Deletion of cyclooxygenase 2 in mouse mammary epithelial cells delays breast cancer onset through augmentation of type 1 immune responses in tumors

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ABSTRACT

Deletion of cyclooxygenase 2 (COX-2) in mammary epithelial cells delays breast cancer onset through augmentation of type 1 immune responses. Inhibition of cyclooxygenase (COX) 2, which is associated with greater than 40% of breast cancers, decreases the risk of tumorigenesis and breast cancer recurrence. To study the role of COX-2 in breast cancer, we engineered mice that lack selectively mammary epithelial cell (MEC) COX-2 (COX-2 KO<sup>MEC</sup>). Compared with wild type (WT), MEC from COX-2 KO<sup>MEC</sup> mice expressed greater than 90% less COX-2 messenger RNA (mRNA) and protein and produced 90% less of the dominant pro-angiogenic COX-2 product, prostaglandin (PG) E<sub>2</sub>. We confirmed COX-2 as the principle source of PG E<sub>2</sub> in MEC treated with selective COX-2 and COX-1 inhibitors. Tumors were induced in mice using medroxyprogesterone acetate and 7,12-dimethylbenz[a]anthracene. Breast cancer onset was significantly delayed in COX-2 KO<sup>MEC</sup> compared with WT (P = 0.03), equivalent in the delay following systemic COX-2 inhibition with rofecoxib. Compared with WT, COX-2 KO<sup>MEC</sup> tumors showed increased mRNA for Caspase-3, Ki-67 and common markers for leukocytes (CD45) and macrophages (F4/80). Analysis of multiple markers/cytokines, namely CD86, inducible nitric oxide synthase (iNOS), interleukin-6, tumor necrosis factor α (TNFα) and Tim-3 indicated a shift toward antitumorigenic type 1 immune responses in COX-2 KO<sup>MEC</sup> tumors. Immunohistochemical analysis confirmed elevated expression of CD45, F4/80 and CD86 in COX-2 KO<sup>MEC</sup> tumors. Concurrent with a role for COX-2 in restraining M1 macrophage polarization, CD86 and TNFα expression were offset by exogenous PGE<sub>2</sub> in bone marrow-derived macrophages polarized in vitro to the M1 phenotype. Our data reveal the importance of epithelial COX-2 in tumor promotion and indicate that deletion of epithelial COX-2 may skew tumor immunity toward type 1 responses, coincident with delayed tumor development.

Introduction

Cyclooxygenase (COX)-2, the inducible COX isoenzyme, has been convincingly linked with colon, prostate, lung and breast cancer (1,2) across basic, animal and human studies. In breast tumors, increased COX-2 expression correlates with parameters of aggressiveness and poor outcome (3). Inhibition or deletion of COX-2 reduced tumor incidence in animal models of mammary tumorigenesis (4–6), whereas targeted overexpression of COX-2 to the mammary epithelium was sufficient to induce tumor formation (7). Conversely, preventive effects of non-steroidal anti-inflammatory drugs, which inhibit both the COX-1 and COX-2 isoforms (8), or COX-2 selective inhibitors (1,9) have been consistently reported. These and other studies have raised interest in targeting COX metabolic pathways, COX-2 in particular, for chemoprevention and/or therapy. Indeed, celecoxib, a selective COX-2 inhibitor, is approved for the prevention and treatment of familial adenomatous polyposis, a condition that frequently proceeds to colon cancer (10). However, clinical use of the COX-2 inhibitors in chemoprevention and/or therapy is limited because of an increased cardiovascular risk associated with this class of drugs (11,12).

COX-2 uses arachidonic acid as a substrate to catalyze the production of the prostaglandins (PGs) and thromboxane A<sub>2</sub>. PGs, a ubiquitous and well-studied product of COX-2, has proven pro-angiogenic roles in many tissues (1). Thus, PGE<sub>2</sub> enhanced intestinal adenoma growth via activation of the Ras–MAPK cascade (13), whereas cell migration was stimulated through activation of the epidermal growth factor receptor (14). PGE<sub>2</sub> also promoted survival and growth of human colon cancer cells via induction of the anti-apoptotic protein Bcl-2 (15) and the Gs–axin–β-catenin signaling axis (16). Recent studies in mice with enhanced mammary COX-2 expression established the role of PGE<sub>2</sub> in the ‘angiogenic switch’ that is necessary for cancer development, growth and invasion (17).

