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Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Characterization of the Cysteinyl Leukotriene 2 Receptor in Novel Expression Sites of the Gastrointestinal Tract

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Cysteinyl leukotrienes (cysLTs: LTC_4, LTD_4, and LTE_4) are pro-inflammatory lipid molecules synthesized from arachidonic acid. They exert their actions on at least two cysLT receptors ($CysLT_1R$ and $CysLT_2R$). Endothelial expression and activation of these receptors is linked to vasoactive responses and to the promotion of vascular permeability. Here we track the expression pattern of $CysLT_2R$ in a loss-of-function murine model ($CysLT_2R$-LacZ) to neurons of the myenteric and submucosal plexus in the small intestine, colonic myenteric plexus, dorsal root ganglia, and nodose ganglion. Cysteinyl leukotriene (LTC_4/D_4) stimulation of colonic submucosal venules elicited a greater permeability response in wild-type mice. In a dextran sulfate sodium-induced colon inflammation model, the disease activity index and colonic edema (measured by wet:dry weights and submucosal thickness) were significantly reduced in knockout (KO) mice compared to controls. Tumor necrosis factor-α levels in colon tissue were significantly lower in KO mice; however, myeloperoxidase activity was similar in both the KO and wild-type groups. Finally, patch-clamp recordings of basal neuronal activity of colonic-projecting nociceptive neurons from dorsal root ganglia (T9-13) revealed significantly higher excitability in KO neurons compared to wild type. These results suggest that a lack of neuronal expression of $CysLT_2R$ in the murine colonic myenteric plexus attenuates colitis disease progression via a reduction in inflammation-associated tissue edema and increases neuronal sensitivity to nociceptive stimuli. ***(Am J Pathol 2011, 178:2682–2689; DOI: 10.1016/j.ajpath.2011.02.041)***

Inflammatory bowel disease (IBD) encompasses mainly two distinct chronic diseases that result in intestinal inflammation: Crohn's disease and ulcerative colitis. According to the Crohn's and Colitis Foundation of America, approximately 1.4 million Americans suffer from IBD, costing the nation 1.8 to 2.6 billion dollars yearly and resulting in significant patient morbidity with symptoms including bloating, abdominal pain, rectal bleeding, diarrhea, constipation, weight loss, and intolerance to specific foods (*http://www.ccfa.org/about/press/ibdfacts*, last accessed December 2, 2011).

Leukotrienes are potent lipid mediators of inflammation that originate from the conversion of arachidonic acid into leukotriene A_4 (LTA_4) via the activity of 5-lipoxygenase and 5-lipoxygenase-activating protein.[1] LTA_4 may then be directed toward the synthesis of leukotriene B_4 (LTB_4) or the cysteinyl leukotrienes (cysLTs: LTC_4, LTD_4, and LTE_4). Leukotrienes exert their actions via G protein-coupled receptors; specifically, cysLTs exert most of their known functions via two receptors, the cysteinyl leukotriene 1 and 2 receptors ($CysLT_1R$ and $CysLT_2R$), which have been localized to the plasma membrane, nucleus, and cytosolic compartment in various cell types.[2–4] It has previously been reported that human IBD colon biopsies show a marked increase in both 5-lipoxygenase and 5-lipoxygenase-activating protein.[5] Moreover, mucosa samples from patients with IBD have been shown to convert almost sixfold more exogenous arachidonate to LTB_4 compared to normal subjects.[6] Although no significant difference in LTC_4 synthase (LTC_4S) between IBD versus normal colons has been reported, mice lacking this enzyme displayed a significant reduction in peritoneal vascular permeability induced by the inflammatory molecule,

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zymosan.[7] Furthermore, in a separate study involving rats, dextran sulfate sodium (DSS) colitis-induced body weight loss and fecal occult blood were significantly attenuated when animals were administered a specific CysLT$_1$R blocker, montelukast.[8] Other studies found that montelukast treatment decreased colonic mucosal degeneration caused by stress.[9]

Previous work by our group[10] revealed a marked increase in both CysLT$_1$R and CysLT$_2$R mRNA levels in myocardium following ischemia/reperfusion injury in a murine model, yet only recently[11] has the role of one of these receptor subtypes (CysLT$_1$R) been examined in comparable gastrointestinal (GI) ischemia/reperfusion injury models despite the important feed-back relationship between IBD progression and vascular dysfunction.[12] CysLT$_2$R shows increased expression in colonic tumors[13] and its concentrated localization to cell nuclei has recently been correlated with a more favorable prognosis in colorectal carcinoma.[14] However, the distribution of CysLT$_2$R in the normal GI tract and its functional relevance to GI physiology has not yet been investigated.

We sought to study the localization of CysLT$_2$R in the GI tract, specifically in the colon, and its role in the progression of a well-characterized colitis model[15] induced by DSS. We report the novel localization of CysLT$_2$R to neurons in the small and large intestinal myenteric plexi, vagus nerve, and dorsal root and nodose ganglia using CysLT$_2$R knockout (KO) mice with an integrated β-galactosidase (LacZ) reporter, as well as its effects on colonic nociception. The lack of CysLT$_2$R reduces exogenous cysLT-mediated submucosal venule permeability, DSS-induced colonic edema, disease progression, and tissue tumor necrosis factor (TNF)-α levels, while increasing excitability in colonic afferent neurons.

