GUT SEROTONIN: REVEALING ITS ROLE IN ANTIMICROBIAL PEPTIDE PRODUCTION

Gut serotonin: revealing its role in antimicrobial peptide production

By

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Abstract

Serotonin (5-hydroxytryptamine [5-HT]) is a key enteric signaling molecule that is implicated in many gastrointestinal (GI) disorders, including inflammatory bowel disease (IBD). Enterochromaffin (EC) cells are a key subgroup of enteric endocrine cells and produce the majority of 5-HT via tryptophan hydroxylase 1 (*Tph1*) in the gut. Recently, we have identified a pivotal role of 5-HT in the pathogenesis of experimental colitis, whereby 5-HT plays as a pro-inflammatory molecule. Gut function as well as pathology rely on interactions with gut microbiota. The intestinal epithelial cells produce antimicrobial peptides (AMPs), maintaining the mucosal barrier by shaping gut microbiota composition. Among the AMPs, β-defensins are the most well investigated subtype in the colon. Aberrant β-defensin expression has been reported in association with various GI disease pathogenesis including IBD. As EC cells are dispersed throughout the intestinal epithelium, it seems possible that 5-HT can modify β-defensin production which can regulate gut inflammation by influencing gut microbial composition. Colitis was induced with dextran sulfate sodium (DSS) in *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ (which have lower amounts of 5-HT in gut). *Tph1*$^{-/-}$ mice exhibited higher levels of β-defensin in the colon, compared with wild-type littermates post-DSS. In addition, increased expression of β-defensin in *Tph1*$^{-/-}$ mice was suppressed by 5-hydroxytryptophan (5-HTP; precursor of 5-HT) treatment. 5-HT treatment resulted in decreased human β-defensin (hBD) 1 and hBD-2 expression in HT-29 cells. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is essential for maintaining β-defensin expression in the colon. GW-9662, PPAR-γ antagonist,

reduced mouse β-defensin (mBD) 1 and mBD-3 (orthologue of hBD-2). Furthermore, disrupting 5-HT_7 receptors, but not 5-HT_3 or 5-HT_4, led to enhanced expression of PPAR-γ via ERK1/2-dependent mechanism. These observations provide us with novel information on pivotal role of gut-derived 5-HT in innate immune response and highlight the potential benefits of targeting 5-HT signaling in various GI disorders such as IBD.

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Table of contents

List of figures

List of tables

List of abbreviations and symbols

5-ASA	5-aminosalicylic acid
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine or Serotonin
5-HTP	5-hydroxytryptophan
5-HTR	5-HT receptor
SERT	Serotonin reuptake transporter
AADC	Amino acid decarboxylase
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
AP-1	Activator protein-1
BPI	Bactericidal/permeability increasing protein
CD	Crohn's disease
CgA	Chromogranin A
Crp	Cryptdin
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNBS	2,4-dinitrobenzenesulfonic acid
DSS	Dextran sulfate sodium
EC	Enterochromaffin

EEC	Enteroendocrine cell
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase-1 and -2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
hBD	Human β-defensin
HD	Human defensin
HNP	Human neutrophil peptide
IBD	Inflammatory bowel disease
iDC	Immature dendritic cell
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
KO	Knock-out
Lcn2	Lipocalin-2
LP	Lamina propria
LPS	Lipopolysaccharide
MAO_A	Monoamine oxidase A
MAMP	Molecular-associated molecular pattern
mBD	Mouse β-defensin

MDP	Muramyl dipeptide
MEK	MAPK ERK kinase
MMP	Metalloproteinase
NF-κB	Nuclear factor-κB
NGN	Neurogenin
NOD	Nucleotide-binding oligomerization domain-containing protein
qPCR	Quantitative polymerase chain reaction
PAMP	Pathogen-associated molecular pattern
*p*CPA	*para*-Chlorophenylalanine
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR-responsive element
PRR	Pathogen recognition receptor
RegIII	Regenerating islet-derived III
RT	Reverse transcriptase
RXR	Retinoid X receptor
s.e.m	Standard error mean
SFM	Serum-free medium
sIgA	Secretory IgA
SLPI	Secretory leukocyte peptidase inhibitor
SPF	Specific pathogen-free

Syn	Synaptophysin
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
Tph	Tryptophan hydroxylase
UC	Ulcerative colitis
UV	Ultraviolet
VMAT1	Vesicular monoamine transporter 1
wt/vol	Weight to volume
WT	Wild-type
α	Alpha
β	Beta
γ	Gamma
θ	Theta
g	Gram
Kg	Kilogram
L	Litre
μg	Microgram
μl	Microlitre
μM	Micromole
ng	Nanogram
nM	Nanomole
nm	Nanometre
pg	Picogram

Declaration of academic achievement

Manuscript titled "Toll-like receptor 2 plays a pivotal role in mediating mucosal serotonin production in gut" has been submitted as a co-first author.

Manuscript titled "Modulation of gut microbiota composition by serotonin signaling influences pathogenesis of colitis" has been submitted as a co-first author.

My supervisor, Dr. Waliul Khan, Lab technician, Huaqing Wang, and myself developed conditional double knockout mice: *Atg7$^{fl/fl}$; villin-Cre;Tph1$^{-/-}$*

Chapter 1: Introduction

1.1. Enteroendocrine System and Enterochromaffin cells

Human gastrointestinal (GI) tract is the largest endocrine organ in the body, which contains an extensive system of specialized epithelial cells, called enteroendocrine cell (EEC)[1]. These cells are interspersed throughout the epithelial layer and not only constitute the largest population of hormone-producing cells in the body[2], but also act as a major gatekeeper against luminal contents, such as food and microorganisms, releasing various biologically active compounds including serotonin (5-hydroxytryptamine; 5-HT)[3].

Amongst many EEC subtypes throughout the GI tract from the gastric antrum to the rectum, enterochromaffin (EC) cell is the most abundant EEC subtype in the colon and rectum, comprising more than 70% of the EEC population in the proximal colon[4]. EC cells originate from stem cells within the crypts andare situated at the base of the intestinal crypts[2]. Down-regulation of the transcription factor for enterocytes (non-secretory lineage), termed Hes-1, inhibits basic helix-loop-helix transcription factors such as Math1 and neurogenin 3 (NGN3), directing stem cells to the secretory and endocrine lineage, respectively[5,6]. Regulation of these key transcription factors is crucial for stem cells in determining differentiation of enteroendocrine cells[7].

EC cells are widely distributed throughout the intestine, taking approximately 0.25–0.50% of the total mucosal volume. EC cell is a pyramidal-shaped cell with apical cytoplasmic extension reaching the gut lumen via short microvilli[8,9]. Immunohistochemical studies of EC cell demonstrated that the cell stains positive for

chromogranin A (CgA)[10,11], synaptophysin (Syn)[12], tryptophan hydroxylase (*Tph*)[8,13], and 5-HT[14]. Amongst these products in EC cell, 5-HT is the main secretory product which is synthesized by EC cell upon stimulation by luminal products and mechanical activity of the intestine[14].

Serotonin

5-HT is a highly ubiquitous signaling molecule, which is widely known for neuropsychological and cognitive functions, such as appetite, mood, pain and sleep[15]. However, the vast majority of the body's 5-HT is primarily synthesized by EC cells[4,16]; while a small proportion of peripheral 5-HT is also synthesized by mucosal mast cells[17], T cells[18], and myenteric neurons[19–21].

In 1937, Vittorio Ersparmer isolated a substance from GI mucosal extracts of various vertebrates, which he called it "enteramine"[22,23]; the substance was an indolalkylamine secreted from EECs. Later, a vasoconstrictor substance was identified from a purified concentrate of bovine serum[24]. An indole nucleus in the substance was indicated by using ultraviolet (UV) spectrophotometer, compared to tryptophan and tryptamine, leading to its name as "5-hydroxytryptamine" (5-HT)[24]. 5-HT was then named as "serotonin"; "Ser-" was obtained from the "serum" and the substance was found to limit or increase the tone ("-tonin") in blood vessels[25]. In 1960, it was discovered that total gastroenterectomy of a rat significantly reduced 5-HT release, demonstrating the gut as the main producer of 5-HT in the body[26]. Furthermore, a considerable decrease in the main metabolite of 5-HT, called 5-hydroxyindoleacetic

acid (5-HIAA), in urine as well as intact 5-HT levels in the brain, suggests that neuronal 5-HT production is independent from the non-neural 5-HT synthesis and central 5-HT accounts for only a small proportion of the body's total 5-HT[26].

5-HT synthesis, release, and metabolism

EC cells synthesize 5-hydroxytryptophan (5-HTP) from dietary amino acid, L-tryptophan, by one of the two isoforms of *Tph*, called tryptophan hydroxylase 1 (*Tph1*)[27,28]. 5-HTP is then converted into 5-HT by L-amino acid decarboxylase (L-AADC) in the cell. Once 5-HT is synthesized, it is packaged into secretory granules and transported to near the apical and basal membrane of the EC cell by vesicular monoamine transporter 1 (VMAT1)[29,30–31]. EC cells sense luminal contents including various chemical and mechanical stimuli[32–34] and microbial-derived metabolites[35–37] by microvilli present on the apical membrane. These physicochemical signals are then converted into biochemical endocrine signals which induce 5-HT release both apically and basolaterally[38]. Upon release, 5-HT can act locally via as many as 22 different receptor subtypes on enterocytes, enteric neurons, and immune cells in the lamina propria (LP), regulating a wide range of GI physiological functions, namely secretion and motility, mucosal blood flow, as well as modulating host immune system[39,40]. The bioactivity of 5-HT is terminated by selective serotonin transporters, called serotonin reuptake transporter (SERT), which sequesters 5-HT into adjacent enterocytes from both sides of the membrane[41]. 5-HT is then further metabolized into 5-HIAA by monoamine oxidase A (MAO_A)[42].

5-HT can also be sequestered by SERT and stored in platelets[42]. Approximately 2% of blood 5-HT enters the general circulation, directly acting as a hormone in various cell types including adipocytes[43,44], cardiomyocytes[45], hepatocytes[46] and osteocytes[47,48]. Platelet-stored 5-HT is released upon injury and/or inflammatory signals, such as platelet activating factor and complement components (C3a and C5a)[49,50], thereby regulating platelet aggregation[51]. There is another isoform of *Tph*, called *Tph2*, which regulates neuronal 5-HT biosynthesis (including enteric neurons)[20,28]. The fact that there are two distinct isoforms of *Tph* regulating and maintaining two distinct serotonergic system can be attributed to the impermeability of blood-brain barrier to 5-HT[52].

1.2. Inflammatory Bowel Diseases

Inflammatory Bowel Diseases (IBD) is chronic idiopathic inflammatory condition that may affect the entire GI lining and mucosa from esophagus to rectum. IBD is emerging as a global disease as the incidence and prevalence have increased, and it is affecting now more than 2.5 million people with European ancestry[53,54]. In addition, there is a recent increase in the incidence of IBD in populations of developing countries as well as migrant populations in Western countries[55,56]. It is reported that the age of the onset of disease is early adulthood, where the peak age is around 30 years[56]. However, the disease may occur at any age as pediatric IBD accounts for approximately 15% of all IBD cases[57,58].

There are two major forms of IBD: Crohn's disease (CD) and ulcerative

colitis (UC). Although the two diseases have overlapping clinical and pathological features, each form has distinct hallmarks. The most common hallmark that distinguishes CD from UC is discontinuous or skip lesions occurring from any region of the GI tract, with common localization in the ileum and the colon[59]. While UC is largely confined to the colon and the rectum with superficial inflammation that is usually limited to the mucosa, CD is a transmural disease involving all layers of the gut[60]. Activated immune cells from both innate and adaptive immune systems infiltrate the mucosa and contribute to extensive ulcerative lesions, which can ultimately lead to abscess or fistula and subsequent fibrosis with intestinal obstruction[61]. Although treatment is far from optimal, many studies from human tissues and animal models of colitis have provided substantial insight into understanding the pathogenesis of IBD, leading to improved therapeutic strategies to control the inflammation. However, the ultimate cause(s) of IBD is still unknown.

Factors Influencing the Development of IBD

IBD is a multifactorial disorder which is thought to be arising in genetically susceptible populations with abnormal mucosal immune responses towards enteric flora and/or various environmental factors[62–64].

Genetic susceptibility

Recent advances in genome-wide association studies (GWAS) and subsequent meta-analyses have provided genetic architecture of IBD-associated genes, which have identified 53 non-overlapping genetic risk loci with 110 loci being shared by

both CD and UC[53,65–67]. However, these risk loci are not exclusively associated with IBD as some genes have contrasting outcomes in different diseases, such as type 1 diabetes and rheumatoid arthritis[68]. *IL23R*, the gene encoding the subunit of the receptor for pro-inflammatory cytokine interleukin-23 (IL-23), was the first gene identified using IBD-based GWAS, and this gene was found to be strongly associated with CD[69]. Most well-known gene associated with CD, however, is nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) or IBD protein 1 (*IBD1*)[70]. *NOD2*, an intracellular pattern recognition receptors, plays an important role in detecting bacterial peptidoglycan product called muramyl dipeptide (MDP), thereby initiating host innate immune responses[71]. It has been suggested that the increased incidence of CD in Western populations of European and Jewish ancestry can be attributed to the three established *NOD2* mutations (Leu1007fsinsC, G908R/2722g>c, and R702W/2104c>t)[72,73]. Although there are studies suggesting that NOD2 mutations are risk factors for CD in African populations, frequency of mutation is far less than in populations of either European or Jewish ancestry[74]. In contrast, NOD2 mutations are absent in Pacific Asian populations of Japanese[75,76], Chinese[77], and Korean ancestry[78]. The remarkable difference in the disease susceptibility across different populations suggests that genetic is not the sole factor associated with IBD; rather genetic and environmental factors interact in the pathogenesis of IBD.

