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Targeted Inhibition of Serotonin Type 7 (5-HT\textsubscript{7}) Receptor Function Modulates Immune Responses and Reduces the Severity of Intestinal Inflammation

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Mucosal inflammation in conditions ranging from infective acute enteritis or colitis to inflammatory bowel disease is accompanied by alteration in serotonin (5-hydroxytryptamine [5-HT]) content in the gut. Recently, we have identified an important role of 5-HT in the pathogenesis of experimental colitis. 5-HT type 7 (5-HT\textsubscript{7}) receptor is one of the most recently identified members of the 5-HT receptor family, and dendritic cells express this receptor. In this study, we investigated the effect of blocking 5-HT\textsubscript{7} receptor signaling in experimental colitis with a view to develop an improved therapeutic strategy in intestinal inflammatory disorders. Colitis was induced with dextran sulfate sodium (DSS) or dinitrobenzene sulfonic acid (DNBS) in mice treated with selective 5-HT\textsubscript{7} receptor antagonist SB-269970, as well as in mice lacking 5-HT\textsubscript{7} receptor (5-HT\textsubscript{7}\textsuperscript{−/−}) and irradiated wild-type mice reconstituted with bone marrow cells harvested from 5-HT\textsubscript{7}\textsuperscript{−/−} mice. Inhibition of 5-HT\textsubscript{7} receptor signaling with SB-269970 ameliorated both acute and chronic colitis induced by DSS. Treatment with SB-269970 resulted in lower clinical disease, histological damage, and proinflammatory cytokine levels compared with vehicle-treated mice post-DSS. Colitis severity was significantly lower in 5-HT\textsubscript{7}\textsuperscript{−/−} mice and in mice reconstituted with bone marrow cells from 5-HT\textsubscript{7}\textsuperscript{−/−} mice compared with control mice after DSS colitis. 5-HT\textsubscript{7}\textsuperscript{−/−} mice also had significantly reduced DNBS-induced colitis. These observations provide us with novel information on the critical role of the 5-HT\textsubscript{7} receptor in immune response and inflammation in the gut, and highlight the potential benefit of targeting this receptor to alleviate the severity of intestinal inflammatory disorders such as inflammatory bowel disease. The Journal of Immunology, 2013, 190: 4795–4804.

The gastrointestinal (GI) tract is the largest producer of serotonin (5-hydroxytryptamine [5-HT]) in the body. About 95% of the body’s 5-HT is located in the GI tract, and enterochromaffin (EC) cells are its main source (1, 2). 5-HT is also found in enteric neurons, but the 5-HT amount present in enteric neurons appears to be very small in comparison with that present in EC cells (~90% of 5-HT is found in EC cells and 10% in enteric neurons) (3). Tryptophan hydroxylase (TPH) catalyzes the rate-limiting step in the synthesis of 5-HT from tryptophan and has been detected prominently in EC cells (4). Recent studies have shown that there are two isoforms of TPH enzymes regulating the 5-HT system. TPH1 is mainly present in EC cells and the spleen, whereas TPH2 predominates in the brainstem and enteric neurons (5, 6). Thus, 5-HT seems to be synthesized independently in EC cells and neurons by two different rate-limiting TPH iso-enzymes. 5-HT is an important enteric mucosal signaling molecule involved in maintaining intestinal homeostasis, and alterations in 5-HT signaling are observed in various GI disorders including intestinal inflammatory conditions (7–9).

Inflammatory bowel disease (IBD) is the most common and chronic inflammatory condition of the human bowel, currently affecting ~1–2 million people in the United States and Canada (10). The incidence and prevalence of human bowel diseases such as IBD have shown a dramatic increase in the last decades (11, 12). Although the exact cause of IBD is unknown, studies have provided evidence that a dysregulated immune response, genetic factors, enteric flora, and environmental factors contribute to the pathogenesis of IBD (13–16). Mucosal changes in IBD are characterized by ulcerative lesions accompanied by a prominent infiltration of activated cells from both the innate and adaptive immune systems. In addition to immune cells, inflammation in the intestine is associated with an alteration in the number of 5-HT–producing EC cells. Changes in the number of EC cells and 5-HT content have been observed in patients with IBD (8, 9, 17–19) and also in experimental colitis (20–22). EC cells are located in very close proximity to or in contact with key immune cells (23); thus, it is very likely that 5-HT plays an important role in immune activation and generation of gut inflammation in various GI disorders including IBD. Recently, we have shown that TPH1-deficient (TPH\textsuperscript{−/−}) mice, which have significantly lower amounts of 5-HT in the gut,
exhibit reduced severity of colitis in two different models of experimental colitis (induced by dextran sulfate sodium [DSS] and dinitrobenzene sulfonic acid [DNBS]), whereas replenishing 5-HT levels upregulated colitis severity (24). A proinflammatory role for 5-HT has also been demonstrated in other studies where chemical-induced colitis or spontaneous colitis associated with an IL-10 deficiency is increased in severity when coupled with the 5-HT–enhancing effects of a knockout of the serotonin reuptake transporter. SERT (25, 26). We have also shown that 5-HT plays a key role in the activation of immune cells to produce proinflammatory cytokines (24, 27). Taken together, these findings suggest 5-HT as an important molecule in the activation of immune cells and in the pathogenesis of colitis.

