IMMUNO-ENDOCRINE INTERACTIONS IN

INTESTINAL INFLAMMATION

Immuno-endocrine interactions in intestinal inflammation

By

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Lay abstract

The gut produces most of the serotonin found in our body, where it regulates many normal functions. A group of special cells, named enterochromaffin cells, produces nearly all of the serotonin in the gut. In diseases of the gut, especially ones that involve inflammation resulting in symptoms like abdominal pain, diarrhea and bleeding, the number of these cells and serotonin concentration are different from that in the normal gut. I found that these changes are controlled by a particular protein produced by immune cells, called interleukin-13, and alteration in serotonin levels, in turn, contributes to the inflammatory process. Our laboratory experiments with cells and animals establish this connection between interleukin-13 and serotonin in gut inflammation. We further confirm this association between interleukin-13 and serotonin in human inflammatory bowel disease. Moreover, we identify a potential genetic cause of these changes in serotonin concentrations which may ultimately result in inflammatory bowel disease.

Abstract

Mucosal inflammation in conditions ranging from infective acute enteritis or colitis to inflammatory bowel disease (IBD) is accompanied by alteration in enterochromaffin (EC) cell numbers and serotonin (5-hydroxytryptamine; 5-HT) content in the gut. Previously we had shown that CD4+ T cells, via production of T helper (Th)2 cytokines, regulate EC cell biology in the *Trichuris muris*-infectious colitis model. I further examined the mechanisms of immuno-endocrine interactions in the context of intestinal inflammation. In chapter 3, utilizing human EC cell line and *Trichuris muris*-mouse model of infectious colitis we identified a critical role of interleukin (IL)-13, a key Th2 cytokine, in increasing EC cell numbers, tryptophan hydroxylase (TPH)1 expression (rate-limiting enzyme of mucosal 5-HT bio-synthesis), and 5-HT production. In chapter 4, we show that IL-13 driven intestinal inflammation is critically dependent on increased 5-HT production using dextran sulfate sodium (DSS) and dinitrobenzene sulphonic acid (DNBS) models of colitis. In DSS-induced colitis, we were the first to identify the increased production of IL-13 and its pathogenic role as IL-13 knockout (IL-13-KO) mice had less severe inflammation compared to wild-type, which was exacerbated following replenishment of 5-HT in IL-13-KO mice. In chapter 5, biopsy examination revealed, higher mucosal IL-13 expression accompanied inflammation in Crohn's disease (CD), which was additionally associated with increased TPH1, 5-HT receptor (5-HTR)3A, 5-HTR7

and decreased 5-HT transporter (5-HTT) expressions. Moreover, CD patients had elevated plasma and platelet-poor plasma 5-HT levels compared to healthy controls (HCs). Furthermore, 5-HTT polymorphism associated genotypes causing inefficiency in 5-HT re-uptake were more common in our patient cohort than HCs. The findings included in this thesis further emphasize the role of immuno-endocrine interactions in intestinal inflammation, which may be a step toward a better diagnosis or management or even a cure for a disease that is of growing concern, and in understanding IBD pathogenesis.

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List of abbreviations and symbols

5-HIAA	5- hydroxyindoleacetic acid	NK cells	Natural killer cells
5-HT	Serotonin or 5-hydroxytryptamine	NKT cells	Natural killer T cells
5-HTP	5- hydroxytryptophan	NLR	NOD-like receptor
5-HTR	5-HT receptor	NO	Nitric oxide
5-HTT or SERT	5-HT transporter or serotonin transporter	NOD	Nucleotide-binding oligomerization domain-containing protein
5-HTTLPR	5-HTT gene-linked polymorphic region	NOX	NADPH oxidase
AADC	Aromatic amino acid decarboxylase	OD	Optical density
Ach	Acetylcholine	OCT	Organic cation transporter
AMP	Antimicrobial peptides	PAF	Platelet activating factor
APC	Antigen presenting cell	PBS	Phosphate buffered saline
ASCA	Anti-Saccharomyces cerevisiae antibodies	PCPA	Parachlorophenylalanine
ATG16L1	Autophagy related 16 like 1	PCR	Polymerase chain reaction
ATM	Adipose tissue macrophage	pDC	Plasmacytoid dendritic cell
ATT	Adipose tissue T cell	PGE2	Prostaglandin-E2
BMDC	Bone marrow-derived dendritic cell	PFA	Paraformaldehyde

cAMP	Cyclic AMP	PI3K	Phosphoinositide 3-kinase
CD	Crohn's disease	PI-IBS	Post infectious IBS
cDC	Conventional dendritic cell	PMAT	Peripheral monoamine transporter
cDNA	Complementary DNA	PP	Peyer's patch
CDP	Common dendritic cell progenitor	PPP	Platelet-poor plasma
CNS	Central nervous system	PRR	Pattern recognition receptor
CRC	Colorectal cancer	qPCR	Quantitative polymerase chain reaction
CRP	C-reactive protein	RA	Retinoic acid
CRP+ or CRP$^-$	C-reactive protein positive or negative	RELM	Resistin-like molecule
CTL	Cytotoxic T lymphocyte	rhIL-13	Recombinant human IL-13
DAI	Disease activity index	RLR	RIG-I-like receptor
DAT	Dopamine transporter	rmIL-13	Recombinant human IL-13
DC	Dendritic cell	ROS	Reactive oxygen species
DMEM	Dulbecco's modified Eagle's medium	RPMI	Roswell Park Memorial Institute medium
DNBS	Dinitrobenzene sulphonic acid	S100A8	S100 calcium-binding protein A8
DSS	Dextran sulfate sodium	S100A9	S100 calcium-binding

			protein A9
DTT	Dithiothreitol	SCFA	Short chain fatty acid
EC cell	Enterochromaffin cell	SCID	Sever combined immunodeficient
ELISA	Enzyme-linked immunosorbent assay	SDS	Sodium dodecyl sulfate
ENS	Enteric nervous system	SEM	Standard error mean
ERK1/2	Extracellular signal-related kinase-1 and -2	sIgA	secretory IgA
ESR	Erythrocyte sediment rate	SLC6A4	solute carrier family 6 member 4
FCAL	Fecal calprotectin	SNP	Single nucleotide polymorphism
GALT	Gut-associate lymphoid tissue	SSRI	Selective serotonin reuptake inhibitor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	STAT	Signal transducer and activator of transcription
GF	Germ-free	Symptom$^-$	Asymptomatic
GI	gastrointestinal	Symptom+	Symptomatic
GM-CSF	Granulocyte-macrophage colony stimulating factor	*T. muris*	*Trichuris muris*
GPCR	G-protein coupled receptor	TCR	T cell receptor
GWAS	Genome wide association study	TFF3	Trefoil factor 3

H&E	Haematoxylin and eosin	Tg	Transgenic
HC	Healthy control	TGF	Transforming growth factor
IBD	Inflammatory bowel disease	Th	T helper
IBS-D	Diarrhea-predominant irritable bowel syndrome	TLR	Toll-like receptor
ICC	Interstitial cells of Cajal	TNBS	Trinitrobenzene sulphonic acid
IDO	Indoleamine 2,3-dioxygenase	TNF	Tumor necrosis factor
IEC	intestinal epithelial cell	TNFSF15	TNF super family member 15
IEL	Intraepithelial lymphocyte	TPH	Tryptophan hydroxylase
IFN	Interferon	Treg cell	Regulatory T cell
Ig	Immunoglobulin	TREM	Triggering receptor expressed on myeloid cell
IHC	Immunohistochemistry	Trp	Tryptophan
IL	Interleukin	TSLP	Thymic stromal lymphopoietin
IL-10R	IL-10 receptor	TWEAK	TNF-like weak inducer of apoptosis
IL-13R	IL-13 receptor	UC	Ulcerative colitis
ILF	Isolated lymphoid follicle	VMAT	Vesicular monoamine transporter

iNOS	Inducible nitric oxide synthase	WT	Wild-type
IP	Intraperitoneal	wt/vol	Weight to volume
IPAA	Ileal pouch-anal anastomosis	ZO	zonula occludens
IR	Intrarectal	α	Alpha
JAM-A	Junctional adhesion molecule-A	β	Beta
KO or -/-	Knockout	γ	Gamma
LP	Lamina propria	δ	Delta
LPDC	Lamina propria dendritic cell	μg	Microgram
LPL	Lamina propria lymphocyte	mol	Mole
LPS	Lipopolysaccharides	mg	Milligram
M cell	Microfold cell	μl	Microliter
MAO	Monoamine oxidase	Kg	Kilogram
MAPK	Mitogen-activated protein kinase	g	Gram
MCP	Monocyte chemoattractant protein	μM	Micromole
M-CSF	Macrophage colony-stimulating factor	ng	Nanogram
mDC	Monocyte-derived dendritic cells	nM	Nanomolar
MHC	Major histocompatibility	nm	Nanometer

	complex		
MLN	Mesenteric lymph node	pg	Picogram
MM	Muscularis macrophage	L	Liter
mol. wt.	Molecular weight	mL	Milliliter
MPO	Myeloperoxidase		
MUC	Mucin		
NET	Norepinephrine transporter		
NFIL3	Nuclear factor-interleukin-3-regulated		
NF-κB	Nuclear factor kappa B		
ngn3	Neurogenin 3		

List of publications and submitted manuscripts *(*as first or co-first author)*

<u>*Peer Reviewed Journal Articles*</u>

Marcus M Manocha*, **Md Sharif Shajib*,** Mushfequr M Rahman, Palanivel Rengasamy, Huaqing Wang, Melina Bogunovic, Manel Jordana, Lloyd Mayer, Waliul I Khan: <u>IL-13-mediated immunological control of enterochromaffin cell hyperplasia and serotonin production in the gut</u>. *Mucosal Immunology*. 07/2012. PMID: 22763407.

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—CHAPTER 1—

LITERATURE OVERVIEW

Serotonin

Serotonin also referred to as 5-hydroxytryptamine (5-HT), is an evolutionarily ancient and phylogenetically conserved monoamine, as it is found in both vertebrates and invertebrates. In 1937, Vittorio Erspamer, an Italian pharmacologist, identified a novel indolealkylamine in the gastrointestinal (GI) tracts of various vertebrates [1]. He found that this new amine causes intestinal contraction and called it "enteraime." Erspamer went on to show that it was the major secretory product of the enterochromaffin (EC) cells of the GI mucosa and had the same structure as 5-HT, a compound discovered by Rapport et al. in 1948, which they termed serotonin, as the bovine serum agent isolated affected vascular tone [2–5]. Since its discovery now over half a century ago, investigations into 5-HT and/or the serotonergic system has revealed biological insights of medical relevance in almost all major organ systems, including the cardiovascular, pulmonary, GI, as well as the central nervous system (CNS).

5-HT perhaps is best known for its role as a neurotransmitter in the brain. Serotonergic neurons, originating from the raphe nuclei of the brainstem, are widely distributed throughout the mammalian brain and form the single largest efferent system of the CNS [6]. In conjunction with other important neurotransmitter systems, such as the catecholaminergic system, 5-HT regulates a broad range of physiological, cognitive and behavioral functions, including sleep,

appetite, mood and sexual behavior [6]. 5-HT also plays an important role in neurogenesis and the development of the brain [7,8]. Separated by the blood-brain barrier, which is relatively impermeable to 5-HT, CNS-derived 5-HT only accounts for a small fraction of this monoamine produced in our bodies. In fact, most of the 5-HT (~95%) is peripheral in origin, where it is an important hormone that plays a central role in the regulation of vascular tone, intestinal motility, cell growth in liver, bone, and pulmonary arteries, as well as the development of heart, and mammary gland [8,9]. Moreover, peripheral 5-HT is a significant regulator of the immune system [6]. The gut by far is the largest producer of 5-HT in the human body, with EC cells contributing the vast majority (~90%) of gut-derived 5-HT [9–15]. The other source of gut-derived 5-HT is the serotonergic neurons found in the myenteric plexus of the enteric nervous system (ENS) and mast cells [16]. However, 5-HT produced by EC cells vastly overshadows all other sources. Nonetheless, in the normal gut mucosal and neuronal 5-HT work synergistically to regulate a variety of intestinal functions including epithelial barrier function, the transport of fluid and electrolytes, the secretion of mucins, as well as motility [16].

EC cells

EC cells belong to a group of specialized epithelial cells of secretory lineage, called enteroendocrine cells (EECs). They are the most abundant of the fifteen

subtypes of EECs that have been identified [17]. EECs constitute less than 1% of the cells in the intestinal epithelium, are found scattered throughout the gut mucosa and comprise what is referred to as the enteroendocrine system [18]. These hormone-secreting cells are sensors of luminal contents, particularly nutrients, which makes them central to the regulation of food intake and the first-line component of the gut-brain axis [18]. EECs act either in a classical endocrine fashion or in a paracrine manner on nearby cells, notably vagal afferent fibres [18]. Numerous hormones released by EECs elaborately control physiological and homeostatic functions of the GI tract, such as postprandial secretion and motility, and consequently makes the gut the largest endocrine organ in the human body [16]. The populations and diversity of EECs vary along the GI tract [17]. Small intestinal EECs display a greater diversity than the ones in the colon, with the highest frequency of EECs, observed in the proximal small intestine, followed by the rectum [17].

EC cells, like all other cells of the intestinal epithelium, arise from stem cells within the intestinal crypts. Notch, Wnt, and bone morphogenetic protein signaling pathways maintain the intestinal stem cell niche and regulate cell lineage [18,19]. Lateral inhibition of Notch signaling down-regulates cellular expression of Hes-1, which enables the expression of transcription factors, such as Math 1 and neurogenin 3 (ngn3), directing a more secretory epithelial cell lineage [18,20–23]. Moreover, this prevents adjacent cells from differentiating into EECs

and expression of ngn3 is essential for the development of EC cells, as ngn3-knockout (KO) mice lack endocrine cells [23,24]. EC cells are found throughout GI tract including the gastric antrum, duodenum, jejunum, ileum and the colon and rectum, as well as the appendix [17]. Though the density of EC cells varies along the GI tract due to the increase or decrease in the number of other EECs, the absolute numbers of EC cells stay fairly constant, particularly in the large intestine [25,26]. Notably, 5-HT content is the highest in the rectum [27]. Approximately 8 micrometers (μm) in size, EC cells have a triangular or pyramidal shape [28]. The microvilli of EC cells project out into the lumen which allows them to transduce luminal physiochemical signals into biochemical-endocrine signals and communicate with surrounding cells, particularly neurons as they do not innervate the lumen, primarily by discharging 5-HT [16]. Other secretory products of EC cells include substance P, chromogranin and guanylin [29,30]. The sensory functions of EC cells arise from their expression of a myriad of ion channels and receptors, including transient receptor potential cation channel subfamily A member 1, taste and olfactory receptors, free fatty acid receptors, Toll-like receptors (TLRs) and toxin receptors such as ganglioside GM1 receptor [28,31–34]. EC cells, similar to other EECs, are marked by the presence of pleomorphic secretory vesicles, but unlike other EECs, these are mostly large dense-core vesicles [27]. Regional morphological differences of EC cells have been observed, such as the size of secretory vesicles [26]. Some EC cells of the

large intestine have basal extensions containing secretory vesicles specialized for neuronal communication, which are not present in the small intestine [35]. It has been shown that secretory functions of EC cells can be regulated, at least in part, by neuronal input [36]. Various somatostatin receptor subtypes have been identified on the naïve human EC cells. It has also been demonstrated that activation of β-adrenergic and pituitary adenylate cyclase-activating polypeptide receptors have stimulatory effects and γ-aminobutyric acid A and cholinergic receptors have inhibitory effects on 5-HT secretion by EC cells [28].

Synthesis and metabolism of 5-HT

5-HT is a derivative of the least abundant essential amino acids tryptophan (Trp) [37]. The initial step in the biosynthesis of 5-HT involves the hydroxylation of Trp to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH) [16]. There are two isoforms of TPH, TPH1, and TPH2. TPH1, the non-neuronal isoform, is predominantly found in EC cells, and TPH2 is the neuronal isoform found in all serotonergic neurons of the ENS and CNS. In a second enzymatic step, decarboxylation of 5-HTP by the ubiquitously expressed aromatic amino acid decarboxylase (AADC) results in the production of 5-HT. Though both TPH and AADC are necessary for the production of 5-HT from Trp, TPH is the rate-limiting enzyme as it has very little affinity for any other amino acids and is only found in cells containing 5-HT [6]. In contrast to AADC inhibition, TPH

inhibition or depletion leads to significantly reduced 5-HT levels. Following the production of 5-HT, it is quickly packaged into vesicles by vesicular monoamine transporter (VMAT).VMAT also has two isoforms VMAT 1 and 2, the former is found in neuroendocrine cells and latter is the neuronal isoform. The 5-HT stored within the vesicles of EC cells can be released both apically and basally via exocytosis in a Ca2+ dependent manner [27]. At physiological pH, the 5-HT molecule has a positive charge and requires active transport to cross cell wall barriers [16]. This function is mainly performed by the 5-HT transporter (5-HTT), or serotonin transporter (SERT). In addition to 5-HTT others, including the dopamine transporter (DAT), organic cation transporter (OCT), norepinephrine transporter (NET) and peripheral monoamine transporter (PMAT) have the capacity to uptake 5-HT, albeit at a lower affinity [6]. Once transported into cells, VMAT is required to sequester 5-HT and protect it from degradation by mitochondrial monoamine oxidase (MAO), primarily MAO-A. 5-HT, like other monoamines, is metabolized via oxidative deamination and the end product of this process, 5- hydroxyindoleacetic acid (5-HIAA), is excreted in urine [6]. Secreted 5-HT that is not taken up by intestinal epithelial cells (IECs) enters the circulation via the dense capillary bed of the lamina propria (LP), and is avidly transported into platelets via 5-HTT [38,39]. Platelets represent the largest reservoir of 5-HT in the human body, but do not produce their own as they lack TPH [40]. Stored within their dense granules, platelets shuttle mucosal 5-HT throughout the body

and propagate the influences of gut-derived 5-HT beyond the gut. Within the gut, five types of 5-HTRs, expressed on various cells, including, neurons, muscle, immune and epithelial cells, regulate the substantial and sometimes confounding influence of 5-HT.
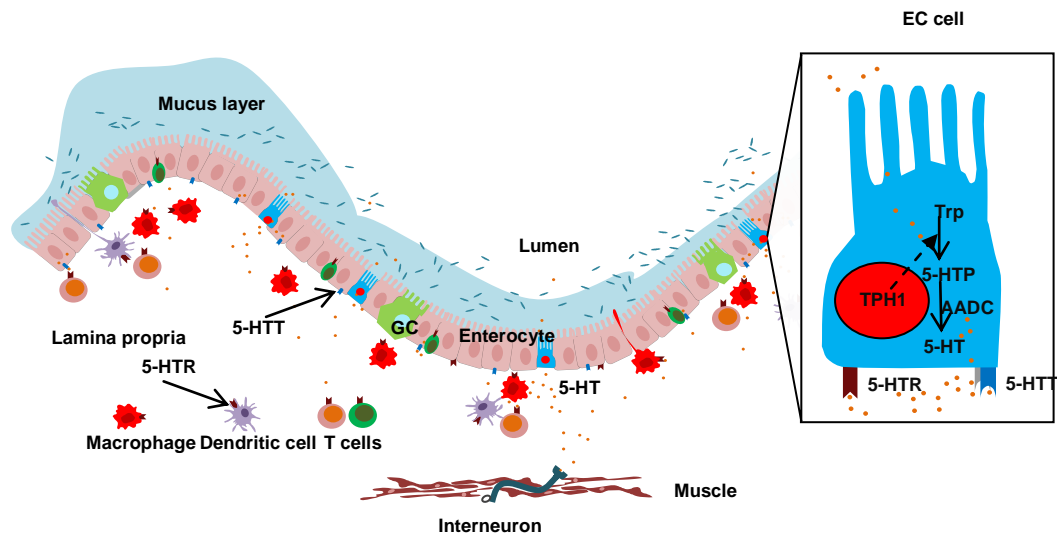
Figure I: Schematic representation of mucosal 5-HT production and functions. Residents of the gastrointestinal (GI) epithelium, EC cells synthesize 5-HT via the rate-limiting enzyme TPH1(inset). They are the main source gut-derived 5-HT, which participates in various GI functions including immune response via its expression of 5-HTRs and 5-HTT on various cells. EC, enterochromaffin; Trp, tryptophan; TPH1, tryptophan hydroxylase 1; 5-HTP, 5-hydroxytryptophan; ADDC, aromatic amino acid decarboxylase; 5-HT, 5-hydroxytryptamine; 5-HTT, 5-HT transporter; 5-HTR, 5-HT receptor; GC, Goblet cell. (adopted from Shajib et al. [16])

5-HTRs and GI functions

An undetermined number of 5-HTRs mediate the diverse effects of 5-HT on a wide range of physiological functions. Eighteen genes encode for at least fifteen mammalian 5-HTRs, which are divided into seven families [41]. With the

exception of 5-HTR 5 and 6 families, all other families of 5-HTRs (1-4 and 7) have been identified in the GI tract. Alternative splicing, RNA editing, and homo- and heterodimerization underpins the extreme biological diversity of 5-HTRs [41]. Receptor heterogeneity is further supplemented by naturally occurring polymorphic variants of 5-HTR subtypes [41]. Excluding 5-HTR3, all other 5-HTRs belong to the G-protein coupled receptor (GPCR) family class A, also known as 7-transmembrane domain receptors [6]. 5-HTRs 1, 2, 4 and 7, like all other GPCRs, signal via intracellular second messenger cascade. G proteins form heterotrimeric complexes of Gα, Gβ and Gγ subunits and the complex is coupled to the C-terminus of the transmembrane 5HTR. GPCRs are capable of assembling homo- and heterodimers as well as oligomers, but 5HTRs preferentially form homodimers [42–46]. Ligation of 5-HTR elicits a conformational change in the receptor inducing signal transduction to facilitate activity. Activation of 5-HTR1 leads to the down-regulation of cyclic AMP (cAMP) due to its negative coupling with adenylyl cyclase [6]. In contrast to that the activation of 5-HTRs 4, and 7 is associated with increased cAMP activity. Up-regulation of inositol triphosphate and diacylglycerol pathways following activation of 5-HTR2 increases intracellular Ca^{2+} release [6]. Unlike other 5-HTRs, 5-HTR3 belongs to the cation-selective ion channel Cys-loop superfamily [6]. The functional 5-HTR3 channel is essentially dependent on the 5-HTR3A subunit for the formation of its

characteristic pentameric ring [47–49]. 5-HTR3 is most permeable to Ca2+, Na+, and K+ [6].

5-HT directly or indirectly participates in various regulatory functions of the digestive process, from transmitting taste information from taste buds to the CNS, regulating pancreatic enzyme secretion to aid in digestion, and in excretion by regulating peristaltic reflex. Once released 5-HT acts not only in an endocrine manner but by neurocrine, paracrine and autocrine means as well [17]. In the gut, 5-HTRs are found on intrinsic and extrinsic afferent neurons, interneurons, inhibitory and excitatory motor neurons, interstitial cells of Cajal (ICC), smooth muscle, goblet cells, enterocytes and EECs including EC cells, mediate the multitude of functions performed by 5-HT [16]. 5-HT via intrinsic sensory neurons regulates peristalsis and secretory reflexes and via extrinsic neurons the sensation of pain, discomfort, and nausea. EC cells release 5-HT in response to acetylcholine, neuronal stimulation, raised intraluminal pressure, and changes in pH [50]. 5-HT relays this information from EC to intrinsic primary afferent neurons, which express 5-HTR1A, 3, 4 and 7 [51]. 5-HT mainly via 5-HTR1B, 3, 4 and 7 influences GI motility which requires the coordinated activity of various cell types including neurons, smooth muscle cells, and ICCs [51–54]. A heterogeneous population of specialized GI cells, ICCs are neither neurocytes nor myocytes but are central to normal motility and express 5-HTR 2B, 3 and 4 [51]. 5-HT also regulates ICC proliferation and network density via 5-HTR2B [54,55].

5-HT regulates the contraction and relaxation of smooth muscles, the former via 5-HTR2B, 3 and 4 mediated stimulation of cholinergic neurons causing acetylcholine (ACh) release, and the latter through 5-HTR1A, 1D and 4 on inhibitory nitrergic neurons and subsequent nitric oxide (NO) secretion [56]. However, it has been shown that neuronal rather than mucosal 5-HT plays a more prominent role in normal GI motility, as TPH2KO but not TPH1KO mice had significantly impaired intestinal transit and motility [57]. 5-HT also stimulates electrogenic fluid secretion throughout the GI tract via 5-HTR1P, 5-HTR2A, 5-HTR3, and 5-HTR4, especially in the terminal ileum, the sigmoid and the ascending colon [58–61]. In response to toxins and pathogens, 5-HT via 5-HTR4 mediates mucus and fluid secretion promoting mucosal protection [62]. As mentioned earlier 5-HT plays an important role in sensation, this is primarily mediated by 5-HTR3 on lumbar splanchnic and vagal afferents [39]. The same receptor also influences food intake by regulating hunger and satiation, which may also involve the hypothalamic expression of 5-HTR2C [63,64]. Though it remains to be determined if the effect on the hypothalamus is mediated primarily by mucosal 5-HT that reaches the hypothalamus via the bloodstream or CNS-derived 5-HT.

Additionally, both depots of enteric 5-HT, neuronal and mucosal, have been implicated in growth and turnover of the intestinal epithelium. Gross et al. demonstrated this in the normal gut, where enteric neuronal 5-HT accomplishes

this feat via 5-HTR2A on submucosal cholinergic neurons and their muscarinic innervations to epithelial effectors [65]. Whereas, Spohn et al. implicated mucosal 5-HT in this process by intraluminal administration of 5-HTR4 agonist (Tegaserod) in experimental models of colitis, demonstrating increased cell proliferation and accelerated healing [66].

5-HTT

Given the multitude of functions performed by 5-HT, its bioavailability is tightly regulated in the GI tract, and in the body in general. The active transport of extracellular 5-HT across the plasma membrane into the cell terminates its actions, and as mentioned earlier this is predominantly performed by 5-HTT, also known as solute carrier family 6, member 4 (SLC6A4). 5-HTT belongs to the sodium: neurotransmitter symporter family and is encoded by the SLC6A4 gene, which is comprised of 14 exons and is located on chromosome 17q11.1-17q12 of the human genome [67]. A 44-bp insertion/deletion polymorphism in the promoter region of SLC6A4 creating two predominant allele variants, a long (L) allele, and a short (S) allele, has been identified and is known as the 5-HTT gene-linked polymorphic region (5-HTTLPR) [67–69]. It has been shown that the S allele is functionally dominant. In comparison with the L/L genotype, the S/S or S/L genotypes (together referred to as the S genotype) are associated with lower levels of 5-HTT mRNA transcripts, and thereby lower levels of 5-HTT expression as

well as reduced 5-HT reuptake efficiency [67]. The functional unit of 5-HTT is a dimer and several posttranslational modifications, including glycosylation followed by insertion of sialic acid into each of two N-linked glycans, leads to its formation. Glycosylation is necessary for the normal functional activity of 5-HTT and the addition of sialic acid molecules ensures dimer formation as well as the association with myosin IIA at the cytoskeleton [70].

5-HTT is a critical component of 5-HT signaling machinery. This is exemplified by the presence of 5-HTT on nerve terminals that release 5-HT and by its expression on every epithelial cell lining the luminal surface of the gut [39]. 5-HTT transcription in the intestinal epithelium differs from that of neurons suggesting tissue-specific regulation of transcription of this protein [71]. In the healthy gut, the expression of 5-HTT varies along the GI tract, with the highest expression observed in the ileum and the lowest in the colon [72]. 5-HTT essentially acts as a selective sponge to remove 5-HT from the interstitial space following secretion by EC cells and serves the same purpose on platelets. It has been shown that up-regulation of 5-HT concentrations initially causes enhancement of 5-HTT density on the platelet cell membrane, but prolonged exposure to increased levels of 5-HT reduces 5-HTT expression below normal [73–75]. As previously mentioned, platelets via 5-HTT take up gut-derived 5-HT that enters the circulation in a fast and saturable process, playing a key role in the maintenance of 5-HT availability in blood circulation. Though these small,

anucleate cells are best known for their role in initiating coagulation and maintaining vascular tone, they are immune capable and are the main benefactor of 5-HT mediated regulation of the peripheral immune system.

5-HT and the immune system

EC cells, via platelets, are the main suppliers of 5-HT for lymphatic tissue and immune cells [6]. Additional but smaller sources of 5-HT for the peripheral immune system include sympathetic neurons which heavily innervate lymphatic tissue, as well as the some immune cells that express TPH1 [6]. It is now well known that 5-HT provokes a wide array of differential effects on immune cells, often in the same cell type [76]. The diverse effects of 5-HT observed on immune cells is potentially due to the local concentration of 5-HT and the expression of various 5-HT signaling machinery, such as 5-HTRs and 5-HTT, with different 5-HT affinity working together to induce specific effects.

5-HT and innate immune system

Most human and rodent innate immune cells, including eosinophils, monocytes (circulating precursor of macrophages and dendritic cells (DCs)), macrophages, DCs, mast cells and natural killer (NK) cells, express 5-HTRs [76,77]. Some of these cells also express 5-HTT, such as monocytes, macrophages, DCs, and mast cells [76,77]. In response injury, 5-HT is rapidly released by platelets, as well as mast cells and basophils, along with other inflammatory mediators, such as IgE

complexes, and platelet activating factor (PAF), leading to the activation of the complement system, chemotaxis, phagocytosis and ultimately inflammation [6,78,79]. Activated platelets can swiftly increase resting plasma 5-HT concentrations from ~10 nM to 10 μM or more [80,81]. 5-HT promotes chemotaxis of human eosinophils via 5-HTR2A and through 5-HTR1A on both human and mouse mast cells [82,83]. The release of platelet-stored 5-HT induces endothelial expression of adhesion molecules, P-selectin and E-selectin, and release of interleukin (IL)-8, which triggers neutrophil rolling, adhesion, and extravasation in acute inflammation [84].

In macrophages, 5-HT aids in the process of antigen-presentation, which is eradicated following para-chlorophenylalanine (PCPA; an irreversible inhibitor of TPH) mediated depletion of 5-HT [85]. Murine peritoneal macrophages, in a nuclear factor kappa B (NF-κB)-dependent manner, increase their secretion of pro-inflammatory cytokines, such as IL-1β and IL-6, following 5-HT stimulation and their phagocytic capacity following 5-HTR1A activation [76,86]. In contrast, 5-HT via 5-HTR2B and 5-HTR7 promotes anti-inflammatory polarization of human monocyte-derived macrophages, which is marked by reduced secretion of pro-inflammatory cytokines IL-12p40 and tumor necrosis factor (TNF)-α, and unaltered secretion of anti-inflammatory cytokine IL-10 [87]. Furthermore, Ménard et al. using cell culture models of human and rat alveolar macrophages demonstrated that 5-HT via the regulation of prostaglandin-E2 (PGE2) down-

regulates TNF-α and IL-12p70, and up-regulates IL-10 and NO production [88]. It has also been shown that 5-HTR2C activation on mouse alveolar macrophages increases intracellular Ca2+ concentration which augments production of CCL2, promoting monocyte recruitment [89]. Human CD14+ monocytes (account for up to 90% of the blood monocytes) under normal physiological conditions require 5-HT for the production of IL-6 and TNF-α, but not IL-10 [90]. In addition to expressing 5-HTT and TPH1, these cells also express 5-HTR1E, 2A, 3, 4 and 7 [89,91,92]. 5-HT via the synergetic actions of 5-HTR3, 4 and 7 up-regulates the secretion of IL-1β, IL-6, and IL-8 by lipopolysaccharides (LPS)-activated monocytes [91]. Soga et al. demonstrated 5-HTR1 and/or 7 activation-associated phosphorylation of extracellular signal-related kinase-1 and -2 (ERK1/2) and NF-κB activation also increase IL-6 and TNF-α secretion by LPS-stimulated monocytes [90]. Whereas, the combined activation of 5-HTR4 and 5-HTR7 reduces TNF-α production by human monocytes, at both resting and LPS-activated states [91]. Activation of these two 5-HTRs on LPS-stimulated monocytes also induces the secretion of IL-12p40, which in turn promote migration of macrophages and bacterially stimulated DCs [91]. 5-HT, via 5-HTR1 and/or 7, was shown to prevent monocytic apoptosis by up-regulating anti-apoptotic proteins, Bcl-2 and Mcl-1, expression as well as by reducing caspase-3 activation [90]. The same receptors have also been implicated in the up-regulation of co-stimulatory molecules CD40, CD80, and CD86 expression on monocytes,

subsequently increasing their stimulatory activity toward allogeneic CD4+ T cells [90]. 5-HTR1A activation on monocytes has been shown to abrogate autologous monocyte-induced suppression of NK cell cytolytic activity and proliferation [93,94]. These innate lymphocytes express 5-HTR1A, and increased number of NK cells have been reported in long-term users of selective serotonin reuptake inhibitors (SSRIs), which also increases their cytotoxic capacity [95–98]. 5-HT in a 5-HTR1 and/or 7-dependent manner alters the phenotype of human monocyte-derived DCs (mDCs; differentiation is dependent on granulocyte-macrophage colony stimulating factor (GM-CSF) [99]) by up-regulating CD14 expression as well as by reducing their capacity to stimulate allogeneic T cells [100]. 5-HT and LPS co-stimulation induces immature mDC migration in a 5-HTR1B and 5-HT2A-dependent manner [100]. However, 5-HT has been shown to inhibit CXCL10 secretion by mature mDCs [100]. 5-HTR7 signaling is required for morphological and migratory modulation of mature bone marrow-derived DCs (BMDCs), the latter via the induction of CCR7 expression [101]. In mature mDCs, 5-HTR3 activation regulates intracellular Ca2+ concentration required for the secretion of IL-8 and IL-1β [102]. 5-HTR4 and 7 also regulate cytokine and chemokine production by these antigen presenting cells (APCs) [102]. Activation of 5-HTR7 on BMDCs increases IL-12/23p40 secretion, in an NF-κB-dependent manner, triggering a more pro-inflammatory T cell response [103]. Furthermore, LPS-induced maturation of BMDCs up-regulates 5-HTT expression by these

cells, suggesting that DCs may release stored 5-HT into the immunological synapse during T cell activation [94].

5-HT and adaptive immune system

As with cells of the innate immune system, expression of 5-HT signaling machinery by adaptive immune cells vary depending on their stage of maturation and/or state of activation. 5-HT directly affects various T cell functions, which is in addition to its influence on T cell priming by APCs. Khan et al. using human K562 lymphocytes implicated 5-HT in T cell maturation in secondary lymphoid organs [104]. 5-HT stimulation, specifically 5-HTR7 activation on naïve T cells induces ERK1/2 phosphorylation and activation of NF-κB, which leads to the expression of 5-HTR1B and 2A [105]. Blockade of 5-HTR2A signaling has been shown to reduce antigen-specific induction of both cytotoxic T lymphocytes (CTLs) and T helper (Th)1 cells as well as IL-2 and interferon (IFN)-γ production [106]. Moreover, antagonism of 5-HTR1B has been shown to prevent the proliferation of Th cells [106]. Increased expression of TPH1 has been reported in activated T cells and disruption of 5-HT synthesis in these lymphocytes prevents autocrine signaling via 5-HTR1A also inhibiting proliferation, as well as IL-2 and IFN-γ secretion by both human and mouse activated T cells [105,106]. In addition to IFN-γ, at steady state human T cells require intracellular 5-HT for their production of IL-10 [107]. 5-HT, more specifically 5-HTR3 signaling, has also

been implicated in activated human T cell localization in tissue [108]. The actions of 5-HT in the adaptive immune system are not limited to T cells, 5-HT along with PAF has been shown to activate B cells [109]. 5-HT in a 5-HTR1A-dependent manner regulates murine B cell proliferation and SSRIs have anti-proliferative as well as pro-apoptotic effects on human leukemic B cells [110,111]. 5-HTT expression has been detected on B cells but is yet to be confirmed on T cells [76]. T cells, however, express other transporters, such as OCT, that enables uptake of 5-HT by these lymphocytes, which is in addition to their aforementioned ability to produce 5-HT [76].

High intracellular 5-HT levels, in addition to surface receptor-mediated regulation, may also affect a myriad of immune functions. Referred to as serotonylation, a high concentration of 5-HT within the cell leads to posttranslational modifications of proteins by transglutaminase, which covalently links 5-HT to small GTPases rendering them constitutively active. This has been implicated in alpha-granule exocytosis and 5-HTT internalization by platelets [112,113]. Serotonylation of small GTPases, such as Rab3a and Rab27a, have also been shown to mediate exocytosis in pancreatic β-cells resulting in insulin secretion [11]. These small GTPases are also found in immune cells and it has been shown Rab27a expression is essential for granular exocytosis by CTLs [114]. Thus, 5-HT via serotonylation has been postulated to be involved in

immune mediator secretion, as well as cytoskeletal reorganization required for antigen presentation.

Given the immunomodulatory properties of 5-HT as well as its important role in almost all major organ systems, changes in 5-HT signaling have been associated various diseases. However to date, its involvement in the pathophysiology of neuropsychological disorders, such as Alzheimer's disease, schizophrenia, depression, and alcoholism, is the most clinically relevant, as components of 5-HT signaling machinery are the targets of many current antipsychotic, antidepressant and anxiolytic medications, as well as the major focus of many CNS drug development [6]. Changes in peripheral serotonergic signaling have also been associated with a myriad of diseases, including myocardial infarction, pulmonary hypertension, asthma, rheumatoid arthritis, osteoarthritis, pancreatitis and a variety of liver diseases [115–125]. Recent work from our laboratory has also implicated peripheral 5-HT with obesity and associated disorders, and obesity, itself has been described as a low-grade inflammation [126,127]. Within the gut, alterations in normal 5-HT signaling are associated with inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, colon carcinoma and various enteric infections [128–148].

