Combination of a Poxvirus-Based Vaccine with a Cyclooxygenase-2 Inhibitor (Celecoxib) Elicits Antitumor Immunity and Long-Term Survival in CEA.Tg/MIN Mice

Hasan E. Zeytin, Arti C. Patel, Connie J. Rogers, Daniel Canter, Stephen D. Hursting, Jeffrey Schlom, and John W. Greiner

1Laboratories of Tumor Immunology and Biology, and 2Biosystems and Cancer, Center for Cancer Research, and 3Cancer Prevention Fellowship Program, Division of Cancer Prevention, National Cancer Institute, NIH, Bethesda, Maryland

ABSTRACT

The present study was designed to determine whether: (a) chronic administration of dietary celecoxib (Celebrex), a potent nonsteroidal anti-inflammatory drug, which targets the cyclooxygenase-2 (COX-2) enzyme, negatively impacts host immunity; and (b) celecoxib can be coupled with a poxvirus-based vaccine to impact tumor burden in a murine tumor model of spontaneous adenomatous polyposis coli. Naïve mice fed the celecoxib-supplemented diets developed eosinophilia with lowered plasma prostaglandin E2 levels and reduced COX-2 mRNA expression levels in their splenic T cells. Responses of splenic T, B, and natural killer cells to broad-based and antigen-specific stimuli were, for the most part, unchanged in those mice as well as COX-2 knockout mice; exceptions included: (a) reduced IFN-γ production by concanavalin A- or antigen-stimulated T cells; and (b) heightened lipopolysaccharide response of naïve B cells from mice fed a diet supplemented with 1000 ppm of celecoxib. When transgenic mice that express the human carcinoembryonic antigen (CEA) gene (CEA transgenic) were bred with mice bearing a mutation in the ApcΔ716 gene (multiple intestinal neoplasia mice), the progeny (CEA transgenic/multiple intestinal neoplasia) spontaneously develop multiple intestinal neoplasms that overexpress CEA and COX-2. Beginning at 30 days of age, the administration of a diversified prime/boost recombinant CEA-poxvirus-based vaccine regimen or celecoxib (1000 ppm)-supplemented diet reduced the number of intestinal neoplasms by 54% and 65%, respectively. Combining the CEA-based vaccine with the celecoxib-supplemented diet reduced tumor burden by 95% and significantly improved overall long-term survival. Both tumor reduction and improved overall survival were achieved without any evidence of autoimmune directed at CEA-expressing or other normal tissues. Celecoxib is prescribed for the treatment of familial adenomatous polyposis in humans, and the CEA-based vaccines have been well tolerated and capable of eliciting anti-CEA host immune responses in early clinical studies. The results suggest that the administration of a recombinant poxvirus-based vaccine is compatible with celecoxib, and this combined chemoinmunobased approach might lead to an additive therapeutic antitumor benefit not only in patients diagnosed with familial adenomatous polyposis but, perhaps, in other preventive settings in which COX-2 overexpression is associated with progression from premalignancy to neoplasia.

INTRODUCTION

Both chemotherapeutic and immunological-based approaches have been independently explored as potential strategies for the intervention of colorectal cancer. One chemotherapeutic/prevention approach that has proven successful in both experimental models and the treatment of familial adenomatous polyposis (FAP) in humans is the selective targeting of the cyclooxygenase (COX) pathway. Two COX isoenzymes have been identified: COX-1 is expressed in most tissues and necessary for healthy mucosa, kidneys, and platelets (1), and COX-2, which is virtually undetectable in most tissues, but induced in response to inflammation, cytokines, growth factors, and other stimuli (2, 3). Both COX isoenzymes convert arachidonic acid to prostaglandin H2, which serves as the substrate for a number of prostaglandin synthetases. Additionally, COX overexpression has been linked with resistance to apoptosis and tumor growth promotion (4). A regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, and sulindac, inhibits both COX enzymes with an associated reduction of cancer risk (5–9). Subsequent findings reported that prolonged suppression of COX-1 activity caused unwanted side effects (10), which underscored the need to develop selective COX-2 inhibitors. COX-2 mRNA and protein are overexpressed in neoplastic epithelial cells (11–13), and COX-2 interruption by pharmacological agents or gene knockout reduced tumor development in a variety of experimental murine models (14–16). Celecoxib, one of the most studied COX-2 inhibitors, reduced the multiplicity and size of intestinal tumors (primarily adenomas) in the multiple intestinal neoplasia (MIN) mouse model (17). In a subsequent clinical study, oral celecoxib (Celebrex) administration reduced the number of colorectal polyps in patients with FAP significantly (18).

A separate investigational path has involved the generation and evaluation of recombinant cancer vaccines directed against carcinoembryonic antigen (CEA), a M, 180,000–200,000 glycoprotein expressed by normal human colonic mucosa. CEA overexpression by a high percentage of human colorectal cancer, colonic polyps (19), and other adenocarcinomas (20, 21) provided an opportunity for the immune system to generate anti-CEA host immune responses and thereby served as the rationale to develop CEA-based vaccines. Using preclinical mouse models expressing the complete human CEA gene as a transgene (22, 23), several different CEA-directed vaccines were reported to overcome CEA immune tolerance by inducing anti-CEA-specific immunity which, in turn, correlated with the regression of CEA-expressing tumors (24–29). Subsequent clinical studies have demonstrated the generation of anti-CEA host T-cell immune responses after vaccination with recombinant poxviruses expressing CEA, three costimulatory molecules (B7.1, ICAM-1, and LFA-3, designated TRICOM), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Refs. 30–32). Phase I clinical trials have shown evidence of some reductions in serum levels of tumor markers and antitumor responses, as well as prolonged survival after vaccination. Studies are under way to determine whether those CEA-specific immune responses are indeed associated with the improved clinical outcome of vaccinated patients.