In recent years, significant progress has been made in understanding the pathogenesis of IBD leading to improved strategies to control inflammation through the use of immunosuppressive drugs and the Ab targeting of TNF-α. However, treatment with these drugs may cause many adverse effects such as toxicity in the case of immunosuppressive agents, acute infusion reactions, and the development of Abs to the anti–TNF-α Abs (28–31). These disadvantages with immunosuppressive drugs or targeting just one proinflammatory cytokine trigger thoughts of development of better therapeutic strategies in IBD by identifying new targets.

Dendritic cells (DCs) play a critical role in orchestrating the primary immune response and perform a key role in the generation of intestinal inflammation (32, 33). Studies have revealed the expression of several 5-HT receptors on DCs, and functional studies have shown that 5-HT alters the cytokine profile of human DCs, enhancing the release of cytokines IL-12 (29). Because DCs express 5-HT receptors, it is reasonable to suppose that if 5-HT signaling is inhibited by blocking specific 5-HT receptor function on DCs, there will be attenuation in immune cell activation and in subsequent inflammation. The 5-HT type 7 (5-HT7) receptor is one of the most recently identified members of the 5-HT receptor family, and DCs express this receptor (34–36).

In this study, we investigated 5-HT7 receptor expression on colonic DCs and the effect of blocking 5-HT signaling in colitis by using antagonists against the 5-HT7 receptor and 5-HT7 receptor–deficient mice with a view to develop an improved therapeutic strategy against intestinal inflammatory disorders. Our study revealed a critical role for the 5-HT7 receptor in the pathogenesis of colitis and demonstrated that inhibition of 5-HT signaling by blocking 5-HT7 receptor function with a pharmacological agent or by targeted disruption of this receptor alleviated inflammation in DSS- and DNBS-induced colitis.

Materials and Methods

Animals

C57BL/6 mice (Taconic) were kept in sterilized, filter-topped cages under specific pathogen-free conditions. Male mice aged 8–10 wk were used for all experiments. Breeding pairs of 5-HT7−/− mice on C57BL/6 background, originally generated as described by Hedlund et al. (37), were kindly provided by Peter B. Hedlund (The Scripps Research Institute, La Jolla, CA). These mice were viable and showed no observed difference in food intake or body weight compared with wild-type (WT) mice. All experiments were approved by the animal ethics committee of McMaster University and conducted under the Canadian guidelines for animal research.

Induction of DSS and DNBS colitis

DSS (molecular mass 40 kDa; ICN; Biomedicals Incorporate, Solon, OH) was added to drinking water for a final concentration of 5% (w/v) for a total of 5 d. For DNBS-induced colitis, mice were anesthetized with isoflurane (Abbott, Toronto, ON, Canada), and a 10-cm-long tubing attached to a tuberculin syringe was intrarectally inserted 3.5 cm into the colon; 100 μl of 5 mg DNBS solution (ICN; Biomedicals) dissolved in 50% ethanol was administered and left for 3 d to induce colitis. Controls received only 50% ethanol for the same time span. Mice given DNBS were supplied with 6% sucrose in drinking water to prevent dehydration. Chronic DSS-induced colitis was induced by adding DSS to drinking water to a final concentration of 5% (w/v) for 5 d followed by 11 d of water. This cycle was repeated twice with 3% DSS.

Drugs

SB-269970 ((2R)-1-[(3-hydroxyphenyl)sulfonyl]yl-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidinehydrochloride) and 5-carboxamidotryptamine maleate (5-CT) were purchased from ToCris Biosciences (Burlington, ON, Canada) and were dissolved in distilled water.

Experimental protocol

For acute colitis, mice were treated with SB-269970 (ToCris Biosciences) or vehicle (saline) in a dosing volume of 20, 40, or 80 mg/kg via i.p. administration (38, 39) for 6 d starting 1 d before DSS exposure and sacrificed on day 5 after DSS. In a separate experiment, colitis was induced in WT (5-HT7+/+) and 5-HT7−/− mice by DSS or DNBS and sacrificed on day 5 after DSS and day 3 after DNBS to assess colitis severity. For chronic colitis, mice were treated with vehicle (saline) or SB-269970 (40 mg/kg i.p.) for 6 d starting 1 d before the beginning of the third DSS cycle. Control mice received saline and water without DSS.