IBD: A multifactorial disease

IBD is characterized by chronic inflammation of the GI tract with interposed periods of remission, defined as complete resolution of symptoms and endoscopic mucosal healing. Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of IBD. The age of onset for these diseases is early adulthood, but they may occur at any age. About 10–15% of patients are diagnosed before the age of 18 years, and pediatric-onset IBD is characterized by distinct phenotypic differences compared to adult-onset IBD [149]. Typical symptoms of IBD include abdominal pain, cramping, diarrhea, bleeding, and extreme fatigue. These are the result of inflammation of the intestine and may be similar in both CD and UC. Up to 40% of IBD patients may also present with non-bowel symptoms, called extra-intestinal manifestations, involving the eyes, joints, skin, bones, kidneys, and liver [150]. Pediatric IBD patients often experience growth problems, without outward signs of an inflamed bowel [149]. Many of the aforementioned symptoms of IBD are non-specific, as the gut has only a limited number of ways to show distress. Thus, these could also be related to other gastrointestinal conditions, including infectious gastroenteritis, IBS, celiac disease, and colorectal cancer [150].

Though CD and UC share common features, they represent two distinct pathophysiological entities. This evident from the nature and location of the inflammation observed. Inflammation in UC is largely confined to the colon and the rectum and is usually limited to the mucosa. The gross appearance of UC can vary with the disease activity and duration. Endoscopically, classic UC starts at

the rectum and progresses proximally, and affect areas up to the ileocecal valve [151]. Inflammation in UC is continuous, the transition from diseased to normal mucosa is usually gradual and only occasionally abrupt, such as in pan-colitis [152]. Unlike UC, inflammation in CD can span the entire GI tract, from the mouth to the anus, and may involve all layers of the gut. The length of the segments involved varies and involvement of the upper GI tract is uncommon [152]. Additionally, macroscopic lesions are apparent both on the mucosal and serosal side of the bowel wall and vary in size [152]. Endoscopically, classic CD appears as "skip lesions" meaning normal healthy mucosa may be found between areas of the inflamed bowel, thus the transition from involved to uninvolved areas in CD is usually abrupt. Over time, periods of active inflammation and remission give rise to the classic "cobblestone" appearance of the mucosa associated with CD.
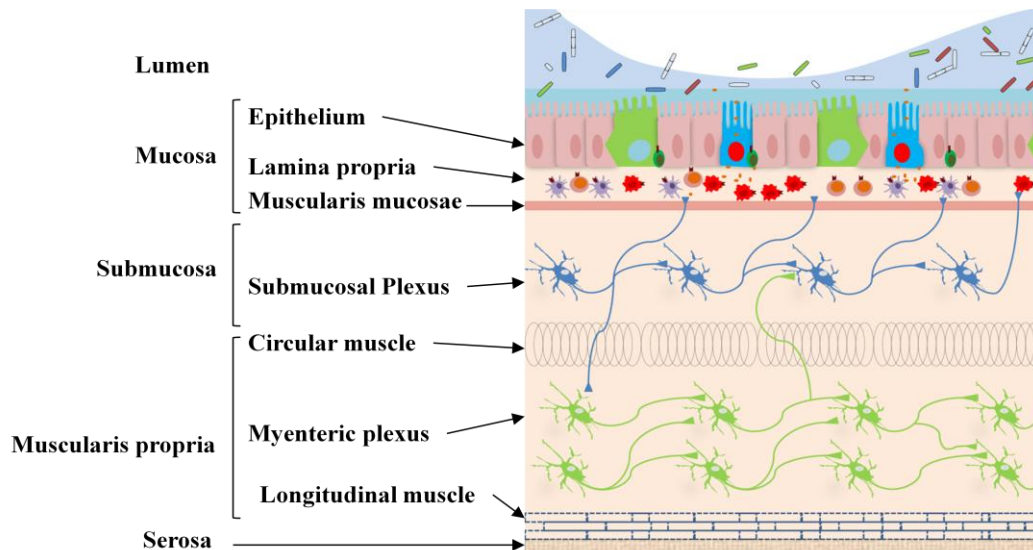
Figure II: A schematic representation of various layers of the gut. Inflammation in Crohn's disease can penetrate all layers whereas in ulcerative colitis it is limited to the mucosa.

Both CD and UC display a tremendous degree of heterogeneity, and like many other diseases, their exact cause is not yet known. IBD is considered to be a multifactorial disease resulting from complex interactions between various environmental factors and host factors including gut microbiota, genetic susceptibilities, and the immune system [153].
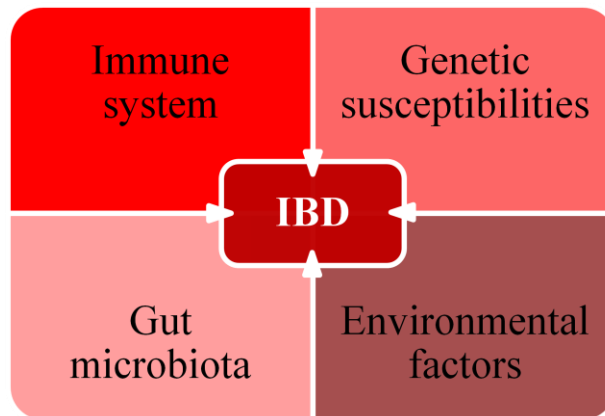
Figure III: Factors involved in IBD. The ultimate cause(s) of IBD is unknown but current research has implicated both host and environmental factors and their complex interplay in IBD pathogenesis. As with many other diseases, the relative importance of nature versus nurture in the development of IBD is yet to be determined.

Environmental factors

Environmental exposures are thought to contribute to the development of IBD, as it has been primarily observed in industrialized nations [154]. Recent data suggests, the prevalence of IBD in the Western world, which includes North

America, Europe, Australia and New Zealand, is approximately 0.5% of the general population, with Canada having one of the highest prevalence (~0.7%) and incidence rates (10, 200 cases/year) for IBD [154,155]. The incidence of IBD has risen steadily through the 20th century in the Western world but was fairly uncommon in developing countries [154]. However, over the past few decades, the emergence of IBD has been documented in the newly industrialized countries of Asia, South America, and the Middle East [156–161]. In these newly industrialized countries, such as India, China, and Brazil, the prevalence of IBD is significantly lower than that in the Western world but the incidence rate of IBD is rising [156,159,160]. In addition to that studies have revealed, first-generation (depending on the age of arrival) and second-generation children of immigrants from regions with low prevalence to countries with higher prevalence are at an increased risk of developing IBD [162–165].

A plethora of theories has been put forth to explain the unknown environmental exposures that may interact with the immune system, resulting in the abnormal inflammatory response observed in IBD. The hygiene hypothesis in the most prominent of these theories and proposes that reduced exposure to microbial antigen in early life increases the risk of immunologic disorders, including IBD in later life [153,166]. This hypothesis has been tested in case-control studies, but these studies relied heavily on patient questionnaires and assumptions based on geographical location and social class [153,154,167]. Several perinatal factors

such as breastfeeding and cesarean section have been linked with pediatric IBD development, with heterogeneous results being reported [168,169]. Repeated use of antibiotics in the early years of life has also been postulated as a possible factor in the development of chronic intestinal inflammation in children and adults [170–173]. Diet and potable water quality have also been studied in the context of IBD. With regards to diet, saturated fats and polyunsaturated fatty acids have been implicated in IBD pathogenesis, whereas refined sugar, carbohydrate, animal protein and dietary fiber intake have shown inconsistent associations [174–183]. A Norwegian study associated iron in drinking water to IBD, which may be related to oxidative stress and bacterial overgrowth [184]. The findings of studies investigating the effect of diet and drinking water on IBD development are often inconsistent and plagued with confounding variables, which highlight challenges of studying the complex effect of these very basic factors in IBD pathogenesis. Interestingly, a paradoxical relationship has been demonstrated between smoking and IBD pathogenesis but is not necessarily consistent across all ethnic groups [154,185,186]. Countries with the highest incidence of smoking were found to have the lowest incidence of IBD, such as South Korea, whereas the highest incidence of IBD actually occurs in countries with a low prevalence of smoking, like Canada [187–189]. A meta-analysis concluded that smokers were less likely to develop UC compared to non-smokers or ex-smokers, and the opposite was observed in the case of CD, as smokers, followed by ex-smokers, were at an

increased risk of developing CD [190]. Environmental factors, including the ones discussed above, have a major impact on gut microbiota composition. The symbiotic relationship between microbes and host is critical in the development of normal physiological processes and imbalances in this interaction may lead to pathophysiology.

Gut microbiota

The gut microbiome plays a major role in health and disease by influencing nutrient absorption, epithelial barrier function, host gene expression as well as in the development and maturation of the immune system [191]. The human GI tract harbors over 100 trillion microbial cells, in addition to affecting physiological aspects of an individual, these microbes also influence behavioral traits [191–193]. Microbial composition of the gut varies with age [194]. The neonatal microbiota has low diversity which gradually increases with time, particularly with the introduction of solid foods [194,195]. It remains in flux up to the age of 2-3 years, then it becomes more stable and converges to resemble that of an adult microbiota [194,196]. The adult microbiota has high inter-individual variability at the species level but is highly conserved at higher-taxa levels [197]. Gut microbiota composition and density also vary along the length of the intestinal tract, this is mainly due to harsher conditions of the proximal intestine. The microbial density in the proximal small intestine can reach 10^3 cells per gram of content and in the distal colon can reach 10^{12} per gram of content [198,199]. The

microbial composition of the large intestine is better studied than that of the small intestine, as most studies have resorted to studying fecal microbiota composition due to more restricted and difficult sampling of small intestinal microbiota. However, it has been shown that the microbiota in the small intestine is less stable than that of the colon [200].

Microbial colonization early in life is essential to the development and maturation of the immune system [198]. This process can be influenced by various factors including birth delivery mode, neonatal antibiotic or probiotic use, and diet [201]. Colonization may aid in the establishment of a symbiotic relationship of tolerance and protective immunity. This is achieved under the reciprocal regulation of the mucosal immune system and the microbiota [202]. The microbiota can directly or indirectly via IEC-microbiota interaction regulate the immune system, conversely, the microbiota is controlled by mediators of IECs and immune cells [198,199,202–204]. It has been demonstrated that certain microbes favor the growth of distinct T lymphocyte subsets in the intestine [202].

Abnormalities in the gut microbial composition, referred to as dysbiosis, has been observed in both UC and CD [205]. Dysbiosis can promote the growth of invasive pathogenic bacteria and bacterial translocation through the mucosal barrier, which may activate the mucosal immune response [205]. IBD patients have been shown to develop antibodies against a variety of microbial antigens, but there is limited evidence of increased cell-mediated immunity [206–210]. Additionally, it remains

to be eluded whether dysbiosis is a primary or secondary phenomenon in IBD. However, the role of microbiota as an important factor in intestinal inflammation is supported by the observations that robust chemical and adoptive transfer models of colitis do not develop in germ-free (GF) mice, as well as by studies in genetically altered murine models of spontaneous colitis, which only develop intestinal inflammation following introduction to luminal microbiota [211].

Genetic susceptibilities

The existence of familial forms of IBD and increased twin concordance highlight the importance of the genetic component in IBD [212,213]. 5-10% of patients have a first-degree family member with IBD, with the relative risk to siblings of patients estimated to be 30- to 40-fold higher for CD and 10- to 20-fold higher for UC [214,215]. The risk of CD in monozygotic twins is 36% and for UC is 16%, in contrast to 4% for both diseases in dizygotic twins [212,216]. In addition to that, certain ethnic groups, particularly of European ancestry, are at an increased risk of disease susceptibility [216]. The known association of CD with nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene variants is found primarily in patients of European or Jewish ancestry, but not in patients of Japanese or Chinese ancestry [217–222]. Single nucleotide polymorphisms (SNPs) associated with increased risk of developing IBD were identified in genes involving microbial detection (NOD2, IRF5, NFκB1, RELA, REL, RIPK2, CARD9, and PTPN22), autophagy (ATG16L1, IRGM) and integration of the

adaptive immune system (IL23R, IL10, IL12, IL18RAP/IL1R1, IFNGR/IFNAR1, JAK2, STAT3, and TYK2) [223]. Of the genetic loci identified by genome wide association study (GWAS) techniques, 110 are associated with both forms of IBD, 30 are specific to CD and 23 are specific to UC [223]. These IBD-associated loci are not all exclusively associated with IBD, are enriched for genes that regulate various functions of the immune system, and the presence of multiple risk loci can significantly increase disease susceptibility.

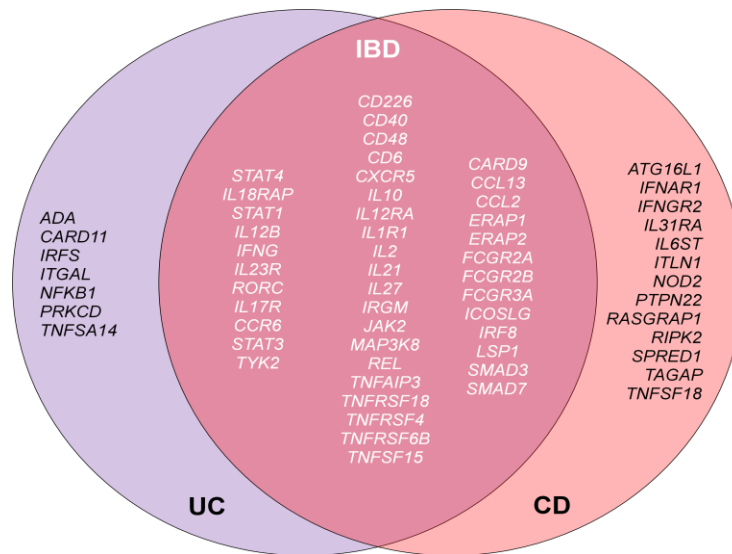**Figure IV: Immune system associated genes whose mutations are linked with increased risk of IBD.** These genes regulate various aspects of an immune response from antigen recognition and presentation to the generation of an adaptive immune response. The majority of them are common to both forms of IBD, while some of these variants are specific for Crohn's disease (CD) and ulcerative colitis (UC).

Intestinal immune system

The GI tract forms the body's largest mucosal interface (approximately 200-400 m^2 of surface area) with the external environment and is constantly exposed to luminal antigens and insults, which include dietary antigens, bacteria, viruses, and fungi as well as pharmacological agents such as nonsteroidal anti-inflammatory drugs [224]. The gut, thus, has adopted a unique state of hyporesponsiveness through carefully balanced local responses. A number of physiological and immunological adjustments enable the gut to maintain this homeostasis, a state of physiological inflammation to fight invading pathogens, but at the same time avoiding the generation of an overwhelming immune response leading to intestinal inflammation [153]. Gut homeostasis is critically dependent on the triadic interaction of luminal antigens, the epithelium, and immune cells. At the epicenter of this complex interface is the single layer of polarized IECs acting as a barrier to foreign antigens [225]. In addition to being an anatomical barrier, IECs perform the main function of the gut, the absorption of nutrients and water [203]. They are also capable of performing immune functions [203]. Through their expression of pattern-recognition receptors (PRRs), such as TLRs, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), IECs can recognize microbial ligands which have a complex influence over physiological and pathophysiological process [203,226,227]. They process and present antigens as well as produce a myriad of signaling molecules, including cytokines, and antimicrobial peptides (AMPs), that can directly affect immune cell functions and

microbial colonization [203,226,228]. The association between increased bacterial translocation and risk of developing IBD is suggestive of a central role of dysregulated epithelial barrier function as either the etiology or the pathology of IBD [229–235]. Increasing evidence also indicates that compromised intestinal barrier function contributes to systemic immune activation [235–239]. The main feature of IBD is inflammation of the mucosal surface, and it is now becoming clear that abnormalities within the epithelial layer and irregularities in IEC-immune cell interactions, may play a key role in IBD pathogenesis [235,240,241].

The epithelium

The proximal and distal ends of the GI tract, i.e. the oral cavity, esophagus and the anus, are lined with stratified squamous epithelium [242]. The epithelial lining of the remainder of the gut, the small and large intestines, is composed of non-ciliated, columnar epithelium [242]. The epithelial monolayer of the intestine has many crypts and villi (only in the small intestine). The intestinal epithelium is continually renewed by pluripotent stem cells found at the base of crypts and all four major cell types in the epithelial layer differentiate from them [242]. Enterocytes, goblet cells, and the aforementioned EECs are found both in the small and large intestines, migrate upwards from the stem cell niche and several days after terminal differentiation undergo programmed cell death (apoptosis) [242,243]. On the other hand, in the healthy gut Paneth cells are found only in the small intestine and have a longer lifespan than the other three major types of cells

[244]. Moreover, they are the only cell type that migrates downwards from the stem cell niche and remains within the crypts after differentiation [243,244]. It is hypothesized that Paneth cells provide continuous antimicrobial protection for the nearby stem niches, as damage to or parasitization of stem cells would have detrimental consequences for the normal digestive epithelium and its functions [244].

As mentioned previously, the primary function of the intestinal epithelium is the absorption of water and nutrients. This accomplished by the breakdown of macromolecules via the combined actions of brush border enzymes produced by IECs, pancreatic enzymes, as well as bile, and finally absorption by enterocytes. Absorptive enterocytes comprise approximately 80% of the epithelial layer and absorption is mediated by the transcellular and paracellular pathways [245]. The transcellular pathway works via selective transporters and channels found on the apical and basolateral surface of IECs and mediates the transport and absorption of nutrients including amino acids, small peptides, fatty acids, vitamins, short-chain fatty acids (SCFA) and sugars [246]. IECs are interconnected by tight and adherens junctional proteins [247]. These proteins maintain the integrity of the epithelium as a selective physical barrier, by regulating the paracellular pathway and controlling the flux of water, ions across the epithelium, as well as cellular polarity [245–247].
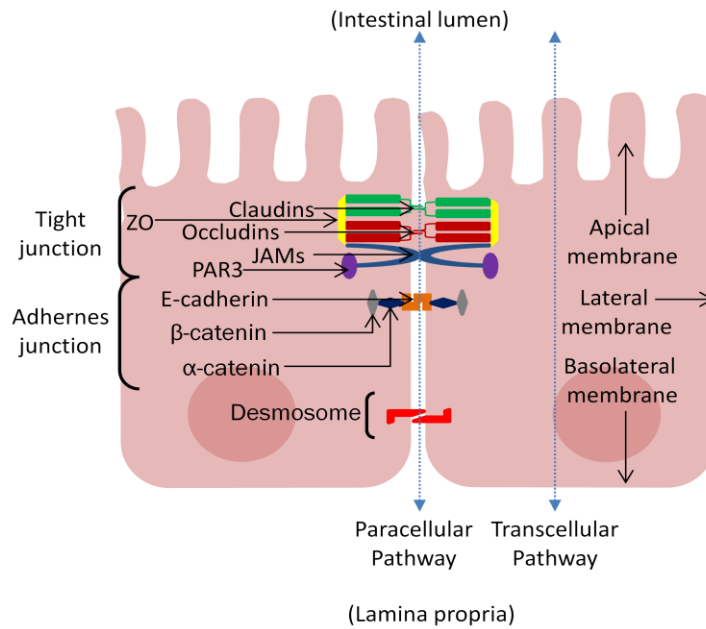
Figure V: Intestinal permeability pathways and overview of junctional proteins. Intestinal permeability is controlled by two major pathways: paracellular and transcellular transport. Paracellular permeability is mainly controlled by tight and adherens junctional proteins. Tight junctions are localized to the apical-lateral membrane junction and consist of three integral transmembrane proteins, claudins, occludin, and JAMs which are connected to the actin cytoskeleton (not depicted) via the ZO-1,2 & 3 or PAR adaptor proteins. E-cadherin is the main transmembrane protein at the adherens junctions and associates with the alpha and beta-catenins. JAMs, junctional adhesional molecules; PAR-3, partitioning defective-3; ZO, zonula occludens.

Defects in these intercellular junctions have been associated with increased epithelial permeability observed in patients with IBD and their first-degree relatives [231,232,248]. It has also been reported that compromised intestinal permeability may predict and possibly cause a relapse of IBD [230]. Altered expressions of tight and adherens junctional proteins, such as E-cadherin, junctional adhesion molecule-A (JAM-A), occludin and claudin, have been

implicated in the barrier disruption observed in IBD [249–252]. Though pro-inflammatory cytokines, such as TNF-α and IL-13, have been implicated in the degradation of the anatomical barrier, the mechanisms involved are not completely understood [250,253–255].

The ability of the intestinal epithelium to perform an array of diverse functions is predominantly due to the presence of the three specialized IECs of secretory lineage [203]. Along with the aforementioned hormone-secreting EECs, mucin-secreting goblet cells, and AMP secreting Paneth cells are specialized for maintaining most GI functions [203]. They also play an important role in the organization of a physical and biochemical barrier of mucus that limits the contact between luminal antigens and the epithelium, as well as the underlying immune cells. Mucus, or mucin glycoproteins, produced by goblet cells are organized in two layers in the colon [240]. Mucin 2 (MUC2), the most abundant of these mucins, plays an important role in the formation of the dense inner layer that prevents microbial contact with the epithelial surface, while the outer layer provides nutrients for mucosal-associated bacteria [256]. The small intestine only has one layer mucus, which is loosely associated with the epithelium and is permeable to bacteria [256]. In addition to mucin, other goblet cell-derived products, such as trefoil factor 3 (TFF3) and resistin-like molecule (RELM)-β, also contribute to the regulation of the anatomical barrier in the intestine. These factors provide structural integrity to mucus through mucin crosslinking and

promote epithelial repair, migration of IECs, resistance to apoptosis as well as MUC2 secretion [257–260]. AMPs are part of the biochemical arsenal that further supplements the intestinal barrier functions. Enterocytes produce some AMPs throughout the small and large intestines [244]. However, Paneth cells of the small intestine specialize in the production of AMPs, including defensins, C-type lectins, cathelicidins, and lysozyme [244]. Notably, it has been shown that C-type lectin, RegIIIγ, is essential for the maintenance of the spatial distance (∼50 μm) between the small intestinal epithelium and bacteria [261]. AMPs disrupt highly conserved and essential features of microbial biology, such as the surface membrane, allowing for the regulation of both commensal and pathogenic microbes [244]. Moreover, IECs via active transcytosis release dimeric immunoglobulin A (IgA) produced by plasma cells in the LP into the intestinal lumen as secretory IgA (sIgA), where it contributes to the separation of luminal antigens from the mucosa [203]. Collectively, these multifunctional cells provide a dynamic barrier to the environment, which protects the host from infection and continuous exposure to potentially inflammatory stimuli.

Secretory products of IECs, such as defensins, IL-33, thymic stromal lymphopoietin (TSLP), and transforming growth factor (TGF)-β and retinoic acid (RA), have immune regulatory properties that are vital in the maintenance of intestinal homeostasis [262]. Thus, it should come as no surprise that alteration in IEC biology and functions has been reported in association with IBD, such as

changes in IL-33 and TLSP expression [263–265]. Studies have reported changes in EEC and goblet cell numbers, as well as Paneth cell metaplasia in the colon, where it is normally absent, in IBD patients [17,266–269]. Furthermore, alterations in the production and expression of secretory products, such as 5-HT, chromogranin A, mucin (including MUC2), and AMPs, has been reported in both CD and UC [228,270,271]. These clinical observations are further supported by studies in animal models. It has been shown that deficiency in gut-derived 5-HT leads to reduced severity of experimental colitis, whereas MUC2-deficient mice are more susceptible and often develop of spontaneous colitis [86,272,273]. Disruption of Paneth cell functions due to defects in autophagy machinery has also been linked to the development of spontaneous disease resembling human IBD [274].

DCs and macrophages

Over two-thirds of all immune cells of the human body are found in gut-associated lymphoid tissue (GALT), which is divided into induction and effector sites [275]. Peyer's patches (PP; only in the small intestine), isolated lymphoid follicles (ILFs; in both the small and large intestines) along with mesenteric lymph nodes (MLNs) are the induction sites of GALT, where antigen-specific immune responses are generated. The effector sites of GALT are the epithelium and the LP, the latter is a network of loose connective tissue and tissue-resident

immune cells [276]. In addition to providing a physical barrier between luminal content and immune cells, the intestinal epithelium includes specialized IECs, mainly microfold cells (M cells), for sampling and presentation of luminal antigens and intact microorganisms to the underlying mucosal immune system [276]. This is critical for the preservation of intestinal homeostasis, as the local immune system must be able to mount an appropriate immune response against invading pathogens while remaining tolerant of innocuous luminal antigens. M cells are found in high numbers in the follicle-associated epithelium overlying PP and ILFs, have increased levels of endocytosis, and are capable of specific receptor-mediated microbial uptake as well as nonspecific antigen uptake from the intestinal lumen [277]. Once antigens are brought in by M cells, they are taken up by APCs, such as DCs. In addition to M cells, small intestinal goblet cells are capable of delivering soluble luminal antigens to subepithelial DCs [278]. Antigens can also be taken up by DC-like mononuclear phagocytes expressing tight junction proteins which sample luminal contents through transepithelial dendrites [279]. Thus, mucosal DCs are uniquely positioned to contribute to the maintenance of mucosal homeostasis or induce inflammation [280].

The exact definition of intestinal DCs is complicated as they are a heterogeneous population of cells with functional diversity [281]. Common DC progenitor (CDP) derived conventional DCs (cDCs) express high levels of CD11c, varying levels of CD8α and CD11b, and reside in secondary lymphoid tissues, such PP

and MLNs [282]. Plasmacytoid DCs (pDCs) and mDCs, the former also arises from the CDP and specializes in the production of type I interferons, are also present in the gut [283]. Most lamina propria DCs (LPDCs) are CD11b+CD8α− cells and along with a subset of CD8α+ pDCs they induce regulatory T (Treg) cell differentiation, playing an active and direct role in maintaining tolerance to innocuous antigens [282,284]. LPDCs can be divided into CD103+ and CD103− populations. IECs in a contact-dependent manner induce CD103 expression in LPDCs via RA and TGF-β [285]. CD103+ LPDCs favor tolerizing Th cell differentiation [286–288]. They have been shown to migrate into the epithelial cell layer and capture bacterial antigens, and their migration to GALT is CCR7-dependent [289–291]. Following antigen experience, mature DCs migrate to T cell-rich areas or zones of the secondary lymphoid tissues [281]. Here they promote differentiation of naïve CD4+ T cells into Foxp3-expressing Treg cells via RA and TGF-β, as well as suppress the differentiation of other Th cells by producing indoleamine 2,3-dioxygenase (IDO) [286–288,292]. Via RA, they also imprint gut-homing properties on T cells during differentiation, so that the mature cells can return to the original site of antigen encounter, the intestinal LP [293,294]. In the secondary lymphoid tissues, interactions between CD4+ T cells and B cells also results in the activation and expansion of B cells to become antibody-secreting plasma cells. CD103− LPDCs are efficient inducers of IgA class switching of B lymphocytes in the PP and LP, and as mentioned earlier sIgA

is a critical component of the biochemical barrier separating the epithelium and microbes [203,282]. CD103− LPDCs are mostly mDCs and support the differentiation of Th17 cells, and these Th cells play an important role in protective immunity of the normal gut [295].

Table I: Murine intestinal DC subsets and their locations in the gut. LP, lamina propria; GALT, gut-associated lymphoid tissue; hi, high; int, intermediate; MHC, major histocompatibility complex; M-CSF, macrophage colony-stimulating factor.

Surface markers	*Location*
CD103+ CD11b+ CD11c^{hi} MHC class II ^{hi} CX3CR1−M-CSF^{low}	LP
CD103− CD11b+ CD11c^{hi} MHC class II ^{hi} CX3CR1^{int} M-CSF^{hi}	LP
CD103+ CD11b− CD11c^{hi} MHC class II ^{hi} CX3CR1−	GALT

Macrophages, a very plastic cell type, have the ability to change phenotype and function according to the immediate environment [296]. Hence, phenotypic distribution of these cells vary along the human GI tract as well as where they are within the layers of the gut [297,298]. Mouse LP macrophages are the progeny of circulating monocytes and are continually replenished [299]. In a CCR2 and macrophage colony-stimulatory factor (M-CSF)-dependent manner monocytes differentiate into gut macrophages, and lose their Ly6C expression while up-regulating other surface markers are, such as CD64, CD11c, F4/80, major

histocompatibility complex (MHC) class II and CX3CR1 [300–305]. This is when they also acquire a tolerogenic gene expression profile, which includes the expression of IL-10 [306]. They also a acquire TNF-α expression, which contributes to their both pro-and -anti-inflammatory activity [301]. IEC secretion of TSLP, TGF-β and semaphorin 7A, has been shown to play an important role in the development of tolerogenic LP macrophages [203,307]. They have an extended half-life and appear to be in a state of anergy with their phagocytic and bactericidal activities intact [301,303,306,308]. These cells represent over 65% of the HLA-DR+ cells in the healthy human colon [309]. In the normal gut, non-migratory CX3CR1[hi] LP macrophages have been suggested to sample luminal antigens and deliver them to CD103+ LPDCs, which then traffic to MLN to prime adaptive immune cells [282,291,300–303]. Once these adaptive immune cells home back to the gut CX3CR1[hi] LP macrophages have been postulated to regulate the balance of LP lymphocytes, via the production of IL-10 contributing to the expansion of Treg cells, regulation of Treg cell functions and subsequently Th1/Th17 immunity [301,303,306,310].

In addition to their contribution to mucosal immune homeostasis, a special subset of CX3CR1[hi] macrophages has been identified, which are implicated in peristalsis [311]. Localized within the muscularis externa from the stomach to the distal colon, muscularis macrophages (MMs) accumulate in layers, and like LP macrophages, are tissue-protective, vigorous phagocytes, and have potent antigen

presentation capacity [311]. At steady state in response to microbial stimulation, MMs directly regulate enteric neurons and in turn GI motility [311]. They have also been implicated in the development of the ENS, though the mechanisms are yet to be determined [281].

Table II: Murine intestinal macrophages subsets and their locations in the gut. LP, lamina propria; hi, high; MHC, major histocompatibility complex.

Surface markers	*Location*
CD64+ CX3CR1^{hi} CD11c+ F4/80+ CD11b+ MHC class II+	LP
CD64+ CX3CR1^{hi} CD11c− F4/80+ CD11b+ MHC class II+	LP
CD64+ CX3CR1+ CD169+ CD11c^{low} F4/80^{low} CD11b+ MHC class II+	LP (close to crypt)
CD64+ CX3CR1^{hi} CD11c^{low} F4/80+ CD11b+ MHC class II^{hi}	Muscularis layer

The ability of IECs to promote tolerogenic differentiation of DCs and macrophages is lost in IBD and is marked by the accumulation of macrophages that have increased expression of co-stimulatory molecules, activating receptors, and TLRs, as well as higher frequency of mature LPDCs with enhanced expression of PRRs [203,263,312–320]. The barrier dysfunction in IBD leads to increased translocation of microorganisms, and CD is marked by macrophages that have defective cytokine secretion and impaired bacterial clearance capacity [240,321]. In acute intestinal inflammation, Ly6C+ monocytes fail to acquire the

quiescent characteristic of gut macrophages and outnumber resident macrophage population [301]. These pro-inflammatory cells secrete IL-12, IL-23, TNF-α, and inducible nitric oxide synthase (iNOS) [301]. A subset of CD14+ inflammatory macrophage population has also been identified in the mucosa of patients with CD [322]. These cells produce large amounts of pro-inflammatory cytokines, IL-23, TNF-α and IL-6, and potentially contributes to IFN-γ production by LP mononuclear cells [322]. Macrophages from CD patients also produce less IL-10, and in UC patients, constitutively produce high levels of the pro-inflammatory cytokine IL-12 [323]. This may be partially due to impaired regulation of TLR-induced inflammatory responses in macrophages. Normal LP macrophages do not express triggering receptor expressed on myeloid cells (TREM)-1, which amplifies TLR-induced inflammatory response of macrophages, but accumulation TREM-1-expressing LP macrophages have been reported in patients with IBD [324–326]. It has been shown that mice lacking phosphoinositide 3-kinase (PI3K) p110δ subunit function, a negative regulator of TLR responses in macrophages, develop spontaneous colitis, have impaired bactericidal activity and lack of p110δ function has been associated with reduced IL-10 secretion [327,328]. Furthermore, it has been shown that IL-10 and microbiota co-regulate the expression of nuclear factor-interleukin-3-regulated (NFIL3) by macrophages, which down-regulates IL-12/IL-23p40 production during host and enteric commensal interactions [329]. NFIL3 expression was found to be significantly

reduced in IBD patients and NFIL3-KO mice have been shown to develop spontaneous colitis [330]. IL-10 signaling is central to the suppression of macrophage-associated inflammation. It has been shown that IL-10 receptor (IL-10R), but not IL-10, deficiency in intestinal CX3CR1+ macrophages leads to increased severity of experimental colitis [306]. This is further supported by the observation of early-onset pediatric enterocolitis in children with nonsense and missense mutations in IL-10R, which reduces IL-10R expression [331]. CX3CR1-KO mice also exhibit increased severity of experimental colitis, with decreased numbers of LP macrophages and increased translocation of commensal bacteria to the MLNs [332]. Inflammation associated changes observed in intestinal macrophages has been suggested to facilitate the loss of tolerance to commensals by activating DCs [333]. Professional APCs, such as DCs, perform three essential functions required for T cell activation, which includes 1) antigen presentation, 2) co-simulation and 3) providing the cytokine milieu. The interplay between secreted cytokines determines the balance between the different types of Th cell differentiation. Accumulation of over-active DCs that has been observed at sites of inflammation in CD, as well as in experimental models of colitis [313,334]. Mucosal DCs from patients with CD and UC have increased expression of TLR2 and TLR4 in comparison with DCs from healthy controls (HCs) [313]. DCs in patients with CD also express higher levels of CD40, produce more IL-12 and IL-6 than normal mucosal DCs [313]. This could promote the differentiation of

Th1/Th17 cells while abolishing the differentiation of Treg cells. It has been shown in a model of T cell mediated colitis that, disruption of DC-T cell interactions via the blockade of CD40/CD40L engagement can prevent intestinal inflammation [334]. DCs in CD also express the chemokine receptor CCR7 and E-cadherin, which leads to their trafficking and retention in the inflamed mucosa, which may contribute to the pathogenesis of CD [320].

T lymphocytes

Depending on their location in the GALT effector sites, lymphocytes of the normal gut are subdivided into intraepithelial lymphocytes (IELs) and LP lymphocytes (LPLs). The latter represents a tightly regulated effector compartment, consisting of IgA-secreting plasma cells and memory T cells [335]. The acquisition and functional regulation of these cells, in the normal gut, by IEC-influenced DCs and macrophages has been briefly described above. In the LP, an overwhelming majority of the T cells are CD4+ T cell receptor (TCR)$\alpha\beta$+ T cells, though CD8$\alpha\beta$+ TCR$\alpha\beta$+ T cells and TCR$\gamma\delta$+ T cells are also present [335]. CD4+ TCR$\alpha\beta$+ T cells help in the functions of plasma and CD8$\alpha\beta$+ memory T cells. At steady state, IL-17 producing Th17 cells and Treg cells are the two most abundant effector T cell types in the LP [336]. Th17 cells promote inflammatory protective immune response and Treg cells suppress excessive or unwanted immune activation. Treg cells are particularly enriched throughout the GI tract,

representing up to 50% of CD4+ T cells found in the colon [336,337]. This is presumably due to the sheer volume of antigens encountered and the need for immune tolerance in the intestine. As mentioned earlier, the gut microbiota, IECs, and innate immune cells are all critical factors that determining the local cytokine environment in the intestine, which plays a key role in determining the balance between T cell subsets, and this, in turn, determines the type of immune response generated.

Nearly all IELs are antigen-experienced T cells that are found interspersed between the epithelial cells lining the intestinal lumen. They play an important role in protective immunity by maintaining gut barrier integrity as well as by preventing pathogen entry and spreading [338]. These specialized immune cells are in an "activated-resting" state and regain their fully-functional status following the presentation of non-classical MHC-I-like ligands by stressed or damaged IECs [339–342]. Further contributing to their role as monitors of barrier functions, IELs produce and can induce the production of AMPs by IECs [343,344]. IELs are categorized based on their expression of αβ or γδ TCRs, with the TCRγδ+ IELs making up a significant portion of the population [338]. They can be further subdivided into natural and induced IELs [345]. Present at birth, natural IELs are either TCRαβ+ or TCRγδ+ and develop as well as acquire their activated phenotype in response to self-antigens in the thymus. In contrast, induced IELs are not present at birth and are the progeny of thymus-derived TCRαβ+ CD4+ or

CD8αβ+ T cells which are activated in the periphery in response to non-self antigens. Most of the natural IELs do not express CD4 or CD8αβ co-receptors but co-express the CD8αα homodimer, which is also expressed by induced IELs [338]. These special effector T cells additionally express both activating and inhibitory types of NK cell receptors, with the majority containing abundant cytoplasmic granules for cytotoxic activity [346–351]. The distribution of various IEL subsets varies in the epithelium of the small and large intestines. There are fewer IELs in the colon compared to the small intestine and the majority of them are CD4+ IELs, a population that is underrepresented amongst small intestinal IELs [338]. The unique physiological conditions encountered and function performed by in the small and large intestines may account for this variance in IEL distribution. Moreover, the microbiota, directly and indirectly, has been shown to play an important role in the maintenance of the IEL compartments [203,343,352,353].

