COX-2 Inhibitors Decrease Expression of PD-L1 in Colon Tumors and Increase the Influx of Type I Tumor-infiltrating Lymphocytes

Denise L. Cecil[1], Ekram A. Gad[1], Lauren R. Corulli[1], Nicholas Drovetto[1], Ronald A. Lubet[2], and Mary L. Disis[1]

ABSTRACT

Colon cancer is initiated under inflammatory conditions associated with upregulation of immune checkpoint proteins. We evaluated immune modulation induced by nonsteroidal anti-inflammatory agents used for colon cancer prevention. Both celecoxib and naproxen inhibited polyp growth in APC Min mice. Treatment of mice with either drug significantly decreased PD-L1 expression on polyps in a dose-dependent manner ($P < 0.0001$ for both). The decrease in PD-L1 was associated with an influx of $CD8^+$ T cells into polyps ($P < 0.0001$, celecoxib; $P = 0.048$, naproxen) compared with lesions from untreated animals and correlated with disease control. Naproxen is a nonselective inhibitor of both COX-1 and COX-2, and we questioned the role of the different cyclooxygenases in PD-L1 regulation. Silencing either COX-2 or COX-1 RNA in the murine colon cancer cell line MC38, reduced PD-L1 expression by 86% in COX-2–silenced cells ($P < 0.0001$) while there was little effect with COX-1 siRNA compared with control. Naproxen could inhibit the growth of MC38 *in vivo*. Naproxen-treated mice demonstrated a significant reduction in MC38 growth as compared with control ($P < 0001$). Both $Tbet^+$ CD4 and CD8 tumor-infiltrating lymphocytes (TIL) were significantly increased ($P = 0.04$ and $P = 0.038$, respectively) without a concurrent increase in $GATA3^+$ TIL ($P > 0.05$). $CD8^+$ TIL highly expressed the activation marker, CD69. Not only was PD-L1 expression decreased on tumors, but $LAG3^+CD8^+$ T cells and PD-1 and LAG3 expression on regulatory T cells was also reduced ($P = 0.008$ and $P = 0.002$, respectively). These data demonstrate COX-2 inhibitors significantly decrease PD-L1 in colonic lesions and favorably impact the phenotype of tumor-infiltrating lymphocytes to control tumor growth.

Prevention Relevance: Nonsteroidal anti-inflammatories (NSAID) are an essential component of any combination chemoprevention of colon cancer. We show NSAID treatment reduces PD-L1 expression on intestinal tumor cells. NSAID regulation of PD-L1 is dependent on COX-2 expression. These data underscore an important immunologic mechanism of action for NSAID in colon cancer prevention.

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Introduction

Colon cancer offers an ideal model for cancer chemoprevention. A variety of agents have shown some level of activity in the prevention of progression of high-risk lesions to invasive cancer in clinical and preclinical models (1). Combination chemoprevention approaches can be synergistic in the ability to prevent colon cancer growth (2, 3). We are interested in developing vaccines designed to intercept and prevent high-risk colon lesions (4). Nonsteroidal anti-inflammatory drugs (NSAID), such as celecoxib and naproxen, have already demonstrated some benefit in colorectal cancer prevention with toxicity profiles that would be amenable to combination with antigen-specific vaccines (5). COX-2 inhibitors have been shown to decrease the expression of PD-L1 on cells of the innate immune system such as neutrophils, macrophage, and myeloid-derived suppressor cells, all of which can inhibit T-cell cytolytic function (6, 7). In preparation for developing combination chemoprevention with NSAIDs and vaccines, we questioned to what extent NSAIDs, as single agents, alter the adaptive immune microenvironment in intestinal polyps and colon cancer in murine models.

