response to tumor challenge. Effective vaccination in this model requires immunization with 10^6 irradiated 3LL cells at weekly intervals for 3

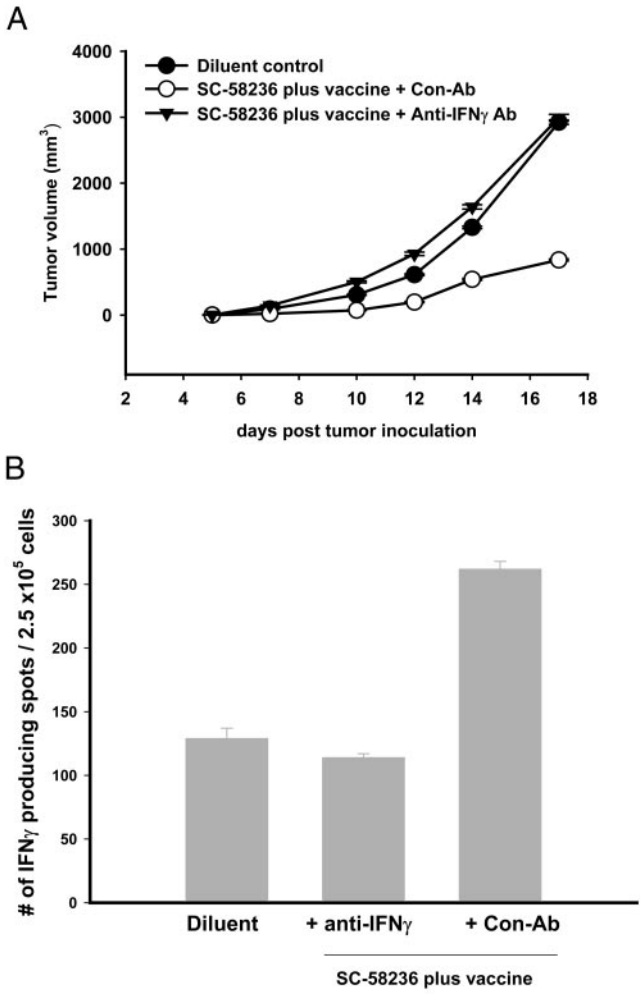

FIGURE 2. *A* and *B*, IFN-γ plays a critical role in the COX-2 inhibitor-mediated enhancement of vaccination. Anti-IFN-γ Ab reverses the vaccination and specific response to autologous tumors. C57BL/6 mice received saline diluent control or three injections of SC-58236 (0.1 mg/kg) i.p. plus one s.c. dose of 5×10^5 irradiated 3LL cells. Following vaccination, mice were treated three times each week with 100 μg/dose of purified monoclonal anti-IFN-γ or appropriate control Abs (rat IgG) by the i.p. route for the duration of the experiment. On day 14, mice were challenged with 10^5 3LL tumor cells, and the tumor volumes monitored or the splenocytes were restimulated for 18 h with irradiated 3LL cells and nonspecific syngeneic controls EL4 and B16 at a ratio of 10:1. Neutralization of IFN-γ in vivo abrogated the COX-2 inhibitor-mediated enhancement in vaccination effect (*A*) and reduced the frequency of tumor specific T cells producing and secreting IFN-γ (*B*; $p < 0.01$ between the control Ab group and the anti-IFN-γ-treated group; $n = 8$ mice/group).