Assessment of colitis severity

Disease activity index (DAI) is a combined score of weight loss, stool consistency, and fecal bleeding, and was blindly assessed using a previously published scoring system (40). This scoring system was defined as follows: weight loss: 0 = no loss, 1 = 1–5%, 2 = 5–10%, 3 = 10–20%, 4 = 20%; stool: 0 = normal, 2 = loose stool, 4 = diarrhea; and bleeding: 0 = no blood, 2 = Hemoccult positive (Hemoccult II; Beckman Coulter, Fullerton, CA), and 4 = gross blood (blood around anus). DAI was measured on all 5 d of DSS treatment. Macroscopic damage scores were blindly scored using a previously published scoring system for DSS (40, 41) and DNBS-induced colitis (42). Formalin-fixed colon segments were paraffin embedded, and 4-μm sections were stained with H&E to assess histological damage. Colonic damage was blindly scored using a previously published scoring system that considers changes in crypt architecture, cellular infiltration, goblet cell depletion, and crypt abscess (41, 43).

Colonic myeloperoxidase activity. Colonic myeloperoxidase (MPO) activity was measured following a published protocol (44). In brief, colonic tissue samples were homogenized in ice-cold 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma). Homogenates were centrifuged and the supernatant was removed, and an aliquot was then added to a solution containing potassium phosphate buffer, O-dianisidine (Sigma), and hydrogen peroxide. The absorbance was measured at 450 nm by a spectrophotometer (model EL808, BioTek). MPO activity was expressed in units per milligram of wet tissue, where 1 U is the quantity of enzyme able to convert 1 μmol hydrogen peroxide to water in 1 min at room temperature.

Colonic tissue cytokine levels. Colon samples were homogenized in TBS containing a protease inhibitor mixture (Sigma). Samples were then centrifuged and the supernatant was frozen at −80°C. Cytokine levels were determined using a commercially available ELISA kit according to the manufacturer’s instructions (Quantikine Murine; R&D Systems, Minneapolis, MN).

Lamina propria CD11c+ DCs isolation and stimulation

Colonic lamina propria (LP) cells were isolated using a previously described method (45) with some modification. In brief, colon tissue from naïve C57BL/6 mice was washed with Ca2+ and Mg2+ free HBSS (Intragen), epithelial cells were dissociated with 5 mM EDTA for 20 min at room temperature, and remaining tissue was digested with 0.25 mg/ml Collagenase IX (Sigma-Aldrich), 0.5 mg/ml Dispase 1 (Roche Diagnostics), and 2μm DNase I (Sigma-Aldrich) in HBSS with 1% FCS for 20 min at 37°C. Digested tissue was filtered through a 40-μm cell strainer (BD Biosciences) and stained for flow cytometric analysis. Alternatively, for in vitro LP DC stimulation, intestine was subjected to the same treatment mentioned earlier and further processed for density gradient centrifugation. Filtered cells were resuspended in HBSS + 2 mM EDTA, layered on Percoll gradient (50 and 25%; density, 1.07 and 1.03 g/ml, respectively), and centrifuged at 1000 g for 40 min at 4°C. Remaining cells were resuspended in HBSS + 2 mM EDTA, layered on Percoll gradient (50 and 25%; density, 1.07 and 1.03 g/ml, respectively), and centrifuged at 1000 × g for 20 min at 4°C. The interface containing mainly mononuclear cells was then pelleted and resuspended in HBSS + 2 mM EDTA and 0.5% BSA. LP DCs were further enriched by positive selection of CD11c+ cells by EasySep mouse CD11c+ selection kit (STEMCELL Technologies, Vancouver, BC, Canada). Purity was routinely >90% using this kit. Purified LP
CD11c+ cells (1 × 10⁵) were resuspended in 200 μl RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 0.1% 2-ME, and 1 mM sodium-pyruvate, and cultured in a CO₂ incubator for 4 h at 37°C in wells of a flat-bottom 96-well plate (BD Biosciences, Mississauga, CA). After 4 h, adherent cells were stimulated for 24 h in triplicate with 5-HT (10⁻⁵ M) and LPS (100 ng/ml) in 200 μl medium as described in the figure legends.

Isolation of DCs from spleens and culture

Mice were sacrificed by cervical dislocation, and spleens were excised, minced, and digested for 30 min at room temperature in Spleen Medium (STEMCELL Technologies). The resulting cell suspensions were strained through a 70-μm nylon mesh filter (BD Falcon) and washed with PBS supplemented with 2% FBS and 1 mM EDTA. Spleen CD11c+ cells were isolated by positive selection using EasySep mouse CD11c+ selection kit (STEMCELL Technologies) according to manufacturer’s guidelines. DCs (10⁶/ml) isolated using CD11c+ selection were incubated for 24 h at 37°C with or without LPS (100 ng/ml; Sigma-Aldrich) in the presence or absence of 5-HT at varying doses (10⁻⁷ and 10⁻⁵ M; Alfa Aesar) and/or NF-kB inhibitor, pyrroline dithiocarbamate (PTTC; 10 μM/L; Sigma), in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (Invitrogen Life Technologies). In a separate experiment, isolated CD11c+ cells were stimulated for 24 h with or without LPS (100 ng/ml) in the presence or absence of 5-CT (at 10⁻⁷ or 10⁻⁵ M). Cells were preincubated with SB 269970 (5 ng/ml) as described in the figure legends. Supernatants were collected after 24 h and analyzed for cytokine levels using commercially available ELISA kits (R&D Systems).