The present study was designed to investigate whether COX-2 and CEA can be simultaneously targeted in a combined chemoinmunobased approach to cancer prevention and/or therapy. Such an approach, perhaps, might lead one to argue that combining a potent anti-inflammatory agent, such as celecoxib, with a proinflammatory vaccine might be counterproductive. Conflicting data exist as to whether a reduction of COX-2 levels in T cells is associated with functional changes (33, 34). To address those concerns, initial studies were undertaken to determine whether chronic exposure to celecoxib,
a strong COX-2 inhibitor, might alter innate and/or adaptive host immune responses. Next, celecoxib and the CEA-based vaccine were combined in the CEA transgenic (CEA.Tg)/MIN mouse model in which mice spontaneously develop numerous intestinal tumors that overexpress CEA and COX-2. Results clearly show that host immunity remains, for the most part, unchanged in mice fed celecoxib-supplemented diets, and treatment of CEA.Tg/MIN mice with celecoxib combined with a CEA-based vaccine significantly reduced tumor multiplicity and prolonged survival. The findings demonstrate that CEA and COX-2 can be simultaneously targeted with a cancer vaccine and a COX-2 inhibitor in a combined chemoimmuno-based approach to cancer prevention/treatment.

MATERIALS AND METHODS

Mice and Celecoxib-Supplemented Diets. Six- to eight-week-old female C57BL/6 (B6; H-2b) mice were purchased from Taconic Farms (Germantown, NY). Mice expressing the gene for human CEA (CEA.Tg, Line 2682, C57BL/6 (H-2b), heterozygous) were obtained from John Thompson (University of Freiburg, Freiburg, Germany). MIN (C57BL/6/J-ApcMIN+/–) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and both colonies were continuously back-crossed with C57BL/6 mice. CEA.Tg/MIN mice were derived and screened for CEA and MIN expression as reported previously (29). Briefly, all CEA.Tg/MIN mice were derived by breeding female CEA.Tg mice with male MIN mice. Fecal and blood samples were taken from the CEA.Tg x MIN F1 offspring at weaning, and the presence of the CEA transgene and the Apc mutation identified by fecal CEA protein levels was detected using a solid-phase, double-determinant, anti-CEA ELISA kit (AMD1, Inc., Tusint, CA), and allele-specific PCR analysis of DNA isolated from the blood, respectively. All of the genotypes were rechecked at the completion of the study. COX-2 +/+ and COX-2 knockout (KO; B6;129P2-Ptgs2–/–) Ref. 35) mice were generously provided by Dr. Robert Langenbach (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Animal care was in compliance with recommendations of the Guide for Care and Use of Laboratory Animals, National Research Council.

Mice received Purina Certified Rodent Chow #5002 supplemented with 500, 1000, or 1500 ppm of celecoxib (Research Diets, Inc., New Brunswick, NJ) beginning at weaning.

Vaccines, Adjuvants, and Injection Schema. Vaccines used were: (a) β-galactosidase (β-gal) protein (100 μg; Prozyme, Inc., San Leandro, CA) emulsified in incomplete Freund’s adjuvant; and (b) recombinant poxviruses (vaccinia (vR) and fowlpox (fxR)) engineered to express the genes encoding bacterial LacZ or human CEA, and three murine costimulatory molecules, B7.1, ICAM-1, and TRICOM (designated rV- , rF-LacZ–, rF- or rF-CEA-TRICOM). Control vaccines contained the three costimulatory molecules alone and were designated either rV- or rF-TRICOM. Details of the construction and production of the recombinant vaccinia vaccinia (36) and avipox (fowlpox) viruses (37) have been published. A description of the construction of the recombinant avipox (fowlpox) virus expressing murine GM-CSF (rF-GM-CSF) has been reported (25).

Fluorescence-Activated Cell Sorter Analyses. Splenocytes were prepared and analyzed using either single or double staining consisting of a FITC-conjugated antibody alone or combined with a phycocerythrin-conjugated antibody (25). FITC-conjugated antibodies used were antiguinea pig Con A (clone 145–2C11), antimouse CD4 (clone RM4–5), and antiguinea pig NK1.1 (clone PK136). Phycoerythrin-labeled antibodies used were antimouse CD19 (clone 1D3), antimouse CD8a (clone 53–6.7), antimouse Ly-6G (Gr-1; clone RB6–8C5), and antimouse CD25 (clone 3C7; Pharmingen, Inc., San Diego, CA). All of the samples also contained 1 μg of the unlabeled 2.2G2 antibody (CD45) to block Fc receptors. Data were gathered from 10,000 cells using a live gate.

Lymphoproliferation. Mouse splenocytes were enriched for either B or T cells by magnetic murine pan T (Thy1.1) or B (B220) Dynabeads (Dynal, A.S., Oslo, Norway) from spleens isolated from mice fed either a control diet or a diet supplemented with 1500 ppm celecoxib. Five million T cells were incubated in complete medium in the presence or absence of 2 μg/ml concanavalin A (Con A) for 24 h. Total RNA was isolated from those cells using the RNAeasy RNA isolation kit (Qiagen, Inc., Valencia, CA). cDNA synthesis and COX-2-specific PCR amplification was performed by using Clontech Titanium One Tube reverse transcription-PCR (Clontech, Palo Alto, CA) with specific COX-2 primers. Briefly, 50 ng of RNA was reverse transcribed and amplified into COX-2-specific 500 bp PCR product by using the sense primer 5′-GGA ACA TGG ACT CAC TCA 3′ and the antisense primer 5′-TAG GCT GTG GAT CTT GCA 3′. A 540-bp murine β-actin-specific PCR product was used as control and amplified by using a sense primer 5′-GGG GCC CGC TCT AGG CAA CCA 3′ and an antisense primer 5′-CCT TTT GAT GTC ACG CAC GAC TAT TCC 3′. DNA amplification was performed using a Perkin-Elmer Gene-AMP PCR System 9600 thermal cycler (Perkin-Elmer, Inc., Boston, MA). The amplification protocol consisted of the following denaturation, annealing, and elongation cycles: 50°C for 1 h, 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 57°C for 30 s, 68°C for 1 min, and 68°C for 2 min. Products were separated by gel electrophoresis on a 1.5–2.0% agarose gel, visualized under UV light using ethidium bromide, and quantified with normalization for β-actin expression using a Kodak 3.0 documentation and analysis system.