Table III: Major murine intestinal T cell subsets. LPLs, lamina propria lymphocytes; IEL, intraepithelial lymphocytes; TCR, T cell receptor; NK, natural killer. The plus symbols in parenthesis represents frequency from low (+) to high (++++).

T cell category	*Location*	*TCR phenotype (frequency)*	*NK receptors frequency*
LPLs	Small intestine	TCRαβ (+++) • CD8αβ+ (++) • CD8αβ+CD8αα+ (+) • CD4+ (+++) • CD4+CD8αα+ (+)	Not expressed

	Large intestine	TCRαβ+ (+++) • CD8αβ+ (++) • CD8αβ+CD8αα+ (+) • CD4+ (+++) • CD4+CD8αα+ (+)	Not expressed
Natural IELs	Small intestine	1. TCRγδ+ (+++) • CD4–CD8– (++) • CD8αα+ (+++) 2. TCRαβ+ (+++) • CD4–CD8– (+) • CD8αα+ (+++)	+++++
	Large intestine	1. TCRγδ+ (+) • CD4–CD8– (++) • CD8αα+ (+) 2. TCRαβ+ (++) • CD4–CD8– (++) • CD8αα+ (+)	+++
Induced IELs	Small intestine	TCRαβ+(+++) • CD8αβ+ (+) • CD8αβ+CD8αα+ (+++) • CD4+ (+) • CD4+CD8αα+ (++)	++++
	Large intestine	TCRαβ+(++) • CD8αβ+ (+) • CD8αβ+CD8αα+ (+) • CD4+ (++) • CD4+CD8αα+ (+)	++

Natural killer T (NKT) cells are a minor subset of T lymphocytes (~1%) found in the gut that express NK cell surface markers (CD 161 in humans and NK1.1 in some strains of mice), and recognize lipid antigens presented by the non-classical MHC class I molecule CD1d a-e [354]. These thymus-derived, innate-like T cells

are an extremely heterogeneous population and their post-thymic education is critically dependent on the gut microbiota [355]. Based on the diversity of their αβ TCR, NKT cells are divided into two subsets, type I and II. Type I NKT cells have a very limited TCR repertoire, whereas the TCR repertoire of type II NKT cells is much more diverse, and more like conventional T cells than type I NKT cells [354]. Unlike conventional naive T cells, NKT cells contain constitutive cytokine transcripts, thus can rapidly produce and release cytokines [356]. In humans, CD1d is expressed by APCs, such as DCs, macrophages and B cells, as well as IECs, which can directly activate NKT cells [357–360]. It has been shown that very low expression of native CD1d on IECs is sufficient to activate NKT cells, and CD1d expression on Paneth cells has implicated NKT cells in the regulation of Paneth cell functions in maintaining intestinal homeostasis [360,361]. NKT cells can also be activated in a CD1d independent manner, which is achieved by the combined actions of the pro-inflammatory cytokines IL-12 and IL-18 [359]. NKT cells are capable of altering their cytokine secretion profile in a context-dependent manner and can produce vast quantities of both pro-and anti-inflammatory cytokines, such as IFN-γ, IL-6, IL-17, TNF-α, IL-13, IL-10, IL-5, and IL-4 [354]. Furthermore, NKT cells also have potent cytolytic capacity marked by the production of perforin, granzymes, and granulysin, as well as by the surface expression of molecules, such as CD95L [362,363].

In IBD, alterations in IEC functions and innate immune responses lead to the induction of an inappropriate adaptive immune response. It has been shown that IECs in IBD lose their ability to induce CD8+ T suppressor cells [364]. Moreover, in UC patient impaired expression of peroxisome proliferator-activated receptor-γ by IECs has been linked with loss of immunoregulation contributing to inflammation [365]. The intrinsic plasticity and adaptability of Th cells to stimuli from the surrounding environment are extremely relevant to the complex process of IBD pathogenesis. Until very recently, CD was described as an inflammatory condition driven by Th1 cells, as elevated production of IL-2, IL-12, and IFN-γ were observed [333]. On the other hand, UC was designated as an atypical Th2 condition, as the production of IL-5, IL-13, but not IL-4, and IL-17 were increased [366,367]. The discovery of various other T cell subsets, particularly Treg and Th17 cells, have challenged this dichotomous archetype of Th1 or Th2. The revised paradigm suggests active IBD is an imbalance of Th and Treg cell numbers and functions [333]. CD has now been redefined as Th1/Th17 condition, as increased levels of IL-17 is detected in the mucosa and serum of most patients with IBD, but is consistently higher in patients with CD than UC [368,369]. As mentioned earlier, a large number of inflammatory DCs and macrophages accumulate in the intestinal tissue of IBD patients. In response to bacterial stimuli, these cells produce vast amounts of IL-23, a potent inducer of Th17 cells [370–373]. It also plays an important role in the expansion and activation of Th17 cells,

and a SNP in the IL-23R gene has been associated with decreased susceptibility to IBD [374]. In addition to that, Th17 cells and a population of IL-17A+ IFN-γ+ T cells were found to be present in large numbers in the inflamed intestine of IBD patients [375,376]. Th17 cells produce multiple cytokines, including IL-21 and IL-22. IL-21, which promotes Th1 signaling and IFN-γ production, was found to be up-regulated in the intestine of CD patients compared to patients with UC and HCs [377,378]. IL-22 expression was also increased in active Crohn's lesions but not in inflammation in UC [379]. In the experimental model of dextran sodium sulfate (DSS)-induced colitis, both of these cytokines are up-regulated and it has been shown that IL-21 KO mice are protected from colitis induced by DSS as well as trinitrobenzene sulphonic acid (TNBS) [378,379]. However, it has been observed that anti-IL-17A antibody, secukinumab, exacerbates inflammation during active CD [380]. This is possibly due to the protective role Th17 cells have as effectors of immunity against invading pathogens [381]. Furthermore, Th17-derived cytokine IL-22 plays an important role in IEC proliferation and migration in DSS-induced colitis, which may contribute to intestinal wound healing [379]. In the normal gut, Th17 cells are restrained by Treg cells, these cells also regulate innate inflammatory mechanisms. Treg cell numbers are found to be elevated in the LP of IBD patients, in fact, inappropriate accumulation of antigen-activated T cells due to impaired apoptosis is a prominent feature of IBD, particularly CD [382–386]. With respect to Treg cells in IBD, it appears their suppressor function

is insufficient as it has been shown that T cells from IBD patients are resistant to TGF-β, which along with IL-10, enforce the regulatory influences of Treg cells [386,387]. IELs, on the other hand, are more prominently associated with intestinal inflammation in celiac disease than IBD [388]. However, several reports indicate that disease severity in patients with IBD correlates well with the number of TCRγδ+ IELs in the intestinal mucosa [389–391]. Moreover, a subset of CD4+ T cells expressing NKG2-D type II integral membrane protein, which recognizes cellular stress markers, have been shown to produce IL-17, TNF-α and IL-22 in CD patients [392]. However, it has also been shown that CD4+ CD8αα+ TCRαβ+ T cells may also help control intestinal inflammation [393]. In the mucosa of UC patients, type II NKT cells have been shown to accumulate [394,395]. These cells are responsible for increased IL-13 secretion observed in UC, which is dependent on recognition of sulfatide glycolipid [250,394,395]. Thus, it has been suggested that UC may be an autoimmune condition in which a self-glycolipid activated LP NKT cells mediate epithelial cell damage [250,394].

IL-13 and gut inflammation

IL-13 is a pleiotropic cytokine which is predominantly secreted by activated Th2 cells [396]. Additional sources of IL-13 include macrophages, eosinophils, basophils, NK cells, TCRγδ+ IELs, NKT cells, mast cells, neutrophils, DCs, and type 2 innate lymphoid cells [396–399]. IL-13 and IL-4 are considered to be the

major cytokines of a Th2 immune response and were among the first cytokines described in the early 1980s [400]. IL-13 and IL-4 has approximately 25% sequence similarity, they share receptor subunits and signaling molecules, thus, elicit some overlapping but also unique biological responses [400]. There are two cognate receptors of IL-13, IL-13 receptor (IL-13R) α1 and α2, which belong to the type I cytokine receptor family. IL-13Rα1 and α2 share 37% homology, and specifically bind to IL-13 with moderate and high affinity, respectively [400]. However, IL-13 predominantly signals through IL-13Rα1. Binding of this receptor to its cognate ligand leads to the recruitment of the IL-4Rα subunit (referred to as type I IL-13R or type II IL-4R), enhancing its affinity for IL-13 and transducing intracellular signals through phosphorylation of signal transducer and activator of transcription (STAT) 6 via JAK [400,401]. Though type I IL-13R pathway mainly signals through STAT6, activation of other second messenger molecules, including PI3K, STAT3 and mitogen-activated protein kinase (MAPK) have been reported in different cell types [402–404]. Since both IL-13 and IL-4 can signal via this receptor complex, it has been postulated that these cytokines may compete for the availability of the ligand binding subunits, and their relative concentration in the extracellular milieu may be an important determinant in this process [400]. It has also been shown that IL-13, in a STAT6-dependent manner, can signal independently of IL-4Rα [405]. IL-13Rα2, also known as type II IL-13R, had previously been described as a negative regulator IL-13 due to the lack

of cytoplasmic tail signaling motifs [401]. However, it has now been shown that IL-13Rα2 can also attenuate IL-4 signaling [401]. IL-13 producing cells can be found in the healthy human LP and the expression of both IL-13 receptors has been identified in a variety of cells of the GI tract, such as IECs, neurons and smooth muscle cells [250,406]. IL-13Rα1 is constitutively expressed in the gut epithelium, while IL-13Rα2 is solely and highly expressed on smooth muscle cells [406,407]. It has also been reported that IL-13Rα1 is uniformly expressed in both the small and large intestines, whereas IL-13Rα2 and IL-13 expressions were significantly higher in the colon in comparison to the small intestine [406]. Thus, IL-13 has been implicated in the regulation of many physiological functions of the gut, such as epithelial cell turnover, maintenance of barrier functions, and motility, as well as in gut mucosal immunity and inflammation, including IBD [396,406–408].

As previously mentioned, there is an increase in the number of IL-13-producing LP NKT cells in active UC, and it has now been identified as an important cytokine effective in fistulizing CD [250,395,409]. The type II NKT cells that are responsible increased IL-13 production in UC are not found in the healthy gut and express IL-13Rα2, which is further up-regulated following lyso-sulfatide glycolipid stimulation [394]. In patients with active UC, mucosal IL-13 mRNA expression is elevated and their macrophages have been shown to produce significantly higher amounts of IL-13 in comparison with HC [321,410,411]. In

pediatric UC patients increased epithelial activation of STAT6 have also been reported [412]. The same group has recently reported that IL-13 and IL-13Rα2, as well as IL-5 and IL-17A expressions, were significantly elevated in treatment-naïve pediatric UC patients in comparison with pediatric Crohn's colitis patients [413]. IL-13 has been shown to induce epithelial cell apoptosis in vivo and in vitro, via the activation of the caspase-3, which also involves TNF-α and TNF-like weak inducer of apoptosis (TWEAK) [410,414]. These proteins were also found to be up-regulated in patients with active UC [410]. IL-13, in both PI3K- and STAT6-dependent manner, detrimentally affects intestinal permeability by inducing claudin-2 expression, and increased expression of claudin-2 has been reported in patients with active IBD [250,251,415]. However, up-regulated expression of this pore-forming tight junction protein is more prominent in patients with active UC compared to patients with active CD [251]. In CD patients, adipose tissue macrophages (ATM) and T cells (ATT) obtained from near active lesions have been shown secrete significantly higher amounts of IL-13 in comparison with ATMs and ATTs from inactive lesions, as well as HCs [416]. Moreover, it has been shown that IL-13 reduces the expression of p53 (a protein that modulates cell cycle, repairs damaged DNA, and initiates apoptosis if damage cannot be repaired) in activated CD4+ T cells and interferes with T cell apoptosis, which as mentioned earlier may be relevant in CD immunopathogenesis [153,417]. These findings suggest, a pathogenic involvement of IL-13 in IBD.

This is supported by the targeted inhibition of IL-13, by IFN-β-1a, yielding promising results in a small group of UC patients responsive to the treatment [418].

In animal models of intestinal inflammation pathogenic role of IL-13 has also been identified. In the oxazolone-induced model of colitis (a model of human UC), IL-13 is up-regulated and type I NKT cells are the source of this increased production [419]. Additionally, it has been shown that neutralizing IL-13 using an IL-13Rα2-Fc fusion protein prevented oxazolone-induced colitis, and IL-4Rα-KO, as well as STAT6-KO mice, are immune to oxazolone-induced colitis [419–421]. In the DSS-induced model of colitis, LP CD4+ T cells have shown to increase their production of IL-13 under the influence of IEC-secreted cytokine IL-33 [422]. Moreover, GATA-3 (the Th2 lineage commitment transcription factor) over-expressing transgenic (Tg) mice were found to exhibit increased severity as well as earlier onset of DSS-induced colitis in comparison with T-bet (the Th1 lineage commitment transcription factor) and RORγt (the Th17 lineage commitment transcription factor) Tg mice [423]. This study demonstrated that up-regulation of IL-13 production by GATA-3 Tg CD4+ T cells, and not IL-4 or IL-5, was responsible for the accelerated onset and increased severity of DSS-induced colitis, which was associated with increased infiltration of F4/80+ macrophages and neutrophils in the colon [423]. Also in the DSS-induced model of colitis, Th1 and Th17 cells were found to produce IL-13 in the absence of canonical Th2

polarizing stimuli [424]. A pathogenic role of IL-13 has also been identified in TNF superfamily member 15 (TNFSF15)-driven small intestinal inflammation, a cytokine that is up-regulated in the active IBD mucosa, moreover, its expression levels correlate with disease severity [425–427]. The study by Meylan et al. used Tg mice (constitutively express TNSF15 in T cells and DCs at levels similar to that when stimulated with pro-inflammatory stimuli) that developed spontaneous small intestinal inflammation and found treatment with anti-IL-13 antibody led to significant amelioration of intestinal inflammation [425]. Notably, authors of this study indicated that the pattern of inflammation observed closely resembled enteric nematode infection and inflammation. In fact, much of our knowledge of this Th2 cytokine in the gut is derived from studies of enteric nematode infection. IL-13 is mostly recognized for its role in the inflammatory reaction to helminthic infections, which is critically dependent on the activation of STAT6 [428,429]. IL-13 by virtue of its pleiotropic nature regulates various mechanisms, including increased mucus production by goblet cells, local release of eotaxin to attract eosinophils, and IgE production to combat enteric infections [407]. It has also been postulated to affect worm expulsion via its regulation of gut motility and epithelial secretion [406–408]. However, depending on the type of nematode utilized to study enteric infection and inflammation, IL-13 may or may not be essential for worm expulsion. In the *Trichinella spiralis*-infection induced model of intestinal inflammation, increased production of IL-13 by intraepithelial NK

cells is observed, but IL-13 deficiency does not impair IL-4 induced worm expulsion [430,431]. Whereas in the *Trichuris muris* model of infectious colitis, insufficient production of IL-13 inhibits worm expulsion despite significant up-regulation in IL-4 levels [430]. We have shown that CD4+ T cells are responsible for this up-regulation in IL-13 levels in *T. muris*-infection induced intestinal inflammation, and adoptive transfer of T cells from infected wild-type (WT) mice can transfer resistance to infection in severe combined immunodeficient (SCID) mice [146]. The critical role of IL-13Rα1 in inflammatory reaction to helminthic infections and worm expulsion has recently been established. Sun et al. demonstrated that IL-13Rα1 is the essential subunit of type I IL-13R/type II IL-4R which regulates Th2 immune response in the *Heligmosomoides bakeri*-infection model, where worm expulsion is critically dependent on IL-4 and not IL-13 [430,432]. The study found that, in comparison with WT mice, IL-13Rα1-KO mice were unable to generate an effective memory Th2 response and STAT6-dependent IL-13-induced muscle hypercontractility required for parasite expulsion, which is at least in part dependent on the increased expression of 5-HTR2A on smooth muscle cells [432,433]. The study also identified the significance of IL-13Rα1 in the regulation of IL-13Rα2 expression, as well as in infection-induced alternatively-activated macrophage (M2) polarization and changes in intestinal permeability [432]. Previous work from our laboratory had established the presence of IL-13Rα1 on murine colonic EC cells [146].

Moreover, by using *T. muris*-infection resistant BALB/C mice and susceptible AKR are mice, which generate Th2 and Th1 immune response, respectively, we have shown that a Th2 immune profile was more capable of generating EC cell hyperplasia and increasing mucosal 5-HT content [147]. These findings strongly suggest that IL-13 from CD4+ T cells may play an important role in regulating 5-HT signaling in the context of intestinal inflammation.

5-HT in gut inflammation

As mentioned earlier, 5-HT is a pleiotropic enteric signaling molecule with immunomodulatory properties. Mucosal inflammation in conditions ranging from infective acute enteritis or colitis to IBD is accompanied by alterations in normal 5-HT signaling [128,133–138,434,435]. This is postulated to stem from the proximity between and the reciprocal regulation of EC cells and immune cells in the gut mucosa [436]. However, at least in the clinical setting, it remains to be determined if the immune response generated during gut inflammation is causing or is caused by these changes. Adding to the degree of complexity of the interactions between the enteroendocrine and the immune systems are the observations that, the gut microbiota can directly influence and be influenced by both of these systems [16]. Moreover, dysbiosis has been associated with various forms of intestinal inflammation, such as in *Clostridium difficile*-infection and IBD [437]. With respect to IBD, there is now enough evidence to support the

notion that IBD is a multifactorial disease, but the prevailing opinion remains that the immune system is 'self-sufficient' in mitigating pathological processes involved. There is no better example of that than the current therapeutic approaches in the management of IBD, despite the failure of biologic therapies in about a third of the patients [438].

In response to inflammatory stimuli, such as IL-1β and LPS, colonic EC cells from patients with CD have been shown to release more 5-HT than the HCs [133]. The activation of IL-1β type I receptor and TLR4 on EC cells induced 5-HT secretion in an NFκB and MAPK-dependent manner. Up-regulation of colonic TPH1 mRNA expression has been detected in CD patients in remission experiencing IBS-like symptoms [134]. Furthermore, 5-HT expressing EC cell hyperplasia has been reported in both ileal and colonic CD, as well as in UC [135,136]. However, Coates et al. observed no such differences in EC cell numbers in rectal biopsy specimens of patients with UC [128]. Moreover, the authors in agreement with Ahonen et al. reported a reduction in the number of EC cells, but this finding was limited to patients with severe UC only [128,137]. The authors also observed a down-regulation of TPH1 mRNA levels, 5-HTT expression, and mucosal 5-HT content but not in mucosal 5-HT secretion [128]. Tada et al. also observed decreased expression of 5-HTT in the inflamed mucosa as compared to the healing mucosa of UC patients, and no significant difference in TPH1 expression [138]. This study additionally showed that in the inflamed

mucosa 5-HTT expression was inversely correlated with IL-8 expression. Furthermore, even in the healing mucosa, residual inflammation marked by up-regulated inflammatory mediator expressions, such as IL-1β, IL-8, and CXCL5, coincided with down-regulated 5-HTT expression [138]. Inflammation associated alterations at the upstream end of the mucosal 5-HT signaling, such as changes in EC cell numbers and TPH1 expression, are not always observed in clinical studies with IBD patients, more specifically UC; however, the general consensus is mucosal 5-HTT expression is decreased. Suppression of 5-HTT function due to reduced expression and/or activity is also observed in other forms of intestinal inflammation including diverticulitis, and giardiasis, the latter is also associated with decreased duodenal EC cell numbers [434,435]. It has been shown that conditioned medium from activated lymphocytes, primarily via pro-inflammatory cytokines IFN-γ and TNF-α, down-regulates 5-HTT mRNA and protein levels, and 5-HT uptake in the Caco-2 human epithelial cell line [439]. In the JAR human epithelial cell line, IL-6 in a STAT3-dependent manner has been shown to regulate the expression and function of 5-HTT [440]. The anti-inflammatory cytokine, IL-10 has been shown to induce a dual effect on 5-HTT [441]. Low levels of IL-10 were shown down-regulate the function of epithelial 5-HTT in the Caco-2 cell line, whereas, at higher concentrations, IL-10 up-regulated epithelial 5-HTT expression and activity, via IL-10R-PI3K signaling pathway [441]. Another anti-inflammatory cytokine, TGF-β1, rapidly enhances 5-HTT function by increasing

its exocytosis via TGF-β1 receptor-mediated activation of PI3K pathways in intestinal epithelial cells [442]. An association between the inhibition of 5-HTT function and the development of microscopic colitis (belonging to IBD, an umbrella term for a group of colitides, such as collagenous colitis and lymphocytic colitis) has been described. Patients without a previous diagnosis of IBD, reported developing chronic diarrhea, followed by IBD after treatment with paroxetine (SSRI) for depression [443,444]. Recently, 5-HTTLPR was identified as a potential candidate gene involved in the pathogenesis of microscopic colitis [445]. The study by Sikander et al. also found increased serum 5-HT levels in patients with microscopic colitis, as well as UC. An up-regulation of 5-HT concentration has also been reported in collagenous colitis [446]. Moreover, 5-HT has been postulated to be a key mediator involved in the inflammatory processes of the pouch, in patients who have undergone restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) surgery [447].

Accumulating evidence indicates that mucosal 5-HT signaling is enhanced in various experimental models of intestinal inflammation. As mentioned earlier, we have shown that the Th2 immune response generated during *T. muris*-infection induced intestinal inflammation is associated with EC hyperplasia and up-regulation of colonic 5-HT content [147]. Up-regulation of mucosal 5-HT signaling via increased EC cell numbers and 5-HT content, as well as decreased 5-HTT expression have been reported in other models of enteric infection, such as

the *T. spiralis,* as well as in chemically induced models of colitis, including DSS, TNBS, dinitrobenzene sulphonic acid (DNBS) [132,138–145]. Moreover, TNBS-induced ileitis has been shown up-regulate EC cell numbers and 5-HT release in non-inflamed regions of the distal colon [448]. In contrast to the aforementioned models, up-regulation mucosal 5-HT signaling in *Citrobacter rodentium* infection is not caused by an increase in EC cell numbers, instead, it is due increased 5-HT release and reduced uptake, the latter resulting from lower 5-HTT expression [449]. Using the DNBS model of experimental colitis, it was demonstrated that a reduction in EC cell numbers was associated with amelioration of colonic inflammation in monocyte chemoattractant protein (MCP)-1-KO mice [144].
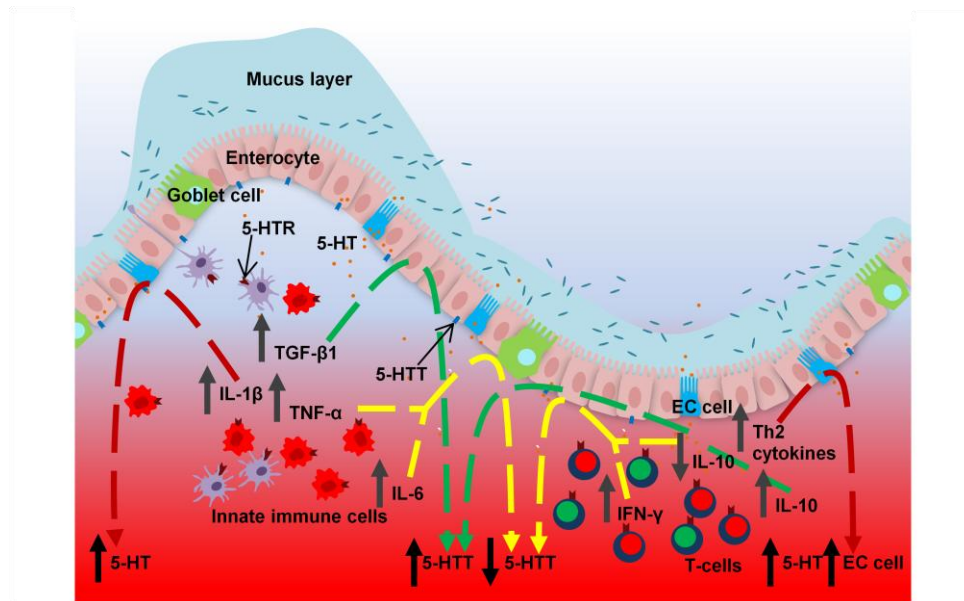

Figure VI: Immunological regulation of mucosal 5-HT signaling. Evidence from clinical and animal studies show that different cytokines influence mucosal 5-HT signaling differently. Pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ

down-regulate 5-HTT expression and/or function, and IL-1β up-regulates 5-HT content. Whereas, anti-inflammatory cytokine TGF-β1 and IL-10 up-regulate 5-HTT expression and/or function. Though IL-10 at low concentration can suppress 5-HTT function. Th2 cytokines have been shown to augment EC cell numbers and 5-HT content. Th, T helper; TNF, Tumor necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; 5-HT, 5-hydroxytryptamine; 5-HTT, 5-HT transporter; 5-HTR, 5-HT receptor; EC, Enterochromaffin. (adopted from Shajib et al. [16])

These animal models of intestinal inflammation also provide clear indication that mucosal 5-HT is a key regulator of intestinal inflammation. Using TPH1-KO mice we have shown that, significantly reduced mucosal 5-HT levels are associated with lower susceptibility to DSS- and DNBS-induced colitis, and is marked by reduced the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α [86]. This study also demonstrated that depletion of 5-HT using PCPA in WT mice and replenishment of 5-HT using 5-HTP in TPH1-KO mice can reduce or exaggerate the severity of DSS-induced colitis, respectively. Though Bischoff et al. took a different approach from us they arrived at the same conclusion. Their findings showed that 5-HTT-KO mice, which have augmented mucosal and neuronal 5-HT, were more susceptible TNBS-induced colitis in comparison with WT mice [450]. Furthermore, in the IL-10-KO model of spontaneous colitis, the severity of inflammation is exacerbated when the IL-10 deficiency is combined with a 5-HTT deficiency [451]. A study by Margolis et al. has shown that reduction of mucosal 5-HT by TPH inhibitors (LP-920540 and telotristat etiprate), which do not affect neuronal 5-HT, reduces the severity of TNBS colitis [452]. We

have made similar observations by blocking peripheral 5-HT using telotristat etiprate in acute and chronic DSS-induced colitis, as well as in the *T.muris* model of infectious colitis [453].

The influence of mucosal 5-HT on the onset, perpetuation, and severity of intestinal inflammation is probably mediated by its diverse effects on various immune cells. We have found that 5-HT regulates F4/80+ macrophage infiltration in DSS-induced colitis [86]. The onset of intestinal inflammation is marked by the adhesion of monocytes to colonic epithelial cells, 5-HT facilitates this process via NADPH oxidase (NOX) 2 derived reactive oxygen species (ROS)-mediated increase in the production of IL-6, IL-8 and MCP-1 [454]. 5-HT has been shown to induce slow rolling of leukocytes on endothelium and as previously stated, this allows mucosal 5-HT to recruit neutrophils by influencing endothelial cells Weibel–Palade bodies in animal models of lung inflammation, acute peritonitis, aseptic skin wounds and endotoxic shock [84,455]. Increased mucosal 5-HT production may also recruit these inflammatory cells to the intestine, however, this is yet to be investigated in the context of intestinal inflammation. We have shown that 5-HT via 5-HTR7 on DCs evokes a pro-inflammatory response by T cells, marked by increased IFN-γ and IL-17 production by these adaptive immune cells [103]. We have implicated this process in leading to the full blown inflammation of the bowel. By inhibiting 5-HTR7 function, by genetic knockdown or by using 5-HTR7 antagonist (SB-269970), we were able to

significantly curb the intensity of immune response generated and reduce the severity of intestinal inflammation in acute DSS- and DNBS-induced colitis, as well as in chronic DSS-induced colitis [456]. Notably, accumulation of 5-HTR7+ mature DCs has been reported in inflamed areas of the colon in CD patients [457]. Antagonists of 5-HTR3, such as tropisetron, granisetron, ondansetron, and ramosetron, however, are by far the most investigated group of pharmacological inhibitors of 5-HT signaling in the gut; and the precise mechanisms of their anti-inflammatory effects remains to be fully understood. Tropisetron and granisetron have been shown to reduce the severity of acetic acid-induced colitis by down-regulating the production of inflammatory cytokines IL-1, IL-6, and TNF-α [458,459]. Tropisetron has been shown to inhibit neutrophil recruitment, IL-2 transcription, and T cell activation [460]. Ondansetron has also been shown to partially inhibit T cell activation and curb the severity of TNBS-induced colitis [461]. Ramosetron and ondansetron, the former a more potent and selective 5-HTR3 antagonist, have been shown to reduce the number of NSAID-associated intestinal lesions in a dose-dependent manner [462]. These two via the suppression of TNF-α and subsequent caspase-3/8 activation have been shown to reduce the severity of intestinal mucositis caused by chemotherapeutic agents [463].

In addition to shaping the immune response generated in intestinal inflammation, functional changes observed in association with gut inflammation further support

the involvement of an altered mucosal 5-HT signaling. Altered bowel habits and abdominal pain are common symptoms of IBD as well as IBS. IBS has been described as a low-grade residual inflammatory condition, and the prevalence of IBS-like symptoms is significantly higher in IBD patients compared to the general population, which frequently precede clinical manifestation of IBD and/or coincide with periods of remission [72,134,464–470]. IBS-like symptoms are reported in nearly one-third of the UC patients, and more than half of the CD patients in clinical remission [467]. Moreover, altered motility causing hypoxia has been shown to induce synthesis and secretion of 5-HT by EC cells in IBD [471]. An episode of acute infectious gastroenteritis can lead to the development of IBS [472]. In *Campylobacter jejuni* enteritis an increased number of T cells and calprotectin-positive macrophages are observed in rectal biopsies of patients, along with 5-HT expressing EC cells hyperplasia which has been identified as an important predictor of post-infectious IBS (PI-IBS) development [473,474]. Plasma 5-HT levels are also significantly increased in patients with PI-IBS and diarrhea-predominant IBS (IBS-D) [475–477]. Reduced expression and function of 5-HTT have also been observed in IBS-D patients [478–480]. The role of 5-HT in IBS-D is further supported by the therapeutic efficacy of 5-HTR3 antagonists, and the promising outcomes observed in phase II clinical trials of LX-1031, a small-molecule TPH inhibitor [481–483]. Furthermore, increased EC cell numbers and 5-HT levels have been reported in irritable pouch syndrome in UC

patients who have undergone IPAA surgery [484]. In addition to that, 5-HTT-KO mice, which as previously mentioned are more susceptible to intestinal inflammation, have alternating bouts of diarrhea and constipation, exhibiting IBS-like symptoms [485]. Dysbiosis has also been observed in 5-HTT-KO mice [486]. These mice, along with their heterozygote (5-HTT$^{+/-}$) littermate, were shown to have an inflammatory-type microbiota compared to WT following exposure to early life stress, such as maternal separation [486]. Thus, up-regulation of mucosal 5-HT signaling may contribute to various pathological aspects of gut inflammation, such as microbial imbalance that may be more prone to inducing inflammation, the generation of an aberrant immune response by regulating immune cell functions, as well as long-lasting changes in motility which may persist even after the resolution of inflammation.

Experimental approaches to studying gut inflammation

Give the difficulty in establishing "cause-effect" relationship in gut inflammation, particularly in human IBD, and its heterogeneous presentation, various models of intestinal inflammation have been developed to study IBD. These animal models are valuable tools to investigate pathophysiological mechanisms and to test emerging therapeutic strategies in the preclinical phase. Three such models were used in this thesis to study intestinal inflammation. The optimized protocols

utilized for these three models, as used in the McMaster University Central Animal Facility, are outlined in detail within the respective chapters to follow.

T. muris model of infectious colitis

T. muris is a natural pathogen of mice and serves as a useful model of *Trichuris trichiura* infection in humans [487]. In addition to that, immunopathology associated with susceptibility to *T. muris* infection is characterized by mucosal and submucosal inflammation resulting in changes in the gut physiology, architecture and transcriptional profile similar to IBD [488,489]. Infective eggs are given by oral gavage and hatch in the distal small intestine, which critically dependent on interactions with the microbiota [487,490]. After hatching, *T. muris* invade the IECs that line the crypts of the cecum and proximal colon and upon maturation the worms release eggs into the environment [487]. A high dose of infective eggs induces a Th2 immune response in resistant strains, such as BALB/c mice, leading to expulsion of worms due to increased mucin production and epithelial cell turnover; whereas in susceptible strains, e.g. AKR/J mice, a Th1 immune response is generated which promotes chronic infections [487]. However, chronic infection can also be induced in resistant strains using a low dose of infective eggs [491]. This is a very useful model for studying T cell-mediated immune response in the gut.

Chemical models of intestinal inflammation

The most commonly used animal models of IBD are chemically induced models of intestinal inflammation. Like all other models they also have limitations, but they resemble some important immunological and histopathological aspects of human IBD.

DSS-induced colitis

DSS is a heparin-like polysaccharide administered to mice in drinking water to induce colitis that is superficial in nature, resembling human UC [492,493]. It is proposed that DSS induces colitis by exacting toxic effects on the epithelium, via increased exposure to luminal antigens through the destruction of mucin or by altering the function of immune cells [494]. The DSS-induced colitis model is very popular in IBD research due to its ease of administration, rapidity, reproducibility, and controllability. By modifying the concentration of DSS and the frequency of administration both acute and chronic models of intestinal inflammation can be produced. In this model, the gut microbiota also plays an important in the development of robust colitis [494]. Acute DSS-induced colitis mostly involves the activation of neutrophils and macrophages and T and B cells are not required, but it should be noted that T cell responses can enhance the inflammatory response generated [211]. Furthermore, lymphocytes are activated in the chronic DSS model of colitis [495]. In acute colitis increased production of Th1/Th17 cytokines, along with IL-13 and IL-5, are observed, whereas, the

chronic model is marked by Th1/Th2 cytokine production [422,496]. DSS-induced colitis alters stool consistency, causes ulceration, colonic bleeding as well as weight loss, and the histopathological damage is marked by granulocyte infiltration and changes in architecture [494].

DNBS-induced colitis

DNBS-induced colitis is a well-characterized transmural, Th1 driven inflammation of the colon [497]. DNBS, like TNBS, is a hapten-induced model of colitis [498]. However, DNBS is currently preferred over TNBS as it is comparatively a less hazardous chemical and considerably less expensive [498]. DNBS is administrated intrarectally (IR) in a solution of ethanol, and produces localized damage to the distal colon [498]. Ethanol is required to break the colonic mucosal barrier to allow the penetration of DNBS into the LP [498]. This haptenizing agent binds covalently with the protein of colonic cell and bacteria rendering them immunogenic and eliciting responses from the host's innate and adaptive immune systems [211]. Histologically, the inflammatory responses include loss of mucosal architecture, infiltration of mucosal and submucosal layers by polymorphonuclear leukocytes, macrophages, lymphocytes, mast cells and fibroblasts [211]. In comparison with DSS-induced colitis, the advantages of DNBS-induced colitis include low cost and rapid onset of intestinal inflammation. However, it does require a greater level of technical expertise, and the need for

anesthesia for rectal installation. Similar to the DSS model, weight loss, and diarrhea are also observed in DNBS-induced colitis, and macroscopic features include adhesion, ulceration, and thickening of the colonic wall [498].

—CHAPTER 2—

HYPOTHESIS AND AIMS

The awareness and understanding of IBD have come a long way in the past few decades, which has led to the development of new therapies (biologics e.g. anti-TNF) in disease management. However, we are still far from fully understanding some of the fundamental aspects of disease pathogenesis, which include but are not limited to the heterogeneity of disease manifestation, as well as the factors involved in disease onset and progression. This is highlighted by the ineffectiveness of these new therapies in a significant proportion of IBD patients, which additionally have serious side effects. Thus, there is a need for identifying new targets for developing new therapies.

Inflammatory conditions of the gut, including IBD, are defined by aberrant mucosal inflammation, where the role of the epithelium and immune cells are intertwined. Specializations within the epithelial cell populations regulate all normal physiological functions of the gut, including its characteristic immunological state. One such population of specialized epithelial cells are EC cells, which are the most abundant EEC type. Their main secretory product, 5-HT is involved in the regulation of a myriad of physiological functions in the normal gut as well as immune response. Moreover, immune mediators have been shown to affect mucosal 5-HT signaling in the context of gut inflammation. IL-13 is an effector cytokine in IBD and increased IL-13 levels have been observed in various models of intestinal inflammation where mucosal 5-HT signaling is also up-regulated. Thus, in view of the important role of 5-HT signaling in the generation

of intestinal inflammation, the ability Th2 cytokines to induce EC cells hyperplasia and increase 5-HT production, and the presence of IL-13Rα1 on mouse colonic EC cells as well as the role of IL-13 described in animal models and human intestinal inflammation, **we hypothesized that the IL-13-EC cell/5-HT axis is important in the pathogenesis of intestinal inflammation whereby inhibition of IL-13 production reduces 5-HT production which in turn attenuates the severity of intestinal inflammation**.

The major findings of this thesis are organized into three aims and chapters, each chapter a published or submitted manuscript.