Materials and Methods

Animals

CysLT$_2$R KO mice with a LacZ knock-in driven by the *Cysltr2* promoter on a C57Bl/6 genetic background were generated as previously described.[16] Wild-type (WT) littermates from heterozygous matings were used as controls. Experimental protocols were approved by the Queen's University Animal Care Committee and conformed to the Guidelines of the Canadian Council on Animal Care.

X-Gal Staining of Tissue

Expression of active β-galactosidase from the LacZ gene allows the use of X-galactose (X-gal) staining as a reporter for native sites of CysLT$_2$R expression. Colons from both sham and DSS-treated (WT) and CysLT$_2$R-LacZ (KO) mice were dissected and either the whole colon or only the dissected muscle layers (with myenteric plexus) were fixed for 15 minutes in cold 2% paraformaldehyde/0.2% glutaraldehyde. X-Gal staining was performed overnight at 37°C in PBS containing 2 mmol/L $MgCl_2$, 5 mmol/L potassium ferrocyanide trihydrate, 5 mmol/L potassium ferricyanide crystalline, 1 mg/mL X-Gal, and 2.5% dimethyl sulfoxide. WT colons were used as negative controls for endogenous β-galactosidase activity. The following day, stained tissues were rinsed in PBS and photographed. In the case of X-gal staining of colon sections, tissues were fixed for 10 to 15 minutes and were snap frozen in optimal cutting temperature medium. Sections (10 μm) were cut and stained with X-gal overnight in a humidified chamber at 37°C.

DSS-Colitis Model

Male mice (6 to 8 weeks old) were housed individually and were provided 3% DSS in their drinking water for 5 consecutive days followed by 2 days of normal drinking water. During this time, the following parameters were assessed daily: body weight, signs of distress/dehydration, diarrhea, bloody stool, and water consumption. At the end of this colitis induction, mice were either injected intraperitoneally with ketamine and xylazine (200 mg/kg and 10 mg/kg, respectively) and used for intravital imaging studies or were overdosed with CO_2 and relevant organs were dissected.

Intravital Imaging

Following DSS treatment, mice were anesthetized intraperitoneally and cannulated in the right jugular vein for administration of fluorescein isothiocyanate (FITC)-albumin or anesthetic, as needed. Proximal colonic submucosal vasculature was imaged as in previous work.[17] Briefly, mice were placed on an acrylic stage adaptor, a laparotomy was performed, the colon was exposed, and the proximal colon was opened along the antimesenteric border by cutting along a pre-cauterized line. The cleared, opened colon sections were secured onto the stage with a double-arteriole clamp and were superfused with a saline solution (131.9 mmol/L NaCl, 4.7 mmol/L KCl, 20 mmol/L $NaHCO_3$, 1.2 mmol/L $MgSO_4$, and 2.0 mmol/L $CaCl_2$) maintained at 37°C and bubbled with 95% O_2/5% CO_2. Submucosal postcapillary venules were localized under a bright field and were then viewed under fluorescence (using a FITC filter) for an initial fluorescence background measurement. FITC-albumin (25 mg/kg BW) was injected intravenously and was allowed to circulate for 5 minutes. Once distributed, fluorescence in the preparation was recorded for the next 15 minutes as previously described.[16] Saline superfusion was stopped and administration of cysLTs (LTC_4 and LTD_4, both at 5 μmol/L in 0.9% ethanol vehicle) was performed while monitoring fluorescence.

Dissection of Colon and Quantification of Disease Progression

The colons were dissected free of adipose tissue with the total length being measured, and the contents were gently cleared with saline-wet cotton swabs. Defined colon samples from individual mice were taken for several different analyses, from proximal to distal: i) myeloperoxidase determination, ii) tissue wet versus dry weight, iii) histology, and iv) TNF-α tissue content.

Determination of Colonic Myeloperoxidase Activity

Proximal colon samples devoid of contents and connective tissue were weighed, flash-frozen in liquid nitrogen, and used to determine myeloperoxidase activity by a method previously described.[18]

Colon Wet versus Dry Weight

Measured segments of proximal colons were dissected free of connective tissue, emptied of contents, rinsed, and blot-dried on a light-duty tissue wipe (VWR International, West Chester, PA). The wet weight of each segment was measured and allowed to air dry in a 37°C oven for 4 days after which its dry weight was recorded. The difference between wet and dry weight (g/cm) was taken as a measure of tissue water content, an indirect assessment of possible tissue edema.