Serum Anti-β-gal and CEA Antibody Titers. β-gal and CEA antibodies were measured by ELISA. Microtiter plates were sensitized overnight at 4°C with 100 ng/well β-gal (Celestogen, Fallbrook, CA), or ovalbumin (Sigma Chemicals, St. Louis, MO). Wells were blocked with DPBS containing 5% BSA, followed by a 1-h incubation of diluted mouse serum (1:10 to 1:31,250). Antibodies bound to the wells were detected with horse-radish peroxidase-conjugated goat antimouse IgG (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD) using an ELISA microplate autoreader at A490 μM. Positive controls for β-gal and CEA were a commercially available mouse anti-human β-galactosidase monoclonal antibody [IgG2a(κ), Promega Corp., Madison, WI] and a murine IgG2a anti-CEA monoclonal antibody, COL-1 (38). Antibody titers were determined as the reciprocal of the serum dilution that results in an A490 nm of 1.0.
Cytokine Production Assays. T cells were isolated from naive B6, COX +/+ , COX KO mice, or B6 mice vaccinated with either β-gal or recombinant poxviruses expressing LacZ or CEA as described previously (25). T cells were incubated in flat-bottomed, 96-well plates in the presence of 5 × 10^5 irradiated syngeneic splenocytes and 10, 1, or 0.1 μg/ml of CEA<sub>26-333</sub> (EAAQNTYLL; D<sup>b</sup>-epitope; Ref. 39), β-gal<sub>10-103</sub> (DAPITYTNV; K<sup>b</sup>-epitope; Ref. 40), or a control peptide, Flu H3N2 influenza A virus nucleoprotein epitope (NP<sub>366-374</sub>) which is also a H<sup>2D<sub>b</sub></sup>-epitope Flu NP<sub>366-374</sub>; Ref. 41). Supernatants were harvested 48 h later, and IFN-γ levels measured using an ELISA assay (Endogen, Inc., Cambridge, MA).

Tumor Scoring, Histopathology, and Immunohistochemical Staining. CEA.Tg/MIN mice were sacrificed by CO<sub>2</sub> inhalation and the entire gastrointestinal tract was removed. The intestine and colon were isolated and analyzed for any macroscopic and/or cellular phenotypic indicators of chronic NSAID administration, eosinophilia (<i>0.2</i> eosinophils/10<sup>3</sup> cells/μl) or total splenocyte number (<i>72</i> million). Furthermore, mice from each diet group were sacrificed, and their spleens were isolated and analyzed for any macroscopic and/or cellular phenotypic indicators of chronic NSAID administration, eosinophilia (<i>0.2</i> eosinophils/10<sup>3</sup> cells/μl) or total splenocyte number (<i>72</i> million).

RESULTS

Celecoxib-Supplemented Diets. At weaning (21–28 days of age), groups of B6 mice were placed on a standard mouse chow diet (control diet) or that same chow supplemented with 500, 1000, or 1500 ppm celecoxib, and their general health status was closely monitored. Two <i>in vitro</i> indicators of chronic NSAID administration, eosinophilia (>0.24 eosinophils/10<sup>6</sup> cells/μl; Ref. 44), and a drop in plasma PGE<sub>2</sub> levels (45), appeared in mice fed the celecoxib-supplemented diets. After >2 months, eosinophilia was present in mice fed diets supplemented with 1000 and 1500 ppm celecoxib. Plasma levels of bicyclo-PGE<sub>2</sub>, a stable metabolite of PGE<sub>2</sub>, were reduced by 11.0%, 54.3%, and 63.9% in mice fed the 500, 1000, or 1500 ppm celecoxib-supplemented diets, respectively (normal plasma bicyclo-PGE<sub>2</sub> levels = ~1.0 ng/ml). Despite those changes, individual weight gain and complete blood count/differential counts were similar for mice in each of the four diet groups (data not shown).

Mice from each diet group were sacrificed, and their spleens were isolated and analyzed for any macroscopic and/or cellular phenotypic changes associated with the celecoxib-supplemented diets. No significant differences were observed for either individual spleen weights (72–77 mg) or total splenocyte number (~100 million). Furthermore,

![Table 1](image)

<table>
<thead>
<tr>
<th>Splenocyte Type</th>
<th>Parameter</th>
<th>Stimuli</th>
<th>Concentration</th>
<th>Control diet</th>
<th>Celecoxib-supplemented diets (+ 500 ppm)</th>
<th>Celecoxib-supplemented diets (+ 1000 ppm)</th>
<th>Celecoxib-supplemented diets (+ 1500 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>Proliferation (cpm/72 h)</td>
<td>Con A</td>
<td>0.5 μg/ml</td>
<td>10151 ± 11372</td>
<td>213104 ± 21575</td>
<td>206773 ± 17573</td>
<td>194860 ± 14752</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-CD3</td>
<td>0.25 μg/ml</td>
<td>17246 ± 9790</td>
<td>152383 ± 11904</td>
<td>149790 ± 11424</td>
<td>154431 ± 8789</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-γ production (ng/48 h)</td>
<td>Con A</td>
<td>0.5 μg/ml</td>
<td>170.0 ± 2.0</td>
<td>15.3 ± 2.2</td>
<td>16.2 ± 1.7</td>
</tr>
<tr>
<td>B cell</td>
<td>Proliferation (cpm/72 h)</td>
<td>LPS</td>
<td>1.25 μg/ml</td>
<td>12687 ± 344</td>
<td>11575 ± 1120</td>
<td>11597 ± 659</td>
<td>11410 ± 752</td>
</tr>
<tr>
<td>NK cell</td>
<td>YAC-1 lysis (%)</td>
<td>E/T</td>
<td>100:1</td>
<td>8.6 ± 1.1</td>
<td>9.8 ± 1.2</td>
<td>8.9 ± 0.4</td>
<td>8.2 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> NK, natural killer; Con A, concanavalin A; LPS, lipopolysaccharide.