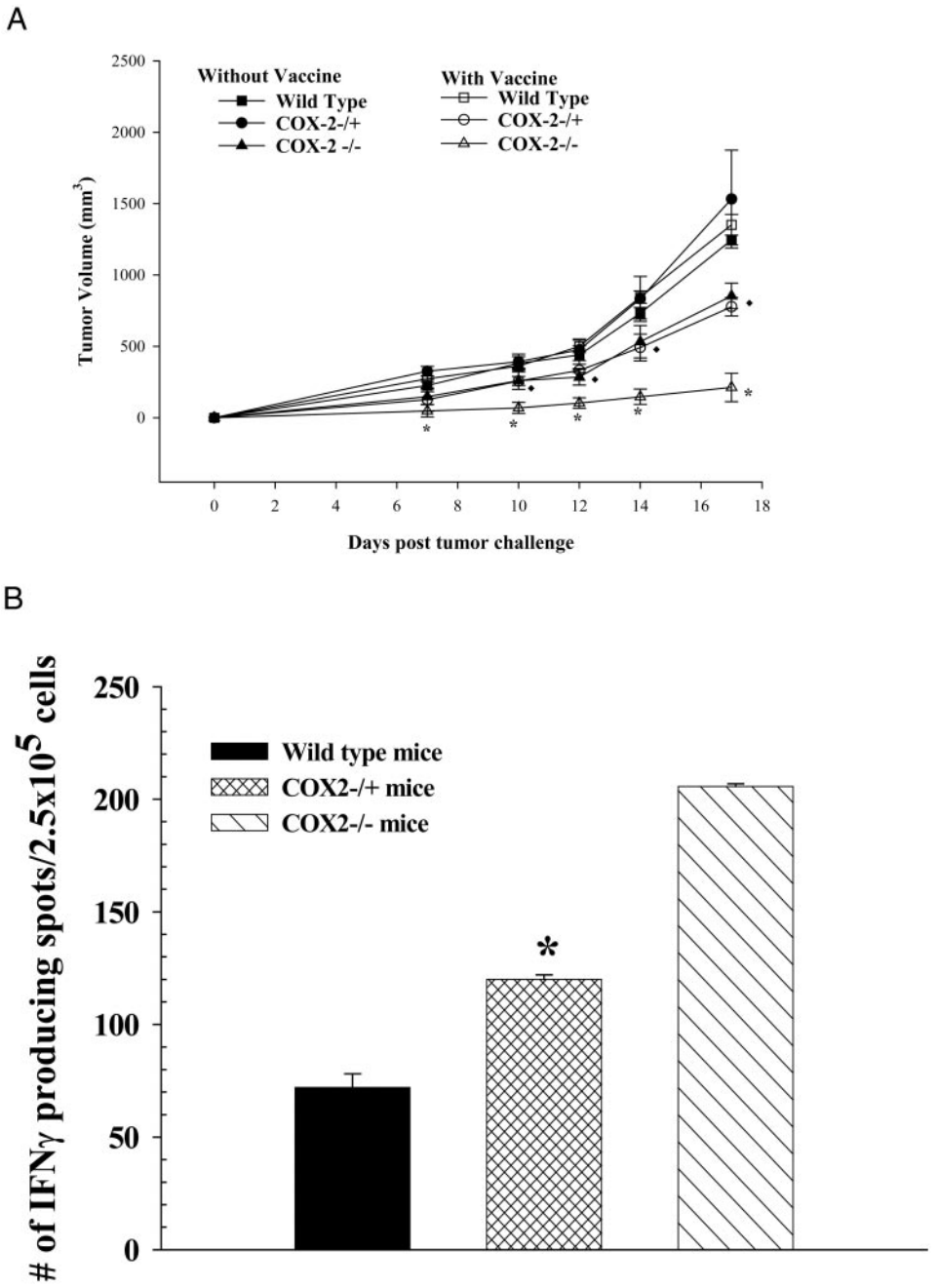

FIGURE 3. *A* and *B*, COX-2 gene knockout mice have enhanced vaccination and specific response to autologous tumors. Vaccination efficacy is enhanced in COX-2 gene knockout mice. Homozygous COX-$2^{-/-}$, heterozygous COX$^{-/+}$, and the control littermates were vaccinated once with 5×10^5 irradiated 3LL cells. On day 14, mice were challenged with 2.5×10^5 3 LL cells, or splenocytes were restimulated overnight with irradiated 3LL cells and nonspecific syngeneic controls EL4 and B16 at a ratio of 10:1. Compared with nonvaccinated wild-type and COX-$2^{+/-}$ heterozygotes, COX-$2^{-/-}$ knockout mice without vaccination had a reduced tumor growth rate (▲, $p < 0.05$). The reduction in tumor growth rate was further enhanced in COX-$2^{-/-}$ knockout mice receiving a suboptimal dose of irradiated tumor cells. COX-2 knockout mice vaccinated with a suboptimal dose of irradiated tumor cells had a significant reduction in tumor growth compared with vaccinated wild-type and heterozygous COX-$2^{-/+}$ mice (∗, $p < 0.05$). Compared with homozygous COX-$2^{-/-}$ knockout mice, heterozygous COX-$2^{-/+}$ vaccinated mice had an intermediate reduction in tumor growth (*A*). Compared with wild-type and COX-$2^{+/-}$ heterozygous mice, COX-$2^{-/-}$ knockout vaccinated mice had a greater frequency of IFN-γ-producing T cells in response to 3LL tumors in vitro (*B*). There were minimal responses to EL4 but a slight increase in IFN-γ in response to B16 tumors (data not shown); $n = 8$ mice/group, $p < 0.01$ between the COX-$2^{-/-}$, COX-$2^{-/+}$, and wild-type mice.

wk that protects 100% of mice on tumor challenge. The doses of the COX-2 inhibitor and irradiated tumor cells were chosen based on our preliminary studies that showed a partial antitumor response with reduced tumor growth rates but without complete eradication of the tumors following administration of these agents alone. The response to a single suboptimal dose of irradiated tumor cell vaccination (5×10^5) was enhanced in mice that received SC-58236 with four of eight mice completely rejecting the tumor challenge dose of 10^5 cells and a significant reduction in tumor growth in the animals that did form tumors on challenge. Studies performed in CD4$^{-/-}$ and CD8$^{-/-}$ mice revealed a requirement for the CD4 T lymphocyte subset for the complete rejection of the tumors following SC-58236 and irradiated tumor cell vaccine administration. Compared with CD4$^{-/-}$ mice, where all animals succumbed to tumors, 40% of the CD8$^{-/-}$ mice and 50% of the wild-type C57BL/6 mice were protected from a tumor challenge.