Histology

Colons were rinsed in PBS (pH 7.4), opened along the antimesenteric border, and loosely pinned (mucosa side up) onto a small piece of balsa wood. Subsequently, they were fixed in 4% paraformaldehyde/3% sucrose for 3.5 hours at 4°C, rinsed 4 × 5 minutes in PBS, and dehydrated in 30% sucrose at 4°C until tissue was quick-frozen in optimal cutting temperature medium and cut into 10-μm sections. Sections were then stained with H&E and viewed under brightfield using a Leica DM IRB microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada). Measurements of submucosal thickness were made in a blinded manner as to the genotype and treatment (sham versus DSS).

TNF-α Tissue Levels-ELISA

Distal colon sections were used to quantify tissue levels of the pro-inflammatory cytokine, TNF-α. Colon samples were dissected free of surrounding tissue, weighed, sonicated in T-PER tissue protein extraction reagent (Thermo Scientific), vortexed, spun at 2000 rpm for 5 minutes, and the supernatant removed and used for quantification of TNF-α. In brief, a pre-coated enzyme-linked immunosorbent assay (ELISA) kit (cat. # 88-7342-29; eBioscience, San Diego, CA) was used according to the manufacturer's instructions. TNF-α levels were determined from a standard curve then normalized to tissue sample size and expressed as picograms TNF-α/mg colon wet weight.

Assignment of Disease Activity Index

Mice were scored with a disease activity unit scale adapted from previous studies,[19] assessed for each of the following symptoms observed during or at the termination of the DSS-treatment period: diarrhea (1.0), colonic adhesions (1.0), blood in the stool/tissue (1.0), scruffy appearance (1.0), and weight loss (1.0). Therefore, a maximum disease activity index score of 5.0 is possible. Scoring was performed in a blinded manner as to genotype of the mice.

Primary Neuronal Culture and Electrophysiological Experiments

Fast-Blue injection was conducted as previously reported.[20] Briefly, mice were anesthetized with ketamine/xylazine and subjected to laparotomy, the colon exposed, and 1 to 2 μL of the retrograde marker, Fast Blue (1.7% w/v in water), injected in multiple sites of the colon wall. Following the injection, the colon was swabbed to remove excess marker and avoid false labeling. The bowel was placed back into the abdomen, the wound was sutured, and the mice were allowed to recover. Seven days postsurgery the WT or KO mice were anesthetized intraperitoneally with ketamine/xylazine. The spinal column was removed and dorsal root ganglia (DRG) from thoracic vertebrae T9 to T13 were isolated bilaterally and placed into ice-chilled GIBCO Hanks' Balanced Salt Solution (Invitrogen, Burlington, ON, Canada).

DRG were then dissociated at 37°C using two 10 minutes sequential enzymatic treatments. The first treatment contained Papain (69 U, Worthington, Lakewood, NJ; activated with 1 mg L-Cys and 3 μL saturated $NaHCO_3$/1.5 mL GIBCO Hanks' Balanced Salt Solution, Invitrogen) whereas the second treatment contained collagenase and dispase (4 and 4.6 mg/mL GIBCO Hanks' Balanced Salt Solution); both were previously warmed and filter-sterilized. Enzyme solutions were removed and DRG washed once with 2 mL pre-warmed F12 medium containing 10% fetal calf serum, spun for 1 minute at $<$200 g and the supernatant was carefully removed. To the pellet containing the digested DRG, 60 μL of F12 were added. Ganglia were then triturated gently $\sim$10 times through a flame-polished Pasteur pipette, and neurons were placed onto round coverslips pre-coated with sterile Laminin-Poly-D-Lys (VWR Scientific, Mississauga, ON, Canada) in 12-well culture plates. Cells were cultured in F12 medium containing 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), and were maintained at 37°C in a humidified incubator with 5% CO_2 until they were used for electrophysiological experiments (short-term cultures $\sim$24 hours). Neuronal activity was measured by patch clamp in its whole-cell configuration (using amphotericin B, 240 μg/mL in an internal solution to achieve the perforated patch). Patch pipettes were made using thin wall glass-capillaries (Warner Instruments, Harvard Apparatus Canada, St. Laurent, QC, Canada) pulled with a Narishige PP-830 puller, and polished with a Narishige (Tokyo, Japan) MF-830 microforge to render a resistance between 2 to 5 MΩ when immersed in bath solution.

Resting membrane activity was recorded from isolated DRG neurons using a Multiclamp 700B (Axon Instruments, Molecular Devices, Sunnyvale, CA) amplifier and Digidata 1440A AD converter (Molecular Devices). Whole-cell activity was recorded and data were analyzed on a PC using pClamp 10.1 (Molecular Devices), GraphPad Prism 5 (La Jolla, CA), and Corel Draw 11. Neurons showing unstable resting membrane potential or <-40 mV were omitted from this study. The standard solutions that were used had the following compositions pipette solution: 110 mmol/L potassium gluconate, 30 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$; external solution: 140