Hypothesis: the IL-13-EC cell/5-HT axis is important in the pathogenesis of intestinal inflammation whereby inhibition of IL-13 production reduces 5-HT production which in turn attenuates the severity of intestinal inflammation.
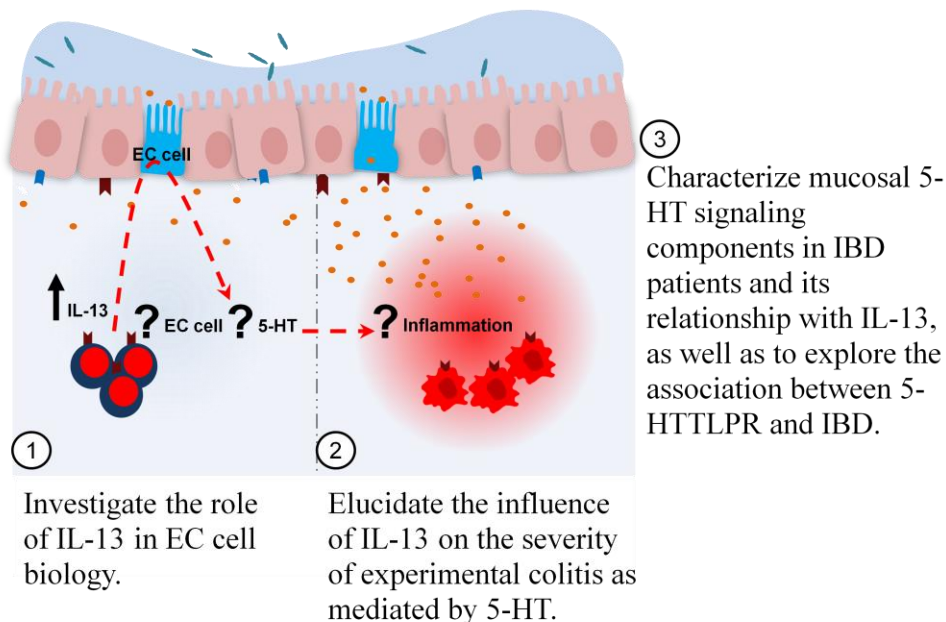

③ Characterize mucosal 5-HT signaling components in IBD patients and its relationship with IL-13, as well as to explore the association between 5-HTTLPR and IBD.

① Investigate the role of IL-13 in EC cell biology.

② Elucidate the influence of IL-13 on the severity of experimental colitis as mediated by 5-HT.

Figure VII: Schematic representation of hypothesis and aims. IL, interleukin; 5-HT, 5-hydoxytryptamine; 5-HTTLPR, 5-HT transporter gene linked polymorphic region; EC, enterochromaffin; IBD, inflammatory bowel disease.

AIM 1: Investigate the role of IL-13 in EC cell biology.

Chapter 3: We investigated the role of IL-13 in EC biology in vivo and in a culture model of human EC cells. These findings were published in the following manuscript:

Marcus Manocha, Md. Sharif Shajib, Mushfequr Rahman, Huaqing Wang, Palanivel Rengasamy, Milena Bogunovic, Manel Jordana, Lloyd Mayer and Waliul I. Khan. Mucosal Immunol. 2013 Jan;6(1):146-55. doi: 10.1038/mi.2012.58.

AIM 2: Elucidate the influence of IL-13 on the severity of experimental colitis as mediated by 5-HT.

Chapter 4: We investigate the effect of IL-13 deficiency associated down-regulation of 5-HT production in the DSS- and DNBS- induced models of chemical colitis. Our findings were published in the following manuscript:

Md. Sharif Shajib, Huaqing Wang, Janice J. Kim, Ivana Sunjic, Jean-Eric Ghia, Emmanuel Denou, Matthew Collins, Judah A. Denburg, and Waliul I. Khan. PLoS One. 2013 Aug 28;8(8):e72774. doi: 10.1371/journal.pone.0072774.

AIM 3: To characterize mucosal 5-HT signaling components in IBD patients and its relationship with IL-13, as well as to explore the association between 5-HTTLPR and IBD.

Chapter 5: We investigated whether up-regulated mucosal 5-HT signaling is associated with enhanced IL-13 expression in IBD patients. Additionally, we explored the potential role of specific 5-HTTLPR genotypes in IBD pathogenesis. These findings have been prepared in a manuscript that has been submitted to European Journal of Gastroenterology and Hepatology.

Md. Sharif Shajib, Usha Chauhan, Salman Adeeb, Yeshale Chetty, David Armstrong, Smita L. S. Halder, John K. Marshall, Waliul I. Khan. In submission at Eur. J. Gastroenterol. Hepatol.

Details regarding author contribution to each individual manuscript can be found in the preface preceding each chapter. Collectively, these manuscripts examine the role of immune and endocrine interactions in the gut and their influence in shaping intestinal inflammation. More specifically, this work shows how IL-13 regulates EC biology and function (Chapter 3), how IL-13 via 5-HT mediates inflammation (Chapter 4) and how our findings relate to human IBD (Chapter 5).

—CHAPTER 3—

IL-13-MEDIATED IMMUNOLOGICAL CONTROL OF

ENTEROCHROMAFFIN CELL HYPERPLASIA AND SEROTONIN

PRODUCTION IN THE GUT

IL-13-mediated immunological control of enterochromaffin cell hyperplasia and serotonin production in the gut.

Manocha M*, Shajib MS*, Rahman MM, Wang H, Rengasamy P, Bogunovic M, Jordana M, Mayer L, and Khan WI.

The material in this chapter was published in Mucosal Immunology (PMID: 22763407).

Mucosal Immunol. 2013 Jan;6(1):146-55. doi: 10.1038/mi.2012.58. Epub 2012 Jul 4.

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Preface: This work was conducted over the period of September 2010- April 2012. This manuscript was an equal contribution (* or 4 in the attached manuscript). WIK (my supervisor), MM (Post-doctorate fellow) and I designed all experiments included in this manuscript. MM led all in vitro experiments, with the exception of reverse transcriptase-PCR of IL-13Rα1 which was performed by MMR (Post-doctorate fellow), and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)-based cell growth assay which I performed. I led all in vivo experiments, with the exception of treatment of naïve IL-13-KO mice with recombinant mouse IL-13 which was performed by MMR while I assisted. MM and I analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. Experimental assistance was provided by PR (Post-

doctorate fellow; western blotting), and HW (Technician; infection with *T. muris*). MB and LM (Post-doctorate fellow and professor, Mount Sinai School of Medicine, New York) provided us with reagents and protocol for immunocytofluorescence of BON cells. IL-13-KO mice were gifted to us by MJ (Professor, McMaster). WIK oversaw all parts of the project and edited the manuscript.

Summary and significance of findings: Herein, we further our previous findings in the *T .muris* model of infectious colitis, by identifying IL-13, and not IL-4, as the key Th2 cytokine involved in EC cell hyperplasia and increased mucosal 5-HT content. We further confirm the role of IL-13 in EC cell biology by replenishing IL-13 in both naïve and *T. muris*-infected IL-13-KO mice, which led to the marked increase in EC cell numbers, TPH1 expression and mucosal 5-HT content as compared to vehicle-treated mice. In BON cells, we identify the expression of IL-13Rα1 and shown that IL-13 also causes hyperplasia in this model of human EC cells, increases TPH1 mRNA expression, followed by protein levels, and subsequently 5-HT production. Collectively these findings shown the IL-13 is an important regulator of mucosal 5-HT signaling.

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ARTICLES

IL-13-mediated immunological control of enterochromaffin cell hyperplasia and serotonin production in the gut

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Enterochromaffin (EC) cells in the gastrointestinal (GI) mucosa are the main source of serotonin (5-hydroxytryptamine (5-HT)) in the body. 5-HT is implicated in the pathophysiology of many GI disorders including functional and inflammatory bowel disorders. Herein we studied the role of interleukin 13 (IL-13) in EC cell biology by utilizing IL-13-deficient (IL-13−/−) mice and BON cells (a model for human EC cells). The numbers of EC cells and 5-HT amount were significantly lower in enteric parasite, *Trichuris muris*–infected IL-13−/− mice compared with the wild-type mice. This was accompanied with increased parasite burden in IL-13−/− mice. Treatment of naive and infected IL-13−/− mice with IL-13 increased EC cell numbers and 5-HT amount. BON cells expressed IL-13 receptor and in response to IL-13 produced more 5-HT. These results provide novel information on IL-13-mediated immunological control of 5-HT in the gut, which may ultimately lead to improved therapeutic opportunities in various GI disorders.

INTRODUCTION

The gastrointestinal (GI) tract is the largest endocrine organ in the human body, producing a wide variety of biologically active products. Many of the enteric endocrine cells reside within the epithelial cells of intestinal mucosa where stimuli from the lumen or surrounding cells can influence their cellular biology.[1] The best-characterized subset of enteric endocrine cells are enterochromaffin (EC) cells, which synthesize and release serotonin (5-hydroxytryptamine (5-HT)) and are the main source of 5-HT in the GI tract.[2,3] 5-HT is an important enteric mucosal signaling molecule that can influence the maintenance of intestinal homeostasis, specifically motor and secretory functions.[4–6] The release of 5-HT increases motility of the gut and is also an extremely important mediator of nausea and satiety.[7,8] Abnormal regulation of 5-HT in the human gut has been implicated with a diverse array of GI disorders such as inflammatory bowel disease,[9,10] irritable bowel syndrome (IBS),[9,11,12] celiac disease,[13] colorectal cancer,[14,15] diverticular disease,[16] and enteric bacterial, viral, and parasitic infection.[17–22] Recently, we have demonstrated a critical role of 5-HT in the activation of immune response and generation of mucosal inflammation in experimental model of colitis.[23,24] Although 5-HT and EC cell biology have been implicated in a plethora of GI disorders, the precise mechanism regulating the observed changes in 5-HT content and EC cell numbers during GI disorder remains unclear.

Considering the location of EC cells in the epithelial layer of the gut mucosa, it is very likely that immune cells play an important role in EC cell biology in the context of various GI disorders. In patients with postinfectious IBS, observation of immune activation (increased number of CD3 + T cells) has been associated with increased EC cell numbers, which is suggestive of a link between these two systems.[25,26] This association between the immune system and endocrine system of the GI tract also holds true in animal models of postinfectious IBS.[20,27] The notion of an immune–endocrine axis in the GI tract is further supported by the findings that EC cells in mucosa of the ileum and colon of rhesus monkeys are in direct or close contact with lymphocytes.[28] Two recent studies from our laboratory have uncovered a pivotal role for the immune system in EC cell biology.[21,22] Using the nematode *Trichuris muris* model of intestinal infection, we have observed upregulation in the

number of EC cells and 5-HT amount following infection.[21] This infection-induced increase in EC cells or in 5-HT was not evident in severe combined immunodeficient (SCID) mice. EC cell numbers and 5-HT amount significantly increased following reconstitution of SCID mice with CD4^{+} T cells from infected mice and this was accompanied by an upregulation of colonic CD3^{+} T cells and T helper type 2 (Th2) cytokines. In addition, laser capture microdissection-based molecular and immunofluorescence techniques revealed the presence of the interleukin-13 receptor α1 (IL-13Rα1) on EC cell.[21] These findings demonstrate an important role for CD4^{+} T cells in EC cell function in enteric infection-induced gut inflammation. Recently, we have also demonstrated a difference in EC cell and 5-HT responses in Th1 and Th2 environments.[22] Higher amount of 5-HT and EC cell numbers were observed in mice that are resistant to infection and expel worms by generating a Th2-type immune response as compared with susceptible mice that developed chronic infection with the generation of a Th1 response. Utilizing Stat4- and Stat6-deficient mice, which are impaired in Th1 and Th2 response respectively, we have also shown impairment in the development of infection-induced EC cell hyperplasia and in 5-HT production in Stat6-deficient mice after *T. muris* infection.[22] These findings suggest the importance of the immunological profile of the inflammatory response in the regulation of EC cell biology in gut mucosa.

The involvement of immune response in the regulation of EC cell hyperplasia and 5-HT production in *T. muris* infection as well as the presence of IL-13Rα1 in colonic 5-HT-expressing EC cells exemplifies the role of IL-13 in EC cell biology. In this study we solidify the role of IL-13 in EC cell biology utilizing IL-13-deficient (IL-13−/−) mice in *T. muris* infection model and a cell culture model for EC cells (BON cells). Our results demonstrate compelling evidence in favor of a role of IL-13 in EC cell biology at the molecular level in both an *in vivo* mouse model and *in culture* human model of EC cells.

RESULTS

IL-13 deficiency inhibits worm expulsion and development of infection-induced EC cell hyperplasia and 5-HT production in *T. muris* infection

BALB/c (wild-type) mice are resistant to *T. muris* infection and can expel almost all the worms within 21 days after infection, whereas IL-13−/− mice on BALB/c background failed to clear the parasitic infection (**Figure 1a**). The clearance of parasite from wild-type mice was associated with significantly higher levels of IL-13 (**Figure 1b**) and IL-4 (**Figure 1c**) in the colon, which is in agreement with previous observations of *T. muris*–infected BALB/c mice.[21] IL-13 levels were elevated on both days 14 and 21 after infection in wild-type mice and, as expected, were not detectable in IL-13−/− mice. IL-4 levels were increased in the colonic tissues of both the wild-type and IL-13−/− mice in comparison with their noninfected controls. Notably, IL-4 levels were significantly higher in the IL-13−/− mice compared with their wild-type counterparts on days 14 and day 21 after infection.
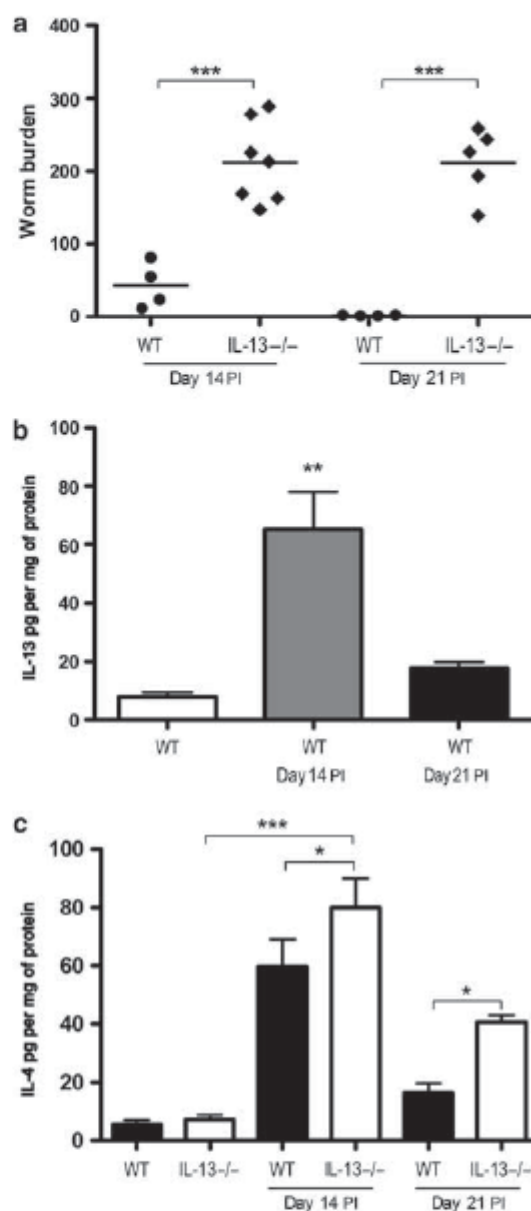

Figure 1 Inhibition of worm expulsion in interleukin-13-deficient (IL-13−/−) mice as compared with the wild-type (WT) BALB/c mice. Both IL-13−/− and WT mice were infected orally with 300 eggs of *Trichuris muris* and killed on days 14 and 21 post infection (PI). (a) Worm burden in infected IL-13−/− and WT mice on days 14 and 21 PI. (b) Colon tissue IL-13 levels in WT mice infected with *T. muris* on days 14 and 21 PI. (c) Colon IL-4 levels in both noninfected and infected IL-13−/− and WT mice on days 14 and 21 PI. Each bar represents mean±s.e.m. of 3–7 mice. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Significantly lower number of EC cells and 5-HT amount was detected in the colon of IL-13−/− mice in comparison with wild-type mice after infection. We found that noninfected wild-type controls had significantly higher number of 5-HT-expressing EC cells compared with IL-13−/− controls and, following infection, a progressive increase in 5-HT-expressing EC cells was observed in the wild-type group on both days 14 and 21 (**Figure 2a**). This increase in 5-HT-expressing EC cell numbers was statistically significant on day 21 when the wild-type
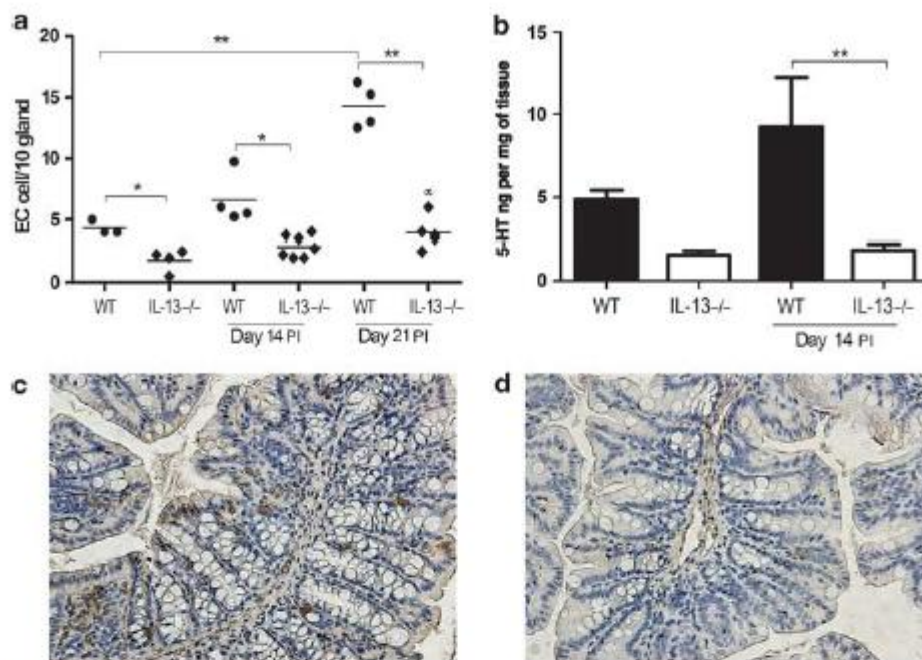
Figure 2 Interleukin-13-deficient (IL-13−/−) mice infected with *Trichuris muris* exhibited significantly less enterochromaffin (EC) cell numbers in comparison with the wild-type (WT) mice. Both IL-13−/− and WT mice were infected orally with 300 eggs of *T. muris* and killed on days 14 and day 21 post infection (PI). (**a**) Number of EC cells in the colon of noninfected and infected mice on days 14 and 21 PI. (**b**) Amount of colonic 5-hydroxytryptamine (5-HT) in noninfected and infected mice on day 14 PI. (**c**) Representative micrograph showing 5-HT-expressing EC cells in the colon of infected WT mice on day 14 PI. (**d**) Representative micrograph showing 5-HT-expressing EC cells in the colon of infected IL-13−/− mice on day 14 PI. Each bar represents mean±s.e.m. of 3–7 mice and micrographs at original magnification ×200. ᵅNot statistically significant. *$P<0.05$; **$P<0.01$.

infected group was compared with its noninfected counterpart. However, minimal changes in 5-HT-expressing EC cell numbers were observed in IL-13−/− mice irrespective of the duration of infection (**Figure 2a**). We have previously established parallels between the number of EC cells and the ability of the mouse to expel the parasite as measured by worm burden.[21] This study also found a similar relationship between worm burden and EC cell numbers (**Figure 1a** vs. **Figure 2a**). Significantly fewer 5-HT-expressing EC cells were found in IL-13−/− mice along with increased worm burden. Restoration of IL-13, by treatment with recombinant mouse IL-13 (rmIL-13) for 5 days, in naive IL-13−/− mice significantly increased both EC cell numbers and 5-HT content in the colon of IL-13−/− mice (**Figure 3a,b**). In addition to that, replenishment of IL-13 in naive IL-13−/− mice resulted in upregulation of TPH1 (tryptophan hydroxylase 1) protein, the rate-limiting enzyme involved in the biosynthesis of 5-HT by EC cells (**Figure 3c**). Upregulation of EC cell number and 5-HT amount was observed in infected IL-13−/− mice treated with rmIL-13 (**Figure 4a,b**). Restoration of IL-13 levels improved the ability of IL-13−/− animals to expel the parasite, as indicated by worm burden (number of worms found was 176±43.2 and 100.5±27.1 in IL-13−/− mice that received vehicle and rmIL-13, respectively). Interestingly, although there was a significantly increased production of IL-4 in the IL-13−/− mice compared with wild-type mice after infection, the number of EC cells remained unchanged (**Figure 1b** vs. **Figure 1a**). These observations provide evidence in favor of a key role of IL-13 in the regulation of EC cell biology and 5-HT production in enteric infection caused by *T. muris*.

IL-13 upregulates 5-HT concentration in both the cell and media of BON cells

Previously, we have shown that 5-HT-expressing EC cells in the mouse colon express IL-13Rα1.[22] In this study we observed that IL-13 plays an important role in promoting EC hyperplasia and 5-HT production. In examining the molecular mechanisms associated with IL-13 and EC cell function, we began by describing the expression of IL-13Rα1 in BON cells (**Figure 5**). BON cells are a human carcinoid cell line derived from a metastasis of a pancreatic carcinoid tumor of EC cell origin.[29] IL-13Rα1 mRNA is expressed in BON cells (**Figure 5a**); in addition, IL-13Rα1 protein was also identified in BON cells using immunocytofluorescence. Treatment of BON cells with recombinant human IL-13 (rhIL-13) resulted in significantly increased intracellular and extracellular 5-HT after 24 h (**Figure 6**). However, IL-13 does not induce the immediate release (after 1 h) of 5-HT from BON cells (**Figure 7**), in comparison with phorbol-12-myristate-13-acetate, a secretagogue known to induce the secretion of hormones from BON cells.[30]

IL-13 induces an increase in TPH1 mRNA expression and amount of TPH1 protein

Recent studies have identified two isoforms of the TPH enzyme: TPH1, which is present in mainly peripheral organs such as the EC cells in gut, and TPH2, which is associated with the nervous system and is present predominantly in the brain stem.[31,32] In this study we have detected TPH1 in BON cells and examined its expression over 24 h. TPH1 expression in BON cells has been
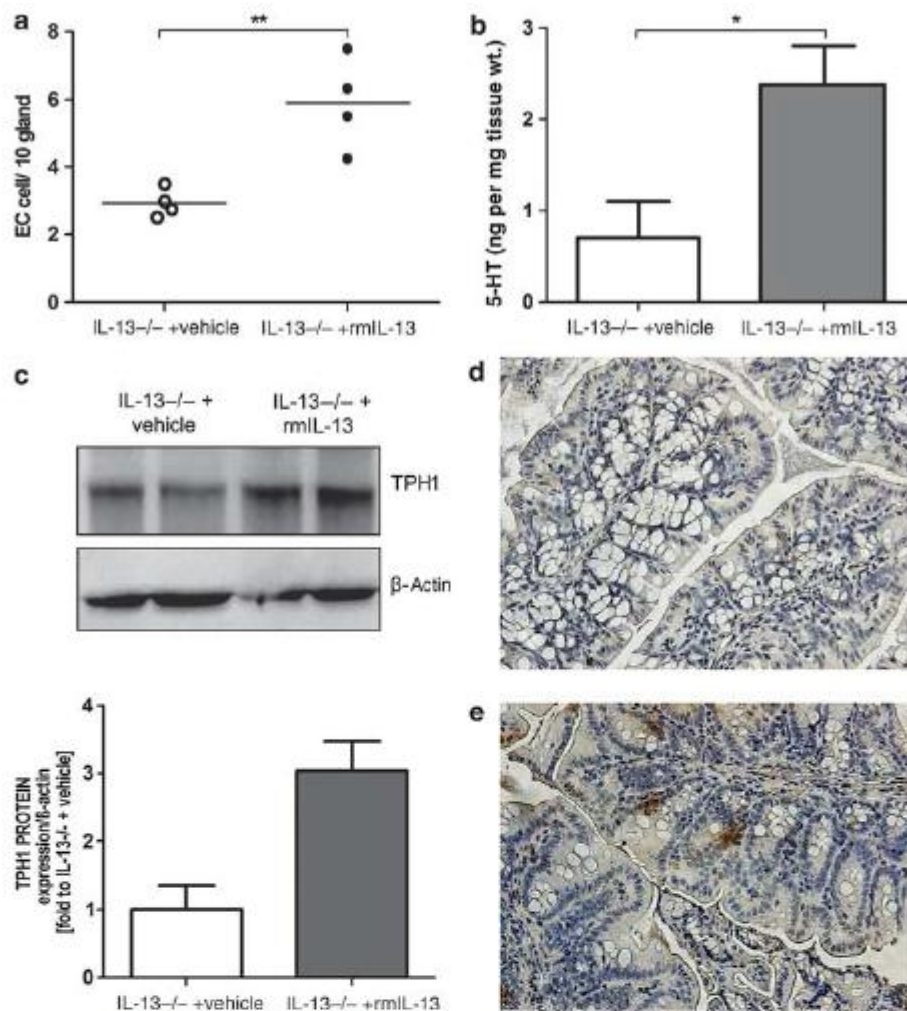
Figure 3 Treatment of naive interleukin-13-deficient (IL-13−/−) mice with recombinant mouse IL-13 (rmIL-13) upregulated the number of enterochromaffin (EC) cells and the amount of 5-hydroxytryptamine (5-HT) found in the colon as well as total tryptophan hydroxylase 1 (TPH1) protein. IL-13−/− mice were given 2 μg of rmIL-13 for 5 consecutive days, and the control group received the vehicle (phosphate-buffered saline (PBS)) by intraperitoneal (IP) injection. (a) Number of EC cells in the colon of IL-13−/− mice injected with vehicle or rmIL-13. (b) Amount of colonic 5-HT in the colon of IL-13 knockout mice injected with vehicle or rmIL-13. (c) Representative western blot and relative band density of TPH1 protein expression in the colon of IL-13−/− mice injected with vehicle or rmIL-13. (d) Representative micrograph showing 5-HT-expressing EC cells in the colon of IL-13−/− mice that received PBS by IP injection. (e) Representative micrograph showing 5-HT-expressing EC cells in the colon of IL-13−/− mice that received rmIL-13. Each bar represents mean±s.e.m. of 4 mice and micrographs at original magnification ×200. *$P<0.05$; **$P<0.01$.

previously described.[33] TPH1 expression in BON cells increases significantly, almost two fold, after 4 h of treatment with rhIL-13, but returns to levels similar to the control at 24 h (**Figure 8a**). However, TPH1 protein levels, as determined by western blot, were increased after 24 h (**Figure 8b**).

IL-13 induces BON cell hyperplasia

The number of BON cells was quantified using two different methods, both of which were found to be significantly elevated in IL-13-treated samples (**Figure 9**). Trypan blue was used to select for live cells; typically, 97–99% of the cells were found to be viable. Cells were counted manually using a hemocytometer (**Figure 9a**). Treatment of BON cells with rhIL-13 (10 ng ml^{-1}) for 24 h resulted in significantly increased fluorescence, as determined by the cell viability reagent alamarBlue, which is a function of increased cell number (**Figure 9b**). The ability of IL-13

to induce BON cell hyperplasia was further confirmed utilizing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, a cell proliferation assay (**Figure 9c**).

DISCUSSION

5-HT is a well-known neurotransmitter of the central nervous system, and traditionally it is known to influence a range of behavioral, physiological, and cognitive functions. However, the vast majority of 5-HT in the body (~98%) is found outside of the central nervous system and is generated mainly by the EC cells situated within the epithelial layer of the gut mucosa, which are the best-characterized endocrine cell population in the GI tract.[1,2] Despite the association of alteration in 5-HT response and a variety of GI disorders such as IBS, inflammatory bowel disease, celiac disease, and colorectal cancer, it is not clear how the changes in 5-HT take place, what role 5-HT has in intestinal
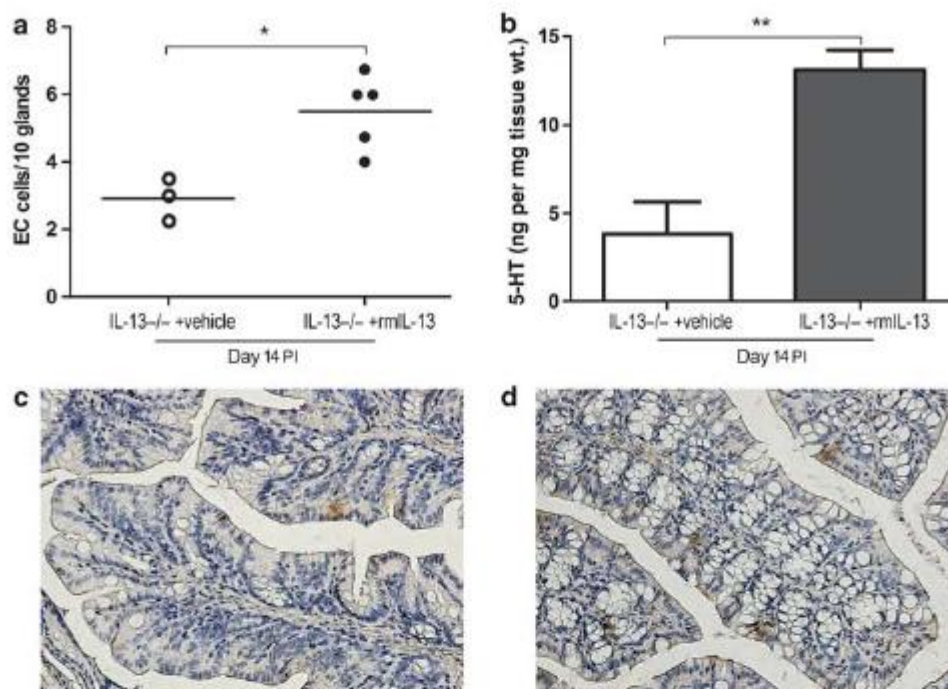
Figure 4 Treatment of *Trichuris muris*–infected interleukin-13-deficient (IL-13−/−) mice with recombinant mouse IL-13 (rmIL-13) increases enterochromaffin (EC) cell number and the amount of 5-hydroxytryptamine (5-HT) found in the colon on day 14 post infection (PI). IL-13−/− mice were given 2 μg of rmIL-13 for 15 days, and the control group received phosphate-buffered saline (PBS) as vehicle via intraperitoneal (IP) injection. (a) Number of EC cells in the colon of infected IL-13−/− mice injected with vehicle or rmIL-13. (b) Amount of colonic 5-HT in the colon of IL-13 knockout mice injected with vehicle or rmIL-13. (c) Representative micrograph showing 5-HT-expressing EC cells in the colon of infected IL-13−/− mice that received PBS by IP injection. (d) Representative micrograph showing 5-HT-expressing EC cells in the colon of infected IL-13−/− mice that received rmIL-13. Each bar represents mean±s.e.m. of 3–5 mice and micrographs at original magnification ×200. *$P<0.05$; **$P<0.01$.

pathophysiology, and whether by modulating 5-HT production and signaling is it possible to elicit a therapeutic effect. In this study, by employing *in vivo* mouse model of enteric infection and *in vitro* model of human EC cells, we have demonstrated a critical role of IL-13 in the development of EC cell hyperplasia and in the upregulation of 5-HT production.

Interactions among immune system, nervous system, and endocrine system have been suggested by a number of studies, and a key player in these neuroimmunoendocrine networks of both human and rodents is 5-HT. The reduced numbers of EC cells in the colon of mice with targeted disruption of IL-2 (ref. 34) and T cell receptor-α,[35] and the presence of EC cells in contact with, or very close proximity to, CD3^{+} lymphocytes[28] strongly suggest the existence of the immunological control on EC cells. It is also shown that interferon-γ has an inhibitory effect on proliferation of BON cells.[36] Recently, the link between EC cells and the immune system during enteric infection has been examined in several studies.[20–22,37] Infection with a nematode parasite produces mucosal inflammation and induces changes in intestinal physiology, and thus these models have considerable importance in exploring the pathophysiology of many GI disorders. It has been shown that infection with *T. spiralis* results in the upregulation of EC cells, and this effect is attenuated in infected T cell receptor knockout mice.[20] Utilizing *T. muris* mouse model of enteric infection, we have previously shown an important connection between immune and endocrine systems in the gut, where secretory products from CD4^{+} T cells interact with EC

cells or their precursors to enhance 5-HT production in the gut via Th2-based mechanisms.[21] We have also observed that EC cell and 5-HT responses induced by the same enteric infectious agent are influenced by Th1 or Th2 cytokine predominance, suggesting the importance of the immunological profile of the inflammatory response in the regulation of EC cell biology.[22] In addition, it is reported that infection-induced reduction of EC cells numbers in *Citrobacter rodenitium* is not evident in SCID mice, further indicating an important role of the immune system in mediating EC cell hyperplasia in the gut.[36] In this study we observed that EC cell numbers were significantly increased in *T. muris*–infected wild-type mice as compared with the control. However, in IL-13−/− mice there was no significant increase in EC cell numbers after the infection as compared with the noninfected controls and this impairment in the development of EC cell hyperplasia is accompanied with delayed worm expulsion from the intestine. Treatment of naive IL-13−/− mice with recombinant IL-13 for 5 days significantly increased both EC cell numbers and 5-HT amount in the colon. Administration of rmIL-13, over a 15-day period, in *T. muris*–infected IL-13−/− mice was also accompanied by upregulation of EC cell numbers and 5-HT content, as well as facilitation of worm expulsion. These observations suggest that IL-13 plays a prominent role in mediating the upregulation of 5-HT-expressing EC cell numbers and in the production of 5-HT in infection-induced inflammatory condition and this may contribute to host defense in this infection.
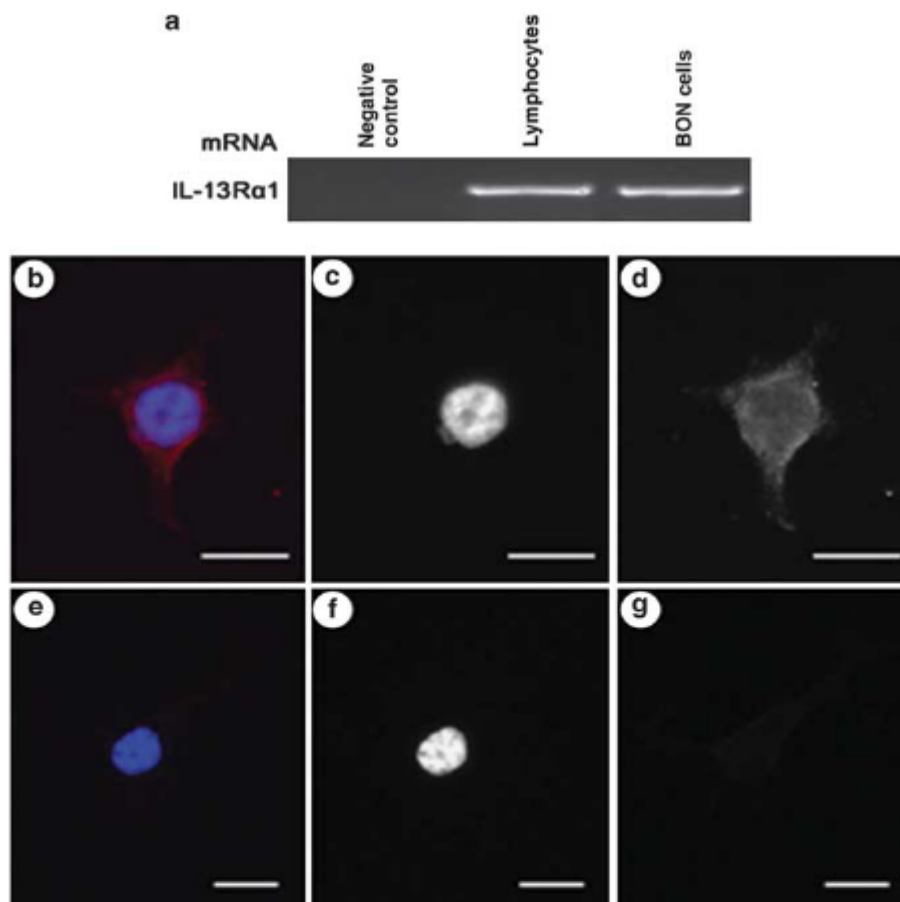
Figure 5 BON cells (a model for human enterochromaffin (EC) cells) express interleukin-13 receptor α1 (IL-13Rα1). (a) Agarose gel electrophoresis of the PCR product from reverse transcriptase-PCR of IL-13Rα1 mRNA. Samples were from a negative control (H_2O), lymphocyte cell population (positive control), and BON cell population. (b–g) Immunocytofluorescence of BON cells using both anti-IL-13Rα1 and mouse isotype control. (b) Overlay of BON cells stained with 4′,6-diamidino-2-phenylindole (DAPI) and (c) IL-13Rα1 (1°: mouse anti-IL-13Rα1 dilution 1:100; 2°: Alexa Fluor 568 F(ab′)$_2$ fragment goat anti-mouse IgG dilution 1:500). (e) Overlay of BON cells stained with DAPI and (f) mouse IgG isotype control (1°: mouse IgG dilution 1:100; 2°: Alexa Fluor 568 F(ab′)$_2$ fragment goat anti-mouse IgG dilution 1:500). White bars represent 10 μm.
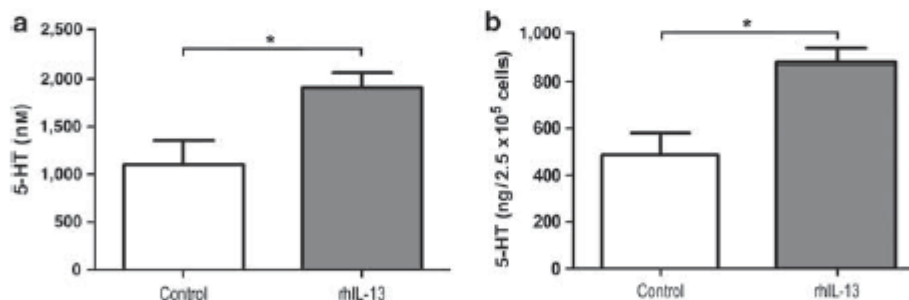

Figure 6 Treatment of BON cells with 10 ng ml^{-1} recombinant human interleukin-13 (rhIL-13) increases the concentration of 5-hydroxytryptamine (5-HT) in both the cell and media after 24 h. (a) Concentration of 5-HT in serum-free media with or without 10 ng ml^{-1} of rhIL-13 after 24 h. (b) Concentration of 5-HT in 2.5×10^5 cells treated with or without 10 ng ml^{-1} of rhIL-13 after 24 h. Each bar represents mean±s.e.m. of four biological replicates (wells). *$P < 0.05$.