Irradiation and bone marrow transplantation

C57BL/6 mice were irradiated with two doses of 5.5 Gy 48 h apart administered via a [³²⁰Cs] gamma irradiation source (Gamma Cell 40; Nordian, Kanata, ON, Canada). Bone marrow cells (BMCS; no fewer than 5 × 10⁶) were harvested from femurs and tibiae of donor mice (5-HT7+/+) and/or NF-κB inhibitor, pyrroline dithiocarbamate (PTTC; 10 μM/L; Sigma), in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (Invitrogen Life Technologies). In a separate experiment, isolated CD11c+ cells were stimulated for 24 h with or without LPS (100 ng/ml) in the presence or absence of 5-CT (at 10⁻⁷ or 10⁻⁵ M). Cells were preincubated with SB 269970 (5 ng/ml) as described in the figure legends. Supernatants were collected after 24 h and analyzed for cytokine levels using commercially available ELISA kits (R&D Systems).

Flow cytometry

For flow cytometry analysis of LP DCs, 100 μl LP cells were preincubated with anti-CD16/32 mAb (FcR block; 2.4G2) for 15 min at 4°C to block nonspecific binding of Abs, then stained for 20 min at 4°C with either matched Ab controls, secondary controls, or with FITC-conjugated anti-CD103 mAb (M290), PE-conjugated anti-CD11c mAb (HL-3), and unlabeled rabbit anti-mouse 5-HT7 receptor Ab (ab13898; Abcam) that recognizes aa 13–28 of the extracellular domain of the 5-HT7 receptor. The unlabeled 5-HT7 receptor Ab was detected by allophycocyanin-labeled anti-rabbit IgG Ab (ab130805) for 20 min at 4°C. It should be noted that given the lack of availability of a suitable monoclonal 5-HT7 Ab to be used for flow cytometry, a polyclonal Ab was used for our purposes. Flow cytometry was performed on FACSCanto (BD Biosciences), and data were analyzed on FlowJo software (Tree Star, Ashland, OR). Splenocyte preparations were surface stained with various Abs (anti-CD11b [clone M1/70], anti-CD11c [clone HL-3], anti-CD80-PerCP-Cy5.5, anti-CD86 [clone GL1], anti-MHC class II [clone 25-9-17], and anti-CD40 [clone 32/23]) and used for flow cytometry assays. All Abs were purchased from BD Biosciences. Data were acquired using a FACSCanto flow cytometer with FACSDiva 5.0.2 software (BD Pharmingen) and analyzed with FlowJo Mac version 6.3.4 software (Tree Star, Ashland, OR).

RNA isolation and quantitative RT-PCR

Real-time PCR was used to quantify transcripts encoding the 5-HT7 receptor in leukocytes isolated from irradiated mice that received bone marrow transplantations from either WT (5-HT7+/+) or 5-HT7-deficient mice (5-HT7−/−). Total RNA and cDNA of each sample was prepared from equal volumes of blood samples using the leukocyte RNA purification kit (Norgen Biotek, St. Catharines, ON, Canada) and the qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD). Following RNA isolation, cDNA was synthesized from 1 μg of total RNA using the iScript cDNA Supermix kit (Bio-Rad). The qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD) was used to perform reverse transcription. The 5-HT7 receptor was amplified using primer pairs: forward 5’-CCCTGCTGAGCGTTCATC-3’ and reverse 5’-GCCAGGCGAGGGGACACT-3’.

Tail vein bleeding model

Tail vein bleeding tests were performed on C57BL/6 mice following a published method (46). In brief, mice were anesthetized with isoflurane (Abbott), and the tail tip (0.7–1.0 cm) was incised using a fresh scalpel. Shed blood was collected onto Whatman #1 filter paper discs every 30 s for 15 min. Tail bleeding times were recorded as the time that it took for mice to stop bleeding. Shed blood collected on filter papers was eluted in water and quantified using OD₄₅₀ and OD₆₅₀ and a standard curve as previously described (46).

Statistical analysis

All data were presented as means ± SEM. Unpaired t test or one-way ANOVA followed by Newman–Keuls multiple comparisons post hoc test or Mann–Whitney U test was performed using GraphPad Prism version 6.0b for Mac OS X (GraphPad Software, La Jolla, CA). An associated p value <0.05 was considered statistically significant.

Results

LP DCs express 5-HT7 receptor

Expression of the 5-HT7 receptor was analyzed by flow cytometry in LP DCs. Colonic LP cells were isolated from naive C57BL/6 mice and stained with mAb for CD11c, CD103, as well as a 5-HT7 receptor Ab (Fig. 1). CD103⁺CD11c⁺ LP DC cells are positive for 5-HT7 receptor staining (Fig. 1). In contrast, CD103⁻CD11c⁺ cells appear negative for 5-HT7 receptor staining. Spleen CD103⁺CD11c⁺ DCs were also positive for 5-HT7 receptor expression (data not shown).