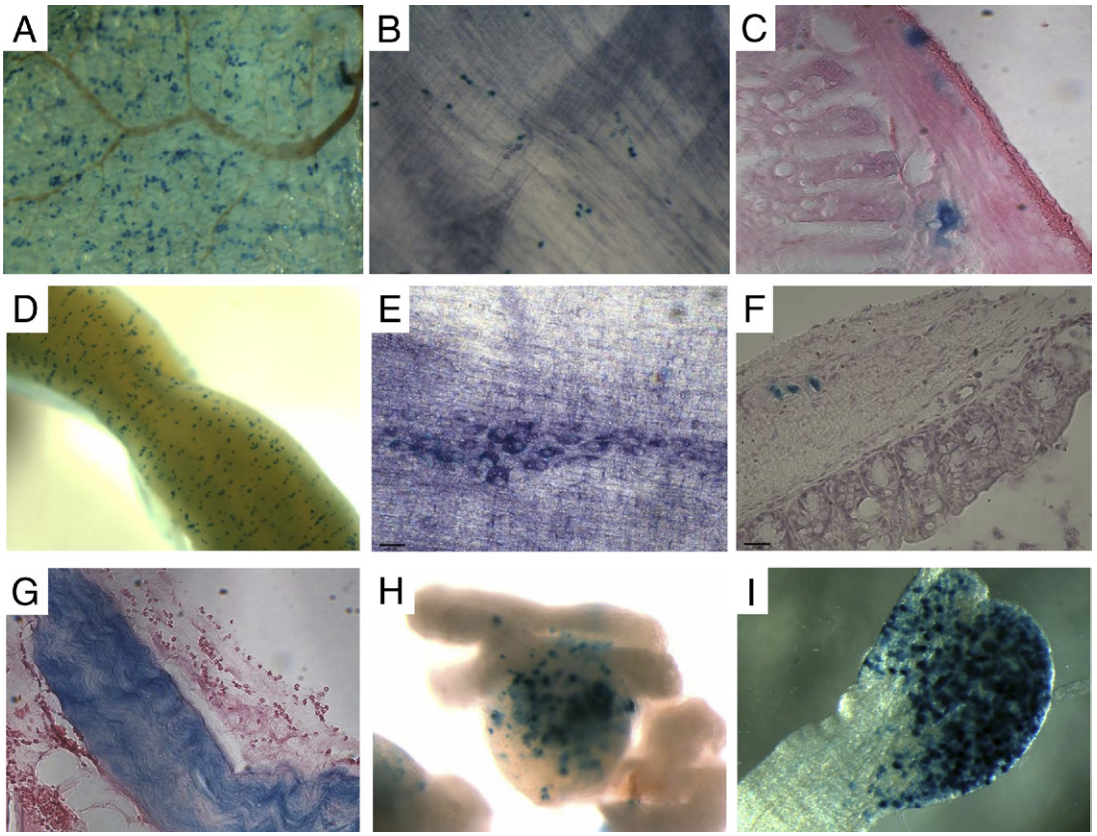

Figure 1. Novel neuronal expression sites for murine CysLT$_2$R. The following tissues were stained with X-Gal (blue). **A:** External small intestine, ×63. **B:** Nitrotetrazolium blue (NTB)-stained small intestine myenteric plexus, ×115. **C:** Modified H&E counterstained small intestine section (10 μm; ×400). **D:** External colon, ×6. **E:** Modified H&E counterstained colon section (10 μm; ×200). **F:** NTB-stained colon myenteric plexus, ×200. **G:** Sectioned vagus nerve, ×50. **H:** Thoracic dorsal root ganglion (representative of staining observed in T9 to T13 ganglia), ×11.5. **I:** Nodose ganglion, ×100.

mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L D-glucose, 1 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$. The pH of solutions was adjusted to 7.25 with 1M potassium hydroxide, KOH (pipette solution) and to 7.3 to 7.4 with 3M sodium hydroxide, NaOH (external solution). The recording chamber was continuously superfused with external solution at a rate of 2 mL/min.

Experiments were performed at room temperature (~23°C). As indicators of neuronal excitability resting membrane potential, rheobase, and action potential number at 2 times rheobase was recorded.

Statistical Analysis

Results are expressed as mean ± SEM. Unpaired *t*-tests were used to calculate differences between treatment groups (DSS versus sham) of mice and genotypes of colons/cells (KO versus WT). *P* values <0.05 were considered statistically significant.

Results

Identification of Novel CysLT$_2$R Expression Sites in the Murine Gastrointestinal Tract

Using a CysLT$_2$R-LacZ mouse strain, in which the reporter enzyme β-galactosidase is used as a surrogate of CysLT$_2$R expression, and by using X-Gal substrate, we have successfully identified the receptor expression in various organs of the GI tract, including the colon and small intestine (Figure 1, A–F). In fact, in the colon and small intestine, the receptor is specifically expressed in neurons of the myenteric plexus (colon) and submucosal plexus (small intestine) as evidenced by co-staining with nitrotetrazolium blue (NTB).[21] Furthermore, neuronal expression sites were also observed in isolated thoracic DRG whose nerve terminals connect to the colon, the vagus nerve, and the nodose ganglion (Figure 1, G-I). In contrast to our previous study in other organs in which the vascular expression was observed,[16] there were no blood vessels that stained for β-galactosidase activity in the colon. No neuronal or vascular staining was apparent in tissues from WT mice (data not shown).