We have previously reported the expression of IL-13αR but not IL-4 receptor in mouse EC cells in the colon.[22] The findings from *in vivo* investigations that recombinant IL-13 can influence the EC cell numbers in the colon and 5-HT content has led us to hypothesize that IL-13 can regulate 5-HT production by acting directly on EC cells and influencing TPH1 (rate-limiting enzyme of 5-HT synthesis) expression and function of EC cells.

In this study we have identified and characterized the expression of IL-13αR in BON cells, a cell culture model of human EC cells. Incubation of BON cells with IL-13 for 24 h results in both increased extracellular and intracellular 5-HT content. TPH1 was originally thought to be regulated at the transcriptional level as slightly elevated nocturnal 5-HT content and TPH1 mRNA levels were found in the rat pineal gland.[38,39] Recent studies have
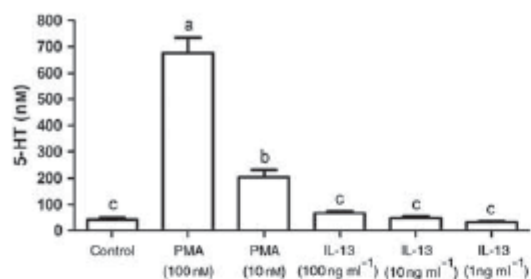
Figure 7 Phorbol ester (phorbol-12-myristate-13-acetate (PMA)) induces the release of 5-hydroxytryptamine (5-HT) from BON cells after 1 h in a dose-dependent manner, but recombinant human interleukin-13 (rhIL-13) at three different concentrations does not. BON cells were treated with serum-free media (Control) containing either PMA or rhIL-13 for 1 h at 37 °C. Media were collected and 5-HT concentration was determined by enzyme-linked immunosorbent assay (ELISA). Each bar represents mean±s.e.m. of six biological replicates (wells). a, b and c represent statistical significance. a is significantly different from b and c, b is significantly different than a and c, and all the conditions with c are not significantly different from each other.
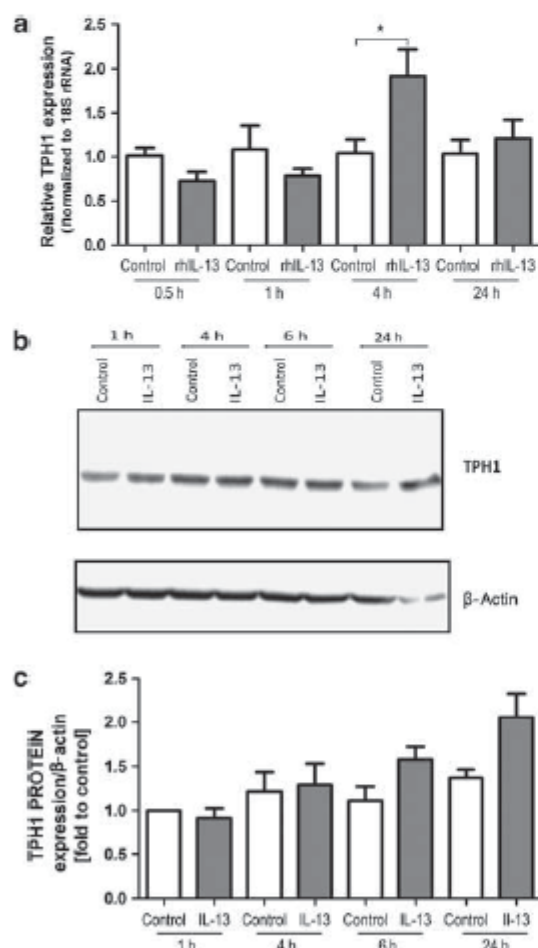

Figure 8 Time course expression analysis of tryptophan hydroxylase 1 (TPH1) in BON cells treated with and without 10 ng ml^{-1} recombinant human interleukin-13 (rhIL-13) at 1, 4, 6, and 24 h. (a) Quantitative real-time PCR analysis of TPH1 mRNA expression in BON cells. (b) Representative western blot and relative band density of TPH1 protein expression in BON cells. Each bar represents mean±s.e.m. of four biological replicates (wells). *$P<0.05$.
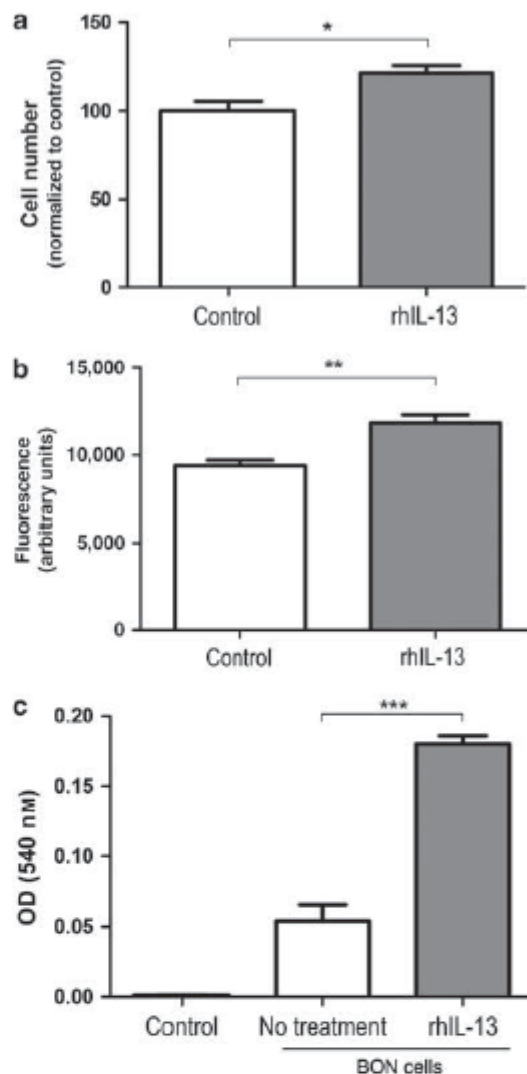

Figure 9 Treatment of BON cells with recombinant human interleukin-13 (rhIL-13) results in increased cell numbers as determined by different methods for quantifying cell numbers. (a) BON cells were treated with and without (Control) 10 ng ml^{-1} of rhIL-13 in serum-free media for 24 h. Cells were lifted from the plate using trypsin and quantified by hemocytometer. Each bar represents mean±s.e.m. of three biological replicates (wells). (b) BON cells were treated with and without 10 ng ml^{-1} of rhIL-13 in serum-free media for 24 h. Media were then replaced with 1× alamarBlue (Invitrogen) in phosphate-buffered saline (PBS) and incubated with the cells for 1 h at 37 °C. Fluorescence was measured using an excitation wavelength of 570 nM and emission wavelength of 585 nm. Each bar represents mean±s.e.m. of six biological replicates (wells). (c) BON cells were treated with and without 10 ng ml^{-1} of rhIL-13 in serum-free media for 24 h and control is media but no cells. The 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)-based cell growth determination kit (Sigma) was utilized and optical density (OD) was measured at 540 nm. Each bar represents mean±s.e.m. of eight biological triplicates (wells). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

suggested that posttranslational regulation of TPH1 may be more important than transcription activation.[39] In BON cells, activation of the phosphatidylinositol-3-kinase/protein kinase B (Akt) signaling pathway results in the loss of TPH1 protein, potentially by posttranslational degradation, with concomitant decrease

in 5-HT synthesis.[40] TPH1 is a target of cAMP-dependent protein kinase, resulting in phosphorylation of serine 58 *in vitro*, and an increase in the catalytic activity of TPH1.[41,42] Our study found that TPH1 mRNA expression increased after 4 h of IL-13 treatment and this was associated with upregulation of protein levels of TPH1 at 24 h. These observations provide evidence that IL-13 regulates 5-HT production from EC cells by enhancing TPH1 activity.

A study by Ishizuka *et al.*[43] showed that 5-HT itself can act as a mitogen on BON cells; however, significant differences were only observed after 4 days. In our study we observed that IL-13 treatment increases the number of BON cells in culture after 24 h, which is associated with an increase in 5-HT content within the media. Potentially, 5-HT and IL-13 may act synergistically to increase the number of BON cells *in culture* as well as a similar mechanism may be responsible for EC cell hyperplasia during a Th2-mediated response to enteric infection.

In conclusion, we have shown that IL-13 plays an important role in the development of EC cell hyperplasia in enteric infection-induced model of gut inflammation. IL-13 secreted from Th2 cells or natural killer T cells[44] may control EC cell biology acting via IL-13 receptor on EC cell. In addition, this study also suggests that 5-HT production and worm expulsion share a common immunologic basis and may be causally linked. Using BON cells, a model for human EC cells, we have shown that IL-13 can influence 5-HT synthesis through modulation of TPH1 activity at both the level of mRNA and protein expression. These observations clearly demonstrate a vital role of IL-13 in EC cell biology and 5-HT production and provide important information on immunoendocrine axis in the gut. Furthermore, by manipulating the host immune response, we are able to modulate the accompanying EC cell response and 5-HT production, and this may have clinical relevance. Alterations in EC cell numbers and 5-HT production occur in a variety of clinical settings associated with inflammation and immune activation such as IBS and inflammatory bowel disease. An understanding of the underlying immunological mechanisms of altered EC cell biology, coupled with the ability to modulate EC cell function by immune mediator as depicted in this study, may ultimately yield new therapeutic strategies in these conditions.

METHODS

Animals. IL-13−/− mice on a BALB/c background (kindly provided by A. McKenzie, MRC Lab, Cambridge, UK[45]) were bred at McMaster University. Male IL-13−/− mice and their age-matched (average age 8–11 weeks) wild-type counterparts were used for the purposes of these experiments. The guidelines implemented by the McMaster University Animal Care Committee, Canadian Council on the Use of Laboratory Animals, and the Home Office Scientific Procedures Act (1986) were strictly followed. All mice were kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities.

BON cell culture. BON cells (obtained from Dr L. Mayer, Mount Sinai School of Medicine, New York, NY) were maintained in Dulbecco's modified Eagle's medium/F-12 media, containing 10% non-heat-treated fetal calf serum at 37 °C in 5% CO_2. Media were changed every 72 h, and all experiments were performed on BON cells from passages 6–14 in this study.

Experimental design for *T. muris* infection. The techniques used for *T. muris* maintenance and infection were described previously.[46,47] Mice were infected with ~300 eggs by oral gavage on day 0 and were killed on days 14 and 21, and at these time points tissue samples were collected. In a separate experiment, IL-13−/− mice were treated with either rmIL-13 (2 µg day^{-1}) or vehicle (phosphate-buffered saline (PBS)) for 15 days, starting 1 day before infection with *T. muris*. The duration of infection in the latter experiments was 14 days, and thereafter the animals were killed and tissue samples were collected. Additionally, in another experiment, naive IL-13−/− mice were treated with aforementioned rmIL-13 dose or PBS and killed on day 5.

Determination of colonic and intracellular BON cell 5-HT content. Segments of colon or ~2.5×10^6 BON cells were homogenized in 0.5 ml of 0.2 M perchloric acid, and then centrifuged at 10,000 g for 5 min. The supernatants were neutralized with an equal volume of 1 M borate buffer (pH 9.25) and centrifuged again at 10,000 g for 1 min. The 5-HT content in the supernatant was analyzed using a commercially available kit (Beckman Coulter, Bera, CA). The 5-HT content of BON cells was expressed in weight (ng) per 2.5×10^6 cells, and colonic 5-HT content was expressed as a function of the colon segment's wet weight.

Measurement of 5-HT concentration in BON cell culture media. BON cell conditioned media were removed from cells and centrifuged at 110 g for 5 min to remove cell debris. Media were placed in fresh tubes and frozen at −70 °C until analysis. The 5-HT content in the media was analyzed using a commercially available kit (Beckman Coulter).

Measurement of cytokines in colonic tissues. Frozen colonic tissues were homogenized in lysis buffer (50 mM Tris-HCl (pH 8); 150 mM NaCl; 1% Triton X-100) containing protease inhibitor cocktail (Sigma, St Louis, MO). The homogenate was centrifuged at 10,000 g for 5 min and the supernatants were collected and stored at −80 °C until analysis.

Colon samples were analyzed using a mouse IL-13 and IL-4 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Total protein concentration was measured by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Cytokine values were expressed as a function of total protein content in each sample.

RNA isolation and reverse transcriptase reaction. RNA from ~1×10^6 BON cells that were treated with or without 10 ng ml^{-1} rhIL-13 was isolated using Total RNA Purification kit from Norgen Biotek (Thorold, Ontario, Canada). Purified RNA was quantified and the integrity of RNA was determined using the Experion bio analyzer with a standard sensitivity RNA chip (Bio-Rad Laboratories). All RNA samples were found to be of sufficient integrity with minimal RNA degradation (RNA quality indicator (RQI) > 8.5). An aliquot of 3 µg of RNA was used for reverse transcription using 0.5 µg of oligo (dT)$_{12-18}$ with the M-MLV Research Transcriptase (Invitrogen, Grand Island, NY) kit. The complementary DNA (cDNA) samples were diluted 1:10, and cDNA pooled samples were diluted in a 10-fold serial dilution to create a standard curve with autoclaved ddH_2O.

Quantitative Real-time PCR. All real-time PCR reactions were performed using the Bio-Rad CFX 96 PCR system (Bio-Rad Laboratories) and amplification reactions (10 µl) were done using 5 µl of EvaGreen PCR Master Mix (Bio-Rad Laboratories), 1 µl of both the 10 µM reverse and forward primers, 2 µl of autoclaved ddH_2O, and 1 µl of diluted cDNA.

The optimal annealing temperature of 58 °C was determined for the TPH1 primers (forward: 5′-TGCCCTTGCTAAGGTCAGCAGGA-3′, reverse: 5′-AGCAAGAGATGGCCCAGACCTCC-3′). The thermal cycling conditions were as follows: initial denaturation of 30 s at 95 °C followed by amplification for 40 cycles of 5 s at 95 °C and 5 s at 58 °C. Using multiple classical internal control genes we assessed the variability of each gene using a random subset of the cDNA samples and assessed the stability of the reference gene using geNorm

(http://medgen.ugent.be/~jvdesomp/genorm/) and as described by Vandesompele *et al.*[48] The reference gene primers that we utilized were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-CCCTCCAAAATCAAGTGGGGCG-3′ and reverse: 5′-ATGACGAACATGGGGGCATCAGC-3′; β-Actin, forward: 5′-ATGTGGCCGAGGACTTTGATT-3′ and reverse: 5′-AGTGGGGTGGCTTTTAGGATG-3′; Ubiquitin C (UBC), forward: 5′-ATTTGGGTCGCGGTTCTTG-3′ and reverse: 5′-TGCCTTGACATTCTCGATGGT-3′; 18S rRNA, forward: 5′-TCCACAGGAGGCCTACACGCC-3′ and reverse: 5′-TTTCCGCCGCCCATCGATGTT-3′.

We found the 18S rRNA gene to be most stable according to geNorm (average expression stability (M)=0.401) and the other reference genes to be less stable (M=0.548 to 0.526), and therefore the 18S rRNA gene was selected for subsequent relative quantification of TPH1 at each time point.

TPH1 western blotting. Colonic tissue samples of IL-13 −/− mice treated with or without rmIL-13 (2 µg day^{-1} for 5 days) were homogenized in the same manner as samples prepared for determination of tissue cytokine levels. Once again, concentration of the total protein in the solution of homogenized tissue was determined using a DC Protein Assay kit (Bio-Rad).

BON cell lysate was prepared in 6 cm dishes and pre-treated with or without rhIL-13 (10 ng ml^{-1}) for different time periods (1, 4, 6, and 24 h). After treatment, the cells were washed with ice-cold PBS, and 300 µl of lysis buffer (50 mM Tris-HCl (pH 8); 150 mM NaCl; 1% Triton X-100) containing protease and phosphatase inhibitors (0.5 mmol l^{-1} Na$_3$VO$_4$, 10 mmol l^{-1} NaF, 1 µmol l^{-1} leupeptin, 1 µmol l^{-1} pepstatin, 1 µmol l^{-1} okadaic acid, and 0.2 mmol l^{-1} phenylmethylsulfonyl fluoride) was added. An aliquot of the cell lysate was used to determine the protein concentration in each sample.

Before loading onto sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis gels, the samples, both tissue and cell, were diluted 1:1 (vol/vol) with 2× Laemmli sample buffer (62.5 mmol l^{-1} Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 50 mmol l^{-1} dithiothreitol, and 0.01% (wt/vol) bromophenol blue). Aliquots of cell lysates and tissue homogenate (equal amount from each group) containing 20 and 30 µg of protein, respectively, were then subjected to SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The total TPH1 protein level was detected using a specific antibody (1:1,000; Abgent, San Diego, CA), and primary antibody detection was performed using horseradish peroxidase–conjugated appropriate secondary antibody. Signal was visualized using enhanced chemiluminescence, and quantified using the ImageJ program (National Institutes of Health, Bethesda, MD).

Immunohistochemistry. Immunohistochemical studies of 5-HT-expressing EC cells were performed on formalin-fixed, paraffin-wax-embedded samples as described previously.[22] Briefly, tissue sections were deparaffinized in CitriSolv (Fisher Scientific, Mississauga, Ontario, Canada), and rehydrated in increasingly less ethanol in PBS. Endogenous peroxide activity was blocked for 30 min (DakoCytomation, Burlington, Ontario, Canada). Antigen retrieval was accomplished using hot 10 mM Citrate Buffer (pH 6.0). Nonspecific binding was reduced using 1% bovine serum albumin in PBS; sections were then incubated with 5-HT rabbit antibody (Immunostar, Hudson, WI; 1:5,000; 1 h at room temperature). Secondary antibody was then applied for 30 min (Envision, DakoCytomation; horseradish peroxidase–coupled anti-rabbit secondary reagent; DakoCytomation). Sections were developed with 3,3′-diaminobenzidine and counterstained with Meyer's hematoxylin. Quantification of 5-HT-positive EC cells was conducted by a blinded observer and expressed as number of positive cells per 10 glands.

Immunocytofluorescence. BON cells were grown in a 12-well dish in the presence of a glass circular cover slip. Media were removed and cell washed with PBS. Fixation with 2% paraformaldehyde in PBS was performed first at room temperature for 10 min. Cells were permeabilized with 0.1% Triton X-100 and 0.1% Saponin in PBS at room temperature

for 15 min. A second fixation step was then performed with 4% paraformaldehyde in PBS. Cells were blocked with 3% bovine serum albumin in PBS for 1 h at room temperature. Primary antibody at indicated dilution was applied to the cells in PBS containing 3% bovine serum albumin, 0.1% Triton X-100, and 0.1% saponin overnight, shaking at 4 °C. The cells were washed 3 times with PBS containing 0.1% Triton X-100, and 0.1% saponin. Secondary antibody was applied at indicated dilution to cells in PBS containing 3% bovine serum albumin, 0.1% Triton X-100, and 0.1% saponin, shaking at room temperature for 1 h. Cells were then washed 3 times with PBS containing 0.1% Triton X-100, and 0.1% saponin. Cover slips were mounted on to slides using ProLong Gold with 4′,6-diamidino-2-phenylindole (Invitrogen).

BON cell proliferation assay. Commercially available MTT based cell growth determination kit (Sigma) was utilized to measure BON cell proliferation. Approximately 1×10^4 cells in 200 µl of aforementioned medium were seeded in triplicates into 96-well culture plates and incubated for 48 h to allow cell adhesion. Thereafter, 200 µl of serum-free medium either with or without 10 ng ml^{-1} of rhIL-13 was added to the wells containing cells and incubated at 37 °C, 5% CO$_2$ for 24 h. At the end of incubation, 20 µl of MTT was added to all wells and incubated for an hour and a half, following which the medium was removed and the plate was dried at 37 °C for 1 h. After 1 h, 200 µl of 0.04 M HCl in isopropanol was added to each well and mixed thoroughly. Optical density was measured at 540 nm using a luminometer (EL 808; Bio-Tek Instruments, Winooski, VT).

Statistical analysis. Comparison of the two groups was accomplished using Student's *t*-test. Comparison of three or more groups was accomplished first using a one-way analysis of variance followed by the Newman-Keuls multiple comparison test unless otherwise indicated. The *P*-values are designated as follows: *0.05, **0.01, ***0.001, and ****0.0001. All statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA).

DISCLOSURE
The authors declared no conflict of interest.

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—CHAPTER 4—

INTERLEUKIN 13 AND SEROTONIN: LINKING THE IMMUNE AND

ENDOCRINE SYSTEMS IN MURINE MODELS OF INTESTINAL

INFLAMMATION.

Interleukin-13 and Serotonin: Linking the Immune and Endocrine Systems in Murine Models of Intestinal Inflammation.

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***Preface*:** This work was conducted over the period of December 2010- July 2013. WIK (my supervisor), JAD (Professor, McMaster) and I conceived the idea for the original experiment of administrating DSS to IL-13-KO and WT mice. All other experiments included in this manuscript stemmed from the original experiment and was conceived by WIK and I. I designed and performed all experiments included in this manuscript with technical assistance from HW (Technician; macrophages isolation experiment), JJK (Graduate student; observer unaware of experimental conditions), IS (undergraduate student; F4/80+ staining), MC (undergraduate student; 5-HT+ staining). I analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. JEG (Professor, University of Manitoba) and ED (Post-Doctorate fellow) contributed scientific input and critically appraised the manuscript. WIK oversaw the project and edited the manuscript.

***Summary and significance of findings*:** In this study, we identified the up-regulation of IL-13 in DSS-induced colitis. In addition to that, we observed decreased severity of DSS-induced colitis in IL-13-KO mice as compared to WT. Furthermore, by treating IL-13-KO mice with mouse IL-13 and 5-HTP we establish that the increased production of mucosal 5-HT mediates the pathologic influence of IL-13 in DSS-induced colitis. We also observed reduced cell proliferation in association with IL-13 deficiency in DSS-induced colitis. Additionally, we found that IL-13 deficiency associated down-regulation of 5-HT production results in decreased severity of DNBS-induced colitis in IL-13-KO mice in comparison with WT. Our findings in this study, establish the inflammatory role of IL-13 as mediated by 5-HT in chemical models of colitis.

Interleukin 13 and Serotonin: Linking the Immune and Endocrine Systems in Murine Models of Intestinal Inflammation

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Abstract

Objective: Infiltration of activated immune cells and increased cytokine production define the immunophenotype of gastrointestinal (GI) inflammation. In addition, intestinal inflammation is accompanied by alteration in the numbers of serotonin (5-hydroxytryptamine; 5-HT) synthesizing enterochromaffin (EC) cells and in 5-HT amount. It has been established that EC cells express interleukin (IL)-13 receptor, additionally IL-13 has been implicated in the pathogenesis of ulcerative colitis. In this study, we investigated the role of IL-13 mediated 5-HT signaling in pathogenesis of colitis.

Methodology: Colitis was induced in IL-13 deficient (IL-13−/−) and wild-type (WT) mice with dextran sulfate sodium (DSS) and dinitrobenzene sulfonic acid (DNBS), as well as in IL-13−/− mice given recombinant mouse IL-13 (rmIL-13) and 5-hydroxytryptamine (5-HTP), the direct precursor of 5-HT.

Principal Findings and Conclusion: Elevated colonic IL-13 levels were observed in WT mice receiving DSS in comparison to control. IL-13−/− mice administered DSS exhibited significantly reduced severity of colitis compared to WT mice as reflected by macroscopic and histological damage assessments. Following DSS administration, significantly lower pro-inflammatory cytokine production and fewer infiltrating macrophages were observed in IL-13−/− mice compared to WT. The reduced severity of colitis observed in IL-13−/− mice was also accompanied by down-regulation of EC cell numbers and colonic 5-HT content. In addition, increasing colonic 5-HT content by administration of rmIL-13 or 5-HTP exacerbated severity of DSS colitis in IL-13−/− mice. IL-13−/− mice also exhibited reduced severity of DNBS-induced colitis. These results demonstrate that IL-13 plays a critical role in the pathogenesis of experimental colitis and 5-HT is an important mediator of IL-13 driven intestinal inflammation. This study revealed important information on immune-endocrine axis in gut in relation to inflammation which may ultimately lead to better strategy in managing various intestinal inflammatory conditions including inflammatory bowel disease.

Introduction

Intestinal inflammation is characterized by mucosal recruitment of activated cells from both the innate and adaptive arms of the immune system; [1] this is exemplified in both Crohn's disease (CD) and ulcerative colitis (UC). CD and UC have distinct immunophenotypes. [2] Whereas CD is due to a T helper (Th)1/Th17 type response, UC is considered to be an atypical Th2 disease. [2,3] Increased levels of interleukin (IL)-13 and IL-5, but not IL-4, have been observed in association with UC and recently, it was identified that a non-invariant subset of natural killer T (NKT) cells are the source of this increased IL-13 production in these patients. [2,4,5] Moreover, targeted inhibition of IL-13 by interferon (IFN)-β-1a yielded promising results in UC patients responsive to the treatment, further implicating IL-13 in the

pathogenesis of UC. [6]. This notion is supported by findings in animal model of UC, namely the oxazolone model. [7,8] These studies reflect the great strides made in mucosal immunology regarding the immunopathogenesis of CD and UC. However, the effects of the distinct immunophenotypes of these inflammatory conditions on the coexisting entero-endocrine system within the gastrointestinal (GI) tract are yet to be fully understood.

The entero-endocrine system is a collection of specialized epithelial cells that establishes the GI tract as the largest endocrine system in the human body. [9] Enterochromaffin (EC) cells are the best characterized enteric endocrine cells and are the main source of serotonin (5-hydroxytryptaime; 5-HT) in the GI tract. 5-HT is considered to be an important enteric mucosal signalling molecule with vital roles in the physiology and inflammatory conditions of

the gut. [10] Changes in EC cell numbers and 5-HT content have been associated with various GI disorders, including CD and UC. [11] Similarly, alterations in normal 5-HT signalling has been reported in various animal models of intestinal inflammation, [1,12,13] and given the strategic location of EC cells in the gut mucosa, [14] it is likely that they are mediators of the cross-talk between the immune and the entero-endocrine systems. Providing evidence for immune regulation of EC cell biology, we have shown that 5-HT production in the gut can be regulated by CD4+ T cells, and EC cell numbers and colonic 5-HT content differ in different cytokine milieu. [15,16] EC cells express IL-13 receptor alpha1-chain (IL-13Rα1); in addition, IL-13 has been shown to up-regulate tryptophan hydroxylase (TPH)-1 protein expression and 5-HT production, both in an *in vivo* model of enteric infection and *in vitro* using a model for human EC cells [16,17].

In view of the critical role of 5-HT signalling in the generation of intestinal inflammation, [10,18] and the connection of IL-13 in pathogenesis of IBD, specifically of UC, in the present study we investigated the role of IL-13 in the pathogenesis of colitis in the context of gut 5-HT production in two different models of experimental colitis [dextran sodium sulfate (DSS) and dinitro-benzenesulfonic acid (DNBS)]. We hypothesized that the IL-13-EC cell/5-HT axis is important in the pathogenesis of colitis whereby manipulation of the immune system to alter IL-13 production will modulate 5-HT production as well as the severity of colitis. Our study revealed an important role of IL-13 in the generation of intestinal inflammation in relation to 5-HT production and demonstrated that IL-13 gene deficient mice, which have less 5-HT, are better protected against these models of inflammation, and *in vivo* replenishment of 5-HT abrogates these benefits. These results identified 5-HT as an important mediator of IL-13 driven intestinal inflammation.

Materials and Methods

Ethics Statement

Animal protocols used for the study were in accordance with McMaster University Animal Care Committee and guidelines set by Canadian Council of the Use of Laboratory Animals. The protocols were approved by Animal Research Ethics Board (AREB)-McMaster University (AUP #11-04-16).

Animals

Male IL-13 deficient mice on BALB/c background (IL-13−/−) [19] (gift from Dr. Manel Jordan, McMaster University, Hamilton, ON) and age matched wild-type (WT) counterparts were used for the purposes of these experiments. All mice were kept in sterilized, filter-topped cages, fed autoclaved food at a temperature of 21–22°C and with 12 h light and 12 h dark cycle in the McMaster University central animal facility. The animals were allowed to acclimatize for 1 week prior to the start of any experiments.

Induction of DSS and DNBS Colitis

DSS (mol. wt. 36-54 kilodaltons: ICN Biomedicals Inc, Soho, OH) was added to drinking water at 5% weight/volume for five days. The average DSS consumption per cage was recorded every day for the duration of the experiment. For induction of DNBS colitis, mice were anesthetized with isoflurane (Abbott, Toronto, Canada) prior to intrarectal (IR) instillation of DNBS (3 mg in 100 μL of 50% ethanol; ICN Biomedicals Inc.), or 50% ethanol as vehicle, via a 10-cm-long PE-90 tubing (ClayAdam, Parsippany, NJ), attached to a tuberculin syringe, inserted 3.5 cm into the

colon. All groups were supplied with 6% sucrose in drinking water to prevent dehydration.

Experimental Design

DSS colitis was induced by orally administering 5% DSS in drinking water for 5 days. In a separate experiment, IL-13−/− mice were injected subcutaneously with 100 mg/kg of 5-hydroxytryptophan (5-HTP) (Sigma–Aldrich, Mississauga, Canada) twice daily for 8 days beginning 3 days prior to induction of DSS colitis; whereas, the control IL-13−/− mice received saline as vehicle. In another experiment, IL-13−/− mice received recombinant mouse (rm)IL-13 (R&D Systems, Minneapolis, MN) at a dose of 2 μg/day, starting 3 days before a 5 day period of DSS administration. In DNBS colitis, mice were euthanized 3 days post-DNBS administration. For all experiments, animals were anaesthetized prior to euthanization via cervical dislocation at the conclusion of each experiment or if they reached a predetermined end point (ie, loss of ≥20% body weight and/or significant deterioration of body condition).

Evaluation of Inflammation

For the duration of all experiments, the weights of the mice were recorded daily, and were expressed as a percentage of body weight prior to induction of colitis. Percentage of body weight lost in combination with stool consistency and feces bleeding comprised the disease activity index (DAI). [20] Macroscopic scoring was performed immediately after the mice were sacrificed using previously established scoring system for DSS and DNBS. [20,21] Categories evaluated for DSS macroscopic scores included, rectal bleeding, rectal prolapse, diarrhea and colonic bleeding, whereas the DNBS macroscopic scores reflect numerical values assigned to the categories, adhesion, hyperaemia, thickness, fecal consistency, number of ulcers and sizes. For the purposes of histological scoring, colonic segments collected during sacrifice were fixed in 10% phosphate buffered formalin and stained with H&E. Colonic damage was assessed based on a published scoring system that considered loss of architectural, degree of inflammatory cell infiltrate, goblet cell depletion, muscle thickening and crypt abscess. [20,21] Myeloperoxidase (MPO) activity was determined using a previously published method and is expressed as unit per mg of tissue [22].

Colonic Immunohistochemistry and Peritoneal Macrophage Culture

Immunohistochemical studies of Ki-67 positive cells, 5-HT expressing EC cells and F4/80 positive macrophages were performed on paraffin-wax-embedded colonic sections, as previously described. [1,10,15,23] Primary antibody used for Ki-67 immunohistochemisrty was a rabbit anti-Ki67 antibody (Abcam, Cambridge, UK), for 5-HT was a rabbit anti-5-HT antibody (Immunostar, Hudson, WI) and for F4/80 was a rat anti-mouse F4/80 antibody (AbD Serotec, Kidlington, UK). Immunostained sections were observed at 200× magnification and the number of Ki-67 positive cells, 5-HT positive EC cells were expressed as number of positive cells per 1 and 10 gland, respectively, and F4/80 positive macrophage areas were quantified using ImageJ (National Institutes of Health, Bethesda, MD) and expressed as a percent of total area.

For macrophage culture, peritoneal cavity cells were collected from WT and IL-13−/− mice with or without DSS treatment and cultured as described previously. [10] Cells were plated at a concentration of 3.0×10^6 cells per millilitre, and treated with either lipopolysaccharides (LPS); (100 ng/mL; Sigma-Aldrich) or

Figure 1. Effects of IL-13 deficiency in DSS-induced colitis. WT and IL-13−/− mice were administered 5% DSS in drinking water to induce colitis. Control mice received water without DSS. (A) Colonic IL-13 levels in WT mice with or without DSS. (B) Disease activity index (DAI). (C) Macroscopic damage score in DSS-induced colitis on day 5 after DSS induced colitis and in mice without colitis. (D) Histological damage assessment on day 5 post-DSS administration. (i) And (ii) Light micrograph of H&E-stained colonic section. DAI data represented as mean ± SEM from 5 mice per group; *represents statistical significance where $p < 0.05$; # significantly lower disease activity in IL-13−/− mice receiving DSS compared to WT mice receiving DSS.
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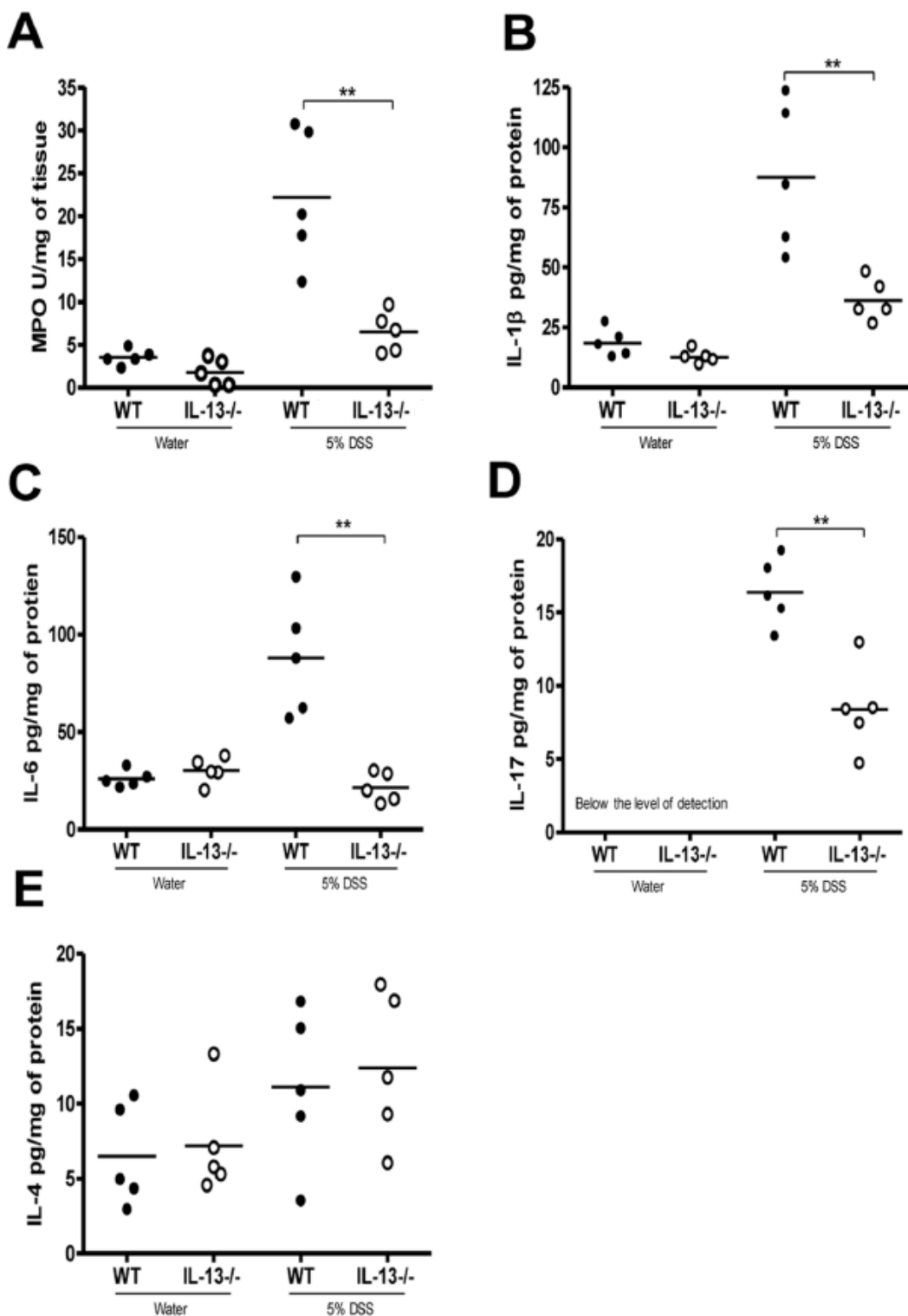
Figure 2. Effects of IL-13 deficiency on MPO activity and colonic cytokine levels in DSS-induced colitis. WT and IL-13−/− mice were given 5% DSS in drinking water to induce colitis and were sacrificed on day 5 post-DSS administration. (A) MPO activity, (B) IL-1β (C) IL-6 (D) IL-17 and (E) IL-4 levels in colonic tissues. *Represents statistical significance where $p < 0.05$.
doi:10.1371/journal.pone.0072774.g002

5-HT (10^{-10} mol/L; Sigma-Aldrich) or both for 24 hours. The culture supernatant were collected and stored in -80°C until determination of cytokine levels using Bioplex protein array system (Bio-Rad Inc, Hercules, CA).