Materials and Methods

Animal models

Work was performed in accordance with the University of Washington Animal Care and Use Committee guidelines in a specific pathogen free environment. Animals were purchased from Jackson Laboratory. APC Min (Strain name: C57BL/6J-ApcMin/J) male mice and AKR/J female mice were bred to produce "F1 Min." Offspring from breeder pairs were genotyped by PCR for the Min mutation using primers: Wild-Type:

[1]UW Medicine Cancer Vaccine Institute, University of Washington, Seattle, Washington. [2]NCI, Bethesda, Maryland.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Corresponding Author: Denise L. Cecil, UW Medicine Cancer Vaccine Institute, University of Washington, 850 Republican St, Seattle, WA 98109. Phone: 206-221-8134; E-mail: dcecil@uw.edu

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5′-GCCATCCCTTCACGTTAG-3′, Common: 5′-TTCCACT-TTGGCATAAGGC-3′, Mutant: 5′-TCCTGAGAAAGACA-GAAGTTA-3′ (8). Both male and female F1 Min mice were included in the study. Female C57BL/6 mice were used for MC38 (cell line derived from a spontaneous azoxymethane-induced colon cancer) implant studies.

Study design

Animals were randomized into treatment groups sequentially at 6 ± 2 weeks of age. Studies were terminated when the F1 Min mice were 7 months old and when the MC38-implanted tumor volume of the naproxen-treated group was statistically significantly less than the control for a minimum of two measurements. With 5 F1 Min mice/group, we calculated an 83% power to detect a significant pairwise difference in the number of small intestinal polyps at the two-sided alpha level of 0.05. Three F1 Min mice died before study termination and were excluded (**Fig. 1**). With 10 C57BL/6/group implanted with MC38 cells, we estimated an 80% power to detect a significant pairwise difference in tumor size at the two-sided alpha level of 0.05. *In vitro* data were generated from a minimum of three independent experiments. All data points for these experiments are included in the analysis.

NSAID administration and assessment of tumor growth

Celecoxib was provided at 30, 75, 125, or 250 ppm and naproxen at 30, 75, 150, or 400 ppm daily, mixed with meal-form, irradiated chow (PicoLab Rodent Diet 20). Samples were confirmed to have ≥94% of the intended concentration of compound by high-pressure liquid chromatography. The small intestine was cut longitudinally and tissues fixed in formalin. Tumors were counted under a Nikon SMZ645 microscope by the same operator. Data are expressed as the total number of tumors in the small intestine.

For tumor challenge, the syngeneic murine colon cancer tumor cell line, MC38 (0.5×10^6 cells; RRID:CVCL_B288; kindly provided by David Threadgill and validated by IDEXX testing) was implanted into the flank. On the same day as implant, mice were provided 400 ppm naproxen mixed with normal chow. Tumors were measured every 2 days as

