Cysteinyl leukotriene 1 receptor influences intestinal polyp incidence in a gender-specific manner in the Apc<sup>Min/+</sup> mouse model

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Abstract

There is emerging literature emphasizing the role of inflammatory eicosanoids, including prostaglandins and leukotrienes, in cancer development. Increased expression of both the cysteinyl leukotriene receptor 1 (CysLTR1) and the enzyme responsible for the production of leukotrienes, 5-lipoxygenase, is associated with poor prognosis in patients with colorectal adenocarcinomas. Apc mutation is an early event in the development of sporadic and hereditary (familial adenomatous polyposis) colorectal cancer. We utilized the Apc<sup>Min/+</sup> mouse model of familial adenomatous polyposis/sporadic colorectal cancer to investigate the role of CysLTR1 in intestinal tumorigenesis by crossing Apc<sup>Min/+</sup> mice with mice lacking the Cysltr1 gene. We could observe a reduced tumor burden in the small intestine of double-mutant female (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) but not double-mutant male mice, compared with gender-matched single-mutant (Cysltr1<sup>+/+</sup> Apc<sup>Min/+</sup>) mice. This reduction was in a Cysltr1-dependent manner, female double-mutant mice having significantly reduced tumor formation compared with control littermates. The female double-mutant phenotype was accompanied with decreased systemic inflammation, as evidenced by significantly reduced serum levels of prostaglandin E<sub>2</sub> and CysLTs, as well as increased CD3<sup>+</sup>CD8<sup>+</sup>T-cell tumor infiltration. Furthermore, the reduced formation of polyps in double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice could in part be explained by the cytotoxic action of CD3<sup>+</sup>CD8<sup>+</sup>T cells in the polyp and reduced nuclear accumulation of β-catenin in the epithelium of small intestinal polyps. Our results stress the important role that CysLTR1 plays in colorectal cancer and its potential as a therapeutic target in cancer therapy.

Introduction

Colorectal cancer is the third most prevalent cancer and the fourth leading cause of cancer-related deaths worldwide (1). Lifestyle factors, such as high red meat intake, excessive alcohol consumption, smoking and obesity are associated with increased risk of developing colorectal cancer (2). Another well-established risk factor of developing colorectal cancer is chronic inflammatory bowel disease, including Crohn’s disease and ulcerative colitis. Various epidemiological studies have estimated a 2- to 5-fold increased risk for inflammatory bowel disease patients to develop colorectal cancer. The risk of developing colorectal cancer correlates with the duration, extent and severity of mucosal inflammation (3,4).
Leukotrienes are lipid inflammatory mediators generated via metabolism of arachidonic acid through the 5-lipoxygenase (5-LOX) pathway. These are involved in airway mucus secretion, increased vascular permeability, eosiophilic chemotaxis and bronchoconstriction (5–8). Cysteinyl leukotrienes (CysLTs; cysteinyl leukotriene C4, D4 and E4) are characterized by their glauathione moiety and were initially discovered as slow-reacting substances of anaphylaxis with the ability to induce bronchial constriction (9). CysLTs have been pre-described to exert their effects via different G-protein coupled receptors in humans, the best characterized are CysLTR1 and CysLTR2 (10). Upregulated expression of CysLTR1 has been observed in several solid tumor cancers and has been positively correlated with poor prognosis in breast and colon cancer (11–16). LTD4-induced CysLT1 signaling has been shown to induce expression of β-catenin and cyclooxygenase-2 (COX-2) (17,18), resulting in proliferation, survival and migration in intestinal epithelial cells through distinct signaling pathways (18,19).

Mutation of the Apc gene is an important initiation factor in human colorectal cancer etiology and is carried by 80–90% of all colorectal cancer patients, which makes the Apc(wt/wt) mouse model an excellent choice for investigation of colorectal cancer (20). We have previously demonstrated the ability of CysLTR1 antagonists to inhibit tumor growth in a colon cancer xenograft model by reducing proliferation, inducing apoptosis and impairing angiogenesis (21). To investigate and further establish the correlation between CysLTR1 and colon cancer development in vivo, Apc(wt/wt) mice were crossed with mice lacking the Cyslr1 gene.