<sup>b</sup> P < 0.05 (versus IFN-γ produced by Con A-stimulated T cells from mice fed the control diet).

<sup>c</sup> P < 0.05 (splenic B-cell proliferative response to LPS from mice fed the control diet and 500 or 1500 ppm celecoxib-supplemented diets).
there were no significant alterations in the percentages of CD19+ (55–60%), CD3+ (31–33%), natural killer (NK) cells (1.9–2.5%), GR-1+ granulocytes/neutrophils (9–11%), CD4/CD25+ (9–11%) splenocytes, or CD4:CD8 ratios among the groups of mice fed either the control or the celecoxib-supplemented diets. No changes were found in the number of dendritic cells (CD11c+/I-Ab+) in any of the diet groups. Several studies have reported that COX-2 is transcriptionally up-regulated after activation of isolated human T cells in vitro, which was subsequently shown to be blocked by the addition of COX-2 inhibitors (33, 46). To analyze COX-2 expression in murine T cells, splenic T cells were purified from mice fed either the control diet or the 1500 ppm celecoxib-supplemented diet and incubated for 24 h in medium alone or medium containing 2 μg Con A/ml (Fig. 1). Reverse transcription-PCR-based analyses of the total RNA from splenic T cells from mice fed the control diet revealed very low COX-2 mRNA levels in unstimulated T cells, which were increased ~9-fold by Con A stimulation (Fig. 1, bottom panel, relative units). In contrast, COX-2 mRNA levels were undetectable in either resting or Con A-stimulated T cells isolated from mice fed the 1500 ppm celecoxib-supplemented diet (Fig. 1).

**Celecoxib Effects on Innate Splenic T, B, and NK Cell Functions.** In separate groups of mice, splenic T, B, and NK cells were purified from mice fed the control or celecoxib-supplemented diets to determine whether dietary exposure to celecoxib might alter their responses to broad-based stimuli. No differences were observed with respect to splenic (a) T-cell proliferative responses to Con A or anti-CD3; or (b) NK cytolysis of YAC-1 cells among the groups of mice fed either the control or celecoxib-supplemented diets (Table 1). In contrast, splenic B cells from mice fed the 1000 ppm celecoxib-supplemented diets had significantly higher proliferative responses to lipopolysaccharide (P < 0.05) than B cells from mice fed the control diet, or diets supplemented with 500 ppm or 1500 ppm celecoxib (Table 1). Splenic T cells from mice fed the celecoxib-supplemented diets did produce lower IFN-γ levels after in vitro stimulation with 1 μg Con A/ml, but no such reduction was observed with 2 μg Con A/ml (Table 1).

**Antigen-Specific Host Immunity in Mice Fed the Celecoxib-Supplemented Diets and COX-2 KO Mice.** Mice fed the celecoxib-supplemented diets or COX-2 KO mice were vaccinated with β-gal protein using the whole β-gal protein in adjuvant or recombinant poxviruses expressing LacZ to examine whether reduction of COX-2 by dietary celecoxib or genetic ablation of the COX-2 gene alters host immune responses to different vaccine types. Strong anti-β-gal serum IgG antibody titers were found in all groups of mice vaccinated with β-gal protein in adjuvant, with significantly higher titers in mice fed the 1500 ppm celecoxib-supplemented diet (Fig. 2A; P < 0.05 versus titers from mice fed the control, 500 ppm, or 1000 ppm celecoxib-supplemented diets). For example, serum anti-β-gal IgG titers in those mice were ~31,000 [1/SD = 1.0 abs (@ A490 nm)]; 5–10-fold higher than the titers measured in mice fed either the control, or 500 ppm or 1,000 ppm celecoxib-supplemented diets. Splenic T cells from those mice were tested for their ability to generate a CD4-specific proliferative recall response to β-gal protein; similar β-gal-specific lymphoproliferative responses were found in all four groups of mice (Fig. 2B). The measurement of IFN-γ and interleukin 4 levels in splenic T-cell supernatants after in vitro stimulation with exogenous addition of β-gal protein indicated a Tγ1-like response (IFN-γ > interleukin 4). Comparison of the relative levels of IFN-γ and interleukin 4 produced by splenic T cells from mice fed the control diet, or diets supplemented with 500 ppm, 1000 ppm, or 1500 ppm celecoxib revealed no significant differences (data not shown).