Administration of 5-HT7 receptor antagonist delays onset and decreases the severity of DSS-induced colitis

Colonic 5-HT7 receptor expression is significantly increased in WT mice on day 5 after DSS as compared with control mice without DSS (Supplemental Fig. 1). Treatment of colitic mice with selective 5-HT7 receptor antagonist SB-269970 (40 mg/kg) resulted in significantly lower clinical disease activity scores (based on weight loss, fecal blood, and consistency) on days 4 and 5 after DSS administration (Fig. 2A). We also observed significantly lower macroscopic scores (based on fecal blood and consistency, rectal bleeding, and erythema) in SB-269970-treated mice as compared with vehicle-
treated mice after induction of DSS colitis (Fig. 2B). H&E-stained colon sections showed increased leukocyte infiltration, loss of goblet cells, and distortion of epithelial cell architecture, as well as thickening of the muscularis mucosa layer in vehicle-treated mice after DSS administration. In mice that received SB-269970, histological damage scores were significantly less severe compared with control mice on day 5 after DSS induction (Fig. 2C, 2D). This decrease in colitis severity was associated with significantly lower colonic MPO activity and lower levels of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) levels. Data are from one representative experiment of two performed. Values shown are mean ± SEM; n > 6 mice/group. ∗p < 0.05.

There was no significant difference in colonic 5-HT levels between groups (4.03 ± 0.26 and 4.57 ± 0.31 ng/mg tissue in WT and 5-HT7−/− mice post-DSS, respectively). There was also no difference in food intake between groups throughout the duration of DSS administration (3.16 ± 0.19 and 3.48 ± 0.25 g/day/mouse for WT and 5-HT7−/− mice, respectively). The macroscopic score was 4.12 ± 0.35 compared with 4.45 ± 0.39 in 5-HT7−/− and WT mice, respectively (p > 0.05). Severity of colitis was significantly less in 5-HT7−/− mice after DSS as compared with the 5-HT7+/+ mice (Fig. 4A, 4B). There were also significantly lower MPO activity along with decreased IL-1β, TNF-α, and IL-6 levels in 5-HT7−/− mice as compared with 5-HT7+/+ mice (Fig. 4C).

Targeted disruption of 5-HT7 receptor decreases the severity of DSS-induced colitis

To further elucidate the role of the 5-HT7 receptor in the generation of gut inflammation, we evaluated mice deficient in this receptor (5-HT7−/−) and WT (5-HT7+/+) mice after DSS-induced colitis. To investigate whether 5-HT can modulate cytokine release in DCs in gut inflammation by acting through the 5-HT7 receptor, CD11c+ DCs were isolated from the spleen of DSS-treated WT (5-HT7−/+) and 5-HT7−/− mice, and stimulated with or without LPS. Supernatant was collected 24 h after stimulation to assess cytokine levels. CD11c+ DCs isolated from 5-HT7−/− mice after DSS produced lower levels of IL-12p40, IL-1β, and IL-6 when stimulated with LPS as compared with DCs isolated from WT (Fig. 5A).

To gain further mechanistic insight on the regulation of DC function by the 5-HT7 receptor, CD11c+ DCs were isolated from
WT mice and stimulated with 5-HT or 5-HT\textsubscript{7} receptor agonist 5-CT in the presence or absence of 5-HT\textsubscript{7} receptor antagonist SB-269970. Stimulation with 5-HT elicited an increase in IL-12p40 release in LPS-matured CD11c\textsuperscript{+} DCs (Fig. 5B). In addition, 5-HT also increased secretion of IL-12p40 in immature (non-LPS–treated) DCs (501 ± 6.60 and 541 ± 17.3 pg/ml in DCs stimulated in the absence or presence of 5-HT [10\textsuperscript{−7} M], respectively). Coincubation with SB-269970 abolished the 5-HT–induced modulation of IL-12p40 release in mature DCs, suggesting that this effect was mediated by the 5-HT\textsubscript{7} receptor. Added together with PDTC, a potent inhibitor of NF-κB, IL-12p40 release was significantly lowered (Fig. 5B). No significant effect of SB-269970 on IL-12p40 release was seen in immature DCs (541 ± 17.3 and 558 ± 6.67 pg/ml in DCs stimulated with 5-HT in the absence or presence of SB-269970, respectively). Stimulation with 5-CT induced secretion of IL-12p40 in non-LPS–stimulated DCs in a non–dose-dependent manner (123 ± 7.62 versus 395 ± 14.7 and 370 ± 16.8 pg/ml in nonstimulated DCs and DCs stimulated with 5-CT at concentrations of 10\textsuperscript{−7} and 10\textsuperscript{−5} M, respectively). 5-HT and 5-CT also increased IL-1β and IL-6 release from mature, but not immature, DCs (Supplemental Fig. 3A–D). Stimulation with 5-HT or 5-CT did not elicit an increase in IL-12p40 secretion in LPS-matured CD11c\textsuperscript{+} DCs isolated from 5-HT\textsuperscript{7}/− mice (Supplemental Fig. 3E).