Materials and methods

Mice

C57BL/6J-Apc(wt/wt) mice were purchased initially from the Jackson Laboratory (Bar Harbor, ME) as founders. The mice with a targeted Cysltr1 gene disruption on a C57BL/6N background were a kind gift from Prof. Frank Austen (Harvard Medical School, Brigham and Women’s Hospital, Boston, MA) (22). Double-mutant mice (Cyslr1−/− Apc(wt/wt) and Cyslr1−/− Apc(wt/wt)−/−) and their control littermates (Apc(wt/wt)) were established and maintained at Lund University Animal Facility, Malmö, according to ethical permit M263-12 approved by the Regional Ethical Committee for Animal Research at Lund University, Sweden.

The breeding colony was genotyped for the presence of Apc(wt/wt)−/− and Cyslr1 alleles by PCR assays using the following six primers: Apc−/− wild-type (IMR0033): 5′-GGCCTCCCTCAGTTAGAC-3′, Apc−/− common (IMR0034): 5′-TTTCACTTGGGATAAGGC-3′, Apc−/− mutant (IMR0758): 5′-TTTCTGAGAAAGACAGATTGA-3′, generating a product of 600 bp for the wild-type and 340 bp for the Apc−/− allele, and Cyslr1 sense: 5′-AAAAAACATAGAGCTGACATATTAAAG-3′, Cyslr1 antisense: 5′-AATCGATATGATCGCGAGATGGAAGGCTGA-3′, Neo antisense: 5′-CTGCCACACTGAGGCGATCCAC-3′, generating a product of 284 bp for the wild-type and 233 bp for the null Cyslr1 allele (Supplementary Figure 1, available at Carcinogenesis Online).

Experimental setup

Gender and age matched 6-week-old mice (n = 14–16/group) were weighed every third day and sacrificed after 8 weeks (14 week old). One hour before animal-sacrifice bromodeoxyuridine was i.p. injected (1mg/100μl) into each mouse. At the experimental end point, cardiac puncture was performed for each mouse, and both the small intestine and colon were cut longitudinally and feces were washed off with ice-cold phosphate-buffered saline.

Approximately half of the experimental animals per group (genotype) had their tissues fixed in 10% buffered formalin overnight and subsequently stored in 70% ethanol. The polyps were counted and their size measured using a dissection microscope (×2). The most distal part of the small intestine (5–6 cm) and the entire colon were subdivided in smaller pieces (~2 cm) and finally embedded in paraffin for immunohistochemistry analysis. The remaining half had their tissues processed for flow cytometry or snap frozen in liquid nitrogen and stored at −80°C.

Immunohistochemistry

Paraffin-embedded sections of small intestine and colon were sectioned (5 μm) for immunohistochemical staining. All procedures were performed using a Dako automatic slide stainer (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The slides were scanned with Aperio ScanScope CS (Aperio Technologies, Vista, CA), and images were evaluated independently, by two observers, in a blinded fashion.

Relative protein expression of COX-2 (Abcam, Cambridge Science Park, Cambridge, UK) and 5-LOX (Cayman Chemical, Ann Arbor, MI) was evaluated as the total percentage of positive stained cells within the polyps. The subcellular localization of β-catenin (BD Biosciences, Franklin Lakes, NJ) for positive stained epithelial cells within polyps was determined. Goblet cells were identified with goat anti-human Mucin 2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The villi (n = 10) selected for cell quantification were similar in size and in near proximity of polyps within the distal part of the small intestine. Infiltrating cells and proliferating cells within polyps were identified with rabbit anti-human CD45 polyclonal antibody and mouse anti-BrdU monoclonal antibody (Santa Cruz Biotechnology, respectively).

Flow cytometry

After sacrificing the mice, the small intestine was dissected and washed with ice-cold phosphate-buffered saline. With the aid of a dissection microscope, the Peyers’ patches were removed and all polyps were identified and separated from non-polyp areas. Polyp areas were cut into smaller pieces and stored in RPMI medium complemented with 10% fetal bovine serum. The tissue was homogenized using gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA) in the presence of 0.5 mg/ml Collagenase P (Roche Diagnostics, Basel, Switzerland).