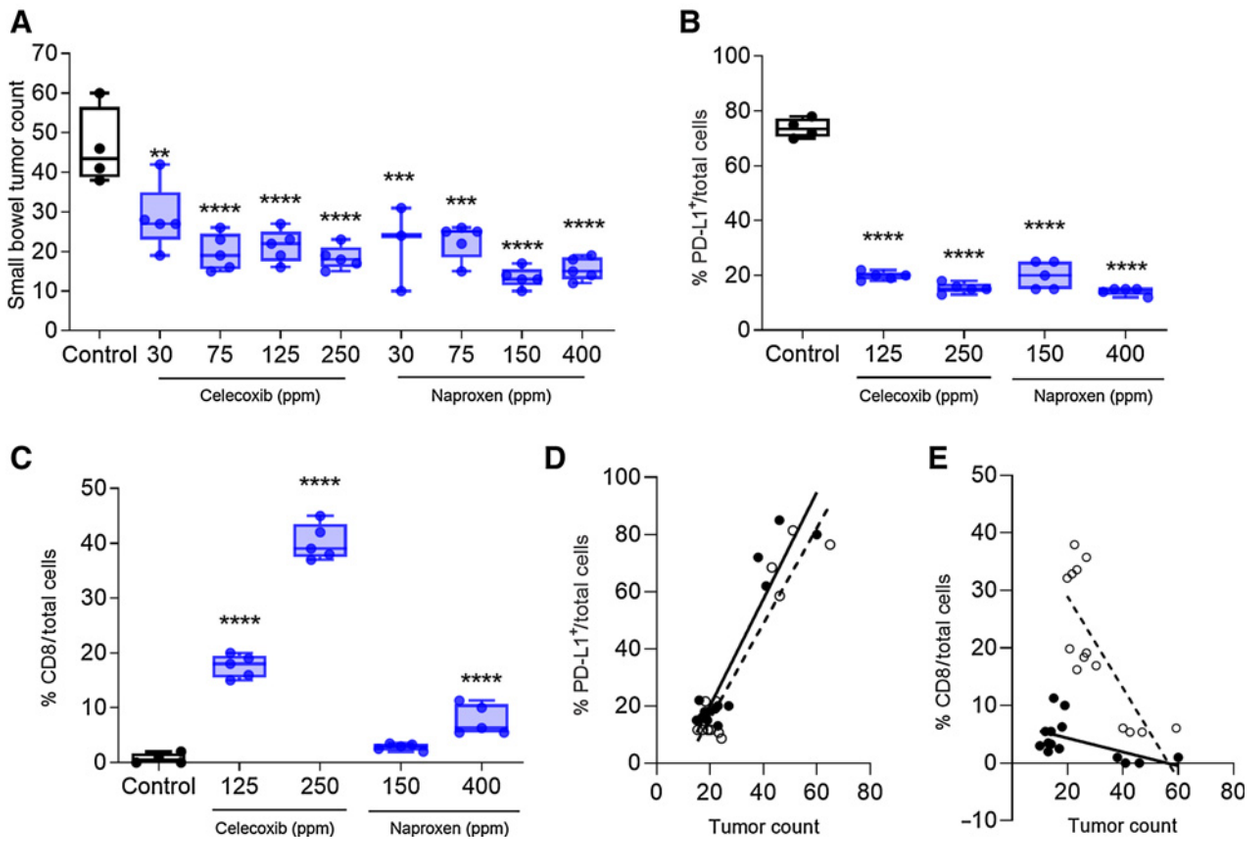

Figure 1.

Treatment with either naproxen or celecoxib significantly inhibited the development of intestinal tumors, decreased tumor PD-L1 expression, and increased infiltrating $CD8^+$ T cells. **A,** The number of small bowel tumors after treatment with the indicated dose (parts per million, ppm) of celecoxib or naproxen. Percent PD-L1 (**B**) or $CD8^+$ T cells (**C**) per total cells after treatment with normal chow (control) or chow containing the indicated dose of celecoxib or naproxen. All data are presented as box and whisker plots, horizontal line at median and whiskers minimum to maximum, showing all points. Linear regression of percent PD-L1 (**D**) or CD8 (**E**) and tumor count for mice untreated or treated with celecoxib (open circles and dotted line) or naproxen (closed circles and solid line). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. $n = 3$–5 mice/group.

previously described during and after naproxen treatment (9). Data are expressed as mean tumor volume ± SEM.

IHC

Tumors removed from the intestine were covered with Tissue-Tek O.C.T Compound in a cryomold and stored at −80°C. Frozen blocks were sectioned and fixed with 75% acetone/25% methanol for 5 minutes. The slides were washed thrice with PBS and 10% goat serum was added for 1 hour at room temperature followed by an anti-mouse PD-L1 (Abcam; clone: MIH6; 1/100 dilution) or anti-mouse CD8 (AbD Serotec; clone KT15; 1/200 dilution) overnight at 4°C. After washing, the slides were incubated with AlexaFluor 488 anti-rat IgG (Abcam; 1:1,000 dilution) for 1 hour at room temperature. Positive cells were counted in three 20× microscopic fields and expressed as the mean percent of total cells.

Cell culture and RNA silencing

MC38, the microsatellite unstable human colon adenocarcinoma cell lines, LOVO (ATCC) and SB10 (Dr. Paraskeva, Bristol, UK), and the CpG island methylator phenotype human colon carcinoma cell, RKO, (ATCC) were used. Cells were seeded at 10^6 cells/well in 6-well plates at 37°C for 24 hours, then treated with naproxen at 200, 400, or 1,000 μmol/L or celecoxib at 50 or 100 μmol/L. PD-L1 expression was examined by flow cytometry at 24, 48, and 72 hours. The maximum decrease of expression in each cell line was determined to be at the naproxen 1,000 μmol/L and celecoxib 100 μmol/L dose at 72 hours. Data are presented as percent PD-L1 expression in total viable cells. PGE_2 levels were measured in the cell culture supernatant by ELISA (R&D Systems) as per the manufacturer's directions. Results are reported as total PGE_2 levels (pg/mL; Supplementary Fig. S1).