Serum IL-13 and Tissue Cytokine Levels

Blood was collected via intra-cardiac puncture from anesthetized (isoflurane) mice following five and eight days of DSS and rmIL-13 treatment, respectively. Colonic samples were homoge-
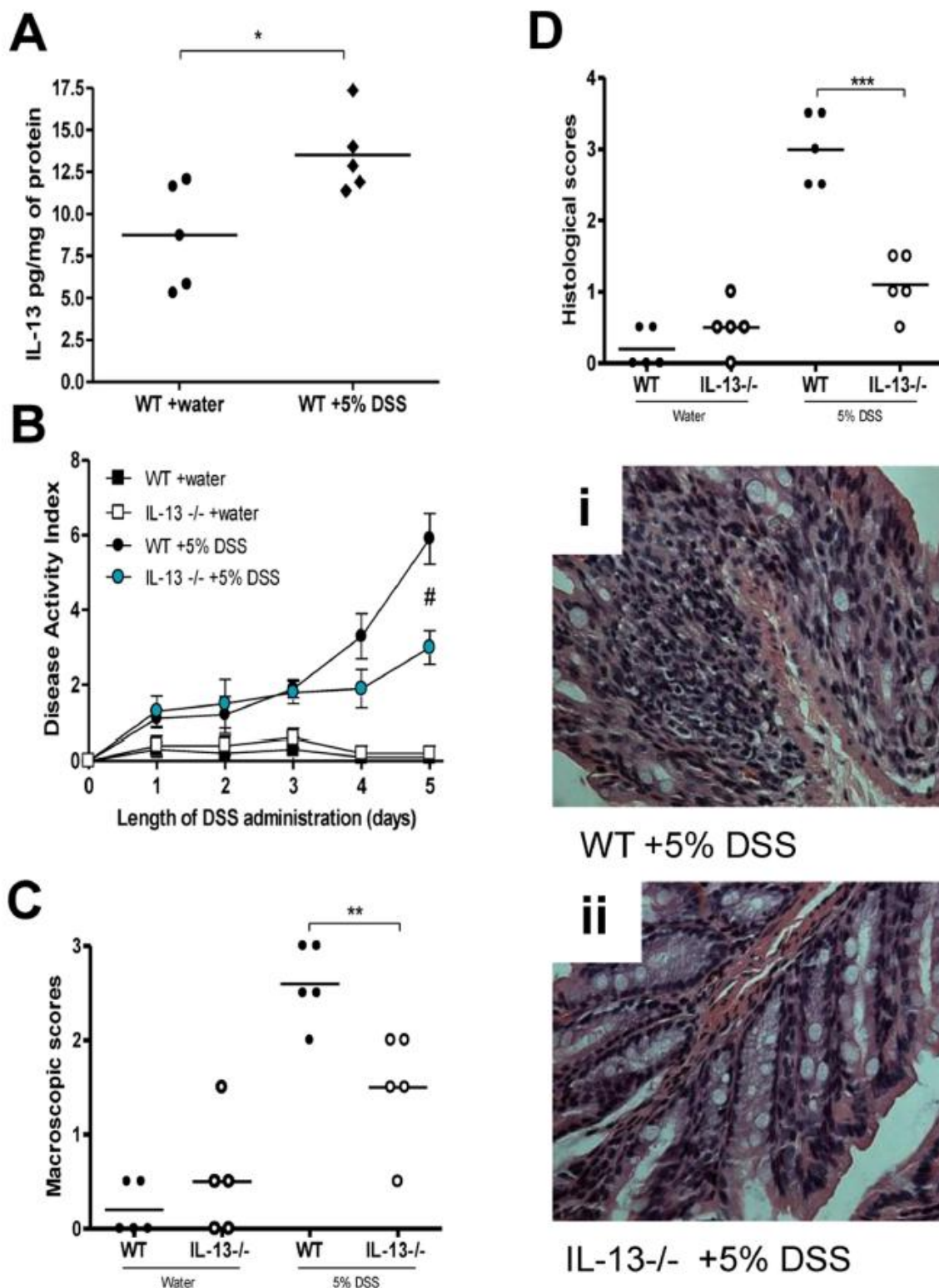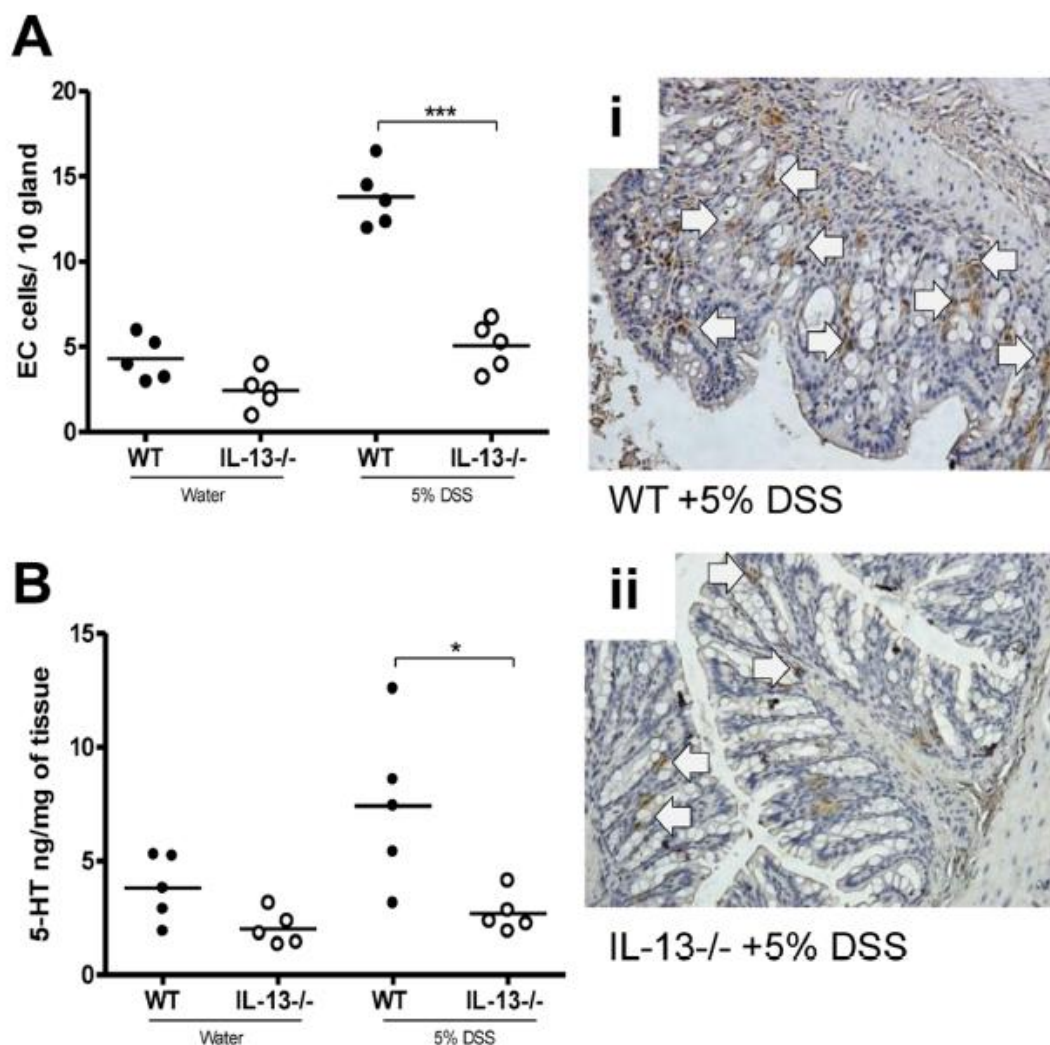
Figure 3. Effects of IL-13 deficiency in DSS-induced colitis. WT and IL-13−/− mice were given 5% DSS in drinking water to induce colitis. (A) Number of 5-HT expressing EC cells per 10 glands and colonic sections immunostained for 5-HT expressing EC cells after 5 days of DSS administration in WT and IL-13−/− mice. (B) Colonic 5-HT amount. (i) and (ii) Representative micrograph and arrows indicate 5-HT expressing EC cells. *Represents statistical significance where p<0.05.
doi:10.1371/journal.pone.0072774.g003

nized in 1 ml of Tris HCl buffer containing protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged and the supernatant was collected and frozen at −80°C until assay. Cytokine levels in tissue and serum were determined using

Table 1. Effects of 5-HT on pro-inflammatory cytokine production.

in vitro treatment	IL-1β		IL-6	
	WT	**IL-13−/−**	**WT**	**IL-13−/−**
Media	15.53±3.13	12.95±1.59	6.88±0.67	4.67±1.96
5-HT	22.07±1.48	15.26±4.33	12.42±0.93	11.83±1.16

Effects of 5-HT on IL-1β and IL-6 production by peritoneal macrophages isolated from WT and IL-13−/− following DSS administration. Data is expressed as percent increase with respect to their controls; mean ± SEM of 4 to 6 mice in each group done in triplicates.
doi:10.1371/journal.pone.0072774.t001

commercial available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

Determination of Colonic 5-HT Levels

Colonic 5-HT levels were determined for all *in vivo* experiments as previously described. [15] Briefly, colonic tissues were weighed and were homogenized in 0.2 N perchloric acid. Following centrifugation at 10,000 g for 5 minutes, the supernatant was collected and the pH was neutralized using 1 M borate buffer. The supernatant was stored in -80°C until analysis of 5-HT levels using commercially available ELISA kits (Beckman Coulter, Fullerton, CA). 5-HT content was expressed as a function of wet weight (ng/mg).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) and results are represented as mean or percentage of control. Student's t-test and one way ANOVA followed by the Newman-Keuls multiple comparison test

WT + 5%DSS   IL-13-/- + 5% DSS

Figure 4. F4/80 positive macrophage infiltration in DSS-induced colitis in WT and IL-13−/− mice. F4/80 positive area, positive stained areas expressed as the percent of the total area. (i) and (ii) Representative micrographs. *Represents statistical significance where $p<0.05$.
doi:10.1371/journal.pone.0072774.g004

were used where appropriate and a p-value of <0.05 was considered statistically significant.

Results

IL-13 Deficiency is Associated with Decreased 5-HT Production and Severity of DSS Colitis

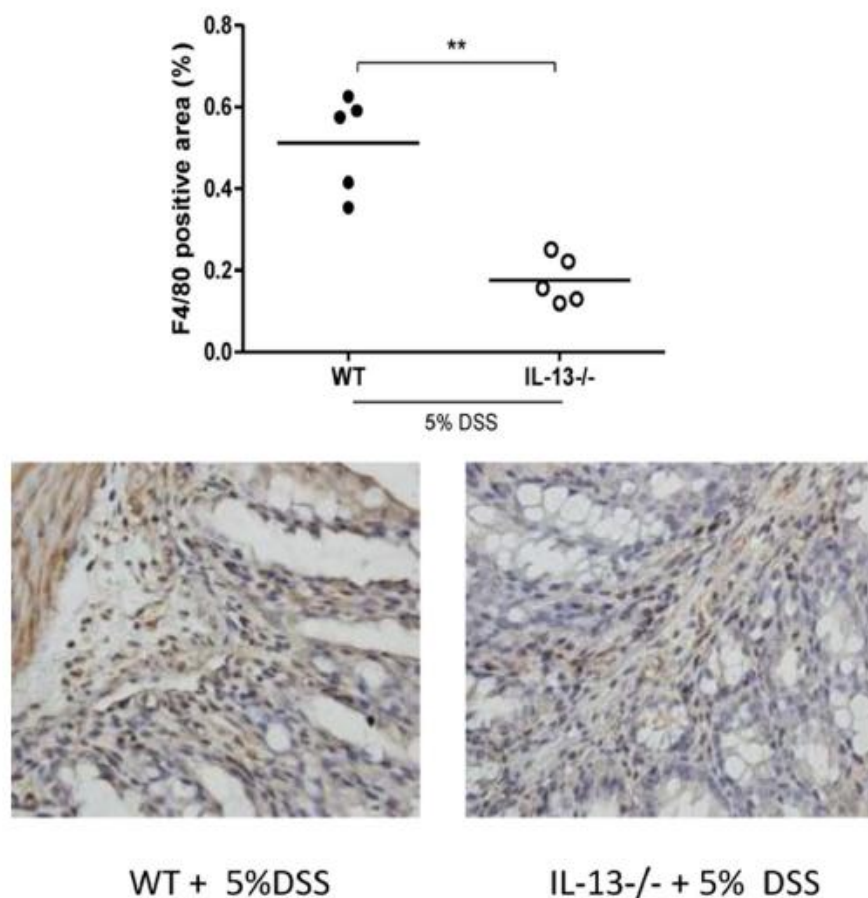Colonic IL-13 levels are significantly increased in WT mice on day 5 post-DSS as compared to controls without DSS (Figure 1A). IL-13−/− mice exhibited significantly reduced disease activity compared to WT mice post-DSS administration (Figure 1B). Reduced disease activity was consistently observed in IL-13−/− mice, compared to WT mice, regardless of litters, cages and time of the experiment (Figure S1). IL-13−/− mice also had marked reductions in macroscopic and histological damage scores (Figure 1C and D). Reduction in the severity of DSS colitis was associated with significantly lower MPO activity, as well as reduced IL-1β, IL-6 and IL-17 production in IL-13−/− mice (Figure 2A–D). However, IL-4 levels observed were not significantly different amongst any of the groups (Figure 2E).

In this study, significantly reduced 5-HT expressing EC cell numbers were observed in IL-13−/− mice following induction of DSS colitis compared to WT mice (Figure 3A). There was also reduced colonic 5-HT content in IL-13−/− mice in comparison to WT mice (Figure 3B). Recently we have identified a role of IL-13 in murine EC cell biology and BON cell hyperplasia, BON cells are human carcinoid cells that secrete 5-HT thus used as a model

of EC cells. [17] In this study, we observed significantly increased in Ki-67 positive cells in WT mice after induction of DSS colitis in WT mice but this DSS-induced up-regulation of epithelial cell proliferation was not evident in IL-13−/− mice (Figure S2). Taken together, these observations suggest that IL-13 plays an important role in EC cell biology and 5-HT production and in generation of inflammation in DSS colitis.

Dampened Response in Cytokine Production by Peritoneal Macrophages and Fewer Activated Macrophages in Mice Lacking IL-13 Following DSS Administration

Reduced 5-HT production in the gut has previously been associated with fewer infiltrating F4/80 positive macrophages in experimental colitis. [10] Consequently, the reduced production of 5-HT observed in the IL-13−/− mice was accompanied by fewer infiltrating macrophages in their colonic segments following DSS treatment in comparison with WT mice (Figure 4). Earlier work in our lab established that, 5-HT stimulation increases IL-1β and IL-6 production by peritoneal resident macrophages. [10]. In the current study, it was observed that macrophages isolated from IL-13−/− mice had dampened cytokine production in comparison to WT mice as revealed when normalized to their respective controls (Table 1) and in culture treatment with 5-HT was able to reverse these effects in IL-13−/− macrophages (Table 1).
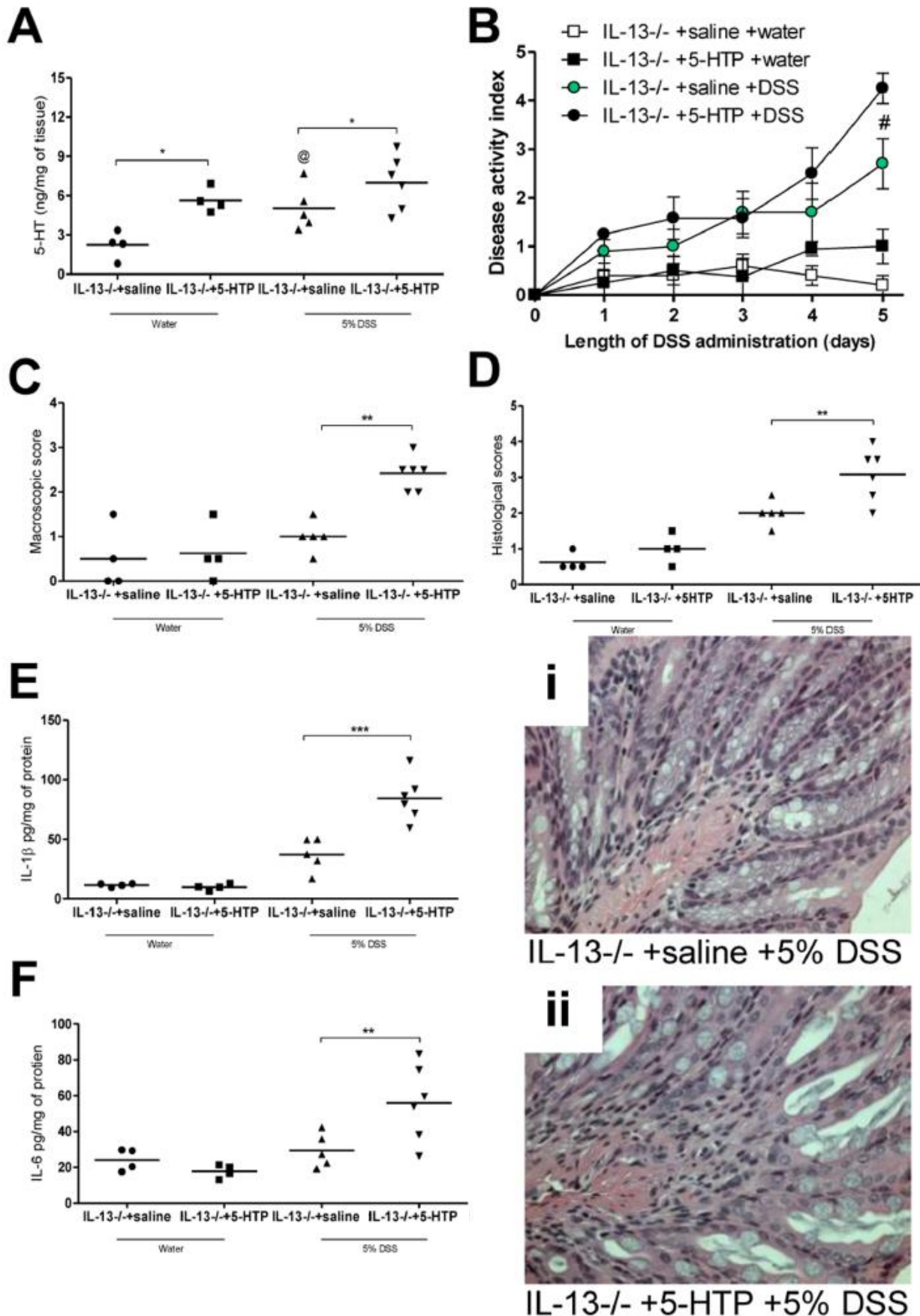
Figure 5. Effects of 5-HTP administration in IL-13−/− mice in DSS-induced colitis. 5-HTP 100 mg/kg treatment started 3 days prior to 5 days of 5% DSS administration, control groups received saline. (A) Colonic 5-HT levels (B) DAI (C) Macroscopic scores (D) Histological scores (i) and (ii) Representative light micrograph of an H&E-stained colonic section. From colonic tissue (E) IL-1β and (F) IL-6 levels. DAI data represented as mean ± SEM from 4 to 6 mice; *represents statistical significance where p<0.05; #significant difference between IL-13−/− +5-HTP and IL-13−/− +saline, both groups receiving DSS; @ indicates no significant difference between IL-13−/− groups receiving saline as vehicle.
doi:10.1371/journal.pone.0072774.g005

Increased Availability of 5-HT Exacerbates Severity of Colitis in IL-13 Deficiency

To evaluate the role of decreased 5-HT production in IL-13−/− mice and its significance in the reduced severity of DSS-colitis in these animals, we treated IL-13−/− mice with 5-HTP, the direct precursor of 5-HT. A significant increase in colonic 5-HT content was observed in IL-13−/− mice receiving 5-HTP compared to IL-13−/− receiving vehicle following induction of DSS colitis (Figure 5A). IL-13−/− mice treated with 5-HTP had significantly higher disease activity scores compared to the vehicle treated group (Figure 5B). Post-mortem macroscopic assessment and histopathological evaluation also revealed significant enhancement of severity of colitis in IL-13−/− mice that received 5-HTP as compared to the IL-13−/− mice that received vehicle (Figure 5C and D). This increase in severity of colitis observed in 5-HTP treated groups was also resulted in increased levels of colonic IL-1β and IL-6 (Figure 5E and F).

Replenishment of IL-13 Levels in IL-13−/− Mice Increases Severity of Colitis

To confirm the effects of IL-13 in 5-HT production and in turn severity of colitis, we reconstituted IL-13−/− mice with rmIL-13. Administration of rmIL-13 significantly increased IL-13 levels in IL-13−/− mice (524.82±120.81 pg/ml) compared to mice receiving vehicle (below the level of detection). Restoration of IL-13 levels in IL-13−/− mice were associated with increased severity of DSS-induced colitis and increased levels of MPO activity (Figure 6A–D). Along with increased severity of colitis, a significant increase in 5-HT expressing EC cell numbers and 5-HT production was observed in the IL-13−/− mice receiving rmIL-13 compared to IL-13−/− mice receiving vehicle (Figure 7 A and B).

IL-13 Deficiency Associated Down-regulation of 5-HT has Protective Effects in DNBS- Colitis

To investigate whether decreased 5-HT production and reduced severity of colitis observed in IL-13−/− mice was restricted to the DSS model; we induced DNBS colitis in IL-13−/− mice. Post-DNBS administration, we observed significantly reduced macroscopic and histological damage in IL-13−/− mice in comparison to WT mice (Figure 8A and B). There was also significant reduction in colonic IL-1β, IL-6 levels and MPO activity observed in the IL-13−/− mice (Figure 8 C-E). In addition, there was a significant down-regulation of 5-HT production observed in the IL-13−/− mice in comparison to WT mice following DNBS-induced colitis (Figure 8F).

Discussion

IBD affects approximately 1.4 million people in North America and costs the healthcare system upwards of $2 billion every year. [24] Although the exact etiology of IBD is not known, studies have provided evidence that dysregulated immune response, genetic factors, gut flora, and environmental factors contribute to the pathogenesis of IBD. [25] Alterations in EC cell population, the main source of our body's 5-HT, have been associated with these disorders. [11] Considering the close proximity between lympho-

cytes and EC cells in the gut mucosa, it is not farfetched to infer that these cells are the gatekeepers at the critical junction of immuno-endocrine interaction, though the mechanism of how the immune and the endocrine systems influence each other and how their interactions may shape the progression or regression of disease is still not well understood. In the current study, using two different models of experimental colitis, we identified 5-HT as an important arbitrator of IL-13-mediated gut inflammation.

IL-13 is a pleiotropic cytokine that exerts its influence on various cell types, including epithelial cells, macrophages, smooth muscle cells and neurons. [26] A plethora of immune cells produce IL-13, [26] and a subset of NKT cells have been identified as the source of increased IL-13 production in patients with UC. [5] Previously, our lab has shown that, in enteric infection driven intestinal inflammation as well as in human model of EC cells, IL-13 plays a vital role in the up-regulation of the rate-limiting enzyme TPH-1 and in turn 5-HT production. [17] It has also been demonstrated that a Th2 immunophenotype is more apt in the regulation of EC cell biology relative to a Th1 immunophenotype. [15] In this study, for the first time, we report a significant increase in colonic IL-13 levels following DSS administration; this is in line with findings in other models of UC. [8] Using IL-13 deficient mice we observed that these mice suffered from reduced severity of colitis and had significantly fewer 5-HT expressing EC cells and lower 5-HT content in the gut. Traditionally, IL-13 deficiency is not associated with depressed inflammatory cytokines production; [27] we postulated that it is the lower colonic 5-HT content that is responsible for the decreased severity of colitis observed in IL-13−/− mice. This became evident when severity of colitis in IL-13−/− mice increased following enhancement of colonic 5-HT amount via 5-HTP treatment, which was also marked by the increased production of pro-inflammatory cytokines.

5-HT is an important neurotransmitter of the central nervous system and is studied extensively for its role in regulating behavior, appetite and energy expenditure. [28,29] What is often underappreciated is that the vast majority of 5-HT in the body is found in GI tract not the central nervous system. Recently, our lab has shown that 5-HT plays a key role in generation of inflammation and immune activation. [10,30] By subjecting TPH-1 deficient (TPH-1−/−) mice, which have significantly reduced colonic 5-HT content, to DSS and DNBS models of colitis, it was demonstrated that the TPH-1−/− group experienced significantly reduced severity colitis and had lower production of pro-inflammatory cytokines. [10] Ghia and colleagues [10] also established the role of 5-HT in recruitment and in the induction of a pro-inflammatory phenotype in macrophages. Given the reduced production of colonic 5-HT observed in the IL-13 deficient group following DSS treatment we anticipated that there would be reduced number of infiltrating macrophages in these animals, and this was confirmed by fewer F4/80 positive macrophages observed in the colonic segments of IL-13−/− mice. In addition, we observed that macrophages isolated from IL-13−/− mice had a dampened response following DSS administration in comparison to WT, and their response was bolstered following in culture 5-HT treatment. Thus, revealing how regulation of 5-HT production by IL-13 may mediate inflammation.

Figure 6. Effects of replenishment of IL-13 in DSS-induced colitis. IL-13−/− mice were treated with rmIL-13 (2 µg/day) injections or vehicle. rmIL-13 treatment started 3 days prior to 5 days of 5% DSS treatment. All groups received 5% DSS in the drinking water to induce colitis. (A) DAI. (B) Macroscopic damage score (C) Histologic scores and (D) MPO activity. (i) and (ii) Representative light micrograph of an H&E-stained colonic section. DAI data represented as mean ± SEM from 4 mice; *represents statistical significance where p<0.05; a, b and c represents statistically significant difference in disease activity scores on days indicated, where p<0.05.
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To further explore the role of the IL-13-5-HT axis in severity of DSS-induced colitis, we replenished IL-13 content in IL-13−/− mice and found that this resulted in a significant increase in severity of colitis as reflected by the disease activity index, macroscopic and histological damage assessment. Elevation of IL-13 levels was once again marked by a significant increase in
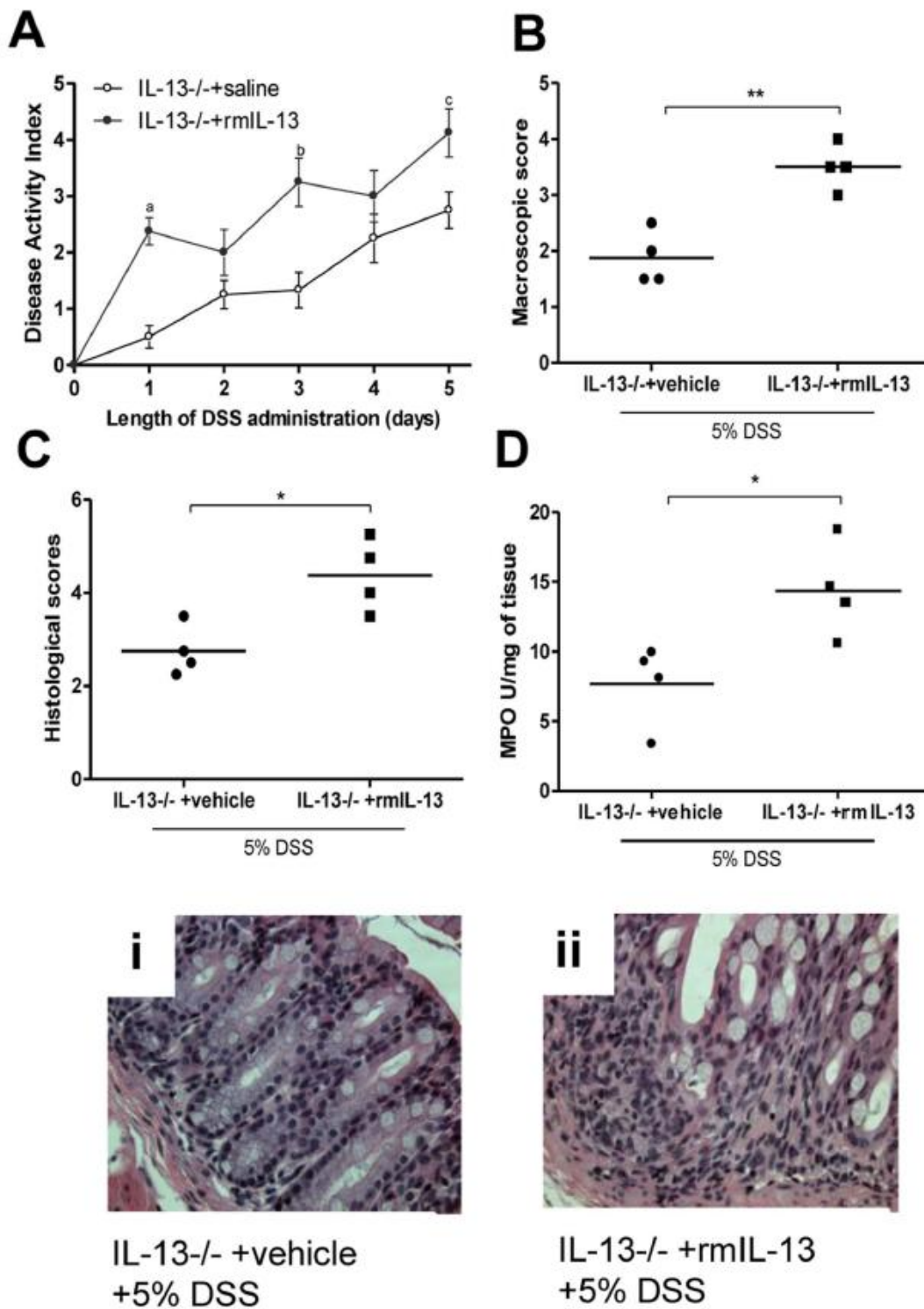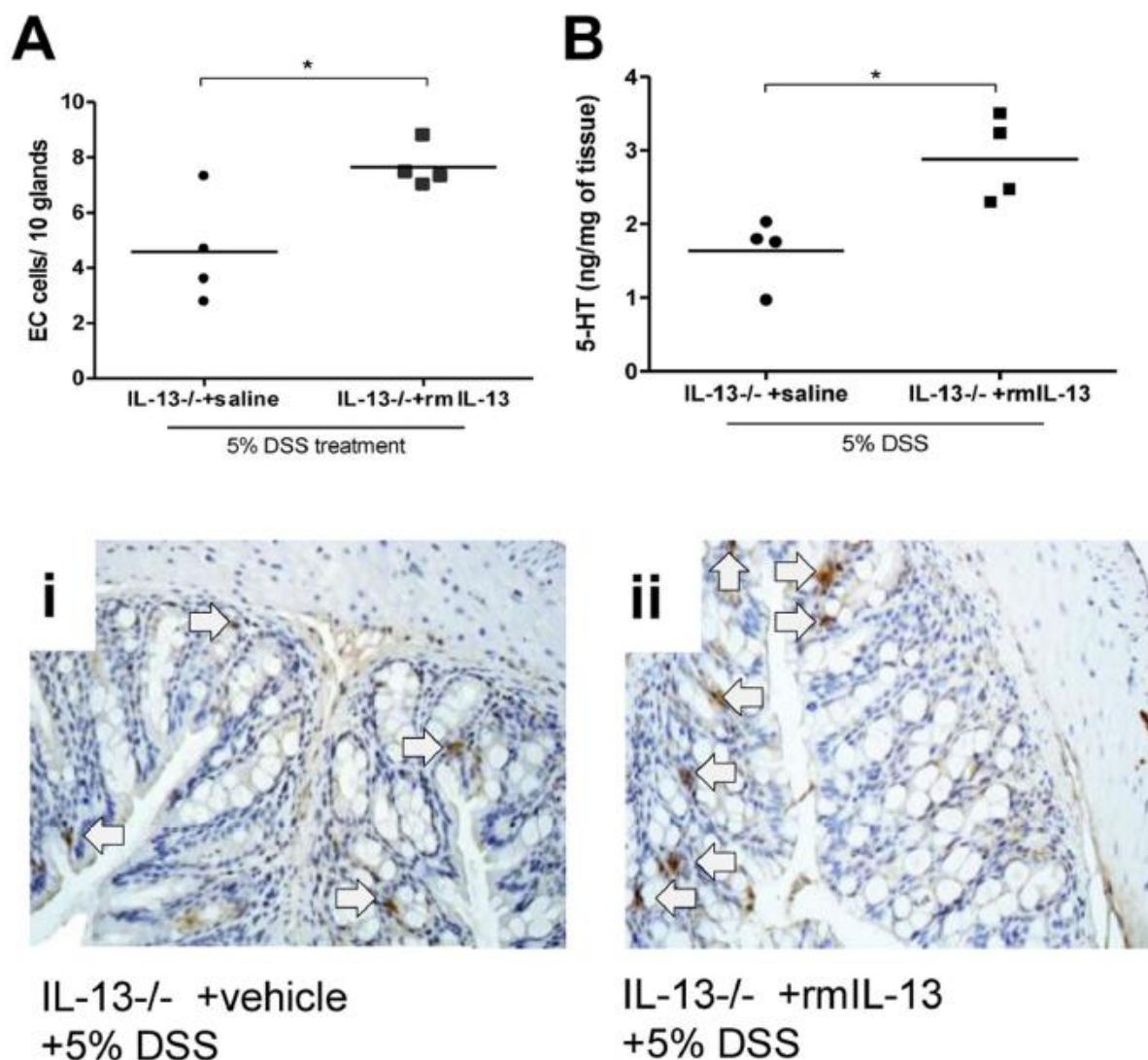
Figure 7. Effects of replenishment of IL-13 in DSS-induced colitis. IL-13−/− mice were treated with rmIL-13 (2 µg/day) injections or vehicle. rmIL-13 treatment started 3 days prior to 5 days of 5% DSS treatment. All groups received 5% DSS in the drinking water to induce colitis. (A) Number of 5-HT expressing EC cells per 10 glands and (B) colonic 5-HT content. (i) and (ii) Representative micrograph and arrows indicate 5-HT expressing EC cells. *Represents statistical significance where $p < 0.05$.
doi:10.1371/journal.pone.0072774.g007

colonic 5-HT content as well as 5-HT expressing EC cell numbers. The increased production of IL-13 and the resulting increase in 5-HT observed in the DSS model of colitis and the role of 5-HT in the other models of colitis previously observed, prompted investigation into the effects of this association in another model of experimental colitis. DNBS-induced colitis is a well-defined Th1 based model of transmural inflammation of the colon that may be considered a model of Crohn's disease. [31], [32] As with DSS-induced colitis, we observed significant reduction in colonic inflammation in IL-13−/− mice following intrarectal challenge with DNBS. The attenuation of DNBS-induced colitis in IL-13−/− mice was observed in macroscopic and microscopic scores as well as MPO activity and pro-inflammatory cytokine production. We also observed a markedly reduced production of 5-HT in IL-13−/− mice. Of note, the 5-HT production observed in DNBS colitis was uniformly lower in all experimental groups compared to levels observed in DSS model. This is most likely due to the differences between the two models of colitis utilized. However,

previously established differences in 5-HT content in Th1 and Th2 environments may also underlie such differences observed [15].