Our data are consistent with the studies of Ribas et al. (46), who showed antitumor responses to DC vaccines in the absence of CD8 T cells. Administration of the COX-2 inhibitor plus the irradiated cell vaccine in the CD8$^{-/-}$ mice induces a protective response to 3LL, which suggests that the CD8$^{-/-}$ mice have developed a mechanism that bypasses the need for CD8 + CTLs. The tumor protection in CD8$^{-/-}$ mice in our model could be due to CD4 T cells enhancing the lytic activity of other effectors such as those with a NK and NK1.1 phenotype. Future studies will be necessary to address the role of these cells in COX-2 inhibitor-mediated enhancement of vaccination responses.

The response to tumor challenge in mice that received SC58236 plus tumor cell vaccine was comparable to mice immunized with mut1mut2 peptide-pulsed DC (10^6). This optimal specific peptide Ag immunization was chosen as a comparative positive control because it has been shown previously by us and others to induce

reliable specific protection (30, 31, 47). In contrast, all mice receiving either tumor cell vaccine alone or SC-58236 alone showed progressive tumor growth. Experiments were also performed with tumor challenge doses of 5×10^5 and 1×10^6 tumor cells. At both these tumor challenge doses, groups of mice that had received COX-2 inhibitor and irradiated tumor cell vaccine had the most significant reduction in tumor growth rate compared with mice receiving the COX-2 inhibitor alone or irradiated tumor cells alone (data not shown). It is possible that if the COX-2 inhibitor was administered for the duration of the experiment or the number of irradiated tumor cells increased a greater percentage of mice would be protected from a tumor challenge.

In ELISPOT assays, at constitutive levels without stimulation with 3LL cells, no difference was noted in the frequency of IFN-γ-producing cells among the treatment groups. However, compared with mice receiving irradiated 3LL cells or SC-58236 alone, mice treated with SC-58236 plus tumor cell vaccine had a greater frequency of splenic T cells producing IFN-γ in response to restimulation in vitro with irradiated 3LL cells. Consistent with the ELISPOT data, mice treated with SC-58236 combined with tumor cell vaccine also showed an enhanced 3LL tumor-specific and B16-nonspecific release of IFN-γ. A significant increase was also demonstrated in response to the nonspecific B16 that was not as great as that demonstrated in response to the autologous tumor. The enhanced response to B16 tumors could be due to responses to differences in minor determinants between 3LL and B16 that may have been enhanced following vaccination. Alternatively, the increase in response to B16 could represent a response to tumor Ags shared with 3LL that have not yet been identified. This would imply that treatment with SC-58236 plus tumor cell vaccine was responsible for generating a response to multiple antigenic epitopes in situ, some of which may be shared among tumors. In previous studies (48), we have found that splenic T lymphocytes from mice vaccinated with DC pulsed with MUT 1 and MUT2 peptide Ags elevated secretion of IFN-γ in response to B16.

Because COX-2 inhibition potently induced IFN-γ, we evaluated the contribution of IFN-γ to antitumor responses in vivo following vaccination. IFN-γ has been documented to be critical for antitumor responses in immune therapies and immunosurveillance (49, 50). During adaptive immune responses, $CD4^+$ and $CD8^+$ T cells are the major source of IFN-γ, the production of which results from Ag-TCR stimulation. An alternative TCR-independent IFN-γ production pathway has also been defined involving IL-12 and IL-18 (51–53). It has been suggested that the type of immune effector function that is induced against a specific target is heavily dependent upon the type and cytokine profile of $CD4^+$ cells that develop (53). IFN-γ has been noted to have dual activities in the development of the Th1 response. First, it enhances both APC IL-12 production and T cell responsiveness in $CD4^+$ cell development (54, 55). Second, IFN-γ blocks the development of the Th2 subset by both inhibiting synthesis of IL-4 and directly inhibiting Th2 cell proliferation. Neutralization of IFN-γ in vivo abrogated the COX-2 inhibitor-mediated enhancement in vaccination effect and reduced the frequency of tumor-specific T cells producing IFN-γ.

Because drugs that inhibit COX may have effects in addition to COX inhibition (36–38), we assessed the involvement of COX-2 expression by performing the vaccination experiments in COX-2 knockout mice. COX-$2^{-/-}$ mice vaccinated with a suboptimal dose of irradiated tumor cells had a significant reduction in tumor growth as well as a greater frequency of IFN-γ-producing, tumor-specific T cells in vitro compared with vaccinated wild-type and heterozygous COX-$2^{-/+}$ mice. Our studies show that COX-2 expression may play an important role in modulating the generation and/or maintenance of the immune response to tumor Ags. Our findings suggest that the combination of COX inhibition with vaccination strategies can serve to enhance the generation of antitumor immunity.

Disclosures

The authors have no financial conflict of interest.

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