After obtaining a single-cell suspension, the cells from the polyp areas were fixed and stained with Alexa Fluor® 647 rat anti-mouse CD3 antibody and subsequently stained with either PerCP-Cy™ 5.5 rat anti-mouse CD4 or FITC rat anti-mouse CD8 antibody. Non-specific staining was reduced by blocking with Mouse BD Fc Block™. Background was determined using isotype-matched antibodies for all stainings. All antibodies used for FACS were from BD Pharmingen, Franklin Lakes, NJ. Flow cytometric measurements were performed using the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), and analyses were performed using Summit v4.3 Software (Dako, Fort Collins, CO).

CysLTs and prostaglandin E2 enzyme immunooassay

Blood was collected by cardiac puncture when sacrificing the mice and the non-selective cyclooxygenase inhibitor indomethacin (Sigma-Aldrich, St Louis, MO) was immediately added to all samples. Serum was separated in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) by centrifugation at 6000g for 2 min. Equal volume of serum from five animals per group were pooled, and separation of CysLTs and prostaglandins was performed by solid-phase extraction Sep-Pak Vac RC (C18-500 mg) cartridges from Water Corporation (Milford, MA). Serum levels of CysLTs and prostaglandin E2 (PGE2) were determined using a competitive enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY) according to manufacturer’s instructions.

Gene expression analysis

Total RNA was extracted from five sections of the small intestine, S1–S5 (S1 being the most proximal and S5 the most distal section), and the
colon of male and female Apc<sup>Min/+</sup> mice (n = 2 for each gender). The RNA was reverse-transcribed using ReverTra Aid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR was performed using Maxima Probe/Rox qPCR (Thermo Scientific). Expression of Cysltr1, estrogen receptor α and β mRNA and also PGE<sub>2</sub>, receptors EP2 and EP4 mRNA were analyzed in males and females for each section of the intestinal tract using the ΔΔCT method and normalizing against Gapdh.

**Statistical analysis**

All statistical analyses were performed in GraphPad Prism version 5.0a (GraphPad Software, San Diego, CA), and the statistical significance of data was determined as P < 0.05. For comparison between two groups unpaired t-test (Student’s t-test), paired t-test or Wilcoxon matched-pairs signed rank test was used. One-way or two-way analysis of variance (ANOVA) was used to compare multiple groups. All values are expressed as the mean ± standard error of the mean (SEM).

**Results**

**Double-mutant female mice develop fewer small intestinal polyps**

No difference in the relative body weight could be observed between Apc<sup>Min/+</sup> mice with differing Cysltr1 genotype (data not shown). Taking into account that the Cysltr1 is carried on the X-chromosome, both female (Cysltr1<sup>+/-</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) and male (Cysltr1<sup>+/-</sup> Apc<sup>Min/+</sup> ) double-mutant mice and their control littermates (Apc<sup>Min/+</sup>) were evaluated for polypl formation separately. Interestingly, the polypl formation was affected in a Cysltr1-dependent manner for the female mice, double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) mice developing significantly fewer polyps in the small intestine compared with their control littermates (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) (36.57 ± 4.735 and 5.859 ± 5.846, respectively, P < 0.05) (Figure 1A). These mice also had a tendency of developing fewer medium-sized polyps (Figure 1C). In contrast, the male mice did not have detectable changes in total polyp number or size distribution between double-mutant mice (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) and their control littermates (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) (Figure 1B and D). The number of colonic polyps did not differ significantly between genotypes within each gender or between the two genders (Supplementary Figure 2, available at Carcinogenesis Online).

Another interesting result is that female Apc<sup>Min/+</sup> mice developed almost three times as many polyps in the small intestine than their male counterparts. In light of this, and that Cysltr1 genotype affected polypl number in females only, we compared the mRNA expression of Cysltr1 between female and male Apc<sup>Min/+</sup> mice across the intestinal tract, including the colon. We found that apart from one midsection of the intestine, females had significantly higher Cysltr1 expression levels than males (two-way ANOVA P < 0.01; paired t-test P < 0.01; Supplementary Figure 3A, available at Carcinogenesis Online). We also compared the mRNA levels of estrogen receptors α and β and found no significant difference between males and females (Supplementary Figure 3B and C, available at Carcinogenesis Online). In conclusion, these results suggest that systemic Cysltr1 expression plays a modifying role in polypl development in female, but not male, Apc<sup>Min/+</sup> mice in the small intestine.