Environment

Since the first case of UC was documented in Western Europe in 1859[79], the incidence of IBD has steadily increased in industrialized countries. Amongst these

countries, Canada has the highest reported incidence (19.2 per 100, 000 for UC and 20.2 per 100, 000 for CD) and prevalence rates (248 per 100,000 for UC and 319 per 100,000 for CD)[54]; in other words, approximately 0.5% of the population in Canada are suffering from IBD[80]. The emergence of IBD in Western countries suggests westernized or modernized lifestyle as a potential environmental risk factor[81,82]. However, emerging evidence has provided other environmental factors associated with the development of IBD, such as diet[83,84], antibiotic use[85,86], hygiene status[80,87], smoking[88], breastfeeding[89,90], vitamin D exposure[91], oral contraceptives[92], and appendectomy[93]. Among these factors, hygiene status is the most well-established prominent factor that explains the increased incidence of autoimmune and allergic diseases in industrialized countries[82], suggesting that ubiquitous microbial exposures early in lifetime is essential for optimal development of the immune system and reducing the frequency of immunological disorders[94].

Gut microbiota

The mammalian GI tract is colonized by 100 trillion (10^{14}) of microorganisms, with the distal ileum and colon being the greatest density populated region with 10^{12} bacteria per gram of feces[98,108]. Finely tuned bi-directional or mutualistic communication exists between the host and intestinal microorganisms through interlinked metabolic networks[95,97,99–101]. In addition to aid in digestion[102,103], commensal microbes are also able to not only efficiently tailor the host immune system[95–97,104,116], but also limit resources available to pathobionts which can hijack the well-adapted consortium[106] once the homeostatic relationship is compromised by

either dietary changes[107,108], antibiotic treatment[109,110] or invasive pathogens[111,112], resulting in normalization of the microbiota and thereby potentially favoring the outgrowth of pathobionts[106,113–116]. This shift in the microbial communities is referred to as dysbiosis, which is strongly associated with the pathogenesis of IBD[106]. There are now abundant studies linking intestinal microbiota with IBD, alluding the important role of the microbiota in intestinal inflammation[117–122]. However, what causes dysbiosis remains to be determined. The disease development may be attributed to perturbation of the delicate balance between host and microbes by mucosal immune system including microbe recognition, barrier function, and antimicrobial effector mechanisms[123].

Immune system

The homeostatic balance between microbes and the host is carefully monitored by the mucosal immune system, which maintains homeostasis by not only inducing physiological inflammation to fend off invading microbes but also preventing chronic mucosal inflammation[73]. Amongst various players of the immune system including innate and adaptive immune cells, intestinal epithelium has recently received growing scientific interest. Intestinal epithelium are the first line of defense at the mucosal surfaces, which consists of a dense mucus layer containing antimicrobial peptides (AMPs) and commensal-specific secretory IgA (sIgA) as well as tight and adherens junction protein complexes that connect individual epithelial cells, forming a continuous, single cell layer[106,124]. There is now increasing evidence suggesting that defect in intestinal epithelial barrier functions is associated with

increased bacterial translocation and risk of developing IBD[53,64,73,96].

1.3. 5-HT receptors

Seven classes of 5-HT receptors have been identified, and they are expressed on various cell types including enterocytes and immune cells. Amongst seven receptor families, 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors are expressed in the GI tract. All 5-HT receptors are G-protein coupled receptor (GPCR) superfamily, except for 5-HT_3 receptor family, which is the only ligand-gated ion channel receptor. 5-HT_3 and 5-HT_4 receptors are involved in regulating GI physiology including motor and secretory functions. On the intestinal epithelial cells, only 5-HT_3, 5-HT_4, and 5-HT_7 receptors have been identified[125]. Previous studies from our laboratory demonstrated that blocking 5-HT signaling with a selective 5-HT_7 receptor antagonist (SB-269970) or genetic deletion of this receptor in C57BL/6 (i.e., 5-$HT_7R^{-/-}$) mice reduced severity of experimental colitis induced by dextran sulphate sodium (DSS) and 2,4-dinitrobenzenesulfonic acid (DNBS)[126]. Interestingly, a recent study demonstrated that 5-HT_4 receptor stimulation via enema administration had a protective effect in experimental colitis, but it was not protective when delivered by intraperitoneal injection[127]. While no data were presented on inflammatory cytokines, this protective effect was shown to be associated with enhanced epithelial cell proliferation and increased motility[127]. These findings suggest that 5-HT may invoke divergent and discrete actions on different cell types. Overall, these studies suggest important roles of 5-HT signalling in pathogenesis of experimental colitis.

1.4. 5-HT in Gut inflammation

5-HT receptors are expressed on a variety of immune cells, such as B and T lymphocytes[128,129], macrophages[130], and dendritic cells (DCs)[131,132]. We have previously shown that DCs isolated from *Tph1*$^{-/-}$ mice produced less IL-12p40 than their WT post-DSS[132]. Co-culture of CD4+ T cells with DCs isolated from DSS-induced *Tph1*$^{-/-}$ mice also resulted in lower levels of IL-17 and interferon-γ (IFN-γ)[132]. We have also highlighted the potential benefit of inhibiting 5-HT$_7$ receptors on dendritic cells to alleviate the experimentally induced colitis[133]. These previous studies altogether suggest that mucosal 5-HT is involved in immune modulation by influencing interaction between innate and adaptive immune cells and potentially generate intestinal inflammation.

There is now abundant evidence suggesting a strong association of chronic intestinal inflammation with aberrant 5-HT signaling in both humans and animal models of colitis. Alterations in EC cell numbers and 5-HT content have been observed in patients with intestinal inflammation[134–137] as well as in various experimental models of colitis[138–141] induced by DSS, DNBS, and 2,4,6-trinitrobenzenesulfonic acid (TNBS); while SERT expression was reduced in these models[139,140]. Previously, in a seminal study, we have demonstrated a critical role of gut derived 5-HT in pathogenesis of colitis in experimental models of IBD by utilizing *Tph1*$^{-/-}$ mice, which have significantly lower amounts of 5-HT in the gut[130]. Upon induction of acute colitis using DSS and DNBS, these mice exhibited a reduced severity of colitis compared with *Tph1*$^{+/+}$, whereby replenishment of 5-HT with 5-

HTP, the immediate precursor of 5-HT, intensified colitis severity[130]. To further support a role of 5-HT in inflammatory states, 5-HT synthesis of *Tph1*$^{+/+}$ mice was halted by *Tph* inhibitor, *para*-chlorophenylalanine (*p*CPA), which delayed the disease onset and severity[130]. This study provides not only a strong evidence that 5-HT released from EC cells acts as an important signaling molecule in the development and course of experimental colitis, but also the basis to further investigate the mechanisms involved in the process. Another study using SERT-deficient mice, which have augmented bioactivity of 5-HT in the lumen and lamina propria (LP), showed that these mice had enhanced colitis severity[142], further supporting a notion that 5-HT plays a key role in pathogenesis of intestinal inflammation. Moreover, our laboratory also provided a strong support on the role of 5-HT in pathophysiology of GI diseases. Blocking peripheral 5-HT synthesis with orally-delivered *Tph1* inhibitor, telotristat etiprate (LX1032/LX1606), significantly delayed the onset and severity of both chemical- and infection-induced intestinal inflammation[126]. Taken together, these findings reveal an alluring role of 5-HT in orchestrating immune system and possible implications in the pathophysiology of intestinal inflammation.

1.5. Antimicrobial peptides

To prevent perturbation of the microbial communities, host plays a crucial role in orchestrating the dynamic interplay with the microbes through production of immunoglobulins (Ig) and secretion of mucins. More importantly, various epithelial cells including colonocytes govern the mucosal barrier through production of

antimicrobial peptides (AMPs), thereby fending off potentially pathogenic microbes colonizing gut mucosal layer[133,143–145]. AMPs represent primitive defense mechanism found in virtually every organism ranging from prokaryotes to humans[146]. There are multiple families of AMPs which are divided into a variety of subfamilies, depending on their amino acid composition and structure[146].

Mechanisms of action of AMPs

AMPs are the first line of host defense and have a wide range of antimicrobial activity towards bacteria, yeasts, fungi, parasites and viruses[114–116,146,147]. Among various types of AMPs, anti-bacterial AMPs are the most studied and well understood AMPs[148]. AMPs vary in their net charge from negative to positive, and changes in their net charge affect the efficiency of antimicrobial activity[148,149]. Cationic AMPs are the largest group of AMPs[148,150]. They are able to effectively kill both gram-positive and gram-negative bacteria through electrostatic attraction towards negatively charged bacterial cytoplasmic membrane and/or cell wall, which is followed by the insertion of the peptides into the membrane and formation of transmembrane pores or 'wormholes'[146,151]. This well-known antimicrobial mechanism is referred to as Shai-Matsuzaki-Huang model[143]. Another important characteristic of AMPs in initiating microbicidal activity is their amphipathic property with overall positive charge and hydrophobic amino acid residues, providing capability to tightly bind to the lipid components and phospholipid head groups of the membrane[152]. This leads AMPs to accumulate on the bacterial membrane, building strains in the bilayer, allowing

peptides to form toroidal pores and subsequently disintegrating the membrane into vesicles[153]. Anionic AMPs, on the other hand, form cationic salt bridges by bringing metal ions, such as zinc (Zn^{2+}), in the vicinity, thereby interacting with bacterial membranes[154]. However, the mechanisms underlying the action of anionic AMPs are still elusive.

It is also important to understand how AMPs differentiate mammalian host from microbes. There are fundamental differences in the membranes of microbes and mammals, which play a crucial part in target specificity of the endogenous peptides. All biological membranes are composed of fluid mosaic proteins and phospholipids. However, the membrane composition differs significantly between prokaryotic and eukaryotic cells. For example, bacterial cytoplasmic membranes are much more electronegative than the mammalian membranes due to higher proportions of anionic phospholipids[155,156,157]. Sterols, such as cholesterol and ergesterol, are generally neutral, and they are rarely detected in prokaryotic membranes[157]. In addition, the electrochemical gradient resulting from the charge separation between the extracellular and intracellular aspects of the membrane provides another degree of selective targeting[157]. Such structural features, therefore, facilitate cationic AMPs to effectively interact with microbial membranes, permeabilizing the membrane. In some studies, it has been reported that Shai-Matsuzaki-Huang model is not the primary mechanism by which the AMPs exert their activity, as there are intracellularly-active AMPs that can kill their target cells without forming pores[158,159]. These peptides can disrupt cellular functions of bacteria by inhibiting DNA and protein synthesis[160,161] or

exert anti-protease activities against microbial proteases within cells[162]. Some of the intracellular AMPs are able to use multiple-hit strategy which can increase the efficiency of antimicrobial activities and prevents potential bacterial resistance against AMPs[163].

Defensins

In humans and other mammals, defensins belong to major group of AMPs with a characteristic β-sheet-fold and a net positive charge on the amino acid residue[146,164]. Defensins are an important component of innate immune response to pathogens. They are small (3-6 kDa) microbicidal molecules[146,165], and they are divided into three subfamilies: α, βand θ[146]. Although they share similar molecular structures, they differ in the conserved pattern of cysteine amino acid residues and the intramolecular disulphide bonds between the cysteine pairs[146,164]. These cationic peptides are induced through recognition of pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) by pathogen recognition receptors (PRR)-activated signaling cascades upon stimulation by their respective ligands[166].

α-defensin

There are 6 types of α-defensins identified in human: human neutrophils peptide (HNP) 1-4 and human defensin (HD) 5 and HD-6. While HNPs are primarily secreted by neutrophils, HD-5 and HD-6 are highly concentrated in the ileum,

particularly in Paneth cells that are strategically located close to the crypts of Lieberkühn[153]. In contrast, mouse neutrophils do not generate α-defensins[167]. Instead, mouse Paneth cells express six α-defensins, termed cryptdins (*crypt d*efens*ins*; Crp 1-6)[168]. The peptide processing is also different between human and mice. While matrix metalloproteinase-7 (MMP-7) cleaves inactive pro-cryptdins (pro-Crps) within the granules of Paneth cells in mice[168,169], HD-5 and HD-6 are cleaved by trypsin[170,171]. However, in both humans and mice, α-defensins are constitutively expressed and confined to the small intestine, maintaining symbiotic relationship with commensal microbiota at the mucosa[147,153].

β-defensin

Unlike α-defensins, β-defensins are primarily expressed by various epithelial cells including colonic epithelial cells. Although α-defensins are sometimes found to be secreted from metaplastic Paneth cells in the colon[172], the major peptides in the colon are β-defensins. Six human β-defensins (hBDs) have been identified, and hBD-1 (*defb1*) and hBD-2 (*defb4*) have been extensively studied. These peptides are expressed in either constitutive or inducible manner. hBD-1 and its orthologue mouse β-defensins 1 (mBD-1) are constitutively expressed at various epithelial sites including colon[173–176]. Even challenges with LPS, pro-inflammatory cytokines (e.g., IL-α, TNF-α, and IFN-γ) and/or bacteria failed to alter hBD-1 expression in human colonic epithelial Caco-2 and HT-29 cells[175]. Unlike hBD-1, hBD-2 and its orthologue mBD-3 are minimally expressed in normal colon. However, these peptides are induced by pro-inflammatory cytokines (e.g., IL-1β and TNF-α), microbes or their

products at sites of infection or inflammation[175]. In addition, β-defensins serve as a bridge connecting innate and adaptive immune responses. They are involved in chemoattracting various immune cells including immature DCs (iDC) and some subsets of T cells, such as CD4+CD45RO and CD8+, to the site of microbial invasion through chemokine receptor, CCR-6[177].