Another approach to examine what role COX-2 may play in host immunity used COX-2 KO mice. Similar to mice fed the celecoxib-supplemented diets, eosinophilia was found in most COX-2 KO mice as well as a slight, but reproducible decrease (5–10%) in plasma levels of bicyclo-PGE2, levels. Other investigators have reported eosinophilia in bronchial lavages of COX-2 KO mice after airway response to an allergen (47). No differences in spleen weights, total splenocyte counts, and B, T, and NK cell counts were apparent in the COX-2 KO mice when compared with those measured in the COX-2 +/- mice (data not shown). COX-2 KO and COX-2 +/- mice were subsequently vaccinated with a bacterial LacZ gene engineered into recombinant poxviruses that expressed three murine costimulatory molecules (rVHrF-LacZ-TRICOM). Both COX-2 +/- and COX-2 KO mice developed equivalent serum anti-β-gal IgG titers (Fig. 3A).
Antitumor Responses in CEA.Tg/MIN Mice by Combining Dietary Celecoxib (1000 ppm) with a CEA-Based Vaccine. The results, thus far, indicated that the administration of an anti-inflammatory agent, such as celecoxib, with a proinflammatory recombinant poxvirus-based vaccine might be compatible in an experimental antitumor model. The CEA.Tg/MIN murine model was chosen to test that hypothesis. CEA.Tg/MIN mice were placed on either the control or celecoxib (1000 ppm)-supplemented diets beginning at 30 days of age. At that time, CEA.Tg/MIN mice also received the primary vaccination comprised of the vaccinia-based vaccine (rV-)- with (rV-CEA-TRICOM) or without (rV-TRICOM) the CEA gene. Mice were administered the appropriate monthly boosts with either of the rF-based vaccines (i.e., rF-CEA-TRICOM or rF-TRICOM). Like the MIN mice, CEA.Tg/MIN mice (29) developed adult-onset anemia accompanied by severe, progressive weight loss, overt changes that are tightly linked with intestinal tumor burden and serve as good indicators of treatment response. Average weight gain of CEA.Tg/MIN mice fed the control diet and administered either the vehicle alone or the non-CEA-based vaccine was 5–6 g/mouse, with severe anemia developing in all of the mice by 150–160 days of age (Table 2). Total number of intestinal tumors in those two groups of CEA.Tg/MIN mice was 38.8 ± 3.2 (Fig. 4A) and 37.8 ± 2.1 (Fig. 4C). As reported previously, CEA.Tg/MIN mice that received the CEA-based vaccine gained more weight (8.5 ± 0.5 g; P < 0.05 versus vehicle or non-CEA-based vaccine-treated CEA.Tg/MIN) and maintained normal hematocrit levels (Table 2); these changes correlated with a significantly lower intestinal tumor burden (21.1 ± 2.6; P < 0.05 versus vehicle or non-CEA-based vaccine-treated CEA.Tg/MIN; Fig. 4B). Adding celecoxib to the diet alone significantly increased the average weight gain with accompanying normal hematocrit levels of the CEA.Tg/MIN mice, whether or not they were vaccinated (Table 2). CEA.Tg/MIN mice on the celecoxib-supplemented diet alone had lower numbers of intestinal tumors (13.4 ± 2.0; Fig. 4D; P < 0.001 versus mice fed the control diet and administered the vehicle alone; Fig. 4A). No additional reduction in tumor multiplicity was achieved with the administration of the non-CEA-based vaccine (P = 0.78; Fig. 4F). Combining the celecoxib-supplemented diet with the administration of the CEA-based vaccine resulted in the highest average weight gain (Table 2); this also correlated with a substantial reduction in the average number of intestinal tumors to 2.1 ± 0.6 (P < 0.001 versus vehicle control-treated mice, CEA-based vaccine, and control diet and celecoxib-treated mice; Fig. 4E). Six of those 16 CEA.Tg/MIN mice receiving the combined treatment regimen remained tumor-free. Importantly, the effects of the CEA-based vaccine and celecoxib treatment appear to be additive as the linear regression coefficient of the combined treatment, i.e., CEA vaccine and celecoxib (β = −12.18), approximate the added effect of each treatment alone, i.e., CEA-based vaccine (β = −8.46) and celecoxib treatment (β = −5.93).

**Table 2** Average weight gain and hematocrit levels in CEA.Tg/MIN mice (150–160 days old) that received the CEA vaccine + celecoxib-supplemented diet

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Diet</th>
<th># mice</th>
<th>Δ body weight [%]</th>
<th>Hematocrit levels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>None</td>
<td>Control</td>
<td>10</td>
<td>5.3 ± 0.6</td>
<td>28.2 ± 3.5</td>
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<tr>
<td>CEA-based</td>
<td></td>
<td>15</td>
<td>8.5 ± 0.5</td>
<td>41.0 ± 5.4</td>
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<tr>
<td>Non-CEA-based</td>
<td></td>
<td>8</td>
<td>5.6 ± 0.4</td>
<td>29.5 ± 2.2</td>
</tr>
<tr>
<td>None</td>
<td>Celecoxib</td>
<td>14</td>
<td>8.6 ± 0.8</td>
<td>45.0 ± 2.0</td>
</tr>
<tr>
<td>CEA-based</td>
<td></td>
<td>14</td>
<td>11.6 ± 0.4</td>
<td>48.5 ± 3.5</td>
</tr>
<tr>
<td>Non-CEA-based</td>
<td></td>
<td>13</td>
<td>8.2 ± 0.4</td>
<td>46.5 ± 2.5</td>
</tr>
</tbody>
</table>

*Maximum body weight gained; mean (g) ± SE.

b P < 0.05 (versus CEA.Tg/MIN mice fed the control diet and administered the non-CEA-based vaccine or the vehicle alone).

* P < 0.05 (versus CEA.Tg/MIN mice fed the celecoxib-supplemented diet combined with the vehicle alone or the non-CEA-based vaccine).

\( \beta \)-Gal-specific lymphoproliferative responses were slightly, but not significantly, lower in the COX-2 KO mice (Fig. 3B). However, IFN-γ production, an indicator of CD8-specific T-cell activation (40), was significantly (P < 0.05) lower as measured in the supernatants from those splenic T-cell cultures from immune COX-2 KO mice when compared with COX-2 +/- mice (Fig. 3C).
Individual tumor sizes were measured for all of the CEA.Tg/MIN mice in the treatment groups and divided as small (<2 mm), medium (2–5 mm), or large (>5 mm), and the percentage of total number of intestinal tumors in those three size categories was compared for each treatment group (Fig. 5). For CEA.Tg/MIN mice fed the control diet (Fig. 5A) and administered the vehicle alone, 12.9%, 65.0%, and 22.2% of the tumors fell into the three size categories. Administration of the CEA-based vaccine slightly reduced the percentage of medium-sized (2–5 mm) tumors to 51.1%, but had no effect on the percentage of larger (>5 mm) tumors. The most dramatic reductions in tumor size were found in CEA.Tg/MIN mice fed the celecoxib-supplemented diets whether or not a vaccine was given (Fig. 5B). In both the celecoxib-supplemented diet alone, and combined with either the non-CEA or CEA-based vaccine, 61–66% of the tumors were small tumors (<2 mm; \( P < 0.001 \)) versus matched treatment groups of mice fed the control diet). Whereas the administration of the CEA-based vaccine did significantly decrease tumor multiplicity in the CEA.Tg/MIN mice (Fig. 4E), the sizes of the tumors that did develop were no smaller when compared with the other mice fed the celecoxib-supplemented diet (Fig. 5B).