To investigate the effect of 5-HT stimulation on CD11c\textsuperscript{+} LP DCs, we incubated freshly isolated LP CD11c\textsuperscript{+} cells overnight at 37°C with or without exposure to LPS and/or stimulation with 5-HT (10\textsuperscript{−7} M). In all conditions, IL-12p40 levels were below the detectable range (data not shown), whereas incubation with LPS slightly enhanced the production of TNF-α and IL-6 by CD11c\textsuperscript{+} LP DCs (Supplemental Fig. 3F). These results agree with previous work done by Takenaka et al. (47) that showed that colonic DCs are less responsive to stimulation with LPS.

Targeted disruption of 5-HT\textsubscript{7} receptor decreases the severity of DNBS-induced colitis

To determine whether the aforementioned changes were specific only to the DSS model of colitis, we used the DNBS model of experimental colitis in WT and 5-HT\textsuperscript{7}/− mice. In WT, DNBS exposure caused significant thickening of the colonic wall, hy-
peremia, observable adhesion between the colon and surrounding tissue, and in some cases, ulcerations. H&E-stained colon sections of WT mice given DNBS showed increased cellular infiltration, loss of goblet cells, and severe mucosal damage. 5-HT\textsuperscript{7−/−} mice had significantly reduced colitis severity and less severe histological scores (less mucosal damage, less cellular infiltration, and goblet cell depletion; Fig. 6A–C). Reduction in colitis severity in 5-HT\textsuperscript{7−/−} mice given DNBS was associated with reduced MPO activity and lower IL-1\textbeta levels compared with control mice (Fig. 6D, 6E).

To further confirm the role of the 5-HT\textsubscript{7} receptor in immune cell activation and in generation of inflammation, we reconstituted lethally irradiated WT mice with BMCs harvested from WT (WT-WT) or 5-HT\textsuperscript{7−/−} mice (5-HT\textsuperscript{7−/−}-WT) via tail vein injections (Fig. 7A). Lymphocyte depletion in BMC preparations was confirmed before injections by flow cytometry. At 8 wk after reconstitution, mice were given 5% DSS ad libitum for 5 d. Mice reconstituted with BMCs harvested from 5-HT\textsuperscript{7−/−} mice (5-HT\textsuperscript{7−/−}-WT) had delayed onset of loose stools, rectal bleeding, and weight loss compared with mice reconstituted with BMCs harvested from WT mice (WT-WT; Fig. 7B). Disease severity and histological scores were significantly lower in mice reconstituted with BMC from 5-HT\textsuperscript{7−/−} mice (5-HT\textsuperscript{7−/−}-WT) after DSS administration (Fig. 7C–E), and this was associated with reduced MPO activity and lower levels of proinflammatory cytokines (Table I). Significant reduction of 5-HT\textsubscript{7} receptor expression in mice reconstituted with 5-HT\textsuperscript{7−/−} BMCs (5-HT\textsuperscript{7−/−}-WT) was verified by leukocyte RNA extraction and analysis by real-time PCR (relative fold expression of 5-HT\textsubscript{7} receptor in irradiated WT mice

**FIGURE 4.** Effect of the lack of 5-HT\textsubscript{7} receptor expression on development of DSS-induced colitis. WT (5-HT\textsubscript{7}+/+) and 5-HT\textsubscript{7−/−} mice were given 5% DSS solution in drinking water to induce colitis. Control mice received water without DSS. (A) DAI was scored daily. (B) Histological scores and representative micrographs of H&E-stained colon sections from WT and 5-HT\textsubscript{7−/−} mice 5 d after DSS. Scale bars, 100 μm. (C) Colonic tissue samples were collected to assess MPO activity and IL-1\textbeta, TNF-α, and IL-6 levels. Data are from one representative experiment of two performed. Values are shown as mean ± SEM; n = 10 mice/group for DSS, n = 3–5 for water-treated groups. *p < 0.05.

**FIGURE 5.** Effect of blocking 5-HT\textsubscript{7} receptor function on cytokine secretion by DCs. (A) DCs isolated from WT (5-HT\textsubscript{7}+/+) and 5-HT\textsubscript{7−/−} mice after DSS were cultured for 24 h with or without LPS. Supernatants were collected and cytokine levels were measured using ELISA. (B) DCs isolated from colitic WT (5-HT\textsubscript{7}+/+) mice were incubated with SB-269970 and stimulated with LPS in the presence of 5-HT (10\textsuperscript{−7} M) with or without PDTC (NF-κB inhibitor). Supernatants were collected 24 h after stimulation, and IL-12p40 levels were measured by ELISA. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
colon sections from WT and 5-HT7 receptor-deficient (5-HT7−/−) mice were blindly assessed. The effects of 5-HT3 receptor antagonists (49, 50) and 5-HT3 receptors, thereby attenuating inflammation (52). More recently, the effect of 5-HT4 receptor agonist (cisapride) was evaluated in IBD, although no significant difference between agonist-treated and nontreated groups was observed (51).