θ-defensin

θ-defensins were first isolated from the leukocytes of rhesus macaques[178]. A lack of evidence regarding the functions of θ-defensin in non-human primates can be attributed to its inability to be translated into a functional protein due to the presence of a premature stop codon[146,178,179].

Other types of AMP

There are more peptides with antimicrobial properties, such as, cathelicidins, Regenerating islet-derived III gamma (RegIIIγ), elafin, secretory leukocyte peptidase inhibitor (SLPI), bactericidal/permeability increasing protein (BPI), lysozyme, lactoferrin, and hepcidin.

β-defensin expression in intestinal inflammation

The well-appreciated notion IBD have is the adaptive immune response plays a major role in the pathogenesis of the disease. However, accumulating evidence now

suggests that abnormal antimicrobial activity leads to alterations in the composition of the microbiota[73,180,181], which provokes devastating consequences on the effectiveness of the intestinal epithelial barrier, thereby resulting in pathological immune responses[182]. In particular, it has been reported that patients with UC have an altered epithelial barrier function[183,184], whereas perturbed innate immune responses, such as β-defensin expression and innate microbial sensing areassociated with CD pathogenesis[185–187]. As compared with UC patients, an impaired induction of hBD has been observed in patients with colonic CD[185,186]. For instance, a decreased expression of hBD-1 and attenuated induction of hBD-2 have been reported in colonic CD patients, while UC patients tend to have increased hBD-1 and hBD-2 expression with increased levels of IL-8 and TNF-α[188,189]. In addition, especially in CD patients, there is an increased prevalence of mucosa-associated *E. coli* compared with UC patients or healthy controls[190]. It is also interesting to note that antibodies against the mycobiota, especially the fungus, *Saccharomyces cerevisiae*, are found in CD patients, and that contributes to colitis severity in a mouse model of colitis[191]. Thus, dysbiosis described in IBD may be caused by defects in mucosal microorganism clearance at the mucosal surface, and that compromised functional antimicrobial activity towards colonizing microbes may result in colonic inflammation.

Table1. Summary of antimicrobial peptide expression profiles in ulcerative colitis (UC) and Crohn's disease (CD)

***Ulcerative Colitis* (UC)**	***Crohn's Disease* (CD) - Colon**
Regular induction of hBD-2	Attenuated induction of hBD-2
Decreased expression of hBD-1	Decreased expression of hBD-1
HD5&6 expression due to metaplasitc Paneth cell	HD5&6 expression due to metaplasitc Paneth cell

1.6. Peroxisome proliferator-activated receptor-gamma

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a ligand-inducible transcription factor that belongs to the nuclear receptor superfamily[192]. Upon ligand binding, two isoforms of PPAR-γ, PPAR-γ1 and PPAR-γ2, heterodimerize with retinoid X receptor (RXR), regulating transcription of target genes by binding on PPAR-responsive element (PPRE) of target gene promoters[193].

Anti-inflammatory role of PPAR-γ in inflamed colon

Besides in hypothalamus[194] and adipocytes[195,196] where PPAR-γ is highly involved in lipid and glucose metabolism, it is abundantly expressed in colonic epithelial cells[197], regulating cell metabolism, cell differentiation and inflammation upon stimulation by luminal microorganisms[198–200]. There is increasing evidence from animal models of colitis and IBD patients that PPAR-γ agonists play as the key inhibitor of colitis by regulating immune activation and inflammation[201–203]. Most recently, it has been shown that PPAR-γ expression is down-regulated in colonic epithelial cells of active UC patients compared with UC patients in remission[204,205], and treatment with PPAR-γ agonist, rosiglitazone, mediated beneficial clinical effect in patients with active distal UC. In addition, the effect of 5-aminosalicylic acid (5-ASA), an anti-inflammatory drug that is widely used for treating colitis, was found to induce PPAR-γ expression[206]. 5-ASA has also been found to bind PPAR-γ, translocating the nuclear receptor from the cytosol to the nucleus[206]. Recently, it has beenfound that PPAR-γ activates colonic expression of β-defensin by directly binding to the *defb1* promoter in human colonic epithelial cell line (HT-29)[200].

Chapter 2: Hypothesis and aims

With basis in the theories presented, the current study examines the role of gut-derived 5-HT in β-defensin production from intestinal epithelial cells. As EC cells are situated in the epithelial layer and epithelial cells express 5-HT receptors (5-HT_3, 5-HT_4 and 5-HT_7), and as 5-HT acts as a pro-inflammatory molecule, we **hypothesize** that 5-HT inhibits β-defensin production from intestinal epithelial cells. By utilizing Tph1-deficient mice and human adenocarcinoma cell line (HT-29), we investigated the role of 5-HT in β-defensin production and underlying mechanisms involved in the process.

Aim 1: To determine the role of 5-HT in modulation of β-defensin production.

Rationale: We have previously shown a critical role of gut-derived 5-HT in the pathogenesis of experimental models of IBD, whereby 5-HT acts as a pro-inflammatory molecule, exacerbating severity of colitis in *Tph1*$^{+/+}$ mice. In addition, preliminary studies from our laboratory has shown that the *Tph1*$^{-/-}$ and their WT littermates have different composition of the gut microbiota, indicating a possible role of 5-HT in β-defensin production. Therefore, in this aim, by using *Tph1*$^{-/-}$ mice and HT-29 cells, we investigated whether 5-HT can modulate β-defensin.

Aim 2: **To elucidate the mechanisms underlying the modulatory effect of 5-HT on the production of β-defensins.**

Rationale: Since it has been shown that HT-29 cells express 5-HT receptors including 5-HT_3, 5-HT_4 and 5-HT_7[125,207], this cell line is an excellent *in vitro* model to study the role of 5-HT. PPAR-γ has been shown to influence secretion of β-defensin in colonic

epithelial cells[200]. In addition, recent studies have shed light on a possible link between 5-HT and the activation of PPAR-γ. 5-HT has been shown to reduce the expression of PPAR-γ through extracellular signal-regulated kinase (ERK)-mediated pathway[208–210]. Thus, it seems likely that 5-HT release from EC cells can inhibit β-defensin expression by down-regulating PPAR-γ.

Chapter 3: Methods

3.1. Animal experiments

Animals

Breeding pairs of *Tph1*$^{-/-}$ and *Tph1*$^{+/+}$ mice were obtained from Centre national de la recherche scientifique (CNRS), France, and were kept and bred under specific pathogen-free (SPF) conditions of McMaster University Central Animal Facilities (CAF). *Tph1*$^{-/-}$ mice on C57BL/6 background were produced by gene mutation as described by Côté et al[45]. These mice are viable and express normal amounts of 5-HT in the brain. All mice were kept in sterilized, filter-topped cages under SPF conditions, fed autoclaved food at a temperature of 21-22°C and with 12:12 h light-dark cycle in the McMaster University CAF. Mice were allowed to acclimatize for at least 7 days prior to the start of any experiments. All experiments were approved by the Animal Research Ethics Board (AREB)-McMaster University and conducted under the Canadian guidelines for animal research.

Animal experimental protocol

Induction of acute intestinal inflammation using dextran sulfate sodium (DSS)

DSS (molecular mass 40 kDa; MP Biomedicals, Solon, OH) was added to drinking water in a final concentration of 5% (w/v) for a total of 5 days. Mean DSS consumption was noted per cage each day. Controls received regular drinking water for the same time span.

Drugs

5-hydroxytryptophan (5-HTP)

As previously described[46], 5-hydroxytryptophan (5-HTP) (Sigma-Aldrich, Oakville, Canada) was administered subcutaneously into *Tph1*$^{-/-}$ mice at a dosage of 50 mg/kg twice a day for 5 days or vehicle (saline).

GW-9662 (PPAR-γ antagonist)

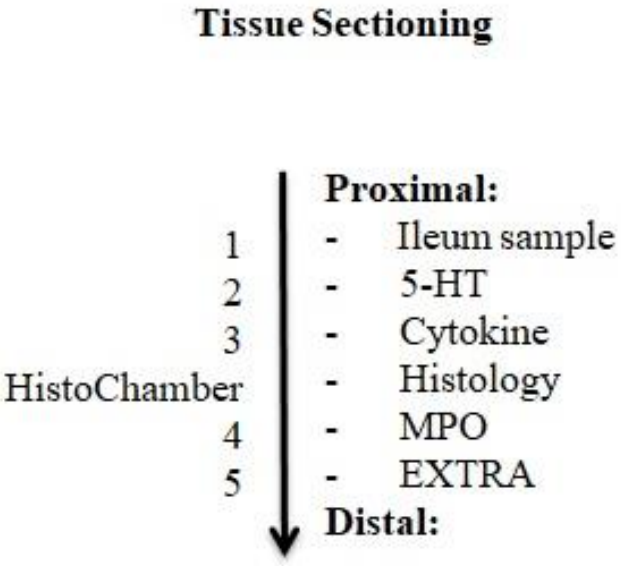
Tph1$^{-/-}$ mice were treated with GW-9662 (Cayman Chemicals, Ann Arbor, MI), or vehicle (dimethyl sulfoxide (DMSO); Sigma-Aldrich) in a dosing volume of 2 mg/kg per day via intraperitoneal (i.p.) administration for 5 days.

Animal tissue sample preparation

Colonic tissues were collected based on the protocol established in the lab (**Figure 1**). For determination of β-defensin levels in colonic tissue samples, colonic section #3 was used.

Figure 1. Guideline for collection of mouse colonic tissues.

Tissues were cut at approximately 1.5 cm in length and stored at -80°C.


Tissue Sectioning
Proximal:
1
- Ileum sample
2
- 5-HT
3
- Cytokine
HistoChamber
- Histology
4
- MPO
5
- EXTRA
Distal:

To assess the level of β-defensin peptides, the colonic sections were weighed and homogenized in 700 μl of Tris-HCl buffer with protease inhibitor cocktail (PIC; Sigma-Aldrich) using a Mixer Mill (MM400; Retsch Inc., Newtown, PA) at 30 frequency (1/s) for 5 min. Total RNA was synthesized using TRIzol™ reagent according to the manufacturer's manual.

In vitro experiments

HT-29 cells, which are isolated from a human colonic adenocarcinoma in a 44-year old Caucasian female, are established by J. Fogh in 1964. These cells are adherent cells and grow well even under serum-free medium up to 48 hours. HT-29 cells were chosen for studying the effect of 5-HT in inhibiting β-defensin production because the cells express 5-HT receptors including 5-HT_3, 5-HT_4, and 5-HT_7 and the nuclear receptor, PPAR-γ. HT-29 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Cell stimulation

HT-29 cells express various 5-HT receptors: 5-HT_3, 5-HT_4, and 5-HT_7, which offer the opportunity to stimulate cells with 5-HT and to elucidate the role of 5-HT in inhibiting β-defensin production from intestinal epithelial cells *in vitro*. Serum-free medium (SFM) is used when stimulating cells because fetal bovine serum (FBS) contains approximately 10 nM of 5-HT[129]. Trypan Blue exclusion assay using Trypan Blue (Thermo Fischer Scientific, Burlington, Canada) is performed to test the viability of drug-treated cells, where blue staining indicates cell death. Cells remained viable

upon stimulation with drug of interest.

Drugs and reagents

IL-1β (Peprotech, Rocky Hill, NJ), 5-hydroxytryptamine (5-HT; Sigma-Aldrich), rabbit polyclonal anti-human β-defensin 1 (Peprotech), rabbit polyclonal anti-beta actin (Abcam, Cambridge, MA), Tropisetron (5-HT_3 receptor antagonist; ToCris Biosciences, Burlington, Canada), RS-39604 (5-HT_4 receptor antagonist; Tocris Biosciences), SB-269970 (5-HT_7 receptor antagonist; ToCris Biosciences), GW-9662 (PPAR-γ antagonist; Caymen Chemicals), and PD98059 (ERK1/2 inhibitor; Cell Signaling Technology, Danvers, MA) were prepared according to the manufacturer's manual.

RNA extraction

TRIzol™ reagent (Thermo Fischer Scientific) can isolate high-quality total RNA, DNA and protein from biological samples of human and animal. This reagent disrupts the cells during homogenization while maintaining the RNA integrity by inhibiting RNase activity. The reagent is very sensitive to light exposure. Aliquots need to be wrapped in aluminum foil prior to use. Total RNA was synthesized according to the manufacturer's manual.

Quantification and purity assessment of RNA by UV-Vis spectrophotometer

NanoDrop™ 2000 (Thermo Fischer Scientific) is a UV-Vis

spectrophotometer which is used to determine the RNA yield and assess the purity of RNA. RNA purity is assessed by the ratio of absorbance at 260/280 nm and 260/230 nm. This wavelength can be used to estimate the concentration of nucleic acid in the sample. Samples with a 260/280 nm ratio and a 260/230 ratio of ~2.0 for RNA are considered for downstream applications. A low value of each ratio indicates contamination by proteins, urea or phenols which can happen during extraction of total RNA from the sample. Unfortunately, genomic DNA (gDNA) contamination cannot be detected since both RNA and DNA absorb UV at 260 nm. As such, further confirmation step should be commenced by including no reverse transcriptase (No RT) control in polymerase chain reaction (PCR) to exclude any potential gDNA contamination in the sample.