For RNA silencing, MC38 cells were seeded at 1.5×10^5 cells/well in 6-well plates. After an overnight incubation, cells were transfected with a pool of four siRNA specific for COX1 or COX2 (Qiagen) at 10 nmol/L/siRNA using Dharmfect (GE Heathcare). Maximum gene silencing occurred at 48 hours incubation. Results are reported as percent expression as compared with mock-transfected cells (Supplementary Fig. S2).

Immunophenotyping

For tumor-infiltrating lymphocyte (TIL) isolation, tumors were cut into 10-mm pieces, incubated in the manufacturer's enzyme mix from the Tumor Dissociation kit (Miltenyi Biotec) then applied to the "37°C mouse TDK1" program on the gentleMACs dissociator. After dissociation, cells were washed with DMEM/10% FBS and resuspended in flow cytometry buffer (PBS/1% FBS/2 mmol/L EDTA), applied to a 70 μm filter, washed through with flow cytometry buffer and red cells lysed. A total of 0.5×10^6 viable cells from samples were blocked with anti-mouse CD16/32 (Mouse BD Fc Block, clone 2.4G2). Prior to surface staining, cells were stained with Fixable viability dye 450 (eBiosciences; 1:1,000) in PBS and then washed in flow cytometry buffer. Intracellular staining was performed using the FOXP3 buffer set. Antibodies were obtained from BD Biosciences or Thermo Fisher Scientific. Receptor expression was documented in the cells or TIL by incubating with anti-mouse-CD3e (BV510; clone 145-2C11), -TIGIT (BV605; clone 1G9), -Tbet (PerCP-Cy5.5; clone 4B10), -PD-L1 (PE, clone MIH5), -CD45 (PE-Cy5; clone 30-F11), -CD4 (PE-Cy5.5; clone RM4-5), -CD69 (PE-Cy7; clone H1.2F3), -FOXP3 (AlexaFluor488; clone MF14), -LAG3 (APC; clone C9B7W), -CD8a (AlexaFluor700; clone 53-6.7), -PD-1 (APC-Cy7, clone J43), -F480 (PE-CF594; clone T45-2342), -CD11b (BV711; clone M1/70), -Ki67 (AlexaFluor488; clone B56) or the appropriate isotype control for 1 hour at 2°C–8°C protected from light. Analysis was performed on FACS Canto RUO and data analyzed using FlowJo software (BD Biosciences). Typically, 2×10^5 events were recorded from the viability gate per sample. Results are reported as a percentage of total cell number or a percentage of a specific cell population.

Statistical analysis

The unpaired, two-tailed Student *t* test was used to evaluate differences between two groups. To compare more than three groups, a one-way ANOVA with Tukey *post hoc* test was used when there was one variable and a two-way ANOVA with Bonferroni *post hoc* was used when there were two variables. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software).

Data availability

The data generated in this study are available within the article and its Supplementary Data files.

Results

Treatment with either naproxen or celecoxib significantly inhibited the development of intestinal tumors, decreased tumor PD-L1 expression, and increased infiltrating $CD8^+$ T cells

Fewer small bowel tumors were observed in mice treated with 30 (mean, 28.6 ± 8.3), 75 (mean, 19.8 ± 4.6), 125 (mean, 21.4 ± 1.2) or 250 ppm (mean, 4.7 ± 0.7) celecoxib as compared with the control (mean, 46.2 ± 9.7; $P < 0.01$ for all; **Fig. 1A**). Similar results were observed when the mice were treated with naproxen. Compared with the control, fewer small bowel tumors were identified in mice treated with 30 (mean, 21.7 ± 10.6), 75 (mean, 22.6 ± 4.5), 150 (mean, 13.4 ± 1.1), or 400 ppm (mean, 15.6 ± 1.2) naproxen ($P < 0.001$ for all; **Fig. 1A**).