**Double-mutant female mice have reduced COX-2 expression but no change in Mucin 2 production or CD45 leukocyte infiltration in the small intestine**

The expression of the inflammatory markers COX-2 and 5-LOX among the epithelial cells of the small intestinal polyps was investigated with immunostaining. The expression of the enzymes is given after calculating the numbers of positive epithelial cells in the polyps. A small decrease in the number of 5-LOX-positive cells could be observed in the small intestinal polyps of double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice, compared with their control littermates (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>; P < 0.05; Figure 2A). However, no reduction in the number of 5-LOX-positive cells was seen in double-mutant mice (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) compared with control female mice (Apc<sup>Min/+</sup>). Also, a significant reduction of COX-2 expression in the small intestinal polyps was detected between double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup> female mice and their control littermates (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>; Figure 2B).

Next, we investigated the status of leukocyte infiltration within small intestinal polyps of Apc<sup>Min/+</sup> mice. Immunostaining of CD45<sup>+</sup> leukocytes revealed no net change in polypl infiltration between double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice compared with single-mutant (Apc<sup>Min/+</sup>) female mice (Figure 2C).

The mucus layer is essential for the protection of the intestinal epithelium against commensal bacteria and other environmental factors in the lumen that can induce inflammation and potentially promote tumorigenesis. We therefore investigated the effect of Cysltr1 gene disruption on mucus production in Apc<sup>Min/+</sup> mouse model by staining for Mucin 2, which is the most predominant mucin expressed in the intestinal mucus layer and a differentiation marker for secretory goblet cells. We could not detect any difference in the number of Mucin 2-stained goblet cells in villi surrounding the small intestinal polyps between females with different Cysltr1 genotypes (Figure 2D).

**Double-mutant female mice have higher membranous and less nuclear expression of β-catenin in the epithelium of small intestinal polyps**

We have previously shown that LTD<sub>4</sub>-mediated CysLTR1 activation induces proliferation in intestinal epithelial cells and that CysLTR1 antagonist treatment inhibits colon xenograft tumor growth by reducing proliferation (18, 21). To investigate the effect of systemic gene disruption of Cysltr1 on proliferation in small intestinal polyps, we performed staining of DNA-incorporated BrdU. A slight reduction in proliferation could be observed in double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice compared with single-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice (Figure 3A). We have previously also demonstrated that LTD<sub>4</sub>-stimulation of CysLTR1 induces β-catenin nuclear accumulation and transcriptional activity in colon cancer cells and stimulates growth (18). Interestingly, we observed a redistribution of the subcellular localization of β-catenin. The double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice expressed more membranous than nuclear β-catenin in the epithelial compartment of their small intestinal polyps, whereas the control (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice exhibited the opposite (Figure 3B).

**Increased CD3<sup>+</sup>CD8<sup>+</sup> lymphocyte infiltration of small intestinal polyps in double-mutant females**

Despite no net change in the CD45<sup>+</sup> leukocyte infiltration of small intestinal polyps of double-mutant mice compared with control mice, we further studied possible differences in the lymphocyte subtype infiltration within small intestinal polyps. A slight difference could be observed in CD3<sup>+</sup>CD8<sup>+</sup> lymphocyte infiltration between double-mutant females (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) and their control littermates (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) (Figure 4A). However, a clear tendency toward increased CD3<sup>+</sup>CD8<sup>+</sup> lymphocyte infiltration was observed in small intestinal polyps of double-mutant (Cysltr1<sup>−/−</sup> Apc<sup>Min/+</sup>) female mice compared with control littermates.
CysLTR1 affects serum levels of PGE$_2$ and CysLTs in Apc$^{Min/+}$ mice