Primary tumor cells are surrounded and strongly influenced by stromal cells, including fibroblasts, adipocytes and immune cells, and a supportive microenvironment appears critical for the tumor to progress toward malignancy (18,19). Environmental cues, tumor-derived stimuli and contact with tumor cells direct stromal cell differentiation and function (20,21). COX-2 contributes to this epithelial-stromal interplay. For example, the pro-tumorigenic effects of fibroblasts and macrophages have been linked to COX-2 upregulation in tumor cells (22,23), whereas COX-2-derived PGE<sub>2</sub> in turn, contributed to matrix remodeling and suppressed immune surveillance (24). Tumor-associated immune responses can be generalized to type 1, in which T<sub>H</sub>1 lymphocytes and M1-polarized macrophages limit tumor progression, and type 2, in which T<sub>H</sub>2 lymphocytes and M2 macrophages favor immune escape and disease progression (25). PGE<sub>2</sub> has been reported to augment pro-tumorigenic type 2 lymphocyte and myeloid cell functions (26,27). However, the precise contribution of COX-2 to tumor-immune responses within the microenvironment remains ill-defined.

Strategies that interrupt global COX-2 function, either via inhibitors or systemic deletion of the COX-2 gene, make it difficult to decipher its role in orchestration of tumor cell’s interaction with its microenvironment. In the present study, we report generation of mice that lack COX-2 only in mammary epithelium. Selective deletion of mammary epithelial COX-2 was sufficient to delay the onset of breast cancer in mice and equivalent in benefit to systemic COX-2 inhibition. This was associated with elevated expression of type 1 inflammatory and immune cell markers/cytokines, indicating a role for epithelial COX-2 in limiting antitumorigenic immune responses in mammary tumors.

Materials and methods

Genotyping

Genomic DNA was extracted from mouse tails and used for polymerase chain reaction (PCR). Primers are listed in supplementary Materials and Methods (available at Carcinogenesis online).

Abbreviations: BMDM, bone marrow-derived macrophages; COX, cyclooxygenase; LPS, lipopolysaccharide; MEC, mammary epithelial cell; mmtv, mouse mammary tumor virus; mRNA, messenger RNA; PCR, polymerase chain reaction; PG, prostaglandin; Q-PCR, quantitative polymerase chain reaction; TAM, tumor-associated macrophages; TNF<sub>α</sub>, tumor necrosis factor α; WT, wild type.
MEC, peritoneal macrophage and bone marrow-derived macrophage isolation and culture

Mouse MEC were isolated and cultured according to the protocol from StemCell Technologies (Vancouver, BC Canada). Reagents used were from StemCell Technologies, unless otherwise stated. Cells were plated on 6- or 12-well plates (50 000 or 100 000 cells/well, respectively) in Epithel-B medium, supplemented with recombinant human epidermal growth factor, recombinant human basic fibroblast growth factor, Heparin and 5% fetal bovine serum; 24 h later, cells were transferred to serum-free Epithel-B medium, to prevent overgrowth by stromal cells. Cells, at 80% confluency, were then used to set up experiment.

For immunohistochemistry, after deparaffinization and rehydration, endogenous peroxidase was blocked with 3% hydrogen peroxide, on 5-μm thick sections. Heat-induced epitope retrieval was performed with 1 mM EDTA. Primary antibody treatments were performed for 1 h at 37°C, followed by immune complex visualization using the Polink-2 HRP Plus AEC System (Golden Bridge International, Mukilteo, WA). Additional details are given in supplementary Materials (available at Carcinogenesis online). Cells were visualized by fluorescent microscopy.