We analyzed tumors from the higher doses of NSAID as these concentrations would be more consistent with the human dose of drug. Tumor cell expression of PD-L1 was reduced when mice were treated with 125 or 250 ppm of celecoxib ($P < 0.0001$; **Fig. 1B**; Supplementary Fig. S3). Similarly, tumor cell expression of PD-L1 was reduced when mice were treated with 150 or 400 ppm of naproxen ($P < 0.0001$ for both doses; **Fig. 1B**; Supplementary Fig. S3). Moreover, celecoxib treatment significantly increased tumor-infiltrating $CD8^+$ T cells as compared with the control ($P < 0.0001$ for both doses; **Fig. 1C**). Naproxen

treatment also increased tumor-infiltrating $CD8^+$ T cells as compared with the control ($P < 0.0001$ for the 400 ppm dose; **Fig. 1C**). A greater number of lesions present in the small bowel was positively correlated with increased PD-L1 expression ($r = 0.928$; $P < 0.0001$ for celecoxib and $r = 0.928$; $P < 0.0001$ for naproxen; **Fig. 1D**) and negatively correlated with increased infiltrating $CD8^+$ ($r = -0.777$, $P = 0.001$ for celecoxib and $r = -0.547$, $P = 0.042$ for naproxen; **Fig. 1E**).

PD-L1 expression is decreased by COX-2 but not COX-1 inhibition

Human colon cancer cells express PD-L1 (LOVO: mean, 83 ± 2%, RKO: mean, 93 ± 4% and SB10: mean, 87 ± 2%; **Fig. 2A**). Naproxen treatment significantly decreased PD-L1 expression on LOVO cells, by 35% ($P < 0.0001$), RKO cells, by 28% ($P < 0.0001$) and SB10 cells, by 16% ($P < 0.0001$; **Fig. 2A**). MC38 cells also express PD-L1 (mean 44 ± 5%; **Fig. 2B**). Naproxen treatment significantly reduced PD-L1 expression (mean, 24 ± 5%; $P = 0.0006$) as compared to untreated cells. Celecoxib treatment similarly decreased PD-L1 expression (mean, 32 ± 3%; $P = 0.007$; **Fig. 2B**) in MC38 cells. We verified that PGE_2 levels were reduced by a mean of 85±2% in the naproxen-treated and a mean of 80 ± 7% in the celecoxib-treated cells ($P < 0.0001$ for both; Supplementary Fig. S1).

We silenced expression of COX-1 by 72% (Supplementary Fig. S2A) and COX-2 by 82% with specific siRNA (Supplementary Fig. S2B) as compared with a mock siRNA transfection ($P < 0.0001$ for both) in MC38 colon cancer cells. Silencing COX-1 did not affect PD-L1 expression (**Fig. 2C**). The percentage of cells expressing PD-L1 in the COX-1–silenced cells (mean, 87 ± 5%), was similar to that observed on the mock transfected cells ($P = 0.07$). However, PD-L1 expression was reduced by 86% in COX-2–silenced cells ($P < 0.0001$; **Fig. 2C**).

Naproxen treatment inhibited the growth of MC38 *in vivo* and increased the influx of activated $Tbet^+$ tumor-infiltrating lymphocytes

MC38 implanted tumors provided a uniform *in vivo* model to evaluate the antitumor effect of naproxen and the adaptive immune infiltrate associated with tumor control. Naproxen-treated mice demonstrated a significant reduction in tumor growth (mean, 180 ± 114 mm^3) as compared with mice fed normal chow (mean, 1,174 ± 125 mm^3; $P < 0.0001$; **Fig. 3A**). The inhibitory effect was transient, however, with disease control continuing in only 30% of mice (3/10) twenty days after cessation of NSAID treatment (Supplementary Fig. S4).

We harvested tumors from naproxen treated and control mice at the timepoint when tumor growth was significantly inhibited in the treatment group to evaluate tumor immune infiltrates elicited by naproxen treatment. While PD-L1 levels were not different between the two groups for $CD45^+CD3^-$ cells ($P = 0.70$; **Fig. 3B**), naproxen treatment significantly reduced PD-L1 expression on $CD45^-$ tumor cells compared with controls ($P = 0.009$; **Fig. 3C**). TIL derived from naproxen-treated mice demonstrated a significant increase in $Tbet^+CD4^+$ ($P = 0.04$; **Fig. 3D**) and $Tbet^+CD8^+$ T cells ($P = 0.038$; **Fig. 3E**). The $Tbet^+CD8^+$ T cells expressed the activation marker CD69 compared with controls ($P = 0.044$). There was no difference between naproxen-treated and control groups in