To investigate the effect of global Cysltr1 gene disruption on systemic inflammation, we measured the serum levels of PGE$_2$ and CysLTs (LTC$_4$, LTD$_4$, and LTE$_4$) using an enzyme immunoassay. The serum levels of CysLTs (Figure 5A) were significantly reduced for double-mutant (Cysltr1$^{−−}$/Apc$^{Min/+}$ and Cysltr1$^{+−}$/Apc$^{Min/+}$) female mice compared with control littermates (Cysltr1$^{++}$/Apc$^{Min/+}$). There was also a significant difference in CysLT serum levels between the male double-mutants (Cysltr1$^{−−}$/Apc$^{Min/+}$) and control (Cysltr1$^{++}$/Apc$^{Min/+}$). However, when comparing CysLT serum levels between males and females, there was only a significant difference between the double-mutant mice (Cysltr1$^{−−}$/Apc$^{Min/+}$ and Cysltr1$^{−−}$/Apc$^{Min/+}$; Supplementary Figure 5A, available at Carcinogenesis Online). Interestingly, serum levels of PGE$_2$ were significantly reduced within and between genders (Figure 5B; Supplementary Figure 5B, available at Carcinogenesis Online). We also looked at PGE$_2$ receptors EP2 and EP4 mRNA expression between female and male Apc$^{Min/+}$ mouse intestines. Although overall prostaglandin receptor levels did not differ significantly between males and females, the mRNA expression profile
EP4 across the intestinal tract between the genders varied significantly (two-way ANOVA; $P<0.01$; Supplementary Figure 3D and E, available at Carcinogenesis Online). The CysLTR1 mRNA expression in control mice was overall lower in males compared with females (Supplementary Figure 3A, available at Carcinogenesis Online). In Supplementary Figure 4, available at Carcinogenesis Online, we show the levels of CysLTR1 expression along the small intestine of these female mice. To summarize, global Cysltr1 gene disruption reduces the number of polyps in the small intestine of ApcMin/+ mice, which could be due to reduced expression of nuclear $\beta$-catenin and COX-2, together with increased CD3+CD8+ lymphocyte infiltration.

**Discussion**

In the current study, we investigated the role of the inflammatory CysLT1 receptor in colorectal cancer using the spontaneous Apc<sup>Min/+</sup> mouse model together with a Cysltr1 gene disruption. Interestingly, the female, but not the male, showed a reduction in polyp formation in the small intestine in a Cysltr1-dependent manner. The double-mutant (Cysltr1<sup>-/-</sup> Apc<sup>Min/+</sup> and Cysltr1<sup>-/-</sup> Apc<sup>Min/+</sup>) female mice had a significantly reduced number of polyps in the small intestine, compared with the control littermates (Cysltr1<sup>++</sup> Apc<sup>Min/+</sup>). A gender-specific difference in polyp count could also be observed among control Apc<sup>Min/+</sup> mice, with females developing almost three times as many polyps in the small intestine as males (Figure 1A and B). Although this result was unexpected, some publications have addressed a gender-specific difference in polyp number in the small intestine reporting that females had a higher polyp count (23, 24). In addition, females generally expressed significantly higher levels of Cysltr1 mRNA across the intestinal tract (Supplementary Figure 3A, available at Carcinogenesis Online). This could be due to the Cysltr1 gene being on the X-chromosome, which therefore means that females have two copies whereas males only have one.

Only one report by Cherukuri et al. (25) has shown a gender-specific reduction in polyp formation due to a gene deletion. They showed that targeted gene deletion of Cox-2 in the intestinal epithelium resulted in reduced polyp formation in the small intestine of female, but not male, Apc<sup>Min/+</sup> mice (25). A reduction in polyp formation has also been observed in Apo<sup>Min/+</sup> mice as a result of global Cox-2 gene disruption and pharmacological inhibition of COX-2 (26, 27). Moreover, PGE<sub>2</sub> treatment of Apc<sup>Min/+</sup> mice has been shown to increase the intestinal tumor burden in a PPARα-dependent manner (28) whereas deletion of mPGES-1,
Figure 3. The effect of CysLTR1 on small intestinal polyp proliferation in female Apc<sup>Min/+</sup> mice. The distal small intestinal part from female Apc<sup>Min/+</sup> mice with different Cysltr1 genotypes from at least five littermates per genotype (Cysltr1<sup>++</sup>, Cysltr1<sup>+-</sup> and Cysltr1<sup>--</sup>) was processed for immunostaining. Representative images (×20 and/or ×40) of (A) BrdU and (B) β-catenin stainings and their corresponding bar diagram showing the percentage of (A) BrdU-positive cells and (B) subcellular localization of epithelial β-catenin within polyps. Arrows indicate nuclear staining. Quantitative data shown are the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way and two-way ANOVA.