The 5-HT7 receptor is the most recent addition to the 5-HT receptor family and has been shown to be positively coupled to adenyl cyclase via Gαs-proteins (53, 54). In the gut, studies have shown that 5-HT induces muscle relaxation by affecting 5-HT7 receptors on smooth muscle cells in addition to neuronal 5-HT7 receptors (55–58). Although the effects of selective 5-HT7 antagonist have been studied in several animal behavioral models where it has been shown to have anxiolytic (59–61), antidepressant (62–64), and antipsychotic-like effects (65, 66), to our knowledge, no current data are available on the effects of 5-HT7 receptor antagonists in IBD.

In this study, we used two different experimental models of colitis, we showed evidence of the importance of the 5-HT7 receptor, expressed on DCs, in immune activation and gut inflammation. Our study, for the first time, to our knowledge, demonstrates that inhibiting 5-HT7 receptor function can act as an effective strategy in alleviation of gut inflammation.

We observed significantly reduced severity of intestinal inflammation in mice that received treatment with 5-HT7 receptor antagonist SB-269970 as compared with vehicle-treated controls. The amelioration of DSS-induced colonic inflammation in 5-HT7−/− receptor antagonist-treated mice was observed in all the parameters examined, including both the macroscopic and microscopic indexes. This was associated with a decrease in MPO activity (an enzyme released by granulocytes such as neutrophils; serving as a surrogate marker of inflammation) (67) and levels of proinflammatory cytokines. Importantly, the beneficial effect of inhibiting 5-HT7 receptor signaling by the antagonist treatment was observed not only in development of acute colitis, but also in chronic colitis induced by DSS. To better understand the role of the 5-HT7 receptor in activation of immune cells and generation of inflammation, we next used mice with targeted disruption of the 5-HT7 receptor (5-HT7−/−). There was also downregulation of inflammation and production of proinflammatory mediators in 5-HT7−/− mice as compared with WT control mice after DSS. One interesting observation noted was the presence of black feces in the cecum of the 5-HT7−/− mice after DSS administration. To rule out a possible difference in bleeding phenotype, we studied bleeding time and did not observe a difference in bleeding time or blood loss between the 5-HT7−/− and WT mice (bleeding times were 402 ± 48.7 versus 367 ± 24.1 s; blood loss was 22.7 ± 3.77 versus 19.2 ± 8.34 μl for 5-HT7−/− and WT, respectively). This suggests that this black stool observed in the 5-HT7−/− mice after DSS might have resulted from bleeding in the upper GI tract, suggestive of modest pathology in the upper intestine of these 5-HT7−/− mice. Nevertheless, exploring the difference between the small and large intestine is outside the scope of this study. More importantly, with pharmacological inhibition of 5-HT7 receptor signaling by antagonist treatment, we failed to see this observation after acute or chronic DSS colitis.

We also investigated the role of the 5-HT7 receptor in a DNBS-induced model of experimental colitis to determine whether the effects seen in the DSS model were specific for this model. DNBS is considered to be a model of Crohn’s disease, and induction

FIGURE 6. Mice lacking 5-HT7 receptor expression show decrease in severity of DNBS-induced colitis. Colitis was induced by administration of DNBS dissolved in 50% ethanol and left for 3 d. Control mice were given 50% ethanol only. A) Macroscopic and B) histologic damage scores were blindly assessed. C) Representative light micrographs of H&E-stained colon sections from WT and 5-HT7−/− mice on day 3 after DNBS. Scale bar, 100 μm. D and E) Colon tissue lysates were used to assess MPO activity and IL-1β levels. Values are shown as mean ± SEM, n = 5–7 mice/group for DNBS, n = 3–5 for ethanol groups. *p < 0.05.

Discussion

5-HT exerts a confounding range of effects in the gut largely because of the presence of multiple receptor subtypes that are present on smooth muscle, enteric neurons, enterocytes, and immune cells (2, 34, 48). Among the seven types of 5-HT receptors (5-HT1−7 receptor subtypes have been identified), subtypes 5-HT1a, 5-HT1c, 5-HT3, 5-HT4, and 5-HT7 have been shown to be expressed within the GI tract, with 5-HT3 and 5-HT4 being the most widely studied. The effects of 5-HT3 receptor antagonists (49, 50) and 5-HT4 receptor agonists (51) have also recently been evaluated in experimental models of colitis. The anti-inflammatory effects of 5-HT7 receptor antagonists, granisetron and tropisetron, were shown to protect against rat colitis and were associated with a decreased inflammatory response in the gut (49, 50). 5-HT3 receptor antagonists may modulate 5-HT release from EC cells (which express 5-HT3 receptors), thereby attenuating inflammation (52). More recently, the effect of 5-HT4 receptor agonist (cisapride) was evaluated in IBD, although no significant difference between agonist-treated and nontreated groups was observed (51).

reconstituted with BMC from WT or 5-HT7−/− mice was 1.00 ± 0.18 versus 0.28 ± 0.03, respectively).