The CysLT$_2$R Knockout Mice Develop Less Severe Colitis

In view of the CysLT$_2$R expression patterns and the role of leukotrienes in inflammation, we sought to examine whether CysLT$_2$R might play a role in IBD. Mice with (WT) or without (KO) CysLT$_2$R expression were subjected to DSS-induced colitis. Genotype did not affect the extent of weight loss (4% to 5% of body weight) resulting from DSS treatment (Figure 2A). However, the

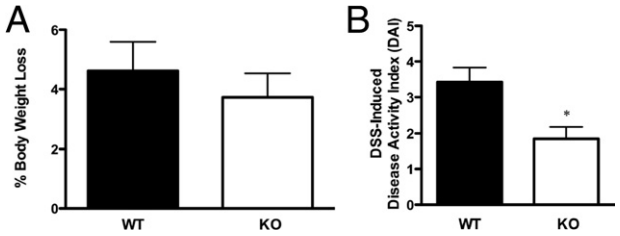

Figure 2. The $CysLT_2R$ knockout mice exhibit attenuated colitis. The 3% dextran sulfate sodium (DSS)-induced body weight loss (**A**) is similar in both knockout (KO) and wild-type (WT) littermates; $n = 16$. Disease activity index (DAI) (**B**) attributing 1.0 point for each of the following: colonic adhesions, diarrhea, blood in the stool, weight loss, and disheveled appearance ($n = 13$). Sham-treatment mice of both genotypes demonstrated none of the previously listed symptoms exhibited by DSS-treated groups (data not shown). $^*P < 0.05$.

severity of colitis was significantly greater in WT mice compared to KO mice (as assessed by the disease activity index, 3.5 ± 0.3 versus 1.8 ± 0.4; $P \leq 0.05$; Figure 2B). WT mice were more likely to develop diarrhea, bloody stool, colonic adhesions, and an overall disheveled appearance after treatment with 3% DSS. Notably, in another set of mice overexpressing the human $CysLT_2R$ in vascular endothelium, these mice were so severely affected by the induced colitis that they were humanely euthanized before the experimental end point was reached, and thus were not included in the study ($n = 4$, results not shown).

Absence of $CysLT_2R$ Results in Significantly Lower TNF-α Production in DSS Colitis

We quantified two hallmark markers of colonic inflammation: leukocyte infiltration, as measured by myeloperoxidase activity assay, and the cytokine TNF-α. Although there was a significant increase in myeloperoxidase activity in all DSS-treated mice, we found no difference in this measured parameter between the two genotypes (Figure 3A). This indicates that at the time of colon processing, $CysLT_2R$ presence did not affect colonic infiltration of proinflammatory leukocytes. However, using a TNF-α tissue content assay, we found that DSS treatment caused a significant twofold increase in TNF-α in WT mice, a response that was virtually absent in KO mice (Figure 3B). Therefore, this implies that the level of inflammation, as measured by TNF-α production, is significantly reduced in the absence of $CysLT_2R$.

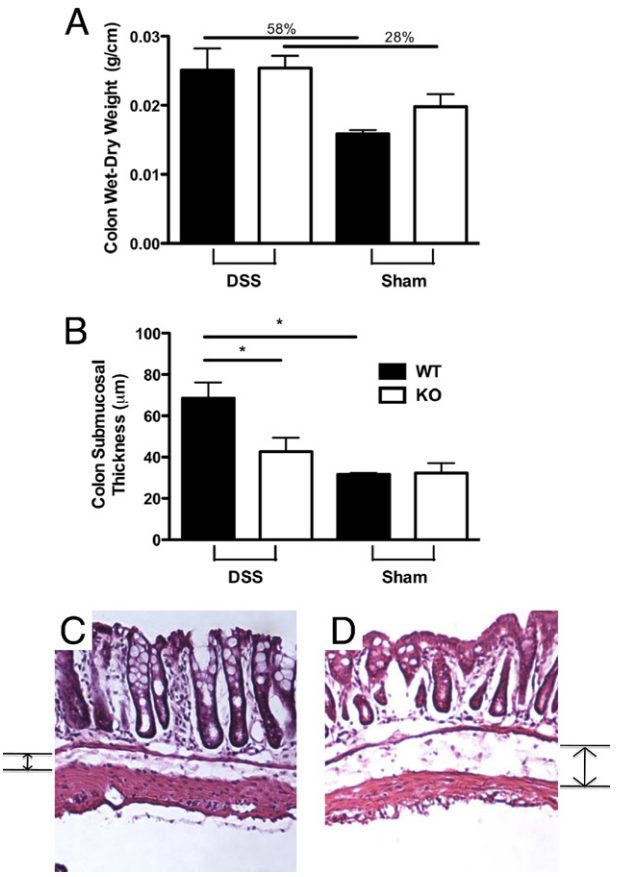

Figure 4. Colonic edema is attenuated in $CysLT_2R$ knockout (KO) mice. Dextran sulfate sodium (DSS) induced an increase in colonic water content (**A**) as measured by the difference between wet and dry tissue weights. The percentage increase of wet-dry weight was enhanced in wild-type (WT) mice (shown in graph as percentage difference) compared to $CysLT_2R$ KO animals. Histological measurement of submucosal thickness (**B**) was significantly augmented in DSS-treated WT mice compared to the KO group ($n = 6$). $^*P < 0.05$. Representative H&E stained KO (**C**) and WT (**D**) 10-μm sections: area denoted by arrows corresponds to submucosa, ×200.