Figure 4. Lymphocyte subtypes within small intestinal polyps of female Apc<sup>Min/+</sup> mice. The small intestinal polyps of 14-week-old female Apc<sup>Min/+</sup> mice with indicated Cysltr1 genotype were double stained with either (A) anti-CD3 and anti-CD4 or (B) anti-CD3 and anti-CD8 and analyzed by flow cytometry. Bar diagrams show the percentage of double-positive cells from the total cell population from three to four separate mouse experiments. Values are mean ± SEM.
a PGE, terminal synthase, in Apc\(^{Min/+}\) mice markedly reduced both the size and number of intestinal polyps with coordinated reduction of COX-2 expression (29).

Non-steroidal anti-inflammatory drugs—non-selective COX inhibitors—and selective COX-2 inhibitors have been shown to reduce the size and number of intestinal polyps (30–32). Selective COX-2 inhibitor celecoxib has also been shown to reduce polyp recurrence in patients (33). However, due to the gastrointestinal and cardiovascular side effects associated with long-term use of non-steroidal anti-inflammatory drugs and selective COX-2 inhibitors, these compounds are not recommended for prevention and/or treatment of hereditary/sporadic colorectal cancer. Other approaches are currently being explored such as dual inhibition of COX-2 and 5-LOX that could provide a more tolerable and efficient alternative. A combined treatment of celecoxib with FLAP inhibitor MK886 or the CysLTR1 antagonist LY171883 in human colon cancer cells augmented anti-proliferative effects and induced apoptosis (34). Similar observations have been made in a xenograft mouse model of colorectal cancer, where the combined inhibition of COX-2 and 5-LOX with celecoxib and AA861, respectively, demonstrated a more pronounced suppression of tumor growth (35). In these studies, however, a shunt of the arachidonic acid metabolism could be observed when targeting either the COX or LOX pathways. Interestingly, our study with a global Cysltr1 gene disruption caused no shunt toward the COX pathway. On the contrary, we observed statistically significant reduced number of COX-2 positive cells in double-mutant (Cysltr1\(^{-/-}\) Apc\(^{Min/+}\)) compared with control mice (Apc\(^{Min/+}\)), a difference not observed for the number of 5-LOX positive cells in small intestinal polyps (Figure 2A and B). However, when we analyzed the serum levels of the products of these two enzymes, PGE, and CysLT, respectively, we found that both of them were significantly reduced (Figure 5A and B). These results suggest that using a CysLTR1 antagonist in combination with a COX-2 inhibitor would be more beneficial than using a COX-2 inhibitor alone as this might reduce the side effects that occur when using COX inhibitors.

In azoxymethane-induced colonic lesions of mice, deletion of mPGES-1 has been shown to block β-catenin nuclear translocation, establishing a link between PGE\(_2\) and activation of Wnt/β-catenin signaling (29). Interestingly, we were also able to observe a diminished nuclear translocation of β-catenin, in addition to reduced serum levels of PGE\(_2\), in double-mutant female mice. Nuclear localization of β-catenin is one of the prerequisites of colorectal cancer progression and readily found in late adenomas and carcinomas (36). Our results confirm a link between CysLTR1, COX-2 and Wnt/β-catenin pathways in the Apc\(^{Min/+}\) mouse, which has also been demonstrated previously in studies by our group (18,19). The EP4 mRNA expression profile varies significantly between male and female Apc\(^{Min/+}\) mice (Supplementary Figure 3E, available at Carcinogenesis Online). Because EP4 is widely expressed in many cancers including colorectal cancer (37–40), it is of note that colonic mRNA levels were higher in males than in females.

Although Muc2\(^{-/-}\) mice were shown to develop colorectal cancer via the β-catenin pathway (41), our results showed no difference in Mucin 2 expression between mice that carried a Cysltr1 gene disruption and their control littermates. Additionally, we did not observe that pronounced tumor formation shifted toward the colon as shown in another study using Muc2-deficient Apc\(^{Min/+}\) mice (42). CysLTR1 antagonists are used in the clinic to treat inflammation in asthmatic patients and have been shown to reduce mucus production in the airways (43). However, the global gene disruption of Cysltr1 in our Apc\(^{Min/+}\) mice did not result in decreased intestinal mucus production.