CD11c+ DCs were isolated from spleens of irradiated mice reconstituted with BMC from WT or 5-HT7−/− mice after DSS. CD11c+ DCs isolated from mice given BMCs from 5-HT7−/− mice produced significantly lower levels of IL-1β and IL-6 in the presence of LPS when compared with CD11c+ DCs isolated from control mice (Fig. 7F). To assess the phenotype of the respective cell populations, we stained splenocytes from both experimental groups for various DC markers and analyzed them by flow cytometry. No significant difference in the expression levels of MHC class II molecules, CD40, or costimulatory molecules CD80 and CD86 were detected (Fig. 7G).

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Irradiated mice reconstituted with BMC isolated from 5-HT7−/− mice show delayed onset and reduced severity of DSS-induced colitis. (A) Irradiated WT mice (5-HT7+/+, n = 20) were reconstituted with BMCs from either WT (WT-WT) or 5-HT7−/− (5-HT7−/−-WT) donors (n = 10 mice/group). Mice were given 5% DSS ad libitum. (B) During DSS administration, DAI was scored. (C) Mice were sacrificed on day 5 after DSS, and colons were scored blindly for macroscopic and (D) histological damage. (E) Representative light micrographs of H&E-stained colon cross sections. Scale bar, 100 μm.

Table I. Irradiated mice reconstituted with BMC isolated from 5-HT7−/− mice produce lower levels of inflammatory markers after DSS colitis

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>MPO, U/mg tissue</th>
<th>IL-1β, pg/mg protein</th>
<th>TNF-α, pg/mg protein</th>
<th>IL-6, pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-WT</td>
<td>2.66 ± 0.88</td>
<td>175 ± 23.6</td>
<td>13.6 ± 1.50</td>
<td>150 ± 12.6</td>
</tr>
<tr>
<td>5-HT7−/−-WT</td>
<td>0.61 ± 0.13*</td>
<td>104 ± 21.5*</td>
<td>6.60 ± 1.30*</td>
<td>95.8 ± 20.9*</td>
</tr>
</tbody>
</table>

WT mice (5-HT7+/+, n = 20) were irradiated with 550 rad on two separate counts within 48 h of each other. Irradiated mice were reconstituted with BMCs from either WT (WT-WT) or 5-HT7−/− (5-HT7−/−-WT) donors within 2 h of the second irradiation (n = 10 mice/group). All mice were given 5% DSS solution. Mice were sacrificed on day 5 after DSS, and colonic tissues were collected to assess for MPO activity and cytokine levels. Values are shown as mean ± SEM. *p < 0.05.

Studies were done with radiation chimeras using BMCs from 5-HT7−/− mice to gain further evidence that 5-HT regulates inflammation by acting on immune cells by acting through the 5-HT7 receptor.
HT7 receptor. It is reasoned that if indeed 5-HT plays a key role in activation of immune cells via the 5-HT7 receptor in relation to inflammation, then recipient mice of BMCs from 5-HT7−/− mice will exhibit reduced severity of inflammation and diminished immune activation. Indeed, we observed that irradiated mice that received BMCs from 5-HT7−/− mice had lower severity of intestinal inflammation when compared with mice given BMCs from WT mice. These results further strengthen the notion that the 5-HT7 receptor plays an important role in immune activation and generation of inflammatory conditions that are associated with alterations in 5-HT signaling in the gut.

In summary, the data presented in this study show that the 5-HT7 receptor plays a critical role in regulation of mucosal inflammation and immune responses, and that targeting the 5-HT7 receptor on DCs may serve as a potential therapeutic strategy to ameliorate mucosal inflammation and intervene in inflammatory disorders such as IBD. In addition to enhancing our understanding of the role of 5-HT on immune cell signaling and pathogenesis of colitis, this study identified a potential therapeutic strategy for intestinal inflammatory disorders, including IBD, by targeting 5-HT signaling. Intestinal 5-HT has not only been shown to be important in modulating gut inflammation, but recent studies have also shown that 5-HT deficiency causes slower growth of colon cancer allografts in vivo (73), and reduction in gut-derived 5-HT synthesis decreases development of osteoporosis by promoting bone formation (74). Thus, in a wider perspective, this data have implications not only in developing strategies in ameliorating GI inflammatory disorders such as IBD, but also in non-GI inflammatory conditions that are associated with alterations in 5-HT signaling in the gut.

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Disclosures
The authors have no financial conflicts of interest.

References