Colon Edema and Submucosal Thickness Are Significantly Reduced in $CysLT_2R$-Deficient Mice

DSS induced an increase in colonic water content (Figure 4A), as measured by differences between wet and dry tissue weights. KO mice demonstrated an increase of 28% compared to the significantly higher 58% seen in WT mice. Furthermore, submucosal thickness, a histological

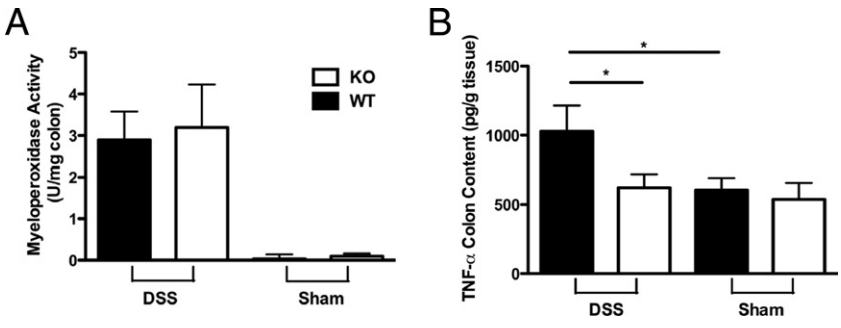

Figure 3. Dextran sulfate sodium (DSS) colitis-induced increase in tumor necrosis factor (TNF)-α levels, but not myeloperoxidase activity, is attenuated in $CysLT_2R$ knockout (KO) mice. **A:** Myeloperoxidase activity within the whole colon was determined using a colorimetric assay. Results are expressed as myeloperoxidase units (1 unit = 1 μmol peroxide degraded/min/mg of wet colon at 25°C ($n = 6$). **B:** TNF-α tissue content as determined via enzyme-linked immunosorbent assay (ELISA). Results are expressed as picogram TNF-α/g of wet colon tissue ($n = 10$). WT, wild type; $^*P < 0.05$.

measure of colonic edema, was significantly greater (almost twofold) in DSS-treated WT mice compared to the KO group (Figure 4, B–D).

Cysteinyl Leukotrienes Stimulate FITC-Albumin Extravasation from Colonic Venules

Mice were intravenously injected with FITC-albumin and following laparotomy, submucosal colonic venules were visualized (Figure 5A). Basal, nonstimulated, extravasation of fluorescence was assessed over a 15-minute period, and no difference was observed between WT and KO littermates (Figure 5B). However, when the tissue was stimulated with a local superfusate of LTC_4/D_4 (5 μmol/L each) and fluorescence was assessed over a 5-minute window, the WT venules were, on average, twice as leaky compared to those of KO mice (Figure 5C).