Results

Characterization of COX-2 KO MEC mice

Mouse genotypes—loxP null (COX-2+/−), heterozygous (COX-2loxP/loxP) or homozygous (COX-2loxP/loxP), and Cre+/− or negative—were determined by PCR (Figure 1A). COX-2+/− mice lacking Cre expression (Figure 1A, lane 4) were considered wild type (WT), whereas COX-2loxP/loxP mice positive for Cre+/− (Figure 1A, lane 3) were considered COX-2 KO MEC. We estimated that >95% of cells cultured from the single-cell suspensions obtained from the mammary glands of WT and COX-2 KO MEC mice expressed either a luminal epithelial cell marker CK18 or a basal/myoepithelial cell marker CK14, or both (Figure 1B) and were negative for a fibroblast marker vimentin (supplementary Figure 1, online), confirming their predominantly epithelial identity. COX-2 expression in cultured MEC appeared to be constitutive and marginally responsive to LPS induction (Figure 1C). This was in contrast to peritoneal macrophages in which COX-2 expression was strongly induced by LPS treatment (Figure 1E). In MEC from COX-2 KO MEC mice, COX-2 messenger RNA (mRNA) and protein expression were dramatically decreased under vehicle or LPS-treated conditions (Figure 1C and D, left panels). COX-1 mRNA and protein were unaltered (Figure 1C and D, right panels). Importantly, COX-2 and COX-1 expression was not different between peritoneal macrophages obtained from WT and COX-2 KO MEC mice, with or without LPS (Figure 1E). COX-2 protein in peritoneal macrophages obtained from WT and COX-2 KO MEC mice expressed either a luminal epithelial cell marker CK18 or a basal/myoepithelial cell marker CK14, or both (Figure 1B) and were negative for a fibroblast marker vimentin (supplementary Figure 1, online), confirming their predominantly epithelial identity. COX-2 expression in cultured MEC appeared to be constitutive and marginally responsive to LPS induction (Figure 1C). This was in contrast to peritoneal macrophages in which COX-2 expression was strongly induced by LPS treatment (Figure 1E). In MEC from COX-2 KO MEC mice, COX-2 messenger RNA (mRNA) and protein expression were dramatically decreased under vehicle or LPS-treated conditions (Figure 1C and D, left panels). COX-1 mRNA and protein were unaltered (Figure 1C and D, right panels). Importantly, COX-2 and COX-1 expression was not different between peritoneal macrophages obtained from WT and COX-2 KO MEC mice, with or without LPS (Figure 1E). COX-2 protein in peritoneal macrophages similarly was not affected by COX-2 deletion in MEC (Figure 1F).

These data confirmed selective loss of COX-2 in MEC.

Genomic or pharmacological inhibition of PGE2 production delays mammary tumor onset

PGE2 in conditioned media from vehicle or LPS-treated WT MEC exceeded other COX-2 products by ~100-fold (Figure 2A). Genomic deletion of COX-2 in MEC led to a 90% reduction in PGE2 production (Figure 2B), despite the presence of COX-1 (Figure 1D). Similarly, treatment with COX-2 selective inhibitor rofecoxib markedly decreased PGE2 production by untreated or LPS-treated MEC (Figure 2B). In contrast, the COX-1 selective inhibitor FR122047 did not affect PGE2 production, whether alone or in combination with rofecoxib, in MEC from WT or COX-2 KO MEC mice. Together these data indicate that COX-1 does not contribute to PGE2 generation in MEC. Systemic COX function, as indicated by urinary prostanooid metabolite levels, was unaltered in COX-2 KO MEC mice compared with WT (Figure 2C).
Despite unchanged systemic PGE2 levels, mammary tumor onset was delayed by 8 weeks in COX-2 KO MEC mice (Figure 2D-1). In the WT group, treatment with the COX-2 selective inhibitor rofecoxib, but not the COX-1 selective inhibitor SC560, similarly delayed tumorigenesis (Figure 2D-2). Pharmacological inhibition of either COX isoform did not modify the delayed tumor onset in COX-2 KO MEC mice (Figure 2D-3). These data indicate a dominant role for epithelial COX-2 in mammary tumorigenesis in this model.

**Fig. 1.** MEC-specific COX-2 gene deletion. (A) PCR detection of COX-2+/+ (lane 1), COX-2flox/+ (lane 2), COX-2flox/flox (lanes 3 and 4), Cre+/− (lower panel, lanes 1 and 4) and Cre−/− (lower panel, lanes 2 and 3) in tail DNA. (B) Immunocytochemistry for CK14 (myoepithelial marker, red) and CK18 (luminal epithelial marker, green) in MEC isolated from WT and COX-2 KO MEC (KO) mice. Background staining in the absence of primary antibody is shown in KO cells (inset) and was similarly negative in WT cells. Nuclei are stained blue with DAPI. Data are representative of similar results from n = 3. (C) Real-time Q-PCR quantification of COX-2 (left panel) and COX-1 (right panel) mRNA expression in untreated and LPS-treated MEC isolated from WT or COX-2 KO MEC (KO) mice (n = 6). (D) COX-2 protein in untreated or LPS-treated MEC (left panel) or COX-1 protein in LPS-treated MEC (right panel) from WT and COX-2 KO MEC (KO) mice. (E) COX-2 (left panel) and COX-1 (right panel) mRNA in untreated and LPS-treated peritoneal macrophages from WT and COX-2 KO MEC (KO) mice (n = 5–6). (F) COX-2 protein in LPS-treated peritoneal macrophages from WT and COX-2 KO MEC (KO) mice. In (C) and (E) data are mean ± SE. *P < 0.005.