Complementary DNA (cDNA) synthesis

iScript^{TM} cDNA synthesis kit (Bio-rad, Mississauga, Canada) is used for synthesizing cDNA from total RNA. The reverse transcriptase enzyme is RNase H+ which is pre-blended with RNase inhibitor, preventing RNA degradation during cDNA synthesis. cDNA was synthesized according to the manufacturer's manual.

Real-time Quantitative Polymerase Reaction Chain (qPCR)

Real-time quantitative polymerase chain reaction is used for quantifying the expression of gene of interest relative to reference genes which are constitutively expressed in all cells. qPCR was performed using a CFX96 real-time PCR system (Bio-rad). Each reaction mixture contains complementary DNA (4 µl), 2 × SsoFast

Evagreen SYBR Green PCR Master Mix (10 µl), and 1 µM of primers (1 µl each). Values of target mRNA were corrected relative to the housekeeping gene coding for human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and mouse 18S. No RT controls were included to exclude gDNA contamination, and the experiment was performed in triplicates. Melting curve analysis was performed to confirm specificity of amplification. Data were analyzed using the $2^{-\Delta\Delta CT}$ methods and expressed as relative abundance (mean ± SEM). PCR sequences for human and mouse primers are listed in **Table2** and **3**, respectively.

Table 2. qPCR human primer sequences

	Forward (5'-3')	Reverse (5'-3')
Gapdh	CTTAGCACCCCTGGCCAAG	TGGTCATGAGTCCTTCCACG
Pparg	AAGGCCATTTTCTCAAACGA	AGGAGTGGGAGTGGTCTTCC
Defb1	Bio-rad qHsaCID0015106, PrimePCR™ SYBR Green Assay	Bio-rad qHsaCID0015106, PrimePCR™ SYBR Green Assay
Defb4	Bio-radqHsaCID0038951, PrimePCR™ SYBR Green Assay	Bio-radqHsaCID0038951, PrimePCR™ SYBR Green Assay

Table 3. qPCR mouse primer sequences

	Forward (5'-3')	Reverse (5'-3')
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Pparg	CTGCTCAAGTATGGTGTCCATGA	ATGAGGACTCCATCTTTATTCA
defb1	GGTGTTGGCATTCTCACAAG	ACAAGCCATCGCTCGTCCTTTATG
defb3	GGATCCATTACCTTCTGTTTGC	ATTTGAGGAAAGGAACTCCAC

Enzyme-Linked Immunosorbant Assay (ELISA)

5-HT ELISA

Colonic 5-HT levels were determined for *in vivo* experiment as previously described. Briefly, colonic tissues were weighed and were homogenized in 0.2 N perchloric acid. Following centrifugation at 10,000 × g for 5 minutes, the supernatants were collected and the pH was neutralized using 1 M borate buffer. The supernatants were used for analysis of 5-HT levels using commercially available ELISA kit (Beckman Coulter, Fullerton, CA). 5-HT content was expressed as a function of wet weight (ng/mg). For *in vitro* experiments, the cell supernatants were diluted 20 times with the dilution buffer provided in the manufacturer's kit. Measured 5-HT concentrations were expressed as nM.

β-defensin ELISA

Secreted hBD-1 and hBD-2 peptides in the cell supernatants as well as mouse total β-defensin peptides in colonic tissue supernatants were quantified using commercially available ELISA kits (Mybiosource, Cedarlane, Burlington, Canada).

Statistical Analysis

All statistical comparisons were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) and results are represented as mean ± SEM. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) with Dunnett's, Bonferroni's multiple comparison test, and two-way ANOVA with Bonferroni's

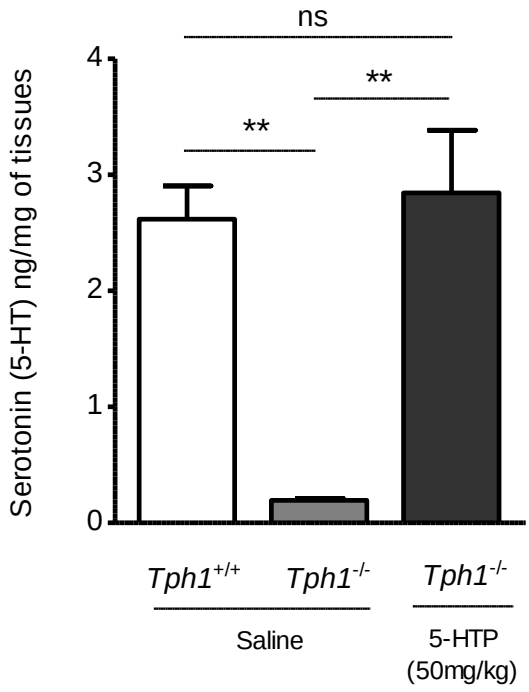
multiple comparison test were used where appropriate. Values of $*P < 0.05$ were considered statistically significant in this study.

Chapter 4: Results

4.1. *Tph1*-deficient mice exhibit an increased expression of murine β-defensins

Our preliminary finding that naïve *Tph1*$^{-/-}$ mice exhibit altered cecal microbiota composition compared with their WT littermates alludes a possible role of β-defensins. Based on this observation and our seminal study, we sought to investigate the expression of β-defensins during DSS-induced colitis. After confirming 5-HT levels in the colon of different groups of mice (**Figure 2**), *Tph1*$^{-/-}$ mice expressed higher levels of total β-defensin in the colon, compared with WT littermates on day 5 post-DSS; while similar trends were also observed in vehicle-treated mice (**Figure3a**). This suggests that 5-HT plays a role in regulation of β-defensin expression, revealing a potential innate immune defect that may contribute delayed onset and severe colitis in WT littermates upon DSS treatment. Interestingly, DSS-treated mice had significantly higher total β-defensin levels, compared with their vehicle-treated counter parts, suggesting that increased β-defensin levels serve as a marker of inflammation.

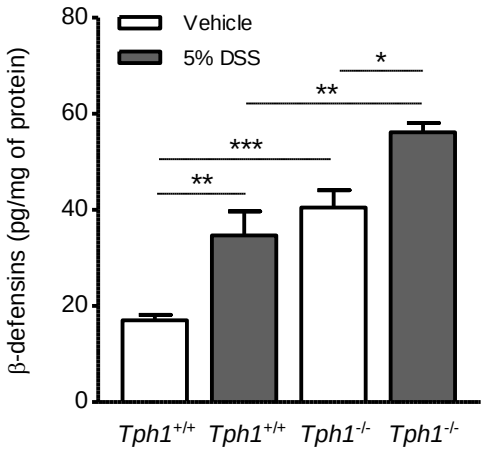
Figure 2. ***Tph1*-deficient (*Tph1*$^{-/-}$) mice have lower amounts of colonic 5-HT**. Assessment of 5-HT levels in colonic tissues of naïve *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, and *Tph1*$^{-/-}$ treated with 5-HTP (n = 4 per group). Data are represented as mean ± s.e.m. *P < 0.05, and **P < 0.01 (One way ANOVA with Bonferroni's post hoc test).


ns
**
**
Serotonin (5-HT) ng/mg of tissues
4
3
2
1
0
Tph1+/+
Tph1-/-
Tph1-/-
Saline
5-HTP
(50mg/kg)

To substantiate whether an increased β-defensin level was due to lack of 5-HT, we restored 5-HT amounts in *Tph1*$^{-/-}$ mice by reconstituting 5-HT synthesis with 5-HTP (50 mg/kg subcutaneously), the product of *Tph1* and an immediate precursor of 5-HT. Administration of 5-HTP reduced the total β-defensin levels in colonic tissues of naïve *Tph1*$^{-/-}$ mice (**Figure 3b**). We further examined whether specific types of β-defensins are influenced by 5-HTP administration by measuring murine β-defensin 1 (mBD-1; *mdefb1*) and 3 (mBD-3; *mdefb3*) expression, which are expressed constitutively and induced under inflammatory condition, respectively. We observed that both mBD-1 and mBD-3 expression were reduced in *Tph1*$^{-/-}$ mice treated with 5-HTP (**Figure 4**). These findings suggest an inhibitory role of gut-derived 5-HT in β-defensin expression.

Figure 3. ***Tph1*-deficient (*Tph1*$^{-/-}$) mice express increased levels of total mouse β-defensin that is reversed by reconstitution with 5-HTP**. (**a**) Total mouse β-defensin in colonic tissues of vehicle- and DSS-treated mice (n = 4 per group). *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test). (**b**) Total mouse β-defensin levels in colonic tissues of naïve *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, and *Tph1*$^{-/-}$ mice treated with 5-HTP (n = 4 per group) relative to total protein. Data are represented as mean ± s.e.m. *P < 0.05, and **P < 0.01 (One way ANOVA with Bonferroni's post hoc test).

a)


Vehicle
5% DSS
β-defensins (pg/mg of protein)
80
60
40
20
0
*
**
***
**
Tph1+/+
Tph1+/+
Tph1-/-
Tph1-/-

b)

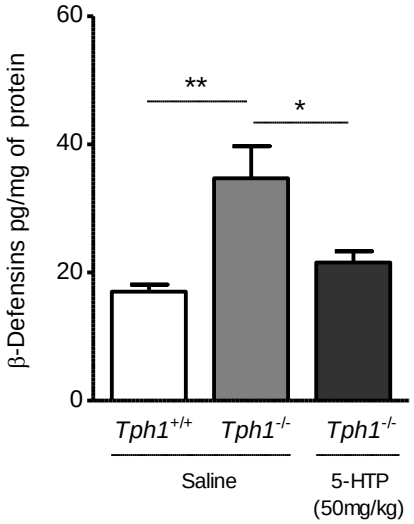
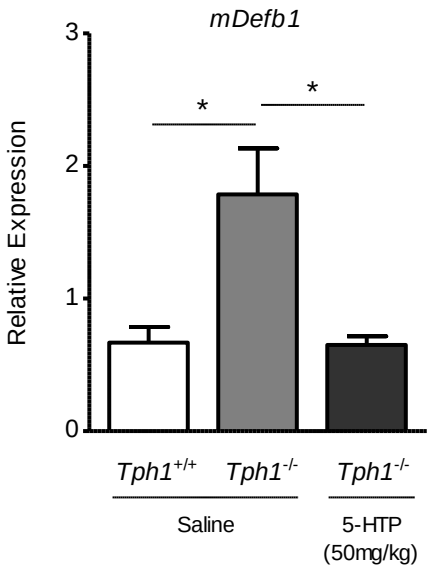
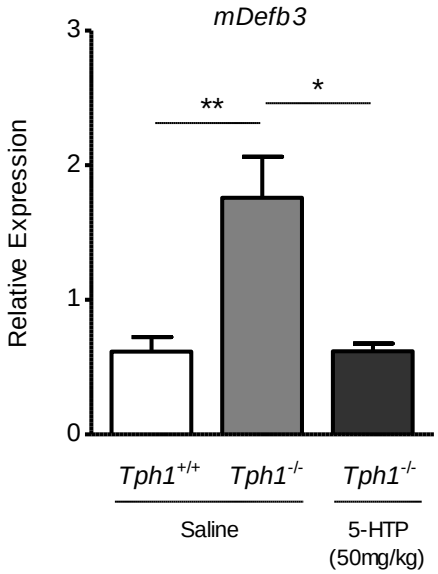

β-Defensins pg/mg of protein
60
40
20
0
**
*
Tph1+/+
Tph1-/-
Tph1-/-
Saline
5-HTP
(50mg/kg)

Figure 4. **Lack of 5-HT leads to higher levels of mouse β-defensin 1 and β-defensin 3 in the colon of *Tph1*$^{-/-}$ mice.** (**a**) Expression of mouse β-defensin 1 (*mdefb1*) and (**b**) *mdefb3* mRNA levels in colonic tissues of naïve *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, and *Tph1*$^{-/-}$ mice treated with 5-HTP (n = 4 per group) using real-time quantitative PCR. Data are represented as mean ± s.e.m. *P < 0.05, and **P < 0.01 (One way ANOVA with Bonferroni's post hoc test).

a)


mDefb1
Relative Expression
3
2
1
0
*
*
Tph1+/+
Tph1-/-
Tph1-/-
Saline
5-HTP
(50mg/kg)

b)


mDefb3
Relative Expression
3
2
1
0
**
*
Tph1+/+
Tph1-/-
Tph1-/-
Saline
5-HTP
(50mg/kg)

4.2. 5-HT directly attenuates β-defensin 1 and 2 production from human colonic epithelial cell line HT-29.

Given the location of EC cells and 5-HT receptors in gut mucosa as well as the fact that 5-HT is released towards the basolateral side of the gut epithelium where 5-HT receptors are localized, it seems likely that 5-HT directly attenuates β-defensin production from intestinal epithelial cells. Herein, the effect of 5-HT on human β-defensin 1 (hBD-1; *defb1*) production was determined by stimulating human colon epithelial cell line HT-29 with increasing dosages of 5-HT (10^{-11}, 10^{-7} and 10^{-5} mol/L) for 24 h. As assessed by real-time qPCR, the expression of *defb1* in 5-HT-treated cells was decreasedin a dose-dependent manner, compared to control (untreated) (**Figure 5**). It has been shown HT-29 cells constitutively express hBD-1, while the expression is not affected by either pro-inflammatory cytokines or bacterial challenge. Similarly, we found that the effect of IL-1β (20 or 40 ng/ml) was not different to that of untreated cells. In contrast to hBD-1, human β-defensin 2 (hBD-2; *defb4*) expression is rapidly induced under inflammatory conditions. Since either untreated or 5-HT treated cells do not express hBD-2 (**Figure 7**), we decided to determine the optimal time point at which IL-1β induces the maximal expression of *defb4* by stimulating cells with increasing dosages of IL-1β (10, 20 and 40 ng/ml) at four different time points: 3, 6, 12 and 24 h. The maximal expression occurred by 6 h after cells were treated with IL-1β (40 ng/ml) (**Figure 6**). We then treated cells with IL-1β and increasing dosages of 5-HT (10^{-11}, 10^{-7} and 10^{-5} mol/L) for 6 h, and found that 5-HT was able to inhibit *defb4* expression in a dose-dependent manner as well (**Figure 7**).