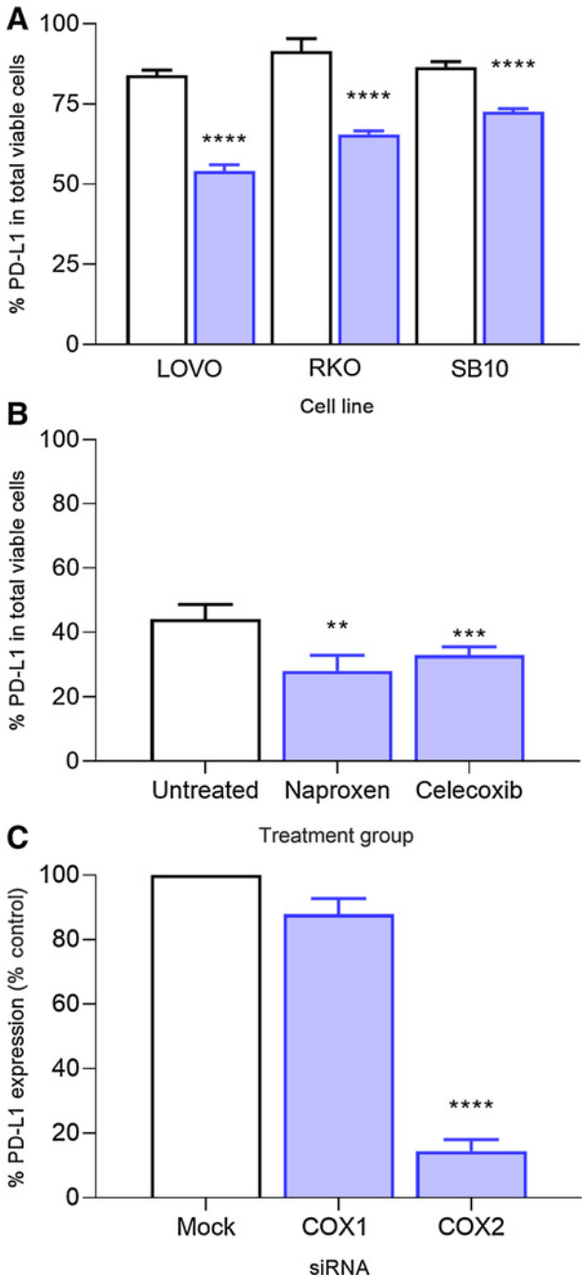

Figure 2.

PD-L1 expression is decreased by COX-2 but not COX-1 inhibition. **A,** Percent PD-L1 on viable cells on LOVO, RKO, or SB10 cells untreated (white bars) or treated with naproxen, 1,000 μmol/L (blue bars). **B,** Percent PD-L1 on viable cells in MC38 cells untreated (white bar) or treated with naproxen (1,000 μmol/L) or celecoxib (100 μmol/L; blue bars). **C,** Percent PD-L1 expression relative to the mock control transfected cells after silencing of the indicated gene in MC38 cells. **, $P < 0.01$; ****, $P < 0.0001$; $n = 3$–4 independent experiments.