Despite unchanged systemic PGE2 levels, mammary tumor onset was delayed by 8 weeks in COX-2 KO MEC mice (Figure 2D-1). In the WT group, treatment with the COX-2 selective inhibitor rofecoxib, but not the COX-1 selective inhibitor SC560, similarly delayed tumorigenesis (Figure 2D-2). Pharmacological inhibition of either COX isoform did not modify the delayed tumor onset in COX-2 KO MEC mice (Figure 2D-3). These data indicate a dominant role for epithelial COX-2 in mammary tumorigenesis in this model.

**Proliferative, apoptotic, angiogenic and extracellular matrix remodeling pathways in mammary tumors from WT and COX-2 KO MEC mice**

To identify the potential mechanism through which COX-2 in MEC promotes mammary tumorigenesis, we next examined expression of a range of genes in WT and COX-2 KO MEC tumors. Messenger RNA levels for both the apoptotic enzyme Caspase 3 and a proliferation marker, Ki67, were increased in tumors from COX-2 KO MEC mice.
(Figure 3A and B), suggesting an increase in cell turnover in KO tumors but not indicating a mechanism for the delay in tumorigen-
esis in these animals. No changes were detected in the expression of
the endothelial marker CD31, either by Q-PCR (Figure 3C) or im-
munohistochemistry (Figure 5K and L), two angiogenic factors vas-
cular endothelial growth factor (VEGF) A and VEGFC (Figure 3D
and E) or their receptors VEGFR 1, 2 and 3 (Figure 3F–H), suggest-
ing that modified angiogenesis did not underlie reduced disease in
COX-2 KO MEC animals. Similarly, matrix metalloproteinase en-
zymes 2 and 9 were unaltered in COX-2 KO MEC compared with
WT (Figure 3I and J), indicating that deletion of COX-2 only in
MEC did not alter extracellular matrix remodeling.

![Graphs showing inhibition of PGE2 production in MEC delays tumor onset in mice.](image)

**Fig. 2.** Inhibition of PGE2 production in MEC delays tumor onset in mice. (A) Prostanoids in conditioned media from cultured MEC treated with or without LPS ($n = 6$). (B) PGE2 in conditioned media from MEC obtained from WT and COX-2 KO MEC (KO) mice and incubated with either rofecoxib, or FR12207, or both, with or without LPS ($n = 4–6$). (C) Urinary prostanoid metabolites measured in WT and COX-2 KO MEC (KO) disease-free 6-week-old female mice ($n = 10–12$). For (A–C), data are mean ± SE, *$P < 0.05$, **$P < 0.0002$. (D) Percent of tumor-free WT and COX-2 KO MEC (KO) mice on regular chow (1, $n = 24–30$) or chow containing rofecoxib (2, $n = 17$) or SC560 (3, $n = 13–14$).
COX-2 deletion in MEC modifies the tumor-immune microenvironment

We observed, by Q-PCR and immunohistochemistry, significant increases in expression of a common leukocyte marker, CD45 (Figures 4A-1 and 5E and F), and a common macrophage marker, F4/80 (Figures 4A-2 and Fig. 5G and H) in COX-2 KO MEC tumors compared with WT. Macrophages can be anti- (M1 phenotype) or pro- (M2 phenotype) tumorigenic, with tumor-associated macrophages (TAM) displaying predominantly M2 characteristics; thus, we examined a range of phenotypic macrophage markers in WT and COX-2 KO MEC tumors. Expression of M2-type macrophage markers Sca1 (Figure 4B-1) and Arginase-1 (Figure 4B-2), as well as the M2-type cytokine transforming growth factor β (Figure 4B-3), were not different between WT and COX-2 KO MEC tumors. Another M2-type macrophage cytokine, interleukin-10, was low/undetectable in tumors from both animal groups. In contrast, expression of the M1-type marker CD86 was increased in COX-2 KO MEC tumors as assessed by Q-PCR (Figure 4C-1) and immunohistochemistry (Figure 5I and J). Similarly, the M1 macrophage cytokine TNFα was significantly higher in COX-2 KO MEC tumors (Figure 4C-2). Two additional characteristic M1 macrophage-associated genes, inducible nitric oxide synthase and interleukin-6, were not significantly altered (Figure 4C-3 and 4C-4) although interleukin-6 correlated strongly with CD86 or F4/80 in COX-2 MECKO, but not WT, animals (supplementary Figure 2 is available at Carcinogenesis online). Taken together, these data are consistent with polarization of macrophages associated with COX-2 KO MEC tumors toward the antitumorigenic M1 phenotype.