For further experiments, we decided to use the minimum effective 5-HT concentration: 10^{-7} mol/L. Consistent with mRNA expression, levels of both hBD-1 and hBD-2 peptide were also reduced in 5-HT-treated cell supernatants (**Figure 8**). These results highlight the direct effect of 5-HT in inhibiting hBD-1 and hBD-2 production from intestinal epithelial cells.

Figure 5. 5-HT directly inhibits human β-defensin (hBD) 1 expression in HT-29 cells. Real-time quantitative PCR analysis of hBD-1 (*defb1*) expression in HT-29 cells treated for 24 h with increasing dosage of 5-HT (10^{-11}, 10^{-7}, and 10^{-5} mol/L) and IL-1β (20 and 40 ng/ml). IL-1β was used as a positive control. Untreated cells were considered as a negative control. Data are representative of three independent experiments (mean ± s.e.m). $^{**}P < 0.01$ (One way ANOVA with Dunnett's post hoc test).

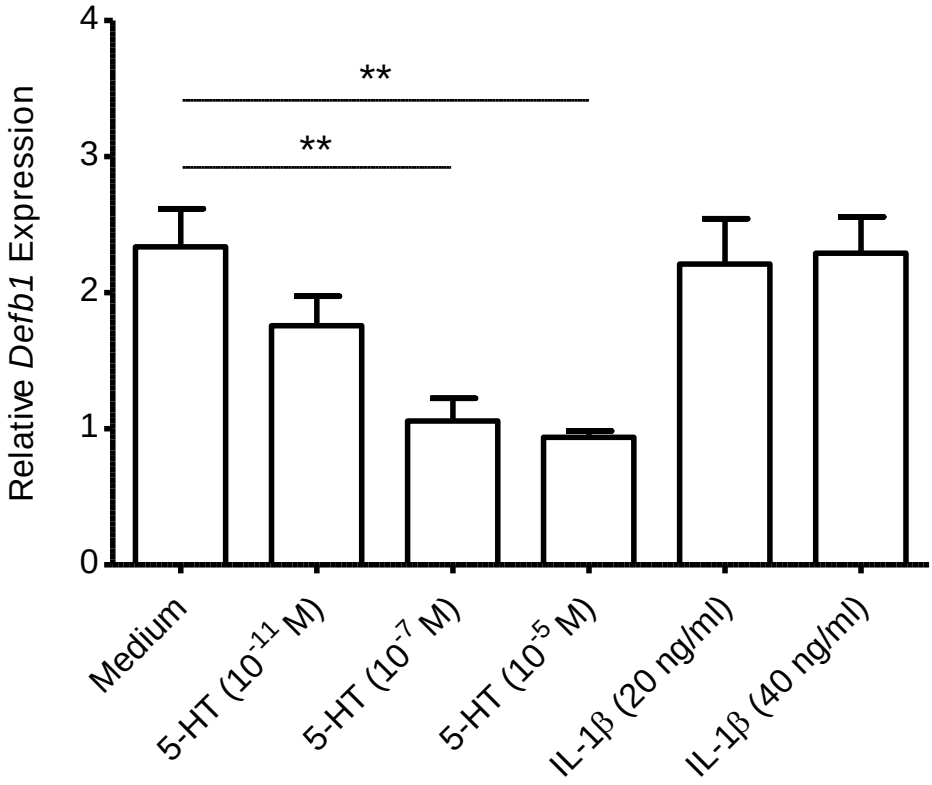

Relative *Defb1* Expression
4
3
2
1
0
**
**
Medium
5-HT (10^{-11} M)
5-HT (10^{-7} M)
5-HT (10^{-5} M)
IL-1β (20 ng/ml)
IL-1β (40 ng/ml)

Figure 6. **Human β-defensin (hBD) 2 are induced by IL-1β in a dose- and time-dependent manner in HT-29 cells.** Real-time quantitative PCR analysis of hBD-2 (*defb4*) expression in HT-29 treated for 3-24 h with increasing dosage of IL-1β (10, 20 and 40 ng/ml). Data are representative of two independent experiments and are represented as mean ± s.e.m. $^{**}P < 0.01$ (One way ANOVA with Bonferroni's post hoc test).

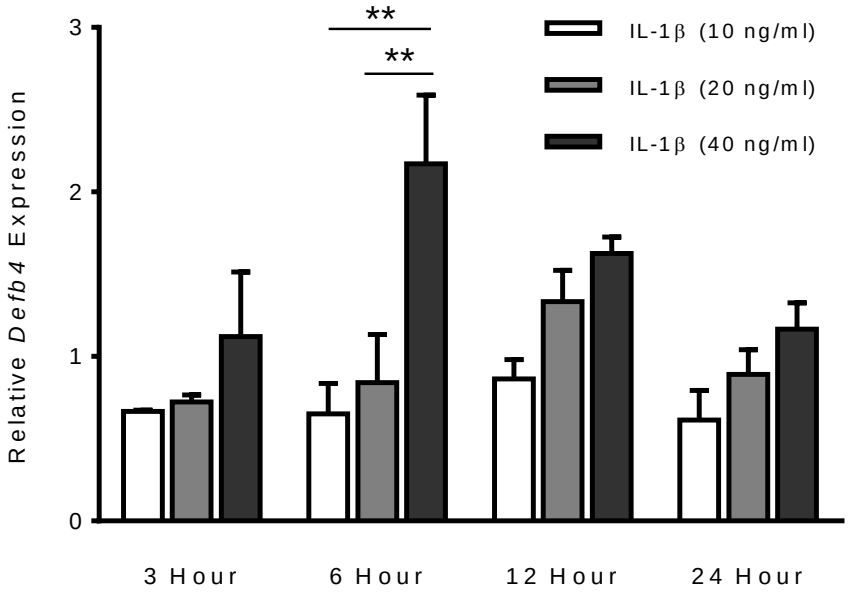

**
**
IL-1β (10 ng/ml)
IL-1β (20 ng/ml)
IL-1β (40 ng/ml)
Relative Defb4 Expression
3
2
1
0
3 Hour
6 Hour
12 Hour
24 Hour

Figure 7. **5-HT down-regulates β-defensin (hBD) 2 expression in HT-29 cells.** Real-time quantitative PCR analysis of hBD-2 (*defb4*) expression in HT-29 treated with either increasing dosages of 5-HT for 6 h (10^{-11}, 10^{-7}, and 10^{-5} mol/L) or IL-1β (40 ng/ml) for 1 h prior to 5-HT treatment for a total of 6 h. Expression of hBD-2 in untreated and 5-HT treated cells were undetected. Data are representative of three independent experiments (mean ± s.e.m). $^{**}P < 0.01$ (One way ANOVA with Dunnett's post hoc test).

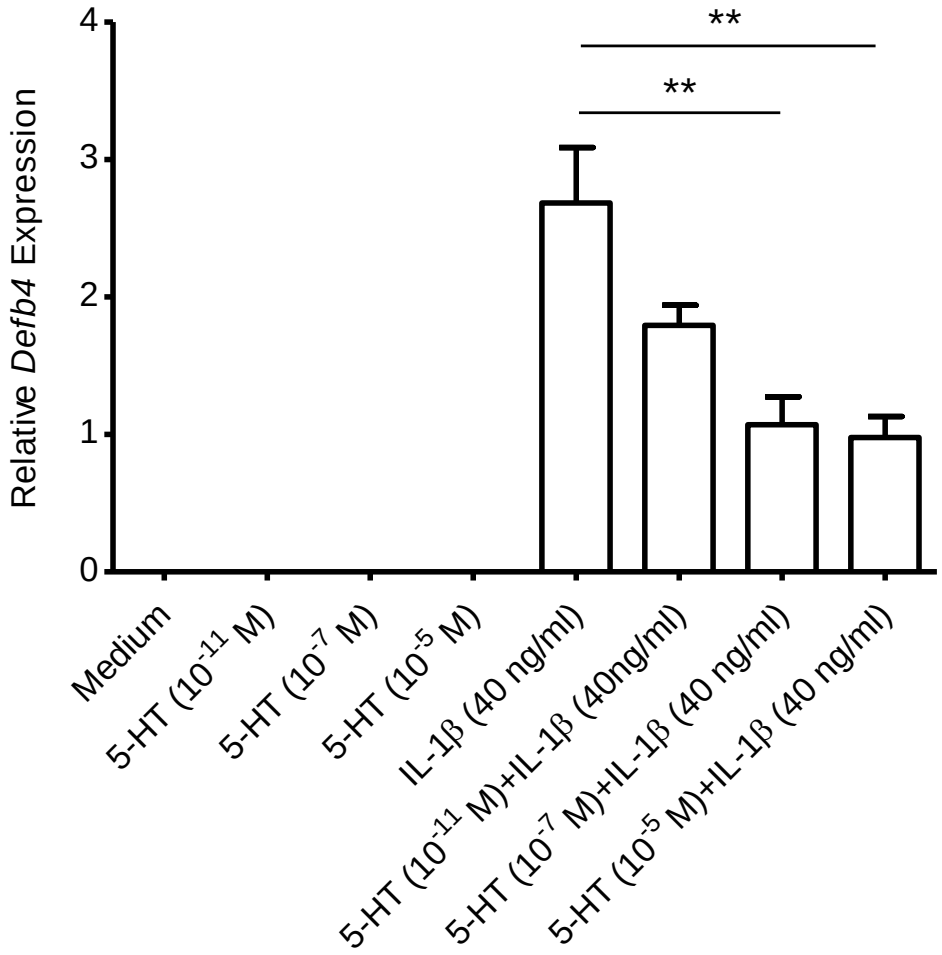
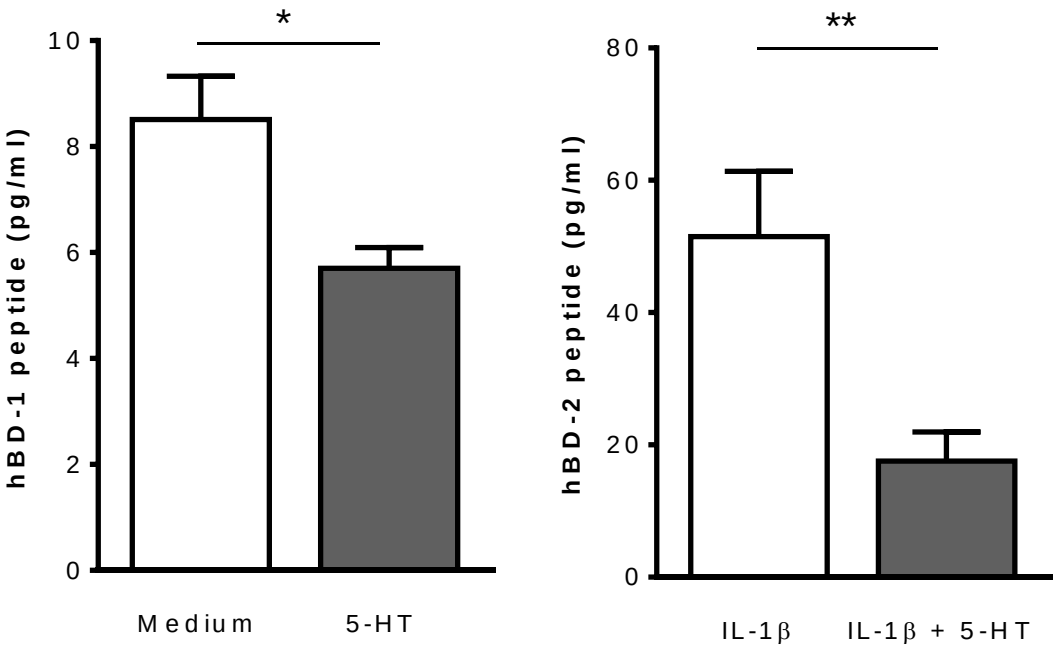

Relative *Defb4* Expression
0
1
2
3
4
**
**
Medium
5-HT (10^{-11} M)
5-HT (10^{-7} M)
5-HT (10^{-5} M)
IL-1β (40 ng/ml)
5-HT (10^{-11} M)+IL-1β (40ng/ml)
5-HT (10^{-7} M)+IL-1β (40 ng/ml)
5-HT (10^{-5} M)+IL-1β (40 ng/ml)

Figure 8. 5-HT inhibits human β-defensin 1 and 2 peptide levels in HT-29 cells. Measurement of secreted (**a**) hBD-1 and (**b**) hBD-2 peptides in cell culture supernatants using commercially available ELISA kit. Data are representative of three independent experiments (mean ± s.e.m). **P* < 0.05, and ***P* < 0.01 (Student's *t* test).