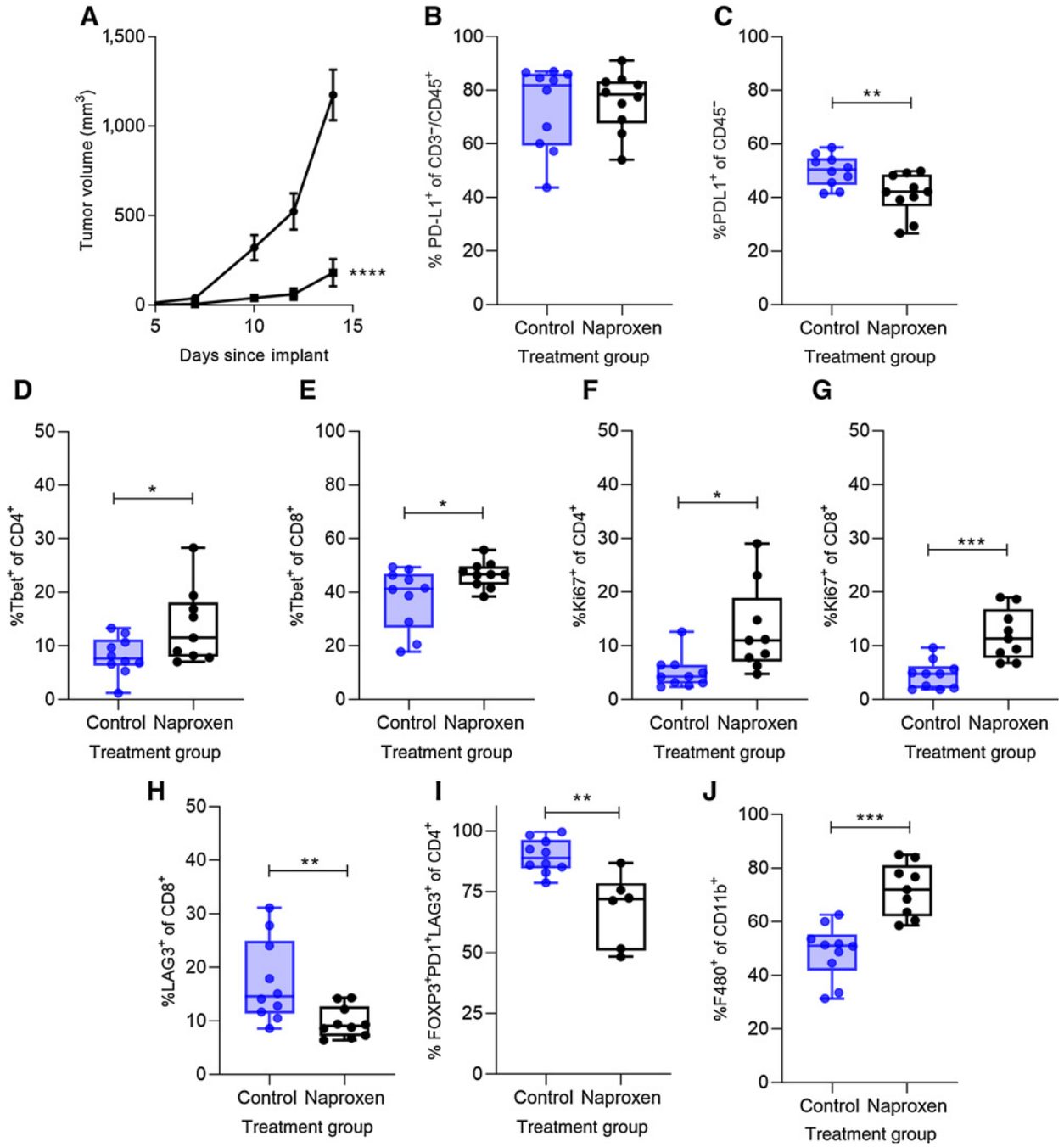

Figure 3.

Naproxen treatment inhibited the growth of MC38 *in vivo* and increased the influx of activated Tbet+ tumor infiltrating lymphocytes. **A,** Tumor volume of mice treated with control diet (●) or diet containing 400 ppm naproxen (■); $n = 10$ mice/group; ****, $P < 0.0001$. Percent of infiltrating $CD45^+CD3\text{-}PD\text{-}L1^+$ (**B**), $CD45^-PD\text{-}L1^+$ (**C**), $Tbet^+CD4^+$ (**D**), $Tbet^+CD8^+$ (**E**), $Ki67^+CD4^+$ (**F**), $Ki67^+CD8^+$ (**G**), $LAG3^+CD8^+$ (**H**), $CD4^+FOXP3^+PD\text{-}1^+LAG3^+$ (**I**), and $F480^+CD11b^+$ (**J**) for the indicated treatment group presented as box and whisker plots, horizontal line at median and whiskers minimum to maximum, showing all points. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ $n = 6$–10 mice/group.

$CD4^+GATA3^+$ ($P = 0.570$) and $CD8^+GATA3^+$ ($P = 0.816$) T cells. Naproxen treatment induced proliferation of both CD4 ($P = 0.011$; **Fig. 3F**) and CD8 T cells ($P = 0.0004$; **Fig. 3G**) in the tumor, an effect that was not observed in controls.