CysLTR1 antagonists pranlukast and montelukast have been shown to reduce vascular permeability in the lungs of mice with allergen-induced asthma (44) and in the peripheral capillaries of mice in a Lewis lung carcinoma metastasis model (45). In a meta-analysis review of clinical studies, it was concluded that the frequency of tumor-infiltrating lymphocytes predicts favorable prognosis and overall survival (46). To investigate the role of CysLTR1 in vascular permeability and immune cell infiltration in our model, we performed immunostaining of the leukocyte marker CD45. We were not able to distinguish any change in the total number of CD45\(^{+}\) cells in small intestinal polyps between double-mutant and control female mice. In a large cohort of human colorectal cancers, the density, location and type of tumor-infiltrating immune cells were found to be stronger prognostic factors than standard TNM classification (47). In our study, the double-mutant female (Cysltr1\(^{-/-}\) Apc\(^{Min/+}\)) mice had an increased CD3\(^{+}\)CD8\(^{+}\) T cell and a slight decrease in CD3\(^{+}\)CD4\(^{+}\) T-cell tumor infiltration, compared with the control littermates (Cysltr1\(^{-/-}\) Apc\(^{Min/+}\)). Interestingly, PGE\(_2\), has been shown to directly and indirectly inhibit the activity and expansion of CD3\(^{+}\)CD8\(^{+}\) T cells, hence contributing to the evasion of the tumor cells from the host adaptive immune surveillance (48). Female double-mutants

![Figure 5](https://academic.oup.com/carcin/article-abstract/37/5/491/1744673/497)

**Figure 5.** Effect of CysLTR1 on serum levels of PGE\(_2\), and CysLTs in female and male Apc\(^{Min/+}\) mice. The concentrations of (A) CysLTs and (B) PGE\(_2\), were determined with enzyme-linked immunosorbent assay for 14-week-old double-mutant (Cysltr1\(^{-/-}\) Apc\(^{Min/+}\) and Cysltr1\(^{-/-}\) Apc\(^{Min/+}\)) female and (Cysltr1\(^{-/-}\) Apc\(^{Min/+}\)) male mice, and their control littermates (Apc\(^{Min/+}\)). Values are mean ± SEM, averaged from triplicates of pooled samples (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA.
had a much lower CD4+/CD8− ratio than control littermates. Diederichsen et al. (49) have shown that low CD4+/CD8− ratio of tumor-infiltrating lymphocytes conveys better prognosis. Another study established that CD8+ T cells within the tumor, and not the invasive tumor margin or the stroma, are significantly associated with colorectal cancer patient survival (50). Because we observed slightly more CD3+CD4+ T cells in control compared with double-mutant female mice, we can speculate that this could be due to an increased level of CD4+Foxp3+ regulatory T cells in the control. Increased frequency of circulating and intratumoral CD4+Foxp3+ T cells (regulatory T cells) are considered to convey negative prog-

osis in colorectal patients (51–53), and studies targeting this sub-
population of immunosuppressive T cells have shown promising results in experimental models of cancer (54).

In conclusion, targeted Cyslr1 gene disruption revealed the role of CysLT1R in intestinal tumorigenesis in female ApcMin/+ mice. The tumor development in the small intestine was reduced in a Cyslr1-dependent manner, female double-mutant (Cyslr1−/−ApcMin/+)/ mice having significantly reduced tumor for-
mation compared with control littermates (ApcMin/+). This pheno-
type was accompanied with decreased systemic inflammation as evidenced by significantly reduced serum levels of PGE2, and CysLTs, and increased CD3+/CD8− T-cell polyp infiltration as well as reduced nuclear accumulation of β-catenin in the epi-

thelium of small intestinal polyps. This study emphasizes the importance of lipid derived proinflammatory mediators in the development of intestinal tumorigenesis and the prospect of targeting the CysLT1R as a novel therapeutic approach.

**Supplementary material**

Supplementary Figures 1–5 can be found at http://carcin.oxford-
journals.org/

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