In conclusion, we have shown that 5-HT plays a pivotal mediating role in IL-13 driven intestinal inflammation. This study is the first to report an important role of IL-13 in generation of inflammation in two different experimental models (DSS and DNBS) of colitis and also identifies 5-HT as a vital factor in pathogenesis of IL-13-mediated colitis. In consideration with the recent reports of elevated IL-13 levels observed in patients with UC [6,33] and alterations in EC cells numbers and 5-HT production accompanying different GI disorders, including IBD, [34] the findings of this study shed light on novel immune-endocrine interactions in the gut that may ultimately lead to improved therapeutic strategies in the battle against pathological intestinal inflammation.
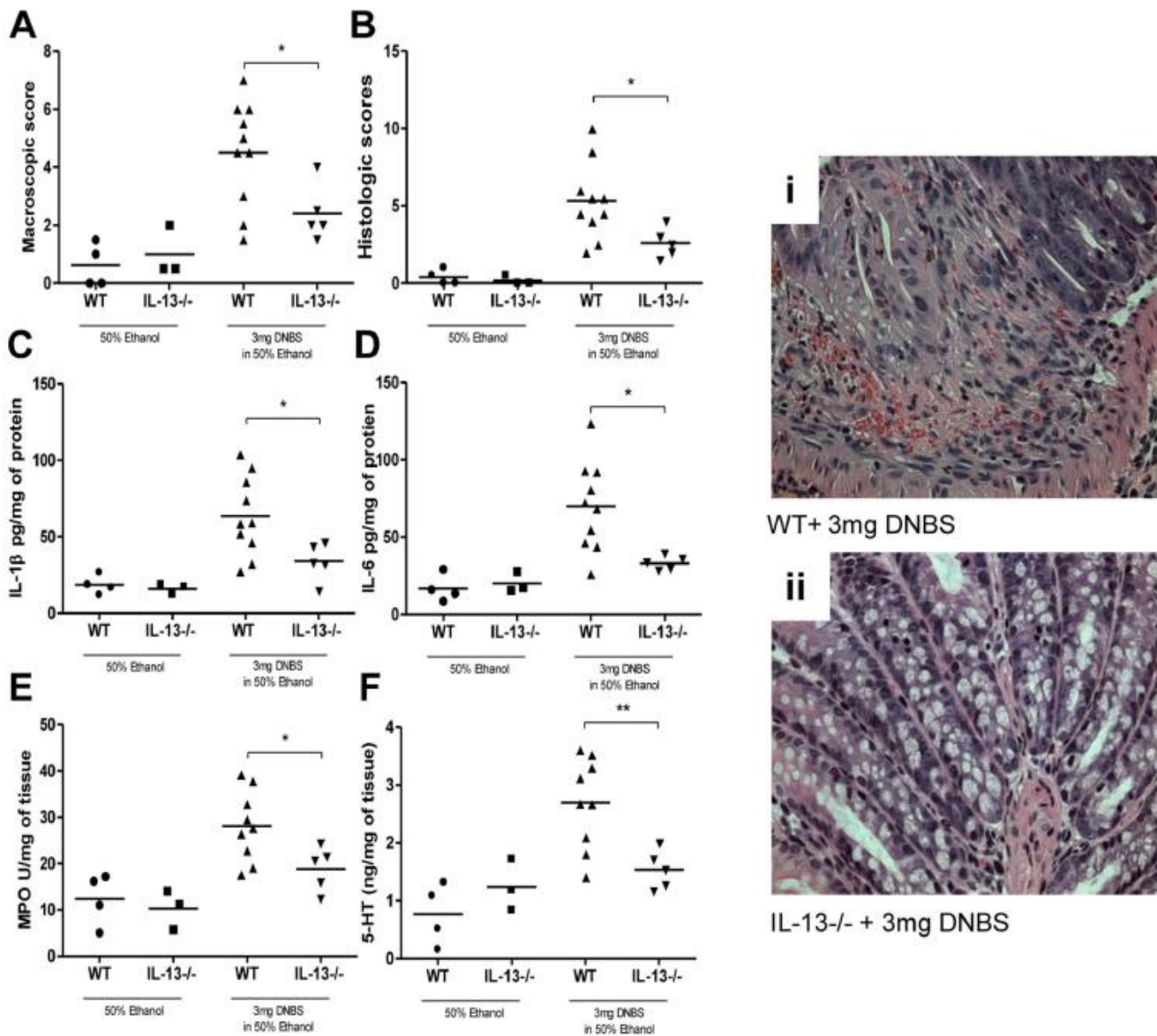
Figure 8. Effects of lack of IL-13 in DNBS-induced colitis. WT and IL-13−/− mice were given DNBS (3 mg/100 μL) in 50% ethanol solution. All controls received 50% ethanol via intrarectal installation. Mice were sacrificed on day 3 following induction of DNBS colitis. (A) Macroscopic scores and (B) Histologic scores. (i) and (ii) Representative micrographs. From colonic tissue (C) IL-1β (D) IL-6 levels (E) MPO activity, and (F) 5-HT levels. *Represents statistical significance where $p < 0.05$.
doi:10.1371/journal.pone.0072774.g008

Supporting Information

Figure S1 Effects of IL-13 deficiency in DSS-induced colitis were not influenced by cages, litters or time. WT and IL-13−/− mice were given 5% DSS in drinking water for 5 days to induce colitis. All control animals received water without DSS. (A) and (B) Disease activity index (DAI) from two separate experiments evaluating the categories weight loss, stool consistency and feces bleeding demonstrate that the DAI did not differ between cages, litters or time. Data are represented as mean ± SEM from 3 to 5 mice for each experiment; # represents statistically significant difference ($p < 0.05$) between IL-13−/− mice and WT mice administered DSS. (PDF)

Figure S2 Effects of IL-13 deficiency in DSS-induced colitis and cell proliferation. WT and IL-13−/− mice were given 5% DSS in drinking water for 5 days to induce colitis. All control animals received water without DSS. Colonic sections of WT and IL-13−/− mice with or without DSS were immunostained with anti- Ki-67 antibody. Representative micrograph of (A) WT mice post-DSS and (B) IL-13−/− mice post-DSS. (C) Number of Ki-67+ cells per gland. * $p < 0.05$; **$p < 0.01$. (PDF)

Author Contributions

Conceived and designed the experiments: MSS JAD WIK. Performed the experiments: MSS HW JIK IS JG ED MC. Analyzed the data: MSS HW. Wrote the paper: MSS WIK.

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Figure S1
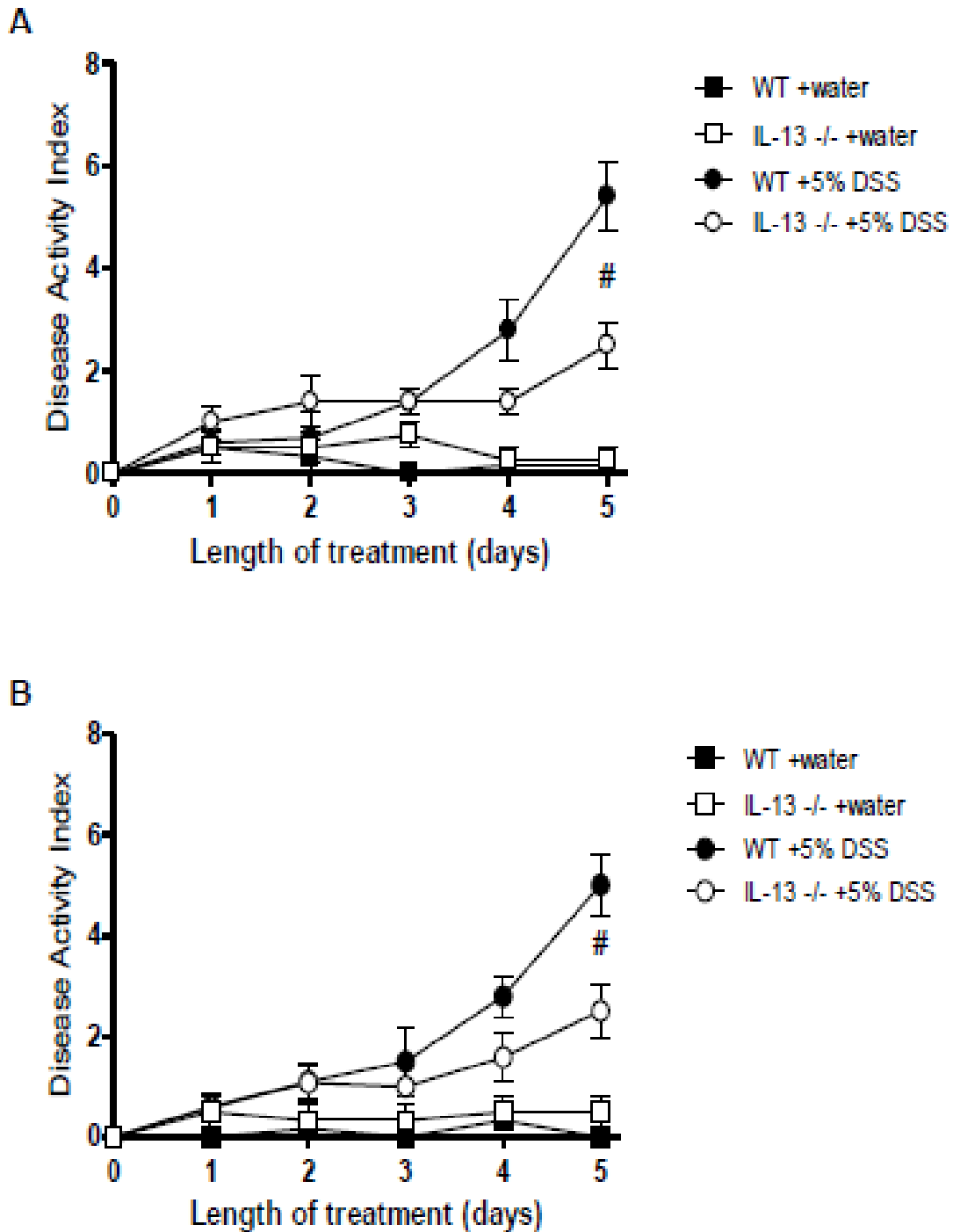
A

B

Effects of IL-13 deficiency in DSS-induced colitis were not influenced by cages, litters or time. WT and IL-13−/− mice were given 5% DSS in drinking water for 5 days to induce colitis. All control animals received water without DSS. (A) and (B) Disease activity index (DAI) from two separate experiments evaluating the categories weight loss, stool consistency and feces bleeding demonstrate that the DAI did not differ between cages, litters or time. Data are represented as mean ± SEM from 3 to 5 mice for each experiment; # represents statistically significant difference ($p < 0.05$) between IL-13−/− mice and WT mice administered DSS.

Figure S2
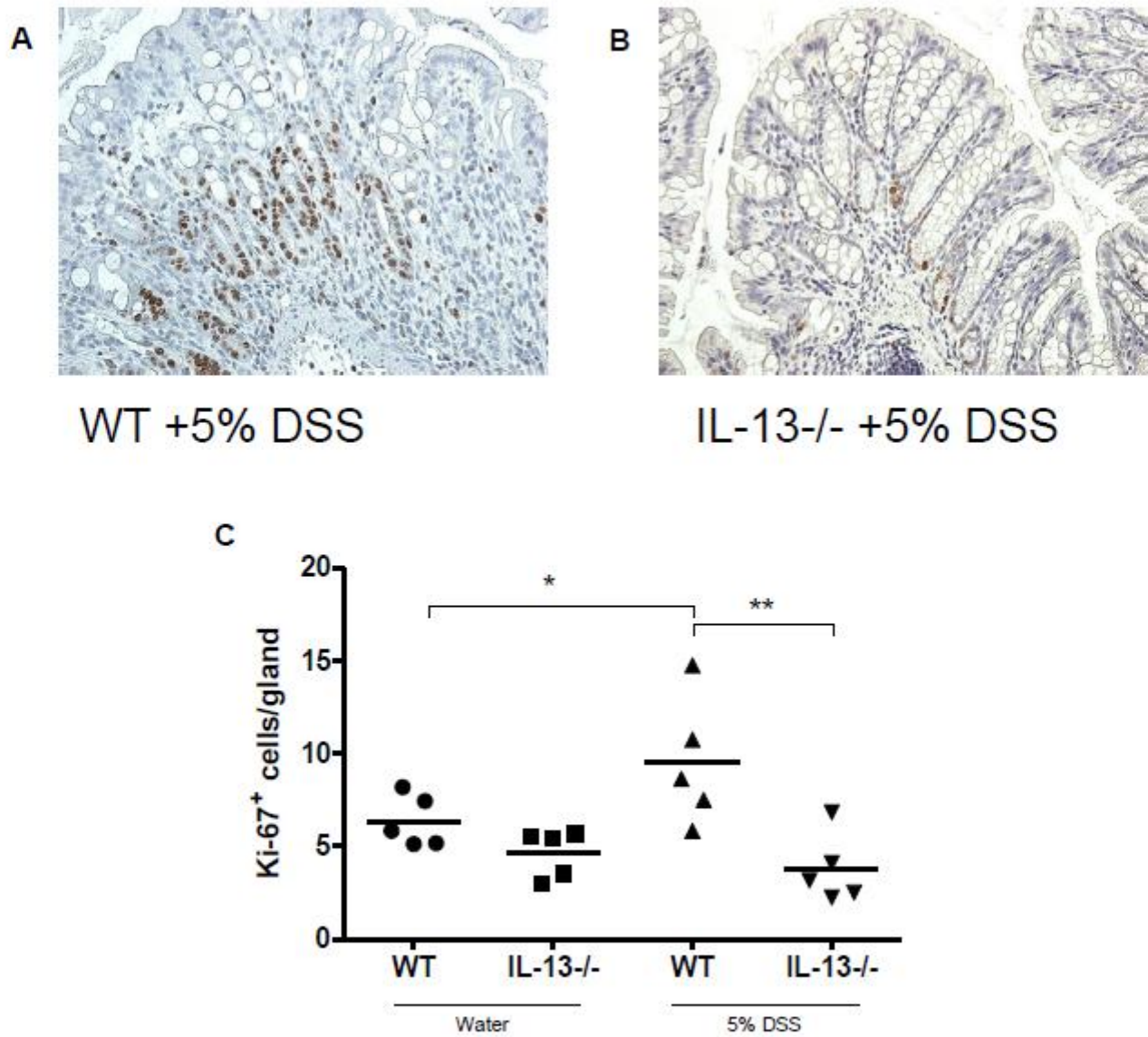
Effects of IL-13 deficiency in DSS-induced colitis and cell proliferation. WT and IL-13−/− mice were given 5% DSS in drinking water for 5 days to induce colitis. All control animals received water without DSS. Colonic sections of WT and IL-13−/− mice with or without DSS were immunostained with anti- Ki-67 antibody. Representative micrograph of (A) WT mice post-DSS and (B) IL-13−/− mice post-DSS. (C) Number of Ki-67+ cells per gland. * $p<0.05$; **$p<0.01$.

—CHAPTER 5—

LINKAGE BETWEEN 5-HT TRANSPORTER PROMOTER

POLYMORPHISM AND 5-HT SIGNALING MACHINERY WITH

CROHN'S DISEASE

Linkage between 5-HT Transporter Promoter Polymorphism and 5-HT signaling machinery with Crohn's disease.

Shajib MS, Chauhan U, Adeeb S, Chetty Y, Armstrong D, Halder SLS, Marshall JK, and Khan WI.

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Preface: This work was conducted over the period of August 2012- April 2017.

WIK (Professor, McMaster; my supervisor), JKM (Professor, McMaster) and I conceived the idea for this project. UC (Nurse practitioners, McMaster University Medical Center), SA and I recruited all study participants. I designed and performed all experiments with assistance from SA and YC (Undergraduate students; assisted with sample preparation). I analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. DA (Professor, McMaster) and SLSH (Assistant Professor, McMaster) allowed me to approach their patients and critically appraised the manuscript. JKM oversaw clinical aspects of the project and edited the manuscript. WIK oversaw all aspects of the project and edited the manuscript.

Summary and significance of findings: Our findings demonstrated that inflammation in CD is associated with increased colonic IL-13 mRNA expression and enhancement of mucosal 5-HT signaling, via up-regulated TPH1 and down-

regulated 5-HTT mRNA expressions. We also characterized mucosal 5-HTR expressions. Moreover, increased PPP and plasma 5-HT levels were observed in patients with CD as compared to HC. In addition to that, we identified 5-HTTLPR as a potential candidate gene involved in CD pathogenesis.

Linkage between 5-HT transporter promoter polymorphism and 5-HT signaling machinery with Crohn's disease.

Short title: 5-HT signaling in CD.

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Background: Tryptophan hydroxylase (TPH)1 catalyzes the biosynthesis of serotonin (5-hydroxytrptamine; 5-HT) in enterochromaffin (EC) cells, the predominant source of gut 5-HT. Secreted 5-HT regulates various gut functions through diverse 5-HT receptor (5-HTR) families, and 5-HT transporter (5-HTT) sequesters its activity via uptake into surrounding cells. In inflammatory bowel disease (IBD) mucosal 5-HT signaling is altered, including up-regulated EC cell numbers and 5-HT levels. Herein, we examined the genetic basis of this altered 5-HT signaling and mucosal signaling components, such as 5-HTRs.

Methods: We investigated 5-HTT gene-linked polymorphic region (5HTTLPR) in forty CD patients and healthy controls (HC) utilizing polymerase chain reaction (PCR), and measured platelet-poor plasma (PPP) and plasma 5-HT concentrations. In the context of inflammation, using quantitative PCR mucosal TPH1, 5-HTT, and 5-HTR expressions were studied in colonic biopsy specimens from HC (n=10) and CD patients (n=15).

Results: Compared to HC, significantly higher S-genotype (L/S+S/S) than L/L genotype and higher PPP and plasma 5-HT levels were observed in CD patients. Moreover, mucosal inflammation was associated with elevated TPH1, 5-HTR3, 5-HTR7 and down-regulated 5-HTT expression, whereas 5-HTR4 expression was up-regulated irrespective of inflammation compared to HC.

Conclusion: Our results suggest 5-HTTLPR may be a potential candidate gene involved in CD pathogenesis. Specific 5-HTTLPR genotype associated decreased efficiency in 5-HT re-uptake and augmented 5-HT production may contribute to CD pathogenesis, through increased mucosal 5-HT availability. These findings revealed important information on various components of 5-HT signaling in intestinal inflammation which may ultimately lead to effective strategies targeting this pathway in IBD.

Keyword: Inflammatory bowel disease; IBD; Crohn's disease; Serotonin; 5-HT; 5-HTTLPR; 5-HT receptor.

Introduction

Inflammatory bowel disease (IBD) is defined by chronic, relapsing inflammation of the gastrointestinal (GI) tract; the two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC) [1]. These lifelong conditions not only affect the physical well-being of the patients but can significantly influence their social and mental well-being [2]. IBD is on the rise across the globe, with Canada having one of the highest incidence rates of CD in the world [3–5]. The etiology of IBD has been extensively studied in the past few decades, but disease pathogenesis is not fully understood. It is believed that IBD results from a continuum of complex interactions among the genetic composition of the host, intestinal microbiota, the immune system, and specific environmental factors [1].

The gut produces approximately 95% of serotonin or 5-hydroxytryptamine (5-HT) found in the human body; where, it is a very important enteric signaling molecule participating in gut motility, sensation, and secretion [6]. The vast majority of the gut-derived 5-HT is produced by specialized epithelial cells of the GI tract, called enterochromaffin (EC) cells [6]. EC cells produce 5-HT from dietary tryptophan, this process involves the rate limiting enzyme tryptophan hydroxylase (TPH)1, once produced this 5-HT can be released into the gut lumen, surrounding tissue and can enter the blood circulation [7]. 5-HT mediates many GI functions by acting on a diverse array of 5-HT receptors (5-HTR) [8]. Five of the seven known receptor families of 5-HT (5-HTR1, 5-HTR2, 5-HTR3, 5-HTR4 and 5-HTR7) are

expressed in the gut. 5-HT is a very tightly regulated molecule in the body. Following secretion, 5-HT is transported into surrounding cells predominantly via 5-HT transporter (5-HTT), also known as serotonin transporter (SERT), and is degraded quickly mainly by monoamine oxidise-A or sequestered by vesicular monoamine transporter [7]. Various cell types express 5-HTT, such as immune cells, enterocytes, and platelets [7]. Stored within the dense granules of platelets enteric 5-HT is shuttled throughout the periphery, and propagates the influence of gut-derived 5-HT beyond the gut. Platelets do not produce 5-HT but rather take it up using 5-HTT while circulating the gut [9]. 5-HTT is encoded by the SLC6A4 gene, which is comprised of 14 exons and is present on chromosome 17q11.2 of the human genome [10]. A 44-bp insertion/deletion polymorphism in the promoter region of SLC6A4 has been reported and is known as the 5-HTTgene-linked polymorphic region (5-HTTLPR). The deletion/insertion in the 5-HTTLPR creates two predominant variant alleles, a short (S) allele, and a long (L) allele, where the former is functionally dominant[10–12]. Compared with the L/L genotype, S/S or S/L genotypes are associated with lower levels of 5-HTT mRNA transcripts, and thereby lower levels of 5-HTT expression and reduced 5-HT reuptake efficiency [10].

5-HT has been evaluated in IBD and in animal models of colitis. An increase in the number of 5-HT expressing EC cells has been observed in IBD patients [13]. Moreover, consumption of selective 5-HT reuptake inhibitors has been associated

with microscopic colitis [6]. An elevated level of 5-HT was also observed in a patient with collagenous colitis [14]. Recently it was shown that TPH1-deficient mice, which have significantly reduced gut 5-HT, exhibit reduced susceptibility to experimental colitis and down-regulation of several pro-inflammatory cytokines [15]. It has also been demonstrated that pharmacologically blocking peripheral 5-HT synthesis reduces the severity of intestinal inflammation in both chemical and infection-induced models [16]. Taken together, there is now compelling evidence of an important role for 5-HT in controlling inflammation in the gut. The implications of 5-HTTLPR polymorphism are of tremendous interest in neuropsychological disorders, such as depression, and in functional GI disorders, such as irritable bowel syndrome (IBS) [10, 17]. Though most clinical studies agree that there is a reduced expression of 5-HTT in IBD, it was only recently that a study by Sikander et al.[18] investigated the association between 5-HTTLPR and IBD. The study explored, 5-HTTLPR association with UC and microscopic colitis in a population of Indian patients, where the prevalence rate of IBD is only now approaching the lower limit of that in North Europe and America [19].

In the present study, we investigated 5-HTTLPR polymorphism along with the key elements of mucosal 5-HT signaling in CD patients for a better understanding of the role of 5-HT in IBD pathogenesis. We observed a higher frequency of the S-genotype (S/S and S/L combined) in patients with CD in our population and this was associated with significantly higher platelet poor plasma (PPP) 5-HT levels.

In a separate group of CD patients, using colonic biopsy samples we observed significant up-regulation of TPH1, 5-HTR3, 5-HTR7 expressions and down-regulation of 5-HTT expression in association with intestinal inflammation. Mucosal 5-HTR4 expression was also markedly elevated in these patients.

Methods

This study was approved by Hamilton Integrated Research Ethics Board (HiREB). All biopsy and blood samples were collected at the McMaster University Medical Center (MUMC) from consenting adults over the age of 18 years (clinicaltrials.gov # NCT01650311).

Blood Samples and 5-HT ELISA

A total of 109 Caucasian participants were approached for blood donation. Of them 12 refused and 97 provided informed consent, which consisted of 55 patients with confirmed diagnosis of CD and 42 healthy controls (HC). Of the 97 consenting participants 80 donated 8 ml of venous blood. HC exclusion criteria included any previous history of GI complaints, long-term use of nonsteroidal anti-inflammatory drugs, steroids, anti-depressants, or any biologics. Samples were collected in EDTA tubes and 4ml of blood was used to measure PPP and plasma 5-HT levels using commercially available ELISA kit (Beckman Coulter, Fullerton, CA). Sample preparation and the ELISA were performed according to manufacturer's instructions.

Genotyping and polymerase chain reaction protocol

The remaining 4 ml of blood was used for DNA isolation using a commercially available kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The insertion/deletion polymorphism in 5-HTT gene was typed by polymerase chain reaction (PCR) using the isolated DNA and a modified version of the methods described by Sikander et al.[18] Briefly, the following primers were used: forward 5'-GGCGTTGCCGCTCTGAATGC-3' and reverse 5'-GAGGGACTGAGCTGGACAACCAC-3'. PCR reactions were performed using CFX96TM Real-Time System (Bio-RAD, Hercules, CA) in a 50 µL reaction containing 1 µl of isolated genomic DNA (25–50 ng), 1 µL of 10 µM of forward primer, 1 µL of 10 µM of reverse primer, 17 µL of Ultra PureTM Distilled water DNAase and RNAse free (Thermo Fisher Scientific, Waltham, MA) , 5 µL of 360 GC Enhancer (Applied Biosystems, Foster city, CA), 25µl of AmpliTaq Gold® 360 Master Mix (Applied Biosystems). DNA was denatured at 95°C for 10 min and subjected to 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, 1 min of extension at 72°C, and final extension was at 72°C for 7 min. PCR products were resolved by electrophoresis on 3% agarose gel with Tris-borate-EDTA as the running buffer and visualized with Syber Green staining (Bio-RAD). The findings of the genotyping experiment were confirmed by the Centre for Applied Genomics (TCAG) operated by the Hospital for Sick Children (SickKids), Toronto, Canada.

Biopsy collection and histopathological assessment

A total of 42 participants were approached and consented to donate colonic biopsy samples for this portion of the study, which consisted of 15 HC and 27 patients with confirmed diagnosis of CD. The HC group comprised of individuals undergoing routine colorectal screening and HC exclusion criteria were as stated earlier. Additionally, participants were excluded from the study if for any reason the gastroenterologist deemed the individual not fit to participate. Two mucosal biopsies were obtained from 10 HCs, 4 were withdrawn as they were declared not fit to participate in the study by the endoscopists, and one participant donated only one biopsy sample thus was not included in the analysis. Two biopsy samples were obtained from both inflamed and non-inflamed regions of the colon as identified by the gastroenterologist, from 15 patients with confirmed diagnosis of CD. Non-inflamed regions were defined as those without any endoscopic features of inflammation and at least 10 cm from any area of active inflammation [20]. 12 patients who had provided consent were withdrawn as they were judged not fit to participate, for example, due to serious comorbid disease or difficult colonoscopy. One of the two biopsy samples was flash frozen, following collection, for analysis of mRNA expression using quantitative PCR (qPCR). The second sample was fixed in 10% buffered formalin and was processed within 24 hours for histopathological evaluation. A pathologist unaware of the diagnosis performed

the histological evaluations and were graded on a numerical scale based on standard criteria [21].

Immunofluorescence of 5-HT expressing EC cells

Immunofluorescent analysis of 5-HT expressing EC cells were performed on paraffin-wax-embedded colonic sections. Sections were deparaffinised in CitriSolv (Thermo Fisher Scientific), and rehydrated through a graded series of ethanol and PBS. After washing, heat-mediated antigen retrieval in citrate buffer was performed. Following which non-specific binding was blocked using 5% normal gat serum in PBS, then the sections were incubated at 4°C overnight with rabbit anti-5-HT antibody (Immunostar, Hudson, WI) at a concentration of 1:3000. Alexa Fluor 594-goat anti-rabbit IgG (Thermo Fisher Scientific) was used as the secondary antibody. ProLong™ gold antifade mountant with DAPI (Thermo Fisher Scientific) was used for nuclear staining and mounting of the sections, which were studied with a Nikon fluorescence microscope. 5-HT positive EC cells were quantified using ImageJ (National Institutes of Health, Bethesda, MD) and expressed as number of cells/ mm^2.

RNA isolation and qPCR protocols

The frozen tissue samples, collected for RNA isolation, were placed in 10x volume of cold RNA later-ice and were allowed to soak for at least 16 hrs in -20°C. Following which the RNA was isolated using RNesay plus universal mini-

kit (Qiagen) the RNA quality and quantity was analyzed using NANOdrop (Thermo Fisher Scientific, Waltham, MA). Complementary DNA (cDNA) was prepared using the quantitect reverse transcription kit, according to manufacturer's instructions (Qiagen). 18s rRNA (M=0.496), forward: 5′-TCCACAGGAGGCCTACACGCC-3′ and reverse: 5′-TTTCCGCCGCCCATCGATGTT-3′, was the reference gene of choice, as it was found to be the most stable in comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; M= 0.548), forward: 5′-CCCTCCAAAATCAAGTGGGGCG-3′ and reverse: 5′-ATGACGAACATGGGGGCATCAGC-3′; and β-Actin (M=0.528) β-Actin, forward: 5′-ATGTGGCCGAGGACTTTGATT-3′ and reverse: 5′-AGTGGGGTGGCTTTTAGGATG-3′. TpH-1 mRNA levels were quantified using previous established methods and primer sequences[22]. Pre-validated primers were used for 5-HTT, HTR1A, 2A, 3A, 4 and 7, as well as for tumor necrosis factor (TNF)-α and interleukin (IL)-13, and S100 calcium-binding protein A9 (S100A9) according to manufacturer's instructions (Bio-Rad).

Statistical analysis

Statistical analysis was performed by means of Chi-square test, two-sided Fisher's exact test, one-way ANOVA with Dunnett's or Newman-Keuls posthoc, as

appropriate, where p<0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA).

Results

Investigating 5-HTTLPR polymorphism in IBD patients

Characterization of study participants

This portion of the study consisted of 40 HC and 40 patients, with 21(52.5%) experiencing symptoms associated with CD. The gender distribution between the two groups was comparable. The demographic information of all participants is shown in Table 1. Additionally, at the time of recruitment 17 (42.5%) patients with CD were C-reactive protein (CRP) positive (CPR+; defined as CRP > 5.1 mg/L),18 (45%) had features of reactive thrombocytosis (RT+; defined as platelet count > 450 $\times 10^9$/L) and 6 (15%) were on SSRI or some other form of antidepressants; thus were excluded from all analysis of PPP or plasma 5-HT levels.

5-HTTLPR polymorphism and CD

The genotypic distribution of the HC and patients with CD was assessed for deviation from Hardy–Weinberg equilibrium using the Chi-square test, and no deviation was observed for the 5-HTTLPR polymorphism. In addition to that, no significant association with gender were observed. Furthermore, no significant

difference in 5-HTTLPR genotype (χ^2 = 5, df = 2, p ~0.08) or allele frequency (Fisher's exact test, two-sided p= 0.05) were observed between CD and HC group. However, when grouped together given the functional dominance of the S allele [11], the S-genotype (L/S and S/S combined) was significantly higher than L/L genotype (Fisher's exact test, two-sided p= 0.04) between CD and HC (Table 2).

PPP and plasma 5-HT levels in CD patients

In comparison with the HC group, significantly higher PPP 5-HT levels were observed in patients with CD (Figure 1a). PPP 5-HT levels were not significantly different among the genotypes of HC and patients with CD (Figure 1b). However, when participants were grouped based the functional dominance of the S allele, PPP 5-HT levels in CD patients with S-genotype was significantly higher compared to HC with L/L and S-genotype (Figure 1c). When the patient group was divided into CRP+ or CRP-negative (CRP$^-$; defined as CRP < 5.1 mg/L) groups, we observed significantly higher PPP 5-HT levels in CRP+ patients in comparison with CRP$^-$ patients as well as with HC (Figure 1d). Additionally, we observed significantly higher levels of PPP 5-HT levels in patients with CD, who reported experiencing symptoms related to IBD at the time of blood donation (Figure 1e). Platelet numbers in CD patients were elevated compared to HC but the difference did not reach statistical significance. However, CD patients with symptoms had significantly higher number of platelets compared to HC (465 x 10^9/L $\pm$ 50 x 10^9/L vs. 367 x 10^9/L $\pm$ 16 x 10^9/L; mean $\pm$ SEM; p<0.05). PPP 5-HT

levels were significantly higher in CD patients irrespective of platelet count, in comparison with HC (Figure 1f). As with PPP 5-HT concentrations, plasma 5-HT concentrations were also found to be significantly higher in CD patients in comparison with HC, as well as in CRP+ and symptomatic patients (Figure 2a-c). However in contrast to PPP 5-HT levels, only patients with platelet count >450 $\times 10^9$/L (RT+), but not <450 $\times 10^9$/L (RT⁻), had significantly higher plasma 5-HT levels compared to HC (Figure 2d).

Investigating mucosal 5-HT signaling in IBD patients

Characterization of study participants

This part of the study included 25 participants, 10 of whom were HC and 15 were patients with CD. The gender distribution of the groups was similar. The demographic information of all participants is shown in Table 3.

Montreal classification, microscopic evaluation and mucosal cytokine mRNA expressions

Montreal classification of the patient cohort is presented in Figure 3a. Histological evaluation of biopsies confirmed the macroscopic assessment of the endoscopist during specimen collection (Figure 3b). For further confirmation, we measured the expression of S100A9, which dimerizes with S100A8 to form calprotectin [23]. Though low expression of tissue S100A9 was observed, it was significantly higher in inflamed tissue compared to healthy and non-inflamed tissue (data not

shown). In patients with CD, TNF-α mRNA expression was significantly higher in comparison with HC (Figure 3c). In a previous study, we had observed that IL-13 up-regulates TPH1 mRNA expression in a model of human EC cells [22]. In this study, we observed a significantly higher expression of IL-13 mRNA at sites of active inflammation in CD patients (Figure 3d).

TPH1, 5-HTT and 5-HTR expressions in CD

In patients with CD, colonic TPH1 mRNA expression was significantly higher in inflamed regions compared to non-inflamed regions, as well as controls (Figure 4a). However, 5-HT expressing EC cell numbers were not significantly different among the groups compared (data not shown). We observed a significant down-regulation of 5-HTT mRNA expression in inflamed tissue in comparison with non-inflamed and control (Figure 4b). We also observed an overall low expression of 5-HTR2A mRNA in colonic tissue, which was significantly lower in CD patients compared to HC (Figure 4c). Conversely, 5-HTR4 mRNA levels were significantly higher in CD patients, irrespective of whether the tissue was collected from inflamed or non-inflamed areas of the colon, in comparison to HC (Figure 4d). Additionally, 5-HTR3A and 7 expressions were significantly higher at sites of active inflammation in CD patients (Figure 4e and f). Furthermore, we observed minimal expression 5-HTR1A in all biopsy samples examined (data not shown).

Discussion

IBD remains incurable and its incidence is increasing, affecting 1 to 2 million people in North America and several million worldwide [24]. Canada is considered to be an epicenter of IBD, with rates of prevalence and incidence among the highest in the world [4]. The present study evaluated the association of 5-HTTLPR polymorphism with CD. Though our sample size is a limitation that must be considered, to our knowledge this is the first study to examine the relation between CD and 5-HTTLPR polymorphism in a Canadian population. We observed that S-genotype was significantly more common than L/L genotype between CD and HC. EC cells of the GI tract produce most of the 5-HT found in the periphery, including the 5-HT found in blood [7]. Once released, 5-HT is transported into surrounding epithelial cells by the 5-HTT and degraded to 5-hydroxyindoleacetic acid [7]. 5-HT originating from EC cells can enter the bloodstream and is quickly taken up by circulating platelets through the action of 5-HTT [9]. Studies have shown that the L/S and S/S genotypes were associated with lower transcriptional activity, resulting in down-regulation of 5-HTT expression, density, and efficiency. Thus, associating the S-genotype with decreased clearance of 5-HT in the circulation as well as synapses [12]. In our study, we observed that CD patients with S-genotype had reduced uptake of 5-HT by platelets resulting in increased availability of 5-HT in PPP. In the current study, we also observed significantly higher levels of PPP and plasma 5-HT in patients with CD in comparison with HC. Alterations in EC cell numbers and various

other machinery of 5-HT signaling have been associated with IBD [6]. 5-HT in the gut stimulates the submucosal sensory branch of the enteric nervous system and participates in GI motility and secretion via interneurons and motor neurons [7]. The increase in 5-HT levels observed in CD would have contributed to accelerated colonic motility and visceral hypersensitivity in patients, which would manifest as symptoms experienced by patients [13]. This is evident from our observation of CD patients experiencing symptoms having significantly higher levels of PPP and plasma 5-HT compared to HC, as well as asymptomatic patients.

In a separate cohort of patients, we also found significant up-regulation of both TPH1 and IL-13 mRNA at sites of active inflammation in patients with CD. IL-13 is a pleiotropic cytokine, that has emerged as an important mediator in UC and fistulizing CD [25]. Additionally, previous work from our laboratory have identified the role IL-13 in increasing TPH1 mRNA and protein levels in BON cells, as well as 5-HT production both in vitro and in animal models of intestinal inflammation [21, 25]. Implicating IL-13 in the increased production of 5-HT via increased availability of the rate limiting enzyme. IL-13 has also been shown to increase intestinal epithelial cell turnover and EC cell numbers [21, 25]. However, in our patient group, we did not observe a significant up-regulation in the number of EC cells (data not shown). This could potentially be due to the fact that EC cell density varies along the length of the colon and the colonic areas from where the

inflamed and non-inflamed tissues were collected also varied within our patient cohort. Moreover, the destruction of epithelial architecture observed in association with inflammation in CD may also be a contributing factor. Other important factors in IBD, such as the gut microbiota, may also influence host 5-HT production via the regulation of TPH1 expression. The role of gut microbiota in 5-HT production has already been established [27]. The findings of Yano et al. [27] are not in the context of inflammation and the precise role of the gut microbiome in IBD pathogenesis is not fully understood, but it is possible that the increase in TPH1 expression may be associated with dysbiosis observed in IBD. Notably, a recent study has also shown that increased availability of 5-HT, via 5-HTT deficiency, is associated dysbiosis [28]. In addition to the up-regulation of TPH1 mRNA at sites of inflammation, we observed a significantly decreased expression of 5-HTT mRNA, which was previously reported in patients with severe UC as well as in the inflamed mucosa compared to the healing mucosa of patients with UC [26, 27]. This may also be a contributing factor in the attenuated clearance 5-HT from the GI tract, which may ultimately be reflected in the patient's PPP and plasma 5-HT levels.