We observed no difference between naproxen-treated and control tumor-infiltrating T cells for PD-1 expression ($CD4^+PD\text{-}1^+$, $P = 0.434$; $CD8^+PD\text{-}1^+$, $P = 0.08$) or expression of TIGIT ($CD4^+TIGIT^+$, $P = 0.415$; $CD8^+TIGIT^+$, $P = 0.109$).

There were, however, significantly fewer $LAG3^+CD8^+$ T cells infiltrating the tumors of mice treated with naproxen as compared with control ($P = 0.008$; **Fig. 3H**). Treatment also significantly reduced the regulatory T-cell subset expressing both PD-1 and LAG3 ($P = 0.002$; **Fig. 3I**). While tumor associated macrophages were significantly increased in number after naproxen treatment ($P = 0.001$; **Fig. 3J**), PD-L1 expression on the macrophage was no different between NSAID-treated and control groups ($P = 0.839$).

Discussion

COX-2 inhibitors, such as celecoxib and naproxen, show partial prophylactic efficacy in murine intestinal tumor models (10, 11). The mechanism by which these drugs inhibit tumor development has been attributed to blocking the synthesis of prostaglandins and COX-2 expression both of which have tumor-promoting effects (12, 13). Recently, inhibition of prostaglandin E_2 or COX-2 has been shown to reduce expression of PD-L1 in innate immune cells, tumor-associated macrophage and myeloid-derived suppressor cells, indicating an immunologic mechanism of action for NSAIDs (7). The role of COX-2 in the regulation of PD-L1 on tumor cells is less well defined. Investigations of a panel of lung cancer cell lines demonstrated that both COX-2 and PD-L1 were upregulated in malignant cells, but incubation of the cells with celecoxib did not impact the level of PD-L1 expression (14). A study of COX-2 expression in a murine model of malignant glioma showed celecoxib could moderately reduce the level of PD-L1 in tumors, but the addition of an anti-PD-1 mAb was required to achieve significant inhibition of COX-2 and an antitumor effect (15). Data presented here shows that a primary effect of NSAID treatment in these intestinal tumor models may be reduction of PD-L1 expression on tumor cells rather than innate immune cells, such as macrophage. Furthermore, NSAID could modulate PD-L1 expression in both polyps as well as colon carcinomas to significant therapeutic benefit.

NSAID treatment resulted in an increased influx of type I T cells into lesions as defined by CD8 T cells and the expression of Tbet, a transcription factor required for optimal type I T-cell function. The presence of increased CD8 T cells in polyps was associated with disease control. Type II T cells, defined by $GATA3^+$ or a regulatory T-cell phenotype are associated with colon cancer initiation and progression (16). Cytokines produced by type I T cells, such as IFNγ, induce the expression of COX-2 in innate immune cells resulting in immune suppression and T-cell anergy (17). Our data suggest that NSAID treatment prevents the development of anergy as evidenced by significant levels of proliferating CD4 and CD8 T cells in the tumors and evidence of T-cell activation. Furthermore, despite the marked influx of type I T cells, there was no evidence of upregulation of PD-1 or other immune checkpoint proteins such as TIGIT. Indeed, LAG-3 an inhibitory receptor which becomes upregulated on activated T cells was also downregulated with NSAID treatment (18).

The mechanism by which COX-2 modulates PD-L1 is not fully understood, but is most likely related to NFκB expression. NFκB regulates PD-L1 gene transcription through p65 binding to the PD-L1 promoter increasing protein expression during an immune response (19). COX-2 inhibitors suppress expression of NFκB in a dose-dependent fashion (19). NSAID therapy, in particular with COX-2 inhibitors, can reverse inflammation-induced immune suppression and support an effective antitumor response. Clinical efficacy can be seen in both intestinal polyp and colon cancer models and represents an additional mechanism of action for this class of agents.

Authors' Disclosures

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Authors' Contributions

D.L. Cecil: Formal analysis, supervision, investigation, visualization, writing–original draft, project administration, writing–review and editing. **E.A. Gad:** Investigation, methodology, writing–original draft. **L.R. Corulli:** Data curation, project administration. **N. Drovetto:** Investigation. **R.A. Lubet:** Conceptualization, writing–review and editing. **M.L. Disis:** Conceptualization, formal analysis, supervision, funding acquisition, writing–original draft, writing–review and editing.

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