We also examined markers for other tumor-associated immune cells. Significantly higher levels of CD2, a shared marker for T-lymphocytes and natural killer (NK) cells, were evident in COX-2 KO MEC tumors (Figure 4D-1). No significant changes were seen in expression of the NK-specific marker CD49b (Figure 4D-2), the regulatory T cell marker CD25 (Figure 4D-3) and the cytotoxic T cell marker CD8 (Figure 4D-4). In contrast, CD4 was increased in COX-2 KO MEC tumors (Figure 4E-1), indicating increased presence of CD4-expressing cells. Among CD4+ cells, T41 cells are known to direct macrophage polarization toward M1 (34) leading us to speculate that the apparent increase in M1-type responses in COX-2 KO MEC tumors could be secondary to the influence of CD4+ T41-derived mediators. Indeed, although expression of T41 markers, TIM3 and TRANCE (35,36) was not significantly different in tumors from COX-2 KO MEC mice compared with WT (Figure 4E-2 and E-3), TIM-3 levels showed almost perfect correlation with both F4/80 and CD86 in COX-2 MEC KO, but not WT, tumors (Figure 4F). Together, these data are consistent with a shift toward type 1 innate and adaptive immune responses in COX-2 KO MEC KO tumors.

PGE2 limits in vitro M1 Polarization of BMDM

Our study suggests that delayed tumorigenesis in COX-2 KO mice is at least partially due to interruption of the limit placed by COX-2-derived PGE2 on type 1 macrophage polarization. We examined whether PGE2 modifies M1 polarization of isolated macrophages. As expected, compared with non-polarized cells, significant increases in mRNA for CD86 and TNFα were evident in M1-polarized BMDM (Figure 6A). PGE2 did not significantly alter M1 markers in non-polarized cells (supplementary Figure 3 is available at Carcinogenesis online). However, concordant with our in vivo data, when PGE2 was included at the beginning of the polarization procedure, the M1-associated CD86 and TNFα mRNA expression, as well as TNFα protein secretion, were substantially reduced in dose-dependent manner consistent with a PGE2-mediated restraint of M1 polarization (Figure 6B and C).

Discussion

Tumorigenesis involves a complex and intricate interplay between tumor and stromal cells (21). Although multiple pro-tumorigenic events are associated with COX-2 (2,37), the contribution of COX-2-derived prostanoids to autocrine and paracrine signaling in tumor cells and the surrounding microenvironment are poorly understood. We used a novel model of COX-2 deletion in mammary epithelium to examine the role of COX-2 in epithelial-stromal interplay and its consequence for mammary cancer. We found that deletion of MEC COX-2 induced a substantial delay in tumorigenesis that was equivalent to systemic inhibition of the COX-2 enzyme. We detected no change in proliferative, apoptotic, angiogenic or matrix remodeling pathways to explain the reduced disease in COX-2 KO MEC mice.
Rather, we observed an increase in markers/cytokines for antitumorigenic M1 macrophages, and possibly Th1 lymphocytes, in COX-2 KO MEC tumors suggesting augmentation of type 1 polarized inflammatory/immune cells in the absence of epithelial COX-2. Consistent with this hypothesis, PGE2, the dominant MEC COX-2-derived prostanoid, restrained M1 polarization of BMDM in vitro. Thus, we conclude that deletion of COX-2 in mouse MECs delayed tumor onset, at least in part, by shifting the tumor inflammatory/immune microenvironment toward antitumorigenic type 1 responses. We propose a role for epithelial COX-2-derived prostanoids in modulating the tumor-immune microenvironment to favor tumor growth and progression.

Multiple prostanoids can be generated via COX-2, and each has been implicated in tumorigenesis. PGE2 is the dominant pro-tumorigenic prostanoid (2,14) although pro-tumorigenic effects of thromboxane A2 have been reported (38). PGI2 and PGD2 are generally considered antitumorigenic (38,39), whereas the role of PGF2α remains...
Deletion of mammary epithelial COX-2 delays tumors

Fig. 5. COX-2 KO\textsuperscript{MEC} tumors show more M1 macrophage infiltration by immunohistochemistry. Tumors, originated from luminal epithelial cells and harvested from WT and COX-2 KO\textsuperscript{MEC} (KO) mice, were selected based on hematoxylin and eosin staining and immune staining for myoepithelial (red staining for CK14, A and B) and luminal epithelial (red staining for CK18, C and D) cells. Red-brown color represents specific staining for CD45 (E and F), F4/80 (G and H), CD86 (I and J) and CD31 (K and L), in tumors from WT (left column) and COX-2 KO\textsuperscript{MEC} (KO, right column) animals. In panels (E-J), areas within dashed squares are shown as insets with arrowheads, indicating positively stained cells. The scale bar in all images indicates \texttimes40 magnification. Images are representative of at least n = 3.

poorly examined (40). In cultured MEC, PGE\textsubscript{2} is the most abundant COX-2-derived mediator (Figure 2A), with little or no contribution by COX-1 (Figure 2B). It is highly likely, therefore, that loss of COX-2-derived PGE\textsubscript{2} in MEC underlies the delayed disease we observed.

Deletion of COX-2 in MEC was equivalent to systemic COX-2 inhibition in delaying carcinogen-induced mammary tumorigenesis (Figure 2D). This is perhaps surprising given the theoretical advantage afforded by depression of PGE\textsubscript{2} generation not only in MEC but also in surrounding stromal cells in rofecoxib-treated animals. Further, reported COX-independent anticarcinogenic effects of rofecoxib (37) would be expected in drug-treated, but not COX-2 KO\textsuperscript{MEC}, mice. The degree to which COX-2 pathways in stromal cells, or COX-independent events, contribute to mammary tumor progression has yet to be elucidated although the pro-tumorigenic effects of fibroblasts (22) and macrophages (23) may be realized through induction of epithelial cell COX-2. The equivalence of mammary epithelial COX-2 deletion with systemic COX-2 inhibition indicates a dominant role of COX-2 in the epithelial cell itself in tumor promotion.

The pro-tumorigenic effects of PGE\textsubscript{2}, which include increased cell proliferation, cell survival, angiogenesis and decreased apoptosis, are well described (2,37). In our COX-2 KO\textsuperscript{MEC} mice, mRNA for the proliferation marker Ki67 and apoptotic enzyme Caspase-3 were both upregulated, indicating increased cell turnover in tumors lacking epithelial COX-2. We sought to determine, by immunohistochemistry, whether epithelial or specific stromal cells were undergoing proliferation or apoptosis. However, Ki67 and Caspase-3 protein localization and expression intensity were very variable across WT and KO tumors, preventing definitive conclusions. Given the established pro-angiogenic role of COX-2, we were surprised to observe similar expression of genes for angiogenic factors and their receptors in WT and COX-2 KO\textsuperscript{MEC} tumors. It may be that mRNA quantitation in heterogeneous tumor samples did not reveal localized changes in angiogenesis. We do not believe this to be the case, however, because no difference was observed between WT and COX-2 KO\textsuperscript{MEC} tumor levels of CD31, an endothelial marker, by PCR (Figure 3C) or immunohistochemistry (Figure 5K and L). Similarly, expression of extracellular matrix remodeling enzyme matrix metalloproteinase 2, regulated by COX-2 in breast cancer (41), was not different between WT and COX-2 KO\textsuperscript{MEC} tumors (Figure 3I). These data imply that non-epithelial cell COX-2 in COX-2 KO\textsuperscript{MEC} tumors was sufficient to maintain angiogenesis and matrix remodeling at levels similar to WT tumors.

Further examination revealed an apparent shift in the immune microenvironment in COX-2 KO\textsuperscript{MEC} tumors. Increased expression of mRNA for the common leukocyte marker CD45, the macrophage marker F4/80, the T cell markers CD2 and CD4 and the M1 macrophage marker CD86 and cytokine TNF\textalpha, was evident in COX-2 KO\textsuperscript{MEC} tumors (Figure 4A-I, -2, C-1, -2, D-1 and E-1). Elevated CD45, F4/80 and CD86 in the KO tumors was also evident by immunohistochemistry (Figure 5E-J). Confirmation of the M1 versus M2 status of TAM, as well as phenotypic analysis of other tumor associated immune cells by flow cytometry, was not possible in this carcinogen-induced model of mammary tumorigenesis because of limitations in the size and number of tumors obtained and the cellular yield per tumor. However, our data from Q-PCR and immunohistochemistry were consistent with the augmented presence of M1-type macrophages in COX-2 KO\textsuperscript{MEC} tumors. We verified a PGE\textsubscript{2}-dependent restraint on in vitro M1 polarization of BMDM, consistent with the lower levels of M1 markers/cytokines in WT versus COX-2 KO\textsuperscript{MEC} tumors. One putative source of a principle M1 polarizing cytokine, interferon \(\gamma\), is the pool of CD4\textsuperscript{+} T\textsubscript{H}1 cells (34,42). In the present study, COX-2 KO\textsuperscript{MEC} tumors also showed increased expression of T cell markers CD2 and CD4, whereas TIM3, a T\textsubscript{H}1 marker, trended toward elevation (\(P = 0.062\)). It may be that a slight increase in the T\textsubscript{H}1 cell population in COX-2 KO\textsuperscript{MEC} tumors was sufficient to shift TAM polarization to the M1 phenotype, although this remains to be verified experimentally. Alternatively, M1 polarization of macrophages in COX-2 KO\textsuperscript{MEC} may be T\textsubscript{H}1 independent, relying instead on mediators elaborated from other cells.

A permissive microenvironment is necessary for the tumors to progress fully to the malignancy (43). The tumor microenvironment consists of many cells and the composition varies depending on the tumor type. Macrophages populate the microenvironment of most if not all tumors. Independent studies have demonstrated a correlation of TAM with poor prognosis, particularly when associated with high microvascular density (44). Further, the local tumor microenvironment may educate macrophages to perform non-immune, trophic functions similar to the
promotion of epithelial outgrowth and invasion during development (21,45). It appears that once they have infiltrated the tumor, the macrophage phenotype is switched from an activated, immunological M1 type to an alternatively activated M2 type that promotes tumor progression (46). However, clinical studies have shown that, in some cases, elevated macrophage infiltration is associated with a beneficial outcome. Thus, resident and newly recruited macrophages displaying M1 characteristics limited the development of peritoneal metastases from colon carcinoma (47). Similarly, in patients with resected non-small cell lung carcinoma, tumor islet macrophage infiltration was identified as a strong favorable independent prognostic marker for survival (48). Further, it was shown that antigen-presenting and cytotoxic functions of M1-polarized macrophages can destroy tumor cells (49). The tumor milieu can direct macrophage polarization to hinder tumor growth and progression. Indeed, in the PyMT mouse model of breast cancer, augmented type 1 polarization of both Th cells and TAM was associated with reduced tumor invasiveness and limited metastasis of the primary disease (42). Our data suggest a putative novel role for COX-2 in contributing to macrophage phenotype in mammary tumors. We believe that MEC-derived COX-2 products, most probably PGE2, restrain M1 polarization of macrophages in tumors, limiting their anti-tumorigenic functions and promoting tumor progression. It is worth noting that macrophages, whose COX-2 expression was strongly inducible by LPS (Figure 1E), may contribute significantly to tumor microenvironmental PGE2 levels with unexplored paracrine and autocrine effects. The contribution of macrophage COX-2 to mammary tumorigenesis is currently under investigation in our laboratory.

The mechanism through which mammary epithelial COX-2-derived products impact immune cell polarization in mammary tumors is not clear. There is evidence that prostanooids can modulate T cell and macrophage differentiation and polarization (27,50,51). Our data in isolated BMDM argue for a direct effect of PGE2 on macrophages although this remains to be verified in TAM. Our current findings support the concept that the COX-2/PGE2 biosynthetic pathway may provide novel targets for therapeutic modulation of the tumor-immune microenvironment. Further, in our head-to-head comparison of mammary epithelial versus systemic interruption of COX-2 activity, similar benefit was seen using the targeted approach. Refined approaches to inhibit specific functions of COX-2 in the tumor microenvironment may yield beneficial antitumorigenic effects while avoiding deleterious systemic events that limit clinical use of systemic COX-2 inhibition in cancer prevention or treatment.

Supplementary material
Supplementary Figures 1–3 and other Materials and Methods can be found at http://carcin.oxfordjournals.org/

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