*
10
8
6
4
2
0
hBD-1 peptide (pg/ml)
Medium
5-HT
**
80
60
40
20
0
hBD-2 peptide (pg/ml)
IL-1β
IL-1β + 5-HT

4.3. Activation of 5-HT_7 receptors, but not 5-HT_3 or 5-HT_4, suppresses β-defensin 1 and 2 expression

There are 5-HT receptors along the colonic epithelium: 5-HT_3, 5-HT_4 and 5-HT_7[125]. We have previously shown that the severity of intestinal inflammation was attenuated in *Tph1*$^{+/+}$ mice received treatment with 5-HT_7 receptor (5-HT_7R) antagonist, SB-269970; while the disease severity was correlated with 5-HT_7R expression level[133]. Thus, we first sought to explore whether 5-HT inhibits β-defensin production by activating 5-HT_7 receptors. HT-29 cells were stimulated with 5-HT and SB-269970 (1 μM), where cells were pre-treated with the antagonist for 1 h prior to 5-HT treatment. The antagonist completely abolished the inhibitory effect of 5-HT on hBD-1 expression at both transcriptional and translational level (**Figure 9**). Similarly, the antagonist was also able to restore hBD-2 expression (**Figure 10**). To investigate the possible role of 5-HT_3 and 5-HT_4 receptors in regulating β-defensins, we pre-treated HT-29 cells with either 5-HT_3 or 5-HT_4 receptor antagonists, Tropisetron (**Figure 11**) or RS-39604 (**Figure 12**), respectively (1 μM), for 1 h prior to 5-HT treatment. Surprisingly, these antagonists were unable to restore both hBD-1 and hBD-2 expression. This suggests that 5-HT attenuates β-defensin 1 and 2 expression by specifically activating 5-HT_7R on colonic epithelial cells, while disrupting the receptor pathway can efficaciously ameliorate the inhibitory effect of 5-HT.

Figure 9. 5-HT down-regulates human β-defensin 1 by activating 5-HT$_7$ receptors in the human epithelial cell lines HT-29. (**a**) Real-time quantitative PCR analysis of hBD-1 mRNA expression and (**b**) measurement of hBD-1 peptides in the supernatants of HT-29 cells treated with 5-HT and 5-HT plus 5-HT$_7$ receptor antagonist, SB-269970 (1 μM). Data are representative of two independent experiments (mean ± s.e.m). *P < 0.05, and **P < 0.01 (One way ANOVA with Bonferroni's post hoc test).

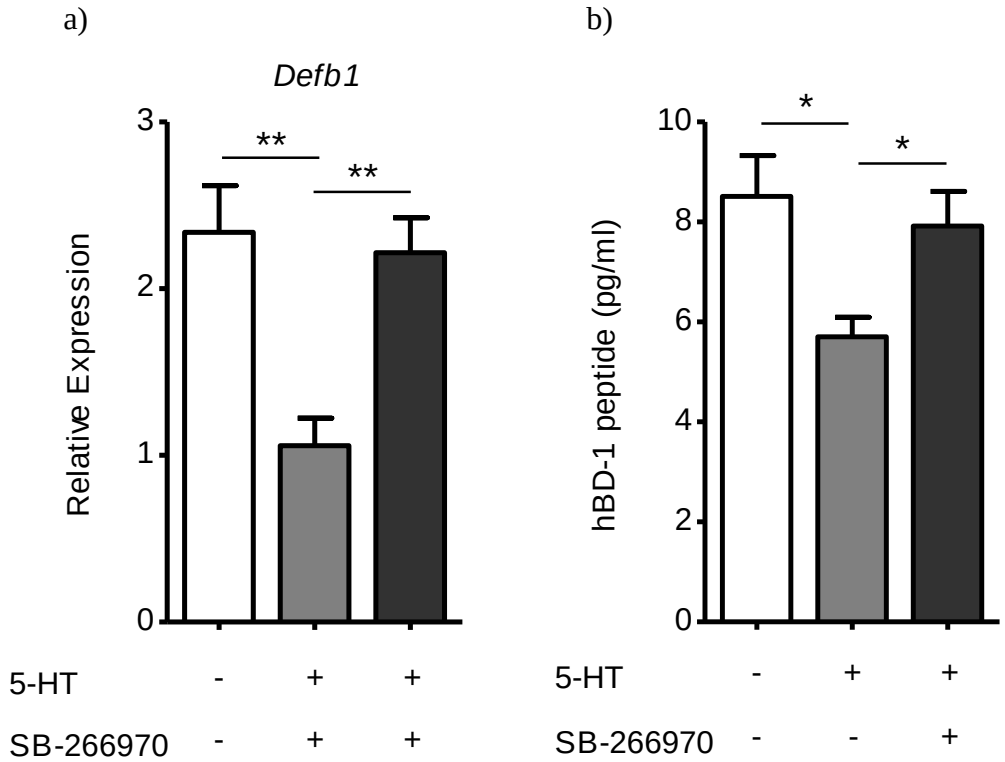

a)
Defb1
Relative Expression
3
2
1
0
**
**
5-HT - + +
SB-266970 - + +
b)
hBD-1 peptide (pg/ml)
10
8
6
4
2
0
*
*
5-HT - + +
SB-266970 - - +

Figure 10. 5-HT down-regulates human β-defensin 2 by activating 5-HT$_7$ receptors in the human epithelial cell lines HT-29. (**a**) Real-time quantitative PCR analysis of hBD-2 mRNA expression and (**b**) measurement of hBD-2 peptides in the supernatants of HT-29 cells treated with 5-HT and 5-HT plus 5-HT$_7$ receptor antagonist, SB-269970 (1 μM). Data are representative of two independent experiments (mean ± s.e.m). ***P*< 0.01 (One way ANOVA with Bonferroni's post hoc test).

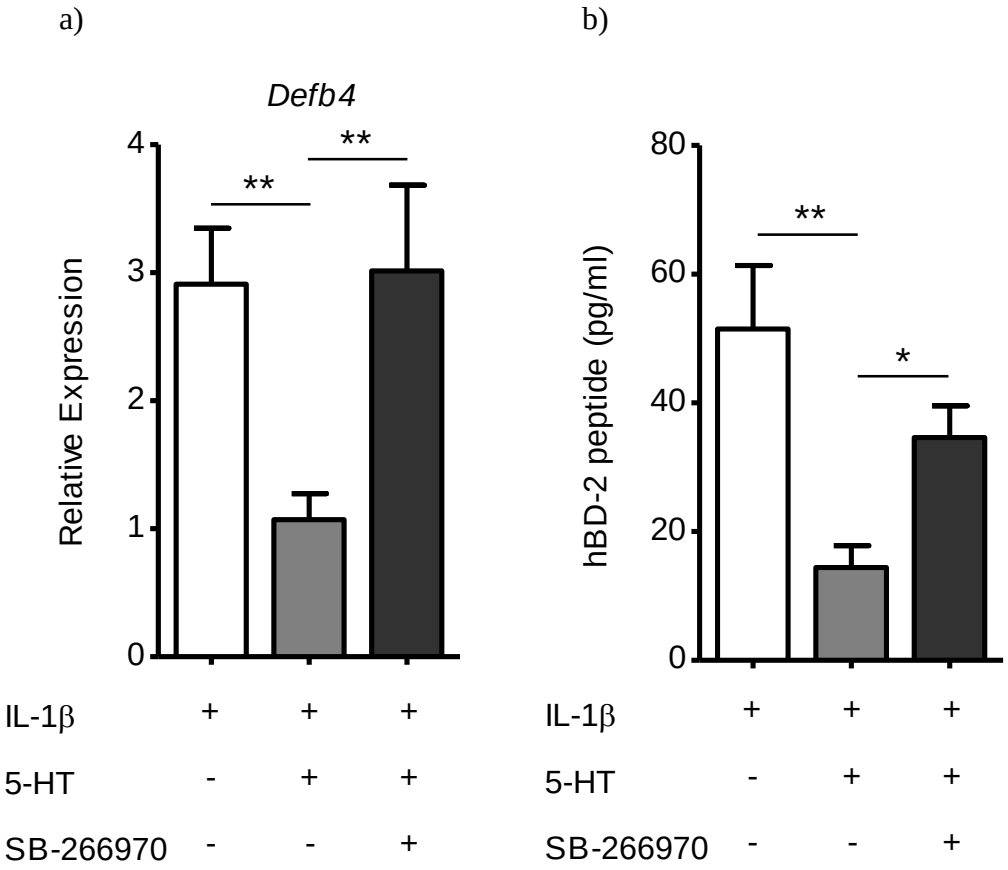

a)
b)
Defb4
Relative Expression
hBD-2 peptide (pg/ml)
**
**
**
*
IL-1β + + +
5-HT - + +
SB-266970 - - +
IL-1β + + +
5-HT - + +
SB-266970 - - +

Figure 11. 5-HT_3 receptors are not involved in inhibiting human β-defensins in HT-29 cells. (**a**) Real-time quantitative PCR analysis of hBD-1 and (**b**) hBD-2 mRNA expressions in HT-29 cells treated with 5-HT and 5-HT plus 5-HT_3 receptor antagonist, Tropisetron (1 μM). Data are representative of two independent experiments (mean ± s.e.m). $^{**}P < 0.01$ (One way ANOVA with Bonferroni's post hoc test).

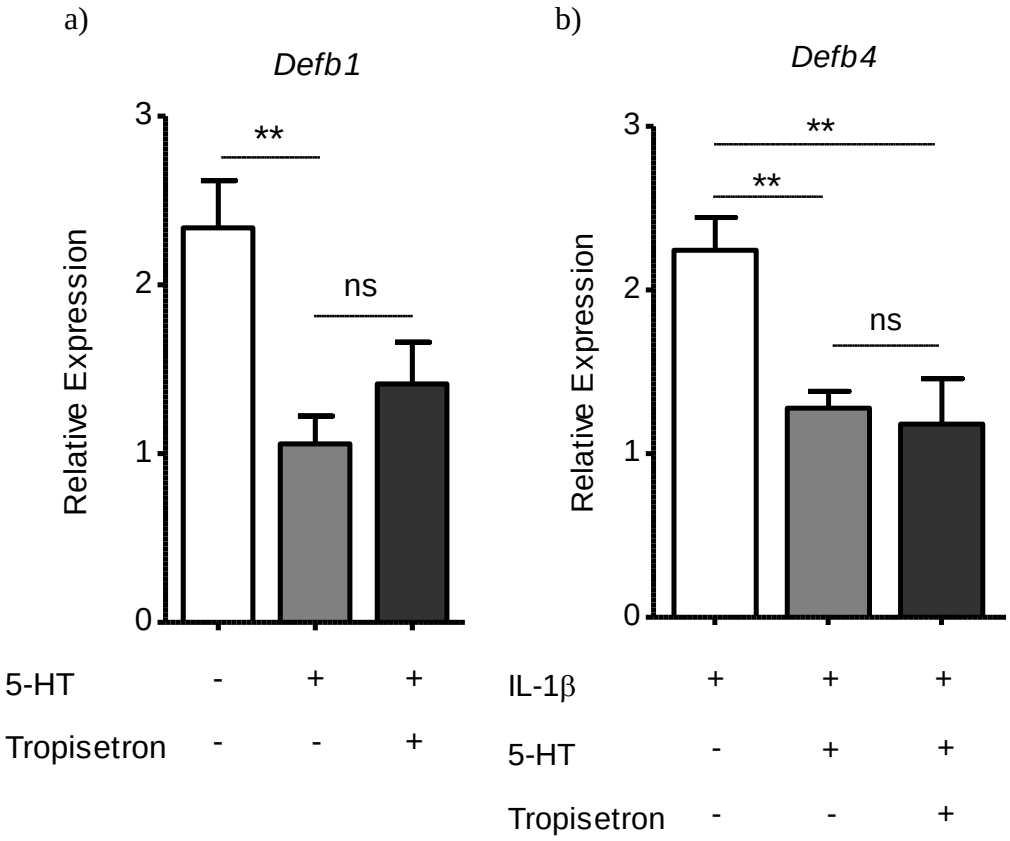
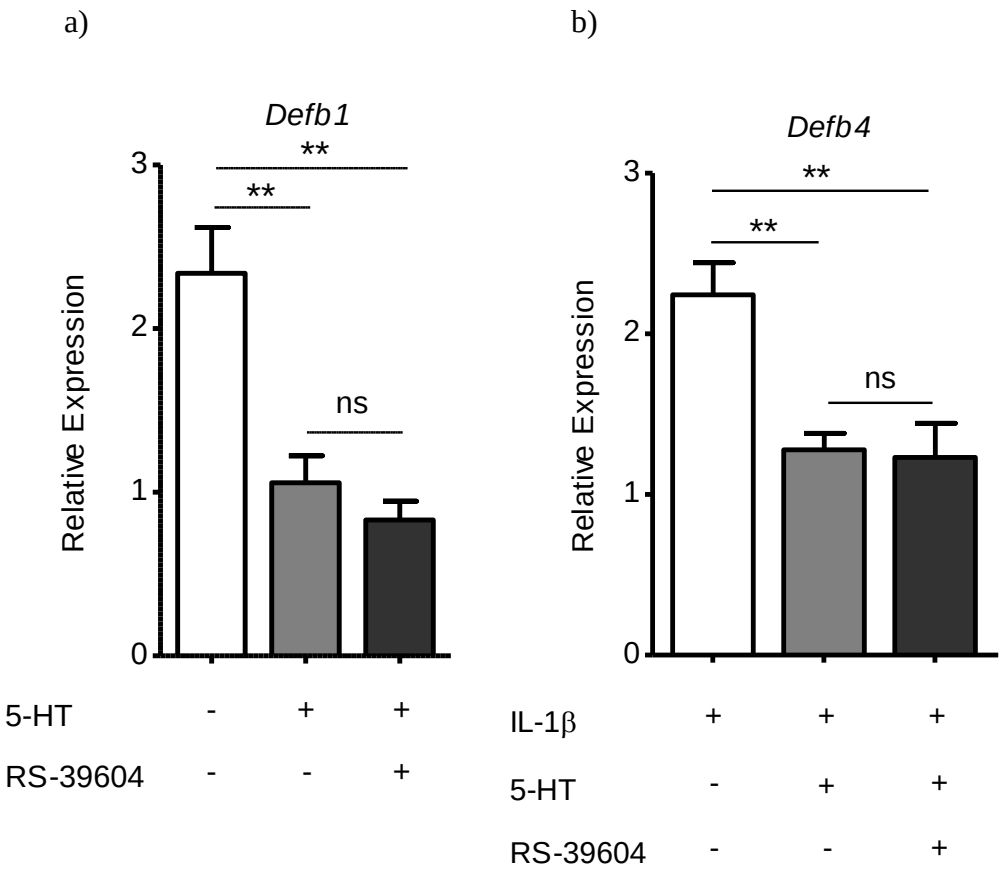

a)
Defb1
Relative Expression
3
2
1
0
**
ns
5-HT - + +
Tropisetron - - +
b)
Defb4
Relative Expression
3
2
1
0
**
**
ns
IL-1β + + +
5-HT - + +
Tropisetron - - +