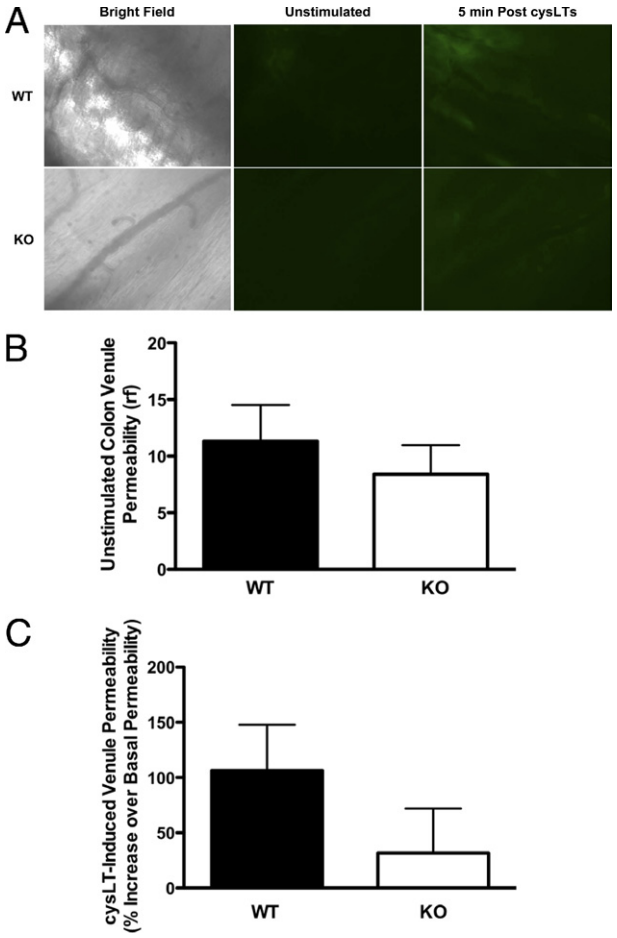

Figure 5. Colonic submucosal venule permeability is lower in CysLT$_2$R knockout (KO) mice. Colonic submucosal venules were viewed via intravital microscopy under brighfield (**A**) before fluorescein isothiocyanate-albumin intravenously injection. Basal, unstimulated, fluorescent leakage from the chosen field of view was assessed over a 15-minute period and graphed as relative fluorescence (rf) units (**B**), as well as over a 5-minute stimulation period with leukotriene C_4/leukotriene D_4 and graphed as a percentage increase over basal venule permeability (**C**); ×250 ($n = 6$). WT, wild type.

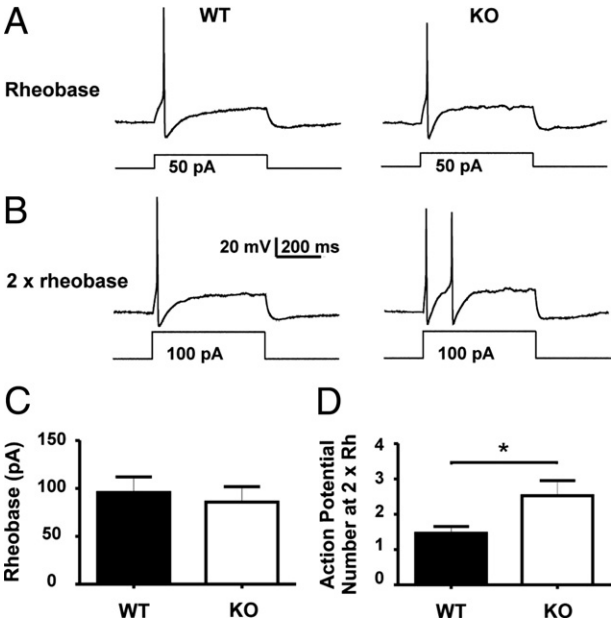

Figure 6. Neuronal activity was assessed in wild-type (WT) and knockout (KO) colon-projecting dorsal root ganglia neurons; these latter cells fired 73% more than WT when stimulated with a pulse equal to twice the rheobase with no difference when stimulated at rheobase. Representative electrophysiological tracings of two neurons are shown (**A** and **B**), and corresponding values are plotted in graphs (**C** and **D**). ($n = 19$). *$P < 0.05$.

Lack of CysLT$_2$R Results in Increased Neuronal Excitation in DRG Neurons

Although the absence of CysLT$_2$R does not alter neuronal membrane potential (−62.6 ± 0.95 mV in WT and −60.6 ± 0.78 mV in KO), neuronal excitation is doubled in KO DRG when the cells are electrically stimulated with a pulse equal to twice the rheobase (Figure 6). These results indicate that CysLT$_2$R may modulate electrical activity of colonic-projecting DRG neurons.

Discussion

IBD patients exhibit an upregulation of enzymes involved in leukotriene synthesis including 5-lipoxygenase, five lipoxygenase-activating protein, and leukotriene A_4 hydrolase[5]; however, the expression level of the key enzyme for cysLT production (LTC_4S) remains unchanged. In the GI tract, cysLTs are produced by mucosal mast cells[22] and potentially by transcellular metabolism of inflammatory cell-donated LTA_4 to endothelial cells and epithelial cells. The cysLTs augment vascular permeability in response to various noxious stimuli,[7,23] and in peritoneal inflammation this vascular leakage has been attributed mainly to CysLT$_1$R,[24] which has also been implicated in the progression of experimental colitis in rats.[8] Conversely, although CysLT$_2$R is upregulated in colonic tumors,[13] there has been little to no elucidation as to its physiological role or cellular localization in the GI tract. Here, in our studies, we have shown for the first time, using CysLT$_2$R-LacZ mice, expression of CysLT$_2$R in neurons of the small intestine, colon, vagus nerve, DRG, and nodose

ganglion with no evidence of expression in the vasculature of the GI tract.