CEA.Tg/MIN mice fed either the control or celecoxib-supplemented diets with or without vaccination were followed with periodic hematocrit readings (Fig. 6) and in a long-term (18 month) survival study (Fig. 7). The disease course in the CEA.Tg/MIN mice fed the control diet and treated with the vehicle alone included the onset of anemia by 90–110 days of age (Fig. 6A) with progressive weight loss that required their sacrifice by 6 months of age (Fig. 7). Administration of the vector-control, non-CEA-based vaccine had little effect on overall survival (Fig. 7). As reported previously (29), administration of the CEA-based vaccine delayed the onset of anemia (Fig. 6C) and significantly improved overall survival (\( P < 0.001 \)) versus either vehicle control or non-CEA vaccine treated mice); 55% of CEA.Tg/MIN mice were alive at 13 months (Fig. 7). The celecoxib-supplemented diet additionally delayed the onset of anemia (Fig. 6, D–F) and prolonged survival (Fig. 7) in all groups of CEA.Tg/MIN mice whether or not they received a vaccine (\( P < 0.001 \)) versus matched treatment groups of mice fed the control diet). CEA.Tg/MIN mice fed either the celecoxib-supplemented diet alone or combined with the control vaccine eventually developed anemia (Fig. 6, D and E) with progressive weight loss beginning at 11 months of age. By 18 months of age, overall survival in those two treatment groups of CEA.Tg/MIN mice was 40–60% of those mice. The most dramatic improvement in general health status (estimated by hematocrit levels) occurred in those CEA.Tg/MIN mice that were vaccinated monthly with the CEA-based vaccine and fed the celecoxib-supplemented diet. Hematocrit levels remained normal (Fig. 6F) and at 18 months of age 100% (12 of 12) of those CEA.Tg/MIN mice were alive, a significant improvement in overall survival (\( \chi^2 = 25.27; P < 0.001 \) versus CEA-based vaccine alone, and \( \chi^2 = 7.76; P = 0.0054 \) versus celecoxib diet alone).

**Anti-CEA Immunity.** Of interest was to examine whether there were any differences in CEA-specific host immunity in CEA.Tg/MIN mice vaccinated with the CEA-based vaccine and fed either the control or celecoxib-supplemented diets. Three age-matched (145–150 days of age) CEA.Tg/MIN mice from each group that had received four vaccinations (1 primary and 3 boosters), and had similar

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**Fig. 5.** Comparison of tumor sizes from CEA.Tg/MIN mice illustrated in Fig. 3. A and B show the percentages (Y axis) of total tumor number from mice fed a control diet or a celecoxib-supplemented diet (1000 ppm), respectively, and administered the vehicle alone (none), the non-CEA-based vaccine, or the CEA-based vaccine (X axis). Within each bar are shown the percentages of tumors that were <2 mm ( ), 2–5 mm ( ), and >5 mm ( ). Number of mice (n) and total number of tumors measured for each group were: Control Diet, vehicle (n = 10, 373 tumors), non-CEA-based vaccine (n = 5, 168 tumors), CEA-based vaccine (n = 13, 273 tumors); Celecoxib Diet, vehicle (n = 12, 161 tumors), non-CEA-based vaccine (n = 6, 56 tumors), CEA-based vaccine (n = 16; 36 tumors).
weight gains and hematocrit levels were analyzed for CEA-specific host immunity. Anti-CEA serum IgG titers and CD4-proliferative responses were significantly lower ($P < 0.05$) in the vaccinated CEA.Tg/MIN mice fed the control when compared with the mice fed the celecoxib-supplemented diet (Table 3). CD8-mediated, peptide-pulsed cytotoxicity was also lower in the CEA.Tg/MIN mice fed the control diet and administered the CEA-based vaccine, but the difference was not significant ($P = 0.09$). Intestinal tumor burden was significantly higher ($P < 0.001$) in the CEA.Tg/MIN mice fed the control diet and administered the CEA-based vaccine than in those mice fed the celecoxib-supplemented diet. No detectable CEA-specific immune responses were found in CEA.Tg/MIN that were fed either of the diets and vaccinated with the non-CEA-based vaccine or the vehicle control.

**Histopathology.** Tables 4 and 5 summarize the histopathological analyses of the CEA-expressing (tongue, trachea, esophagus, stomach, intestine, caecum, and colon) and CEA-negative tissues. No macroscopic or microscopic abnormalities were found in the tongue, trachea, esophagus, and stomach of 4 individual CEA.Tg/MIN mice fed the celecoxib-supplemented diet and administered 5–6 injections of the CEA-based vaccine. Where indicated, adenomas and intraepithelial neoplasms were identified in the intestine and colon. In those GI tissues free of neoplasms, histopathological analyses found normal tissues with an occasional area of gut-associated lymphoid tissue hyperplasia. Gut-associated lymphoid tissue was also noted in CEA.Tg/MIN mice that were administered either the CEA-based vaccine or celecoxib as single agents. In a single CEA.Tg/MIN mouse treated with the CEA-based vaccine and the celecoxib, and found to be tumor-free, the entire GI tract was judged to be within normal histological limits.

In most tumor-bearing mice, enlarged spleens and hepatic extramedullary hematopoiesis were evident due to increased extramedullary hematopoiesis associated with anemia (Table 5). Subacute liver inflammation, the presence of a lipogenic pigment in the adrenals, lung lymphocytic infiltrate, and melanosis of a heart valve were also noted. Because those abnormalities were found in all of the treatment groups, their appearance did not seem to be associated with the administration of either the celecoxib-supplemented diet or the CEA-based vaccine.