Figure 12. 5-HT$_4$ receptors are not involved in inhibiting human β-defensins in HT-29 cells. (**a**) Real-time quantitative PCR analysis of hBD-1 and (**b**) hBD-2 mRNA expressions in HT-29 cells treated with 5-HT and 5-HT plus 5-HT$_4$ receptor antagonist, RS-39604 (1 μM). Data are representative of two independent experiments (mean ± s.e.m). $^{**}P < 0.01$ (One way ANOVA with Bonferroni's post hoc test).


a)
b)
Defb1
Defb4
Relative Expression
0
1
2
3
**
**
ns
5-HT
RS-39604
IL-1β
- + +
- - +
+ + +
- + +
- - +

4.4. 5-HT inhibits β-defensin production by attenuating peroxisome proliferative-activated receptor gamma via 5-HT$_7$ receptors

PPAR-γ plays a crucial role for colonic expression of β-defensin. In addition, it is recently reported that 5-HT induced inhibition of PPAR-γ expression in pulmonary artery smooth muscle cells[196,208,210], thereby providing a crucial link between 5-HT and PPAR-γ regulation. Thus, we first sought to investigate whether naïve *Tph1*$^{-/-}$ mice express altered levels of PPAR-γ as compared with their WT mice. Colonic tissues of *Tph1*$^{-/-}$ mice expressed significantly higher levels of PPAR-γ, while replenishing 5-HT amount in these mice by 5-HTP decreased the expression (**Figure 13**). To further elucidate whether an increased expression of both mBD-1 and mBD-3 in *Tph1*$^{-/-}$ mice is mediated through PPAR-γ activation, these mice were treated with vehicle (DMSO; 5% in dH_2O) or PPAR-γ antagonist, GW-9662, in a dosing volume of 2 mg/kg per day via intraperitoneal (i.p.) administration for 5 days. Colonic tissues of the antagonist-treated *Tph1*$^{-/-}$ mice exhibited an attenuated expression of both mBD-1 and mBD-3 (**Figure 14**), suggesting PPAR-γ activation is essential for maintaining β-defensin expression. Next, we examined whether 5-HT is directly involved in regulating PPAR-γ expression. We treated HT-29 cells with 5-HT or SB-266970 (1 μM) for 1 h prior to 5-HT treatment. 5-HT inhibited PPAR-γ expression and the inhibitory effect was diminished by SB-266970 (**Figure 15**). These findings reveal that 5-HT down-regulates PPAR-γ expression by activating 5-HT$_7$ receptors.

5-HT activates MEK-ERK pathway via 5-HT$_7$ receptor in a wide array of cell types[211–214]. The phosphorylated ERK1/2, in turn, leads to phosphorylation of PPAR-γ,

which results in rapid degradation of PPAR-γ that is reversed by mitogen activated protein kinase (MAPK) ERK kinase (MEK) inhibitor[215]. In addition, it has recently been shown that 5-HT inhibited PPAR-γ expression through induction of extracellular signal-regulated kinase-1 and -2 (ERK1/2) pathway in pulmonary artery smooth muscle cells[210], providing a crucial link between 5-HT_7 receptor and its downstream cascade. Thus, we sought to investigate the intracellular pathway responsible for β-defensin expression. We inhibited ERK1/2 activation in HT-29 cells by a MEK inhibitor (PD98059; 40 μM) for 1 h prior to 5-HT treatment. We found that ERK1/2 activation inhibits hBD-1 and hBD-2 expression as the inhibitor was able to mask the inhibitory effect of 5-HT (**Figure 16**). These *in vitro* findings suggest that 5-HT down-regulates β-defensin expression via 5-HT_7 receptors by activating ERK1/2 and thereby suppressing PPAR-γ expression.

Figure 13. Higher levels of PPAR-γ expression in *Tph1*$^{-/-}$ mice. Real-time quantitative PCR analysis of PPAR-γ mRNA in colonic tissues from *Tph1*$^{+/+}$, *Tph1*$^{-/-}$ and *Tph1*$^{-/-}$ plus 5-HTP (n = 4 per group). Data are represented as mean ± s.e.m. ***P < 0.01 (One way ANOVA with Bonferroni's post hoc test).

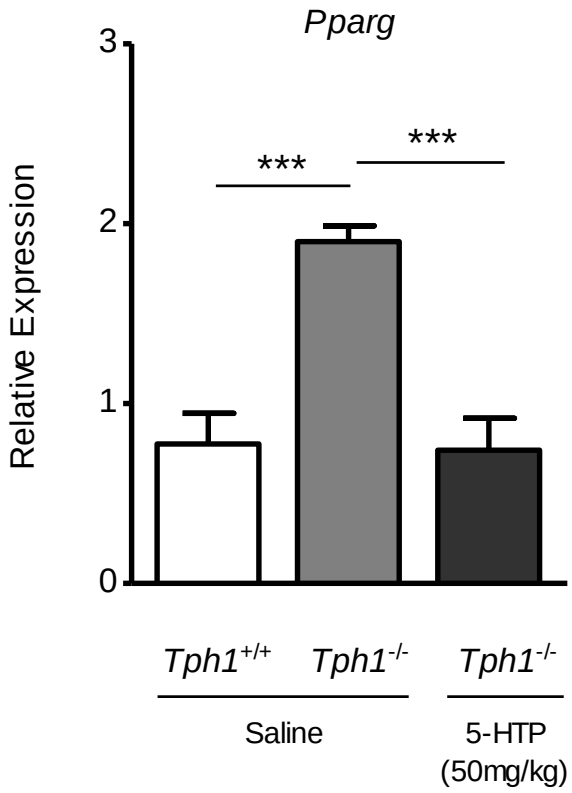
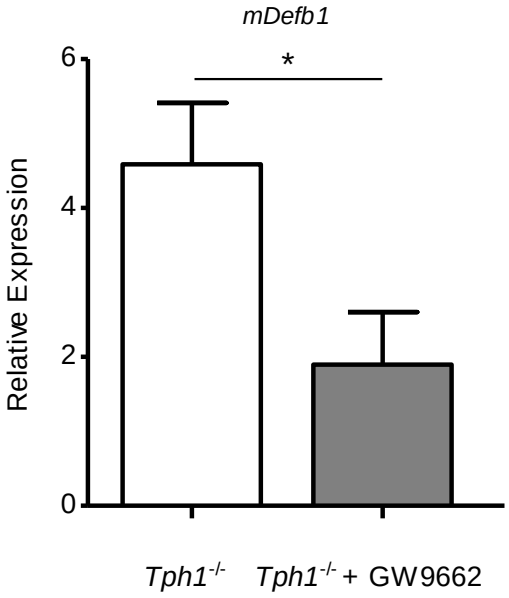

Pparg
Relative Expression
3
2
1
0
***
***
Tph1+/+
Tph1-/-
Tph1-/-
Saline
5-HTP
(50mg/kg)

Figure 14. PPAR-γ expression is essential for maintaining mouse β-defensin (mBD) 1 and mBD-3 expression in *Tph1*$^{-/-}$ mice. Real-time quantitative PCR analysis of (**a**) mBD-1 and (**b**) mBD-3 mRNA in colonic tissues from *Tph1*$^{-/-}$ mice intraperitoneally (i.p.) treated with PPAR-γ antagonist, GW-9662 (2 mg/kg) for 5 days or *Tph1*$^{-/-}$ with vehicle (DMSO) (n = 4 per group). Data are represented as mean ± s.e.m. *$P < 0.05$ (Student's t-test).

a)


mDefb1
*
Relative Expression
0
2
4
6
Tph1-/-
Tph1-/- + GW9662

b)

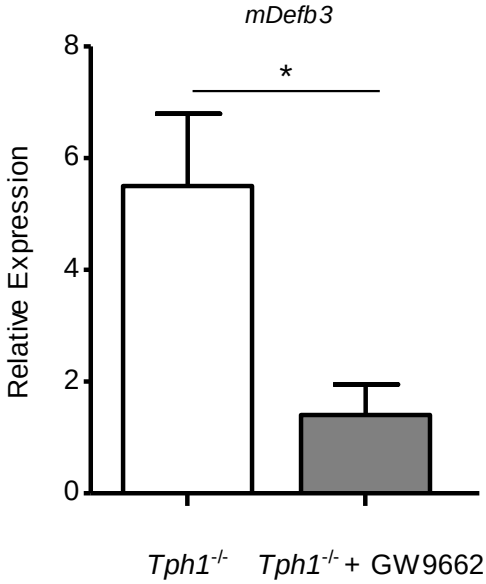

mDefb3
*
Relative Expression
0
2
4
6
8
Tph1-/-
Tph1-/- + GW9662

Figure 15. 5-HT_7 receptor activation suppresses PPAR-γ expression in HT-29 cells. Real-time quantitative PCR analysis of PPAR-γ mRNA in HT-29 treated with 5-HT and 5-HT_7 receptor antagonist, SB-269970 (1 μM) for 24 h. Data are represented as mean ± s.e.m. **P* < 0.05 (One way ANOVA with Bonferroni's post hoc test).

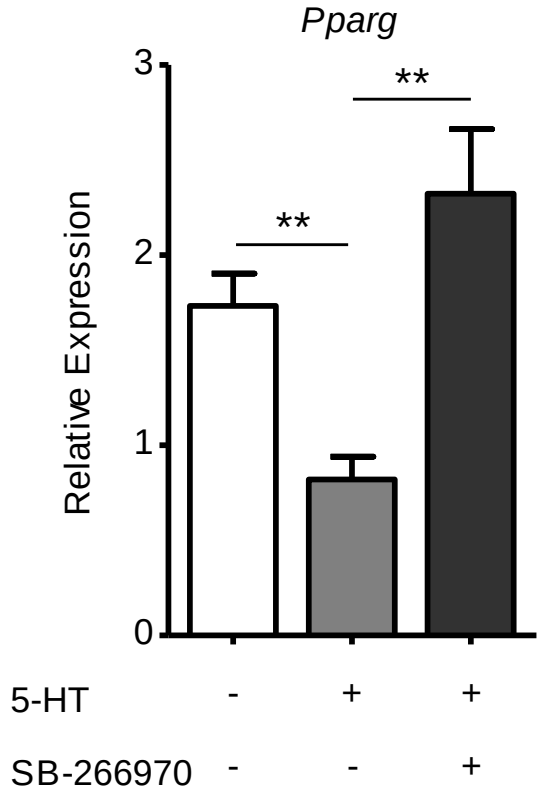
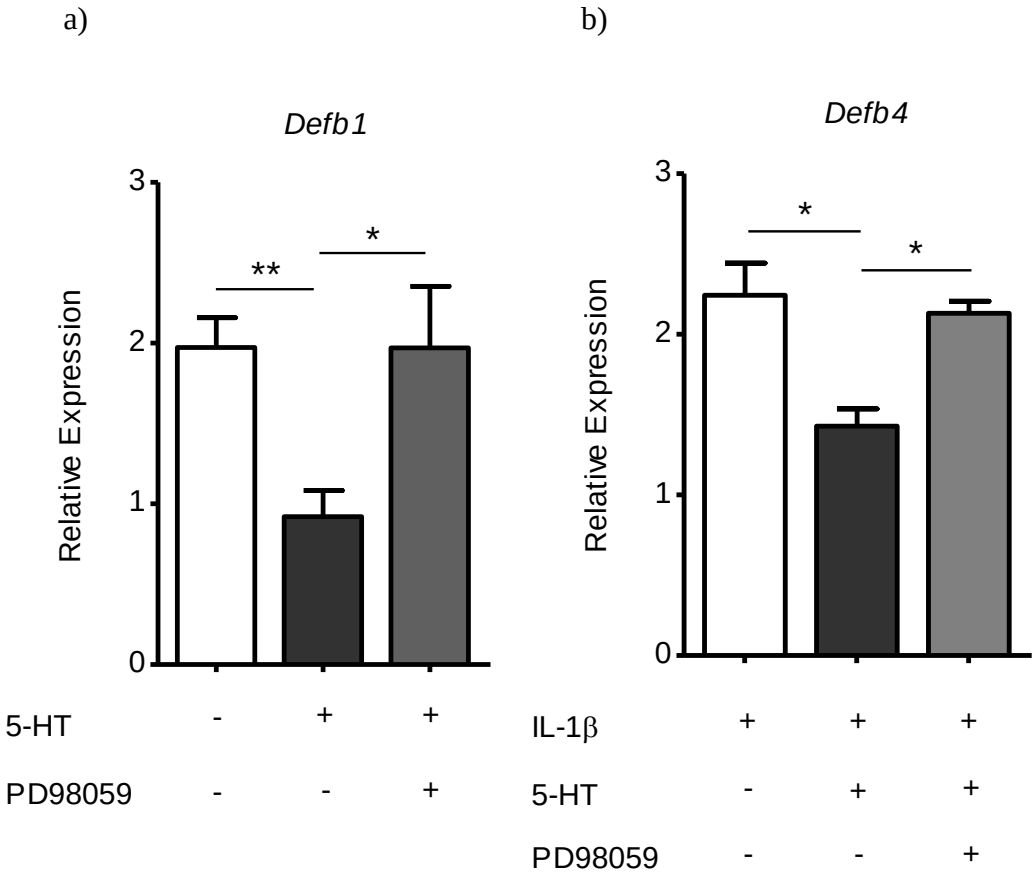