Previously, we mapped $CysLT_2R$ expression to the microvascular endothelium of several organs, including that of the cremaster muscle.[16] In the latter site, the receptor is capable of mediating cysLT-induced vascular permeability via a transendothelial vesicle transport mechanism, thus suggesting its localization to the plasma membrane in this tissue and cell type. In the well-established model of colonic inflammation, DSS-induced colitis, absence of $CysLT_2R$ reduces exogenous cysLT-mediated submucosal venule permeability, as measured by extravasation of FITC-albumin, and significantly reduces submucosal thickness and colon wet-dry weight in DSS-treated mice compared to a sham treatment group. This suggests that $CysLT_2R$ is important in colonic fluid reuptake as its functional absence results in decreased tissue edema, at least during an inflammatory state. This selective effect is likely due to the already augmented leukotriene production seen in colitis,[25] which sensitizes the tissue to leukotriene-mediated effects. Interestingly, infusion of cysLTs also exacerbates ethanol-induced damage to the gastric mucosa, although this effect does not appear to be due to an increase in vascular permeability.[23] The mechanistic relationship between $CysLT_2R$ and colonic edema observed in DSS colitis has not been established here, but there are a number of potential avenues remaining to be explored. For instance, given that $CysLT_2R$ is not expressed in gut endothelial microvasculature, the permeability-promoting mechanism likely differs from the transcytotic mechanism seen in cremaster vasculature. Moreover, because $CysLT_2R$ is expressed in myenteric plexus neurons, it is possible that activation of these circuits indirectly modulates the release of factors influencing vascular permeability (eg, vascular endothelial growth factor). Future experiments confirming the subcellular compartmentalization of the $CysLT_2R$ in these neurons could help elucidate a mechanism of action. It is important to note that vascular permeability is further augmented in transgenic mice overexpressing human $CysLT_2R$ in the vascular endothelium[26] resulting in DSS colitis of such severity that the animals reached humane endpoints before the termination of the study. This increase in vessel permeability and exacerbation of tissue damage is in accordance with the observed effects of applying proinflammatory stimuli *in vivo* to either the cremaster muscle[16] or during cardiac ischemia/reperfusion.[10]

Although we did not see an effect on colitis-induced body weight loss, mice lacking $CysLT_2R$ demonstrated fewer symptoms (i.e., diarrhea, colonic adhesions, blood in stool/tissue, and dehydration/lack of grooming) than their WT littermates. Whether these effects are secondary to differences in vascular permeability remains to be determined. Interestingly, pharmaceutical blockade of $CysLT_1R$ with montelukast has previously been shown to reduce DSS-induced fecal occult blood, although it also prevented body weight loss.[8] However, the efficiency of $CysLT_1R$ blockade in attenuating colitis severity appears to be dependent on both the antagonist used and the method of colitis induction.[27] Synergism of potential beneficial effects of $CysLT_1R$ and $CysLT_2R$ pharmacological or genetic blockade on colitis would be worth exploring in future experiments.

Apart from affecting vascular permeability, the absence of $CysLT_2R$ leads to a dramatic attenuation of TNF-α levels in DSS-induced colitis without altering neutrophil influx (as measured by myeloperoxidase activity). Cytokines, interferon-γ in eosinophils,[28] IL-4 in mast cells,[29] and TNF-α in intestinal epithelial cells[30] are all known to upregulate $CysLT_2R$ transcription, thereby suggesting that positive feedback mechanisms may play a role in the cytokine-leukotriene receptor relationship. For instance, in IL-4-primed mast cells, cysLTs are able to elicit a greater response, resulting in augmented TNF-α production[31] via a $CysLT_1R$-dependent mechanism. Our study demonstrates that $CysLT_2R$ expression may also be involved in a putative positive feedback relationship, as its absence correlates with significantly lower DSS-induced TNF-α content in colon tissue. Whether $CysLT_2R$ activation and TNF-α expression directly or indirectly regulate one another and the nature of any interconnection remains to be determined.

Another interesting finding of our studies is that $CysLT_2R$ KO primary cultured DRG neurons appear to be hyperexcitable when stimulated with a suprathreshold pulse. In contrast, others have shown that pharmacological stimulation (with leukotrienes) of enteric small intestinal neurons has excitatory actions that are unaffected by dual $CysLT_1R$/$CysLT_2R$ antagonism with the compound Bay-u9773.[32] These seemingly contradictory findings raise many questions. Is the observed hyperexcitability in DRG nociceptive neurons due to a lack of functional $CysLT_2R$ or to its physical absence in $CysLT_2R$-LacZ KO mice? Is the lack of $CysLT_2R$ activity/expression promoting compensatory activity in other leukotriene receptors or other pathways that are known to be involved in the sensitization of nociceptors?[33] Interestingly, the B leukotriene receptor subtype 2 (BLT_2) receptor was recently found to be protective in DSS-induced colitis progression.[34] How does this alteration of DRG nociceptive neuron excitability correlate with the changes in vascular permeability? It is also plausible that the $CysLT_2R$ has different roles in the two GI neuronal subpopulations: DRG versus myenteric plexus. Previous studies have demonstrated that cysteinyl leukotrienes promote gut motility[35] and pharmacological inhibition of the $CysLT_1R$ alleviates indomethacin-induced gastrointestinal hypermotility.[36] However, although leukotrienes might evoke similar responses in two neuronal subpopulations within the same plexus, the effector response can be completely different depending on the type of neuron (Dogiel type I excitatory motor neuron versus type II interneurons) and the total number of neurons being stimulated.[32] Future research in our laboratory and others will seek to address answers to these important questions.

In summary, we demonstrated that $CysLT_2R$ is involved in the DSS-induced progression of colitis, particularly by increasing colonic edema, submucosal venule permeability, and TNF-α production, and by potentially altering DRG neuron excitability.

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