**DISCUSSION**

This study was designed: (a) to examine whether the chronic administration of an anti-inflammatory NSAID (celecoxib) might impair the ability of the murine-adaptive immune system to respond to...
broad-based stimuli as well as to generate host immune responses to different antigens; and (b) to evaluate whether targeting CEA and COX-2 in the CEA.Tg/MIN mouse model with a CEA-based cancer vaccine and celecoxib may be efficacious as a chemoimmuno-based approach to tumor therapy. Previous results have reported that COX-2 mRNA and protein levels in human T cells rose after activation (33). However, after the in vitro addition of celecoxib, several events associated with T-cell activation, such as CD25/CD71 expression, cytokine production, and proliferation, were reduced (33, 46), suggesting that celecoxib and other COX-selective NSAIDs may down-regulate T-cell activation resulting in immune suppression. Upon additional examination, however, it was apparent that the in vitro celecoxib levels needed to inhibit T-cell activation were supra-physiological (10–100 μM) and, in a more recent study, down-regulation of COX-2 mRNA by the in vitro addition of COX-2 antisense oligonucleotides did not impede T-cell activation (34).

In the present study, B6 mice were fed diets supplemented with 500 ppm, 1000 ppm, or 1500 ppm celecoxib, levels shown previously to reduce intestinal tumor multiplicity and size in MIN mice (17). Blood celecoxib levels in the mice fed those diets have been reported to be 0.09, 0.26, and 1.80 μg/ml, respectively (17), comparable with the 0.5–2.0 μM concentrations in patients diagnosed with FAP and treated with 200 or 400 mg celecoxib twice/day (19). Mice fed the 1000-ppm and 1500-ppm celecoxib diets developed eosinophilia, which often coincides with chronic NSAIDs administration. Reduction in plasma PGE2 levels, as measured by plasma bicyclo-PGE2 levels, was also present in all groups of mice fed the celecoxib-supplemented diets, another indication of the in vivo bioactivity of celecoxib. Thus, the present study provides a physiologically relevant setting to examine celecoxib effects on COX-2 expression in T cells and the ability of the host to mount antigen-specific immune responses during chronic celecoxib administration.
The findings in *vivo* celecoxib administration completely suppressed COX-2 mRNA levels in resting and Con A-stimulated splenic T cells are in accord with those from previous studies reporting suppression of COX-2 mRNA and protein levels after an acute *in vitro* exposure of human T cells to celecoxib (33). Moreover, the findings are also consistent with the hypothesis that COX-2 expression is regulated by a positive feedback loop, and any disruption within the pathway would be expected to suppress COX-2 expression levels (48). Despite the virtual absence of COX-2 mRNA, those splenic T cells responded normally to broad-based proliferative stimuli (*i.e.*, Con A and anti-CD3; Table 1). Interestingly, splenic B cells from mice fed the 1000-ppm celecoxib diet had a heightened (*P < 0.05*) *in vitro* response to lipopolysaccharide, which was not seen for splenic B cells from mice fed either the 500- or 1500-ppm celecoxib diets. No apparent differences in NK-mediated cytolyis were observed in mice fed the celecoxib-supplemented diets. In other experiments, B6 mice fed the celecoxib-supplemented diets as well as COX-2 KO mice responded normally in mounting host immune responses after vaccination with either a whole protein (*β*-gal in incomplete Freund’s adjuvant) or recombinant poxviruses engineered to express the LacZ gene and three costimulatory molecules (rV*-rF-LacZ-TRICOM). In fact, mice fed the 1500-ppm celecoxib diet and vaccinated with the *β*-gal protein-based vaccine generated more robust anti-β-gal serum IgG titers than mice fed the control, or 500 or 1000 ppm celecoxib diets (Fig. 2A).

At this point, the data suggested that disruption of the COX-2 isoenzyme by dietary celecoxib or by genetic ablation of the COX-2 gene resulted in some alterations in host immunity. On the one hand, IFN-γ production by T cells was reduced after (*a*) *in vitro* Con A stimulation of naive T cells from celecoxib-treated mice (Table 1); and (*b*) β-gal peptide-specific immune T cells from COX-2 KO mice vaccinated with the recombinant poxviruses (Fig. 3C). In contrast, more robust anti-β-gal serum titers were found in mice fed the 1500-ppm celecoxib-supplemented diet and vaccinated with the *β*-gal protein in adjuvant. However, those increases were not observed in COX-2 KO mice, indicating that other celecoxib-sensitive pathways, such as Akt activation (49), may play a role. Whereas the initial data suggest that dietary celecoxib may mediate a modest shift from a Th1 to a Th2 response, preliminary evidence found no significant changes in the cytokine profiles from antigen-stimulated T cells from mice vaccinated with β-gal in adjuvant. Finally, in an experimental lung tumor model, abrogation of COX-2 expression, which reduced PGE2 levels, restored the balance between Th1 and Th2 cytokines (*i.e.*, interleukin-10 and interleukin-12) resulting in normal dendritic cell function and more effective antitumor responses (50, 51). Those observations underscore the complexity of the interactions among COX enzymes, prostaglandin production, and host immunity, as well as the need to design future mechanistic studies focused on those questions.

Our immediate interests were to investigate the compatibility of the combined use of celecoxib and a tumor vaccine in an experimental mouse tumor model. The CEA.Tg/MIN spontaneous intestinal tumor model was chosen for those studies because (*a*) CEA.Tg/MIN mice develop multiple spontaneous GI tumors (MIN genotypes), which overexpress CEA (29) and COX-2 (17), and (*b*) treatment with either the CEA-based vaccine or dietary celecoxib alone was known to significantly reduce tumor multiplicity along the GI tract (17, 29). For the CEA-based vaccine to induce CEA-based antitumor host immunity, immune tolerance, due to the constitutive CEA along the GI tract, must be overcome. If the reduced IFN-γ production by Con A or β-gal peptide-stimulated splenic T cells from naive or immune mice might be linked with a diminution in antitumor immunity, that linkage might be best tested in a setting that requires the immune system to initially overcome immune tolerance against a weak, self-antigen. As reported previously (17, 29), administration of either the CEA-based vaccine or dietary celecoxib as single agents reduced the number of intestinal tumors in CEA.Tg/MIN mice by 54% and 65%, respectively. Combining the CEA-based vaccine with dietary celecoxib (1000 ppm) led to an additional reduction in intestinal tumor multiplicity, 95% reduction when compared with untreated CEA.Tg/MIN mice. In fact, 6 of 16 CEA.Tg/MIN mice administered monthly injections of the CEA-based vaccine and dietary celecoxib were tumor-free at 150–160 days of age. Another cohort of CEA.Tg/MIN mice that was administered the combined treatment regimen continued their weight gain with normal hematocrit levels resulting in a significant improvement in survival. At 18 months of age, 100% (12 of 12) of CEA.Tg/MIN mice that continued to receive monthly booster vaccinations of rF-CEA-TRICOM combined with rF-GM-CSF, as well as being maintained on the 1000-ppm celecoxib-supplemented diet, remained alive.