Pparg
Relative Expression
3
2
1
0
**
**
5-HT
SB-266970
-
+
+
-
-
+

Figure 16. 5-HT activates 5-HT$_7$ receptors and ERK1/2, down-regulating human β-defensin 1 and 2 expression by inhibiting PPAR-γ expression in HT-29. Real-time quantitative PCR analysis of (**a**) hBD-1 and (**b**) hBD-2 mRNA expression in HT-29 cells treated with MEK inhibitor, PD98059 (40 μM) for 1 h prior to 5-HT treatment for 24 h and 6 h, respectively. *P < 0.05 and **P < 0.01 (One way ANOVA with Bonferroni's post hoc test).


a)
b)
Defb1
Defb4
Relative Expression
3
2
1
0
**
*
*
*
5-HT - + +
PD98059 - - +
IL-1β + + +
5-HT - + +
PD98059 - - +

Chapter 5: Discussion

Since the discovery of 5-HT by Erspamer in 1937, it has been most intensively studied regarding its role in the regulation of various neuropsychological and cognitive functions[15]. There is now abundant evidence favoring that 5-HT play a crucial role in gut physiological function and pathogenesis of various GI diseases including IBD. EC cells are responsible for synthesizing vast majority of 5-HT in the body and are dispersed among the epithelial cells in the mucosal layer throughout the GI tract[16]. The strategic location of EC cell in the mucosal layer makes EC cells interact with luminal microorganisms and respond to changes in gut contents to synthesize and release 5-HT in the context of gut health[216]. 5-HT released from EC cells mediate many GI functions through 5-HT receptors. AMPs are endogenous antibiotics produced by various epithelial cells including intestinal epithelial cells. AMPs are crucial members of the innate immunity, which shape the composition of luminal microorganisms, thereby maintaining the balance between commensal and pathogenic microbes. Reduced levels of AMPs, especially β-defensins, in the colon of IBD patients[185–187] highlight the important role of these effector molecules in regulating the microbial community while maintaining homeostatic state at the mucosal barrier. As several 5-HT receptors are expressed on colonic epithelial cells and our preliminary data that 5-HT is involved in regulating gut microbiota composition, it seems possible that 5-HT released from EC cells plays a pivotal role in β-defensin production from the epithelial cells. In this thesis, we demonstrated the

importance of 5-HT-β-defensin axis for gut homeostasis and outlined intracellular pathways responsible for β-defensin production from intestinal epithelial cells.

EC cells comprise the largest endocrine cell population in the gut, which are responsible for synthesizing the vast majority of the body's 5-HT[4,16]. Besides the role of 5-HT in mediating many GI functions, such as motility, secretion, through activation of a diverse range of 5-HT receptors[40], 5-HT has been implicated in pathophysiology of various GI disorders including IBD. Aberrant 5-HT signaling have been observed in IBD[134–137] and changes in EC cell numbers and 5-HT content have been in association with both UC and CD[134,135]. In various experimental models of intestinal inflammation, increased EC cell numbers and 5-HT levels have been observed[138,140,141]. In a seminal study, we have previously shown that the severity of intestinal inflammation induced by DSS and DNBS was significantly reduced in mice lacking the rate-limiting *Tph1* enzyme or *Tph1*$^{+/+}$ mice treated with *Tph* inhibitor *para*-chlorophenylalanine (*p*CPA), compared with their WT mice; while reconstitution of 5-HT synthesis in *Tph1*$^{-/-}$ mice with 5-HTP exacerbated the disease severity[130]. Recently, we have also shown that peripheral *Tph* inhibitor (LX1032/LX1606) effectively reduced 5-HT synthesis in EC cells, but not in the brain, thereby suppressing severity of chemical- and infection-induced intestinal inflammation[126]. In addition, we have also shown that immune cells, such as DCs and macrophages, from *Tph1*$^{-/-}$ mice generated lower levels of IL-12p40 and IL-1β, along with less stimulatory activity towards CD4+ T cells and decreased macrophage infiltration in these mice on day 5 post-DSS, respectively[132]. These observations

suggest a pivotal role of 5-HT in the pathogenesis of intestinal inflammation by exerting its pro-inflammatory actions by modulating immune cells in LP.

Trillions of microorganisms in the GI tract regulate many GI physiological functions and develop immune system without harming the host, thereby maintaining intestinal homeostasis[37,98,108]. It is increasingly recognized that gut microbiota dysbiosis plays a key role in the pathogenesis of various GI diseases including IBD. The healthy human gut is the home of approximately 64% of *Firmicutes*, 23% of *Bacteriodetes*, 8% of *Proteobacteria*, and 3% of *Actinobacteria*[122]. In particular, an unusual expansion of *Proteobacteria* is positively correlated with the severity of IBD in both human and mice[217,218]. On the other hand, a decreased abundance and biodiversity of the dominant phylum *Firmicutes* has been implicated especially in CD patients[120,121]. In addition, it has been reported that *Faecalibacterium prausnitzii*, a major member of *Firmicutes*, is an anti-inflammatory commensal bacterium as it increased IL-10 and decreased TNF-α and IL-12 secretion in mice post-TNBS; while the reduction has been reported in association with an increased risk of post-operative recurrence of ileal CD[117]. These observations suggest that regulatory mechanisms responsible for maintaining host-microbe interactions are compromised. AMPs are crucial part of the regulatory mechanisms in the innate immune system as they contribute to an intact mucosal barrier and a beneficial host-microbe homeostasis in the gut by shaping microbial communities. Indeed, dysregulation in AMP production has been implicated in a number of GI disorders, such as IBD[182,185,186,200]. β-defensins are the primary AMPs expressed by colonic epithelial cells[146]. It has been shown that

diminished antimicrobial activity and subsequent weakening of the mucosal barrier due to attenuated hBD-1 and hBD-2 expression is associated with colonic CD patients[182,186]. The widely held hypothesis on reduced levels of hBDs in these patients compared with healthy individuals and UC patients is that highly polymorphic DNA copy number of the β-defensin gene cluster on chromosome 8p23.1 leads to a lower *defb4* gene copy number, which predisposes to colonic CD[187]. Besides β-defensins, many studies allude that other types of AMP play a pivotal role in enteric infection as well as the pathogenesis of experimental colitis. Recently, it has been shown that mice lacking lipocalin-2 (Lcn2) exhibited aggravated colitis[219]. In addition, ablation of gut microbiota in these mice had lower severity of colitis, indicating that *Lcn2*$^{-/-}$ mice are colonized by a colitogenic microbiota[219]. Another study using mice lacking RegIII gamma (Reg3γ) has reported an increased bacterial contact with the ileal epithelium along with enhanced mucosal inflammatory responses due to recruitment of monocytes and neutrophils[220]. Together, these observations provide a strong support that intestinal inflammation results from reduced mucosal antimicrobial activity.

However, to the best of our knowledge, no current data are available on the effect of gut-derived 5-HT in the production of any AMPs. Based on our seminal study showing 5-HT as the major determinant factor in the development of experimental colitis as well as our preliminary finding revealing *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice have different microbial composition, we investigated the potential role of 5-HT in β-defensin production in the colon. In this thesis, by utilizing both *in vitro* and *in vivo* model systems, we demonstrate that 5-HT released from EC cells inhibits β-defensin production from intestinal epithelial cells.

We observed significantly increased total β-defensin levels in the colon of both vehicle- and DSS-treated *Tph1*$^{-/-}$ compared to their WT littermates while reconstitution of 5-HT synthesis in naïve *Tph1*$^{-/-}$ mice with 5-HTP attenuated total β-defensin production. It is important to mention that the increased β-defensin levels in DSS-induced mice suggest an activation of the mucosal innate immune response toward a pro-inflammatory state. We then sought to investigate specific subtypes of β-defensin, such as mBD-1 and mBD-3 in naïve *Tph1*$^{-/-}$ mice. We observed that these mice expressed higher levels of both mBD-1 and mBD-3 whereas replenishing 5-HT synthesis with 5-HTP reduced mBD-1 and mBD-3. These findings suggest a role of 5-HT in regulation of β-defensin production in the colon in the context of innate immune response, whereby diminished enzyme-mediated generation of 5-HT results in enhanced expression of β-defensins. As intestinal epithelial cells express several 5-HT receptors including 5-HT_3, 5-HT_4, and 5-HT_7 receptors, we elucidated whether 5-HT directly inhibits β-defensin production by utilizing HT-29 cells. Upon 5-HT treatment, these cells produced significantly lower hBD-1 and hBD-2 levels in a dose-dependent manner. To verify whether 5-HT has toxic effects on the cell viability, preliminary tests were performed by using trypan blue exclusion assay. We have not found any evidence of increased cell death. To better understand how 5-HT inhibits β-defensin production, we treated HT-29 cells with selective 5-HT receptor antagonists. We observed that activation of 5-HT_7 receptors suppressed hBD-1 and hBD-2 expression in HT-29 cells, thereby discounting the involvement of 5-HT_3 and 5-HT_4 receptors and providing a strong support for our recent work in which 5-HT_7 receptors play a pivotal role in regulation of mucosal inflammation. Humans have three

isoforms of 5-HT_7 receptors: 5-HT7(a), 5-HT7(b), and 5-HT7(d)[221]. Further experiments should be performed to shed further light on elucidating which specific 5-HT_7 receptor subtype is involved and how alternative splicing affects 5-HT_7 receptor activation for regulating β-defensin production. Taken together, these findings demonstrate a crucial role for gut-derived 5-HT signaling via 5-HT_7 receptors in regulation of β-defensin production from intestinal epithelial cells.

We next examined intracellular signal transduction pathway that is responsible for the inhibitory effect of 5-HT in β-defensin production. PPAR-γ is highly expressed in colonic epithelial cells[199] and plays a crucial role for maintaining intestinal homeostasis through regular induction of β-defensin from the epithelial cells[200]. We showed that *Tph1*$^{-/-}$ mice expressed significantly higher levels of PPAR-γ in the colon whereas administration of 5-HTP reduced PPAR-γ expression in these mice. To further understand the role of PPAR-γ in β-defensin production, *Tph1*$^{-/-}$ mice were intraperitoneally administered with GW-9662. We observed a significant reduction in both mBD-1 and mBD-3 expression. Blocking 5-HT_7 receptors resulted in restoration of PPAR-γ expression in HT-29 cells, demonstrating that 5-HT inhibits PPAR-γ expression. It has been reported that TNF-α down-regulates adipocyte PPAR-γ expression in nuclear factor-κB (NF-κB)- and activator protein-1 (AP-1)-dependent mechanisms[222]. However, to our knowledge, no current data are available on how 5-HT down-regulates the mRNA expression of PPAR-γ in the intestine. It seems possible that higher levels of TNF-α indirectly down-regulates PPAR-γ in intestinal epithelial cells. Indeed, it has been shown that TNF-α significantly decreased SERT

mRNA expression, resulting in reduced 5-HT uptake in Caco-2 cells (another *in vitro* model for human colonic epithelial cells)[223]. Future studies on the mechanism or nature of the interaction between 5-HT and PPAR-γ would be interesting. Investigations on the downstream signaling events revealed an important role of PPAR-γ signaling in β-defensin production. Previously, it has been shown that 5-HT induces phosphorylation of ERK1/2 in a variety of cells[129,211–214]. Another study also demonstrated that ERK1/2 phosphorylates PPAR-γ[215], which resulted in reduction of PPAR-γ expression and activity[224,225]. We observed that treatment of MEK inhibitor (PD98059) resulted in restoration of both hBD-1 and hBD-2 expression in HT-29 cells. Taken together, these findings not only provide direct evident that 5-HT is critically involved in β-defensin regulation but also further strengthen the notion that 5-HT plays an important role in colitis pathogenesis.

In summary, the data presented in this thesis show that 5-HT released from EC cells down-regulates PPAR-γ via 5-HT_7 receptors and subsequently inhibits β-defensin production. In addition to enhancing our understanding of the role of 5-HT on innate immune response, this thesis not only identifies a novel strategy for modulating β-defensin production, but also provides important information that disruption of 5-HT-β-defensin axis may play an important role in regulating gut microbiota composition and subsequent colitis susceptibility.

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