A vast number 5-HTRs mediate the diverse effects of 5-HT in the gut and five of the seven families of 5-HTR, with the exceptions of 5-HTR5 and 5-HTR6, are expressed throughout the human GI tract [7]. However to date, very few studies have investigated alterations in 5-HTRs expression in IBD. In our study, we found

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that CD is associated with up-regulation of 5-HTR4, 5-HTR7 and 5-HTR3A (essential for the formation of a functional 5-HTR3 channel [7]), with the latter two up-regulated only in the inflamed regions of the colon. Our observation of increased 5-HTR7 expression associated with inflammation in CD is in agreement with previous findings by Guseva et al [31]. The changes observed in 5-HTR expression may contribute to various symptoms experienced by patients with IBD, including abnormal gut motility and sensation of pain. These 5-HTRs, in addition to being expressed on various GI cell types, are found on immune cells and alterations observed in the expression of these receptors may potentiate the pro-inflammatory influence of 5-HT [7]. The role of 5-HT as a pro-inflammatory mediator in animal models of intestinal inflammation is well established. It has been shown that antagonism of 5-HTR3 by tropisetron and granisetron can reduce the severity of acetic acid-induced colitis in rats. Tropisetron also reduces the severity of trintrobezenesulfonic acid (TBNS)- induced colitis in rats [29–31]. We have made similar observations by antagonizing 5-HTR7 in the dextran sulfate sodium (DSS) and dintrobezenesulfonic acid (DNBS)-induced colitis models [35]. In addition to targeting receptors of 5-HT, blocking the synthesis of peripheral 5-HT using telotristat etiprate, a TPH inhibitor, has been demonstrated to attenuate intestinal inflammation [16].

In conclusion, a significant association was observed between S-genotype 5-HTTLPR polymorphism and CD. 5-HT levels were significantly higher in

patients with CD, which may be due to the increased expression of TPH1 or reduced clearance of mucosal 5-HT due to decreased expression of mucosal 5-HTT associated with the S allele of 5-HTTLPR. An increase in 5-HT availability in mucosal layer due to impairment in clearance of 5-HT associated with specific genotypes may play an important role in the pathogenesis of intestinal inflammation. We also discovered up-regulation of mucosal 5-HTR3A, 5-HTR4 and 5-HTR7 which in addition to playing a role in the manifestation of symptoms associated with CD, may perpetuate intestinal inflammation. Our findings revealed new and important information on the key elements of 5-HT signaling in CD and support the need for more studies with larger populations investigating 5-HT signaling in IBD patients with both CD and UC, which may ultimately lead to the development of new diagnostic tools and/or therapeutic strategies in the management of IBD.

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Table 1: Demographic data of healthy controls and patients with Crohn's disease in the investigation of 5-HT transporter gene-linked polymorphic region (5-HTTLPR) in inflammatory bowel disease.

	Healthy controls (HC)	Crohn's Disease (CD)
Number of participants	40	40
Mean age (range) in years	32.8 (19-62)	37.8 (19-77)
% Female	50	57.5
Mean disease duration (range) in years	-	11.6 (2-34)
Symptomatic (% of group)	-	21 (52.5)

Table 2: Distribution of 5-HT transporter gene-linked polymorphism in the healthy controls and patients with Crohn's disease.

Genotype distribution	Healthy controls (HC) n=40 (%)	Crohn's disease (CD) n=40 (%)
Wild-type (L/L)	16 (40%)	7 (17.5%)
Heterozygous (L/S)	16 (40%)	21 (52.5%)
Homozygous polymorphism (S/S)	8 (20%)	12 (30%)
S-genotype (L/S + S/S)	24 (60%)	33 (82.3%)*
Allele (no. and %)		
Allele (L)	48(60%)	35(43.8%)
Allele (S)	32 (40%)	45 (56.2%)

Healthy controls vs. patients with Crohn's disease

*Fisher's exact test, two-sided p=0.04

Table 3: Demographic data of healthy controls and patients with Crohn's disease in the investigation of mucosal 5-HT signaling in inflammatory bowel disease.

	Healthy controls (HC)	Crohn's Disease (CD)
Number of participants	10	15
Mean age (range) in years	61 (50-76)	36.5 (19-68)
% Female	40	47
Mean disease duration (range) in years	n/a	10.3 (1-28)

Figures and legends
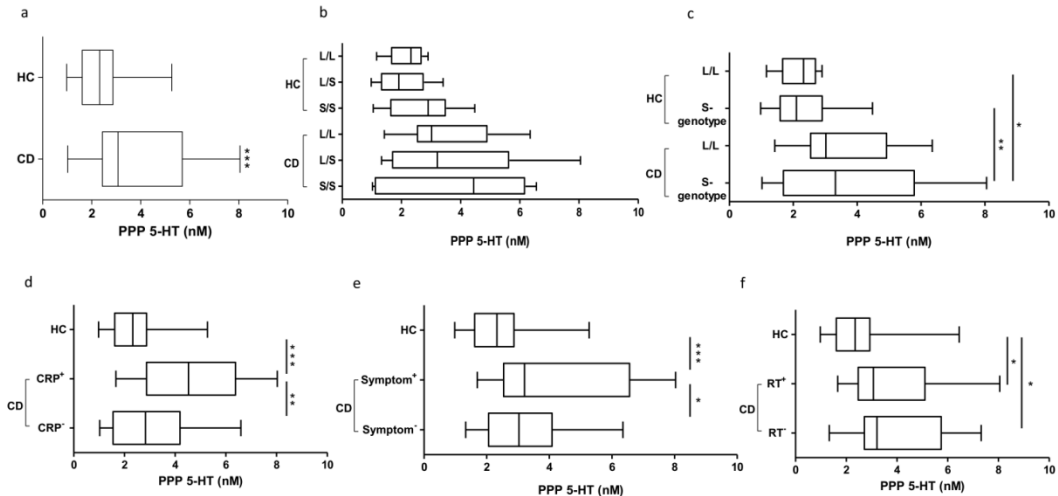
Figure 1: Comparison of platelet-poor plasma 5-HT levels in HC and patients with inflammatory bowel disease. (a) Between HC and patients with CD. (b) Among different genotypes of HC and patients with CD (c) Among L/L and S-genotype of HC and patients with CD. (d) Among HC, C-reactive protein positive (CRP+; CRP > 5.1 mg/L) and negative (CRP⁻; CRP <5.1 mg/L) patients with CD. (e) Among HC, symptomatic (Symptom+) and asymptomatic (Symptom⁻) patients with CD. (f) Among HC, CD patients with platelet count greater than 450x109/L (RT+) and CD patients with platelet count less than 450x109/L (RT⁻). Box-whisker plot representing mean, maximum and minimum. *p<0.05, **p<0.01, ***p<0.001. HC, healthy controls; CD, Crohn's disease; PPP, platelet-poor plasma; 5-HT, 5-hydroxytraptamine. RT, reactive thrombocytosis.
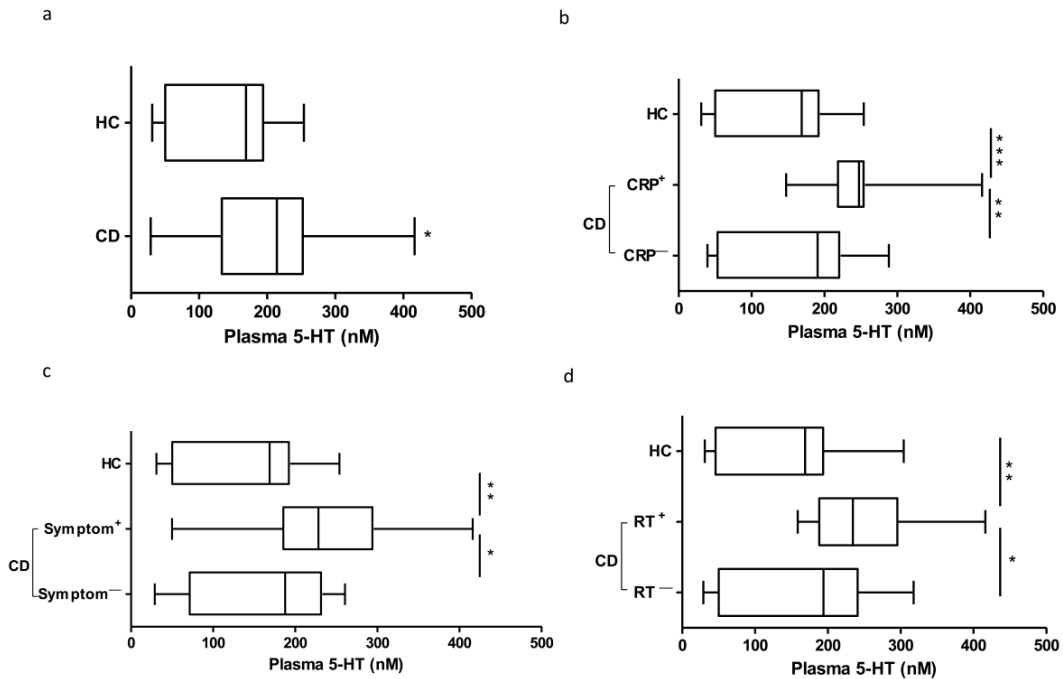
Figure 2: Comparison of plasma 5-HT levels in HC and patients with inflammatory bowel disease. (A) Between HC and patients with CD. (B) Among HC, C-reactive protein positive (CRP[+]; CRP > 5.1 mg/L) and negative (CRP[-]; CRP <5.1 mg/L) patients with CD. (C) Among HC, symptomatic (Symptom[+]) and asymptomatic (Symptom[-]) patients with CD. (D) Among HC, CD patients with platelet count greater than 450×10^9/L (RT[+]) and CD patients with platelet count less than 450×10^9/L (RT[-]). Box-whisker plot representing mean, maximum and minimum. $*p<0.05$, $**p<0.01$, $***p<0.001$. HC, healthy controls; CD, Crohn's disease; 5-HT, 5-hydroxytraptamine; RT, reactive thrombocytosis.
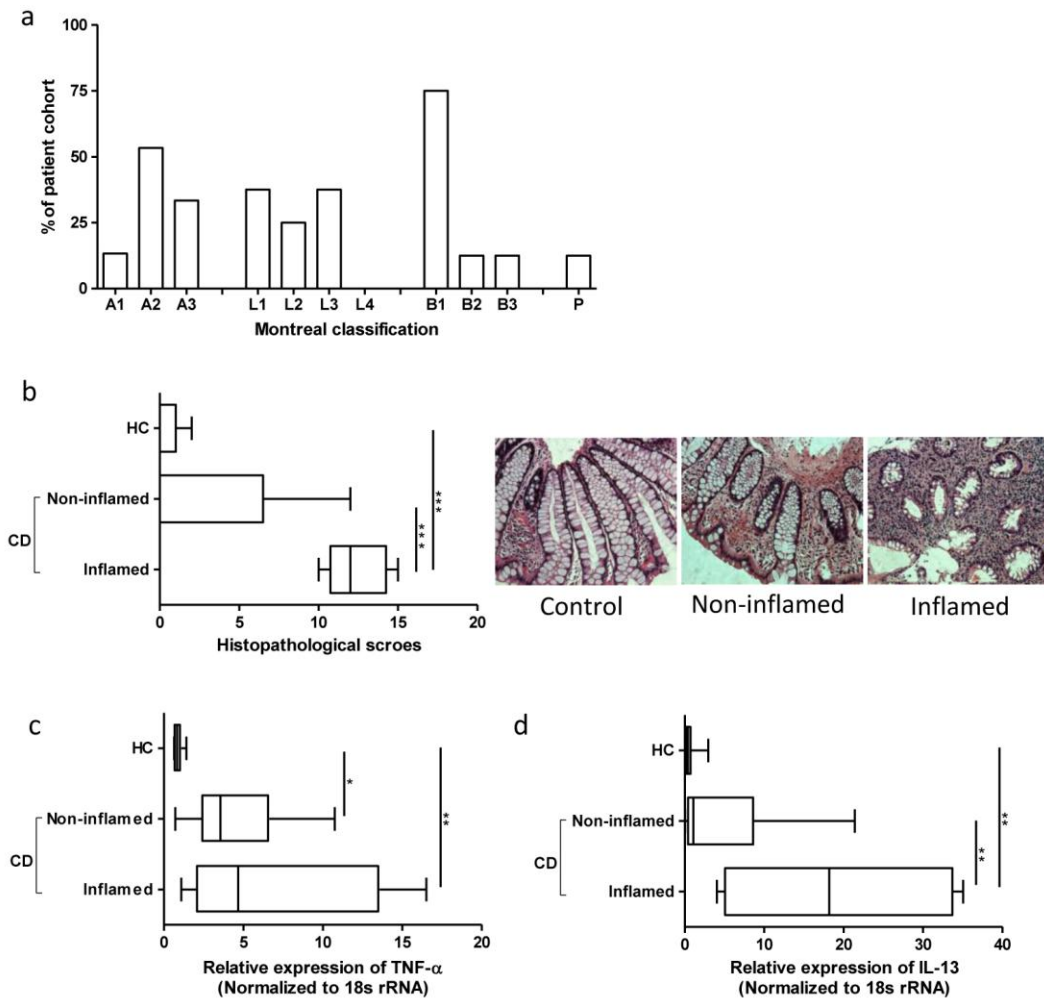
Figure 3: Montreal Classification, colonic histopathology and cytokine mRNA expression in patients with CD. (a) Montreal classification of CD patient cohort (n=15). (b) Histological assessment confirming endoscopists' assessment during biopsy collection and representative micrographs. mRNA expression of cytokines (c) TNF-α and (d) IL-13 in inflamed and non-inflamed regions of the colon. Box-whisker plot representing mean, maximum and minimum, where *p<0.05 and **p<0.01. HC, healthy controls; CD, Crohn's disease; TNF, tumor

necrosis factor; IL, interleukin.
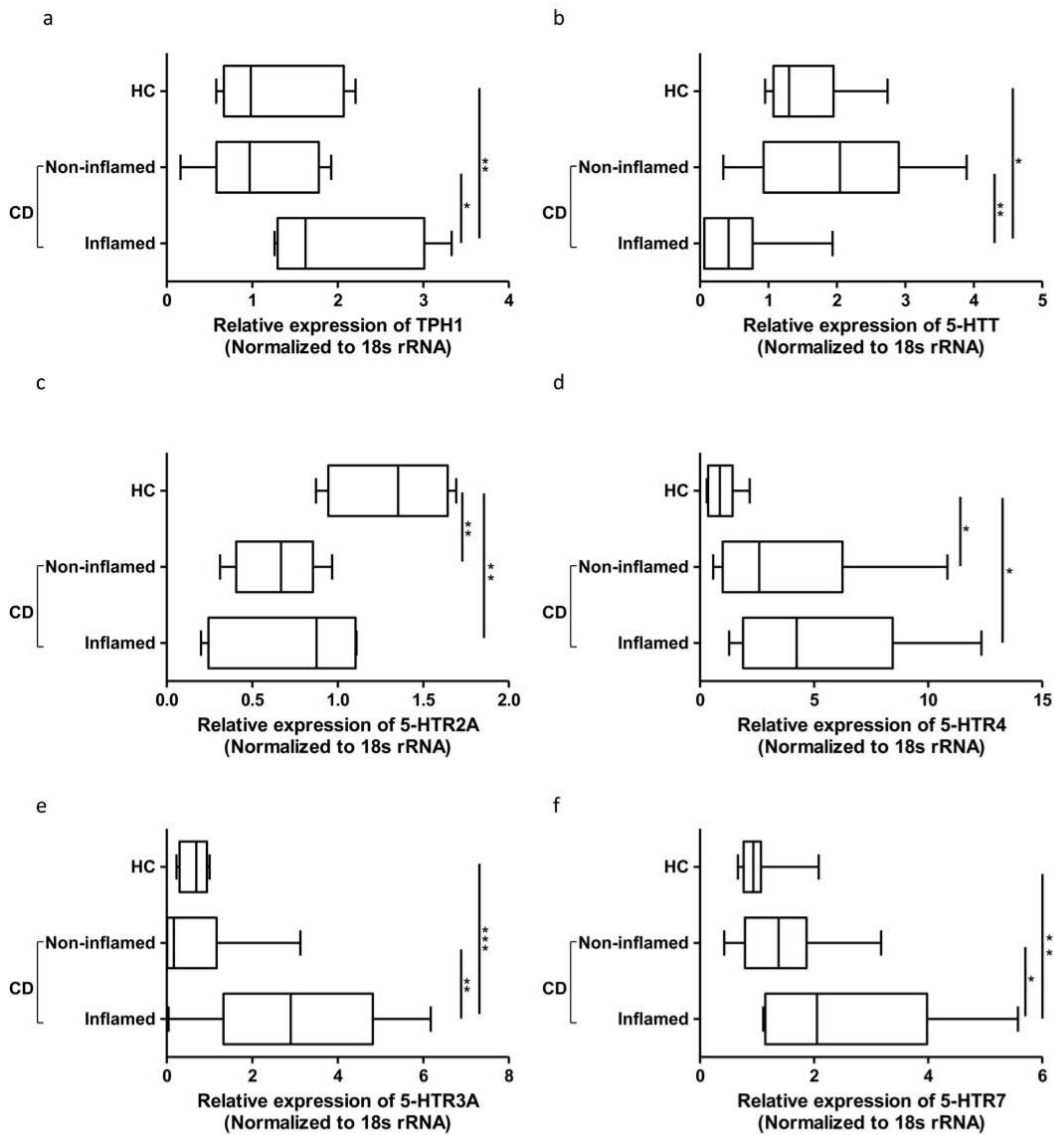
Figure 4: Alterations in key components of mucosal 5-hydroxytraptamine signaling machinery. Differences in mRNA expression of (a) TPH1 (b) 5-HTT and 5-HTR (c) 2A (d) 4 (e) 3A as well as (f) 7 in inflamed and non-inflamed

regions of the colon in patients with CD and HC. Box-whisker plot representing

mean, maximum and minimum, where $*p<0.05$, $**p<0.01$ and $***p<0.001$. TPH,

tryptophan hydroxylase; 5-HTT, 5-HT transporter; 5-HTR, 5-HT receptor.

—CHAPTER 6—

GENERAL DISCUSSION

Since its discovery in 1937 by Vittorio Erspamer, our understanding of the roles of 5-HT has come a long way. Though it is more popularly referred to as serotonin and is a known as the "happy" molecule, Erspamer's prediction of 5-HT playing an important role in gut functions is now being vindicated. Given the diversity and complexity of the 5-HT signaling machinery many more questions are being generated, in addition to the ones that remain to be answered.

Implication of our findings

The central question of this dissertation was, how immuno-endocrine interactions influence gut inflammation? To that end, we have explored how an immune mediator, IL-13, regulates an enteroendocrine mediator, mucosal 5-HT, and how this affects intestinal inflammation. Our findings showed that in *T. muris* infection-induced immune response, the Th2 cytokine IL-13 up-regulates EC cell numbers and colonic 5-HT content. IL-13 also increases TPH1 protein expression in vivo and in vitro up-regulating the bio-synthesis of 5-HT (Chapter 3). We were the first to identify the elevation of IL-13 levels in the DSS-induced model of colitis (Chapter 4). EC cell hyperplasia and increased 5-HT content had previously been reported in DSS-induced colitis [499,500]. Using IL-13-KO mice we have shown that, the increase in IL-13 underlies this up-regulation of EC cell numbers and 5-HT content observed in this model. By treating IL-13-KO mice with 5-HTP, which bypasses the function of TPH, we were able to establish that

increased 5-HT levels drive IL-13 associated intestinal inflammation in DSS-induced colitis. Other studies from our laboratory have shown that inhibiting 5-HT signaling ameliorates DSS-induced colitis [453,456]. Additionally, inhibition of peripheral 5-HT synthesis, without affecting IL-13 levels, up-regulates regulatory cytokine IL-10 production resulting in accelerated worm expulsion by *T. muris*-infected mice [453]. In Chapter 4, we have shown that 5-HT mediated recruitment and regulation of immune cell functions contributes to intestinal inflammation, which is in agreement with our previous findings as well as others [86,454]. The translational value of our findings arises from the observation that IL-13 mRNA expression is also up-regulated in inflammation associated with IBD, in conjunction with augmented TPH1 mRNA expression (Chapter 5). Our findings further implicate IL-13 in the pathogenesis of IBD, which had previously been reported by others [250,395,409]. Additionally, in a different cohort of IBD patients were able to confirm that platelet-poor plasma (PPP) and plasma 5-HT concentrations were significantly higher in comparison with HC. We also observed that PPP and plasma 5-HT concentrations in symptomatic patients were increased compared to HC and asymptomatic patients. In this cohort, we were unable to directly associate this finding with disease activity in the intestine, it does, however, indicate that increased 5-HT production may play a role in patients' experience of IBD-associated symptoms. Reactive thrombocytosis is a nonspecific response to inflammation which is known to occur in chronic

inflammatory conditions [501]. It is now well established that thrombocytosis is related to IBD activity and severity, and we found that plasma 5-HT levels were up-regulated in patients with reactive thrombocytosis compared to patients without and HC [502]. In this study, we also characterized various 5-HTR mRNA expression in the colonic biopsy specimens of patients with CD. Even though 5-HTRs are critical components of 5-HT signaling only scattered observations about 5-HTR in IBD had been previously reported by a handful of studies [457,503]. Moreover in agreement with previous studies, we observed a down-regulation of mucosal 5-HTT expression in association with inflammation in IBD [128]. We additionally observed that the S-genotype was more common among our patient group than HC, which indicates inefficiency in 5-HT re-uptake may contribute to IBD, more specifically CD, onset and/or pathogenesis. However, more studies are needed, and some of the limitations of our clinical study, such as sample size, will have to be addressed in a future study with a larger population or via meta-analytic approaches.

Our findings clearly show that IL-13 is an important regulator of the upstream end of mucosal 5-HT signaling. Multiple cellular sources of IL-13 have been identified in various models of intestinal inflammation and in human IBD [16]. Most of these cells modulate their IL-13 production in response to various IEC-derived factors, such as TSLP and IL-33 [504–506]. Increased IL-33 expression, in particular, has been associated with IBD, as well as various models of intestinal

inflammation [506]. In our study following DSS administration, we also observed

significantly up-regulated mucosal IL-33 and TSLP levels, in both WT and IL-13-

KO mice. However, the fold change in the former was much greater than the latter

(unpublished data). In addition to IL-13, altered production of various other

cytokines that affect mucosal 5-HT signaling has been reported in IBD, such as

IL-1β, TNF-α, IL-6, IL-10, and IFN-γ. It can be postulated that enhancement of

mucosal 5-HT signalling in gut inflammation may be the result of both increased

production and secretion of 5-HT influenced by inflammatory cytokines such as

IL-13 and IL-1β, as well as decreased uptake due to reduced expression/function

of 5-HTT caused by increased levels of inflammatory cytokines, i.e. TNF-α, IL-6,

IFN-γ, and low levels of regulatory cytokine, such as IL-10. Lower expression of

5-HTT or reduced efficiency associated with 5-HTTLPR may also predispose an

individual to intestinal inflammation via increased availability of 5-HT. Various

studies, including studies from our laboratory, have established that 5-HT via its

repertoire of receptors on immune cells can regulate the immune response [6]. It

is also possible, that changes in 5-HTT expression may contribute to the

breakdown of IEC-immune cell communications, which may involve

serotonylation, leading to an aberrant immune response. More studies will have

conducted to ascertain the role of serotonylation in immune and epithelial cell

mediator secretion and antigen presentation. As mentioned earlier, El Adiy et al.

have implicated diminished 5-HTT expression with dysbiosis, which when

combined with adverse early life events results in a shift toward an inflammatory-type microbiota [486]. Thus, specific 5-HTTLPR genotype associated reduced expression/function of 5-HTT may further increase the risk of developing intestinal inflammation via microbiota modulation.

IL-13/5-HT axis and other factors in intestinal inflammation

A holistic understanding of the effects of immuno-endocrine interactions in gut inflammation, particularly in IBD, will have to consider how other mitigating factors, such as the microbiota, can influence or be influenced by immuno-endocrine interactions. The gut microbiota has a critical influence on the normal development of the immune system as well as IECs. It has been shown that post-thymic education of NKT cells, is dependent on the diversity of the gut microbiota and lack of diversity may be related to gut inflammation [507,508]. IL-13 along with other inflammatory cytokines, such as TNF-α and IFN-γ, promote IEC apoptosis and detrimentally influence permeability [250,509–511]. A compromised barrier increases the risk of an inappropriate immune activation and inflammation which is the hallmark of IBD and may also lead to associated dysbiosis. Recently, it was demonstrated that the presence of two bacteria (*Escherichia coli* and *Serratia marcescens*) and one fungus (*Candida tropicalis*) was significantly higher in patients with IBD, more specifically CD, compared to their healthy relatives [512]. Additionally, it was shown that these three

microorganisms work together to produce a biofilm that adheres to portions of the intestine triggering inflammation in CD [512]. It is possible that the IL-13/5-HT axis may play an important role in microbial colonization of the normal gut as well as in the context of inflammation. As previously mentioned, 5-HTT-KO mice (increased 5-HT levels both in the gut and the brain) have a more inflammatory-type gut microbial composition and recent work from our laboratory indicates that 5-HT can regulate AMP production by colonocytes [486,513]. We observed that naïve TPH1-KO mice had significantly higher colonic production of AMPs, such as mouse β-defensins, than their WT counterparts. In addition to that, using HT-29 cells (model of human IECs), we were are able to demonstrate that 5-HT in a dose-dependent manner inhibited the production of human β-defensins [513]. CD has been associated with reduced production of AMPs, including β-defensins in Crohn's colitis [514–517]. IBD associated changes in AMP production has been linked to with pathogen recognition and autophagy, thus, future studies will have to investigate the connection between already identified IBD risk loci (NOD2, ATG16L1, etc.) and 5-HT. Significant up-regulation of calprotectin forming proteins, S100A9 and S100A8, in inflamed tissue of UC patients in comparison with non-inflamed tissue has been demonstrated [518]. Additionally, it has been reported that inflammation specific up-regulation of tissue calprotectin levels may not be reflected in the measurement of this protein in stool [519]. In recent years, FCal has gained popularity as a biomarker of inflammation in the gut.

Calprotectin is an AMP produced by neutrophils, monocytes, and IECs, which has bacteriostatic and fungistatic properties. In agreement with Reinisch et al. [518] we found that S100A9 mRNA expression was significantly up-regulated only in the inflamed areas of the colon of CD patients, just as IL-13 and TPH1 (Chapter 5). However, future studies will have to determine if the changes observed in the local enhancement of 5-HT signaling and calprotectin are causative or associative. In addition to the ability of 5-HT to effect AMP production, it can regulate mucin production, the major component of the intestinal biochemical barrier which can affect microbiota composition as well as outcomes in intestinal inflammation. We have found that pharmacological inhibition of peripheral 5-HT production in models of intestinal inflammation was associated with up-regulated MUC2 production, potentially via increased IL-10 production [453]. Conversely, evidence of microbial modulation of mucosal 5-HT signaling has been found. In the normal gut, specific gut microbes via short-chain fatty acids, such as acetate and butyrate, can up-regulate TPH1 expression in EC cells and 5-HT production as well as down-regulate 5-HTT expression [520,521]. Though this is yet to demonstrated in the context of inflammation, it is plausible these inducible and reversible modulation of 5-HT production by gut microbiota (due to dysbiosis) can occur during intestinal inflammation. Both the gut microbiota and mucosal 5-HT signaling are extremely sensitive to important environmental factors, such as diet. Studies have shown that fat rich western diet may be causing a leaky gut

with increased permeability and changes in the microbiota, contributing to the high incidence of IBD [522–524]. In animal models, high-fat diet has been shown to increase TPH1 expression and EC cell numbers and decrease intestinal 5-HTT expression, as well as to exacerbate DSS-induced colitis [525–528]. Up-regulation of mucosal 5-HT signaling, inflammatory biomarkers, as well as upper GI tract histopathology have also been reported in individuals with obesity [528–533]. Furthermore, Pendyala et al. [533] demonstrated that diet-induced weight loss in obese individuals is associated with down-regulation of pro-inflammatory cytokine production in the gut, and we have shown that inhibition of peripheral 5-HT by genetic deletion of TPH1 or by pharmacological means prevents diet-induced obesity and associated disorders [126]. In addition to that, TPH1 SNPs have been linked to with measures of obesity including BMI, waist circumference in nondiabetic controls, and weight gain during pregnancy [534]. In a future study, we plan to explore the association between TPH1 SNPs and IBD.

IL-13 and 5-HT in the management of intestinal inflammation

Clinical trials attempting to interfere with IL-13 signaling have been conducted in patients with active UC, with most having limited success. As mentioned earlier, Mannon et al. in a pilot study demonstrated that suppression of IL-13 in patients with moderate to severe UC (Short Clinical Colitis Activity Index; SCCAI ≥ 5) by IFN-β-1a (Avonex) led to better outcomes in disease activity [418]. This was a

small study with 16 participants, and the primary outcome evaluated was a drop in the SCCAI of three or more points (clinical response). 11 of the 16 patients reached the primary outcome and had significantly reduced production of IL-13 by LP T cell, whereas, five were non-responsive to treatment and there were no observable changes in IL-13 production. Moreover, seven patients reported remission of symptoms during the 12 week-long trial. The study also identified that UC patients with increased IL-17 and IL-6 production represent a subset who are less receptive to IFN-β therapy. In a phase IIa clinical study with moderate to severe UC patients, tralokinumab (a potent IL-13 neutralizing antibody) was administered as an add-on therapy for whom standard treatments were failing [535]. The study failed to reach statistical significance between the placebo and treatment groups for the primary (clinical response) and secondary (clinical remission and mucosal healing) endpoints measured during the 8-week long trial. However, a higher percentage of patients receiving tralokinumab attained clinical remission and mucosal healing in comparison with placebo. In another phase IIa clinical study with patients with mild to moderate UC, Anrukinzumab (an antibody that binds to IL-13 and inhibits signaling by preventing attachment to IL-4Rα) failed to achieve a significant difference in the primary endpoint measured [518]. For this study the primary endpoint was a significant reduction in FCal levels compared to baseline. This study used three different doses (200, 400 and 600 mg) and found worse outcomes at higher doses. Additionally, the lowest

dose tested, demonstrated lower FCal levels relative to placebo at all time points except for week 14 (statistically significant at weeks 4, 8 and 12), these patients also had the most improved rectal bleeding subscores. Patients receiving 400 mg of anrukinzumab had the highest rate of mucosal healing and the largest proportion of patients with improved Mayo endoscopic subscores at week 14. All the studies highlighted above were not powered to detect statistical differences in the clinical endpoints, thus the small positive association between clinical remission and treatment conditions observed need to be interpreted with caution. However, they bring to attention several key points, 1) there is a tremendous degree of heterogeneity in IBD, 2) they provide proof of concept that IL-13 is involved in IBD pathogenesis, 3) targeting IL-13 alone in the management of IBD is not sufficient, and 4) multiple inflammatory mediators will have to be targeted for better management of IBD. The last observation is further supported by the failure of the revolutionary anti-TNF therapy in approximately a third of IBD patients [438].

To date, a TPH1-specific inhibitor does not exist and no clinical studies have been undertaken to determine the safety and efficacy of blocking mucosal 5-HT synthesis in IBD management. However, non-specific TPH inhibitors that cannot enter the nervous system, which therefore act as TPH1 inhibitors, have been generated. As mentioned earlier, work from our laboratory and others have shown that these inhibitors significantly deplete 5-HT from the small and large intestine

without affecting neuronal 5-HT in various models of intestinal inflammation [452,453]. Moreover, these TPH inhibitors were shown to reduce the severity of colitis as measured by symptoms, histopathology score, and pro-inflammatory cytokine production. The high rate of side effects and toxicity of currently available medication for IBD are major issues in IBD management [438]. For example, side effects of anti-TNF therapy range from minor symptoms such as headaches and joint pain to increased risk of serious infections and cancer. A well tolerated orally administered peripheral TPH inhibitor (LX-1031) has already been shown to be effective in phase II clinical trial for non-constipating IBS, without any serious side-effects [482]. Even though this new class of drugs is at the preclinical stage, the low rate of adverse events reported is promising as they can potentially be implemented in IBD management. Taken together, the findings of the basic and clinical studies provide proof of concept of a potential new therapy for IBD, and a justifiable reason to further evaluate this novel class of drugs as a treatment for IBD.

Furthermore, increased concentrations of blood 5-HT levels in association with IBD has been reported by us (chapter 5) and others [446,445]. As nearly all of this 5-HT originates from the gut, measurement of blood 5-HT levels may have potential diagnostic value as a marker of gut-specific inflammation. Future studies will have to address many questions before blood 5-HT concentrations can be included in the array of diagnostic tools used in IBD assessment and management.

Some of the questions that will have to be answered include are differentiating levels of 5-HT concentrations for different forms of IBD, the range associated with active and inactive disease, if there are differences in 5-HT levels depending on the location of the disease, etc.

Concluding statements

This thesis advances our understanding of immune-endocrine interactions that generate or perpetuate intestinal inflammation, mainly that 5-HT has a pivotal mediating role in IL-13 driven intestinal inflammation and that enhancement of mucosal 5-HT signaling may have a genetic basis. Nevertheless, more information is needed to better define and expand upon these findings in the basic science and clinical realms. I have discussed some of these objectives and implications here, which may ultimately lead to the development of new therapeutic and/or diagnostic strategies in combating intestinal inflammatory disorders, including IBD.

—APPENDIX I—

THE ROLE OF SEROTONIN AND ITS RECEPTORS IN ACTIVATION OF

IMMUNE RESPONSES AND INFLAMMATION

The role of serotonin and its receptors in activation of immune responses and inflammation.

Shajib MS, and Khan WI.

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ACTA PHYSIOLOGICA

REVIEW

The role of serotonin and its receptors in activation of immune responses and inflammation

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Abstract

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter and hormone that contributes to the regulation of various physiological functions by its actions in the central nervous system (CNS) and in the respective organ systems. Peripheral 5-HT is predominantly produced by enterochromaffin (EC) cells of the gastrointestinal (GI) tract. These gut-resident cells produce much more 5-HT than all neuronal and other sources combined, establishing EC cells as the main source of this biogenic amine in the human body. Peripheral 5-HT is also a potent immune modulator and affects various immune cells through its receptors and via the recently identified process of serotonylation. Alterations in 5-HT signalling have been described in inflammatory conditions of the gut, such as inflammatory bowel disease. The association between 5-HT and inflammation, however, is not limited to the gut, as changes in 5-HT levels have also been reported in patients with allergic airway inflammation and rheumatoid arthritis. Based on searches for terms such as '5-HT', 'EC cell', 'immune cells' and 'inflammation' in pubmed.gov as well as by utilizing pertinent reviews, the current review aims to provide an update on the role of 5-HT in biological functions with a particular focus on immune activation and inflammation.

Keywords 5-HT, 5-HT receptors, colitis, immune response, inflammation, serotonin.

The phylogenetically conserved monoamine serotonin or 5-hydroxytryptamine (5-HT) was discovered 60 years ago, first as a vasoconstrictor released by platelets during the coagulation process and soon after in the gut, as a substance that causes smooth muscle contractions (Rapport *et al.* 1948, Erspamer 1986). The gastrointestinal (GI) tract, blood platelets and the central nervous system (CNS) are the main locations of 5-HT in the mammalian body and are functionally implicated in most major organs systems including the GI tract and the CNS (Berger *et al.* 2009). Although 5-HT is best known for its role in the CNS, the vast majority of 5-HT (~95%) in the body is found in the periphery and gut-resident enterochromaffin (EC) cells are the primary source of peripheral 5-HT (Gershon & Tack 2007). 5-HT, a very basic molecule, is tightly regulated in the body as 99% of it is stored intracellular (Mohammad-Zadeh *et al.* 2008). 5-HT in interaction with its receptor families regulates various aspects of cognition, behaviour and physiology, including mood, sleep, energy balance, tissue regeneration, platelet coagulation, GI functions, as well as immunity (Meneses 1999, Baganz & Blakely 2012). The importance of 5-HT is further highlighted by the

association between deregulation of serotonergic signalling and pathogenesis of various diseases ranging from psychiatric and neurological disorders, such as depression (Coppen 1967, Artigas *et al.* 1996) and Alzheimer's disease (AD) (Tohgi *et al.* 1992, Kepe *et al.* 2006), to functional and inflammatory disorders of the GI tract, such as irritable bowel syndrome (IBS) (Bearcroft *et al.* 1998, Coates *et al.* 2004, Spiller 2007) and inflammatory bowel disease (IBD) (Bishop *et al.* 1987, El-Salhy *et al.* 1997, Coates *et al.* 2004, Khan & Ghia 2010).

Here, we review how 5-HT signalling modulates various biological functions, with a particular focus on its contribution in immune responses and inflammation.

5-HT production and metabolism

5-HT is an indolamine [3-(β-aminoethyl)-5-hydroxyindole] (Kim & Camilleri 2000) that functions as a neurotransmitter, both in the gut and in the brain, and as a paracrine messenger in the gut (Gershon & Tack 2007), as well as a hormone in the periphery (Gershon 2013). The total amount of 5-HT found in the human body is derived from only 5% of the essential amino acid tryptophan (Tyce 1990). The biosynthesis of 5-HT from tryptophan occurs in two enzymatic steps. In the first step, tryptophan is hydroxylated by tryptophan hydroxylase (TPH) to produce 5-hydroxytryptophan (5-HTP) (Walther & Bader 2003). In the second step, the newly formed 5-HTP is decarboxylated by aromatic amino acid decarboxylase (AADC) yielding 5-HT (Walther & Bader 2003). Although both TPH and AADC are necessary for the production of 5-HT from tryptophan, TPH is the rate-limiting enzyme as it has very little affinity for any other amino acids and is only found in