The antitumor mechanisms of the combined treatment of CEA.Tg/ MIN mice with the CEA-based vaccine and dietary celecoxib are currently under study. The combined treatments began at 30 days of age, when the intestinal neoplasms found in most MIN mice are aberrant crypt foci (ACFMIN), which are believed to be preneoplastic lesions that later form adenomas (52). Whether COX-2 is expressed by the ACFMIN is unknown. In ApcMIN mice, however, COX-2 expression was found in polyp stromal cells and polyps >1 mm, not in polyps <1 mm, in diameter (53). If a similar COX-2 tissue expression profile exists in the CEA.Tg/MIN mice, then the target of celecoxib seems to be the polyp stroma, not the epithelial cells. Despite the dietary supplementation of celecoxib, which reduces tumor multiplicity, intestinal tumors do emerge and are attacked by CEA-specific immune cells. That combined intervention successfully reduced tumor multiplicity by 95%. Table 3 provides a direct comparison of the relevant strengths of the CEA-specific immune response in CEA.Tg/MIN mice fed either the control or celecoxib-supplemented diet and vaccinated four times with the CEA-based vaccine. Interestingly, in the CEA.Tg/MIN mice fed the celecoxib-supplemented diet and administered the CEA-based vaccine, serum anti-IgG and CD4-proliferative responses were significantly higher than in those CEA immune mice fed the control diet. Whereas the increase in serum anti-CEA IgG levels agreed with previous findings, the increase in CEA-specific lymphoproliferative responses seemed to contradict previous results. One possible explanation is that the presence of intestinal tumors in CEA.Tg/MIN mice fed the control diet and vaccinated with the CEA-based vaccine might dampen CEA-specific host immunity. Tumor-associated suppression of host immunity is well established and considered one of many tumor escape mechanisms (54). In this study, no direct evidence is presented that a CEA-specific immune response generated by the CEA-based vaccines combined with the antitumor properties of celecoxib resulted in the additive reduction of tumor multiplicity in CEA.Tg/MIN mice. However, in previous studies, that same vaccine regimen reduced the growth of CEA-expressing, transplantable s.c. tumors in those same CEA.Tg mice. Furthermore, antibody-based *in vitro* depletion studies revealed a requirement for CD4 and CD8 T cells, as well as NK cells to elicit the antitumor effects of the vaccine (55). Subsequent studies will investigate the temporal relationships between CEA and COX-2 expression in the tumor tissue. In particular, the intestinal tumors that form in CEA.Tg/MIN mice after the administration of the CEA-based vaccine alone were fewer in number, but the same size as those in the unvaccinated mice. Additional study will address whether the administration of the CEA-based vaccine results in an outgrowth of intestinal tumors with altered CEA and/or class I antigen expression, which may impact overall tumor growth rate. Furthermore, studies will
investigate whether administration of the CEA-based vaccine alters COX-2 expression or other celecoxib-modulated biological actions (i.e., blocking Akt activation, antiangiogenesis, prostaglandin production, and so forth).

Those results provide another example of a CEA-based vaccination protocol that generates substantial antitumor immunity with little or no evidence of autoreactive T cells resulting in autoimmune pathology. Histological analyses of CEA-expressing normal tissues of the gastrointestinal tract in CEA.Tg/MIN mice that received 5–6 vaccinations revealed normal tissue architecture. As reported previously (29), the most prevalent pathological finding was splenic and hepatic extramedullary hematopoiesis, a compensatory response to developing anemia. The reasons(s) that the CEA-based vaccine elicits strong antitumor responses without accompanying autoimmune involvement remains an intriguing observation. Some possible explanations that offer avenues for future studies are: (a) a selective susceptibility of tumor tissue to immune attack due to a combination of the disruption of tissue architecture and CEA overexpression; and (b) a “braking” action of tolerizing antigen-presenting cells and/or regulatory T cells on autoreactive T cells (56).

Finally, the data provide a strong argument for future experimental as well as clinical efforts to combine cancer vaccines with NSAID targeting of COX expression. A recent study reported that COX-2 inhibition enhanced the efficacy of an adenovirus-based intratumoral therapy (57). The most immediate population that may benefit from a combined CEA-based vaccine with celecoxib is patients diagnosed with FAP. Celecoxib (Celebrex) is being prescribed for FAP patients based on a 30% reduction in polypl burden (19). In several early clinical studies, administration of the CEA-based vaccine has been well tolerated and able to generate anti-CEA host immune responses (30–32). One question that remains is whether CEA overexpression is present in the polyps of the FAP patients. CEA levels, as measured by quantitative analyses, were 2–6-fold higher in tubulovillous adenomas and hyperplastic polyps when compared with CEA levels in normal mucosa from healthy donors (18). A larger study is needed to not only examine CEA expression in colorectal polyps, but also to determine the tissue expression of COX-2. A high percentage of other carcinomas, including colorectal (FAP), hereditary nonpolyposis colorectal carcinoma, and sporadic, gastric, pancreas, breast (invasive and ductal carcinoma in situ), small cell lung, cervical, and head and neck (58–63) overexpress both CEA and COX-2. Experimental studies are needed to determine the breadth of combining a cancer vaccine with targeting a COX isoenzyme. Whereas the present results encourage the use of this combined treatment to inhibit the progression from premalignancy to cancer, the effectiveness of treating overt cancers with such a combined chemoimmuno-based approach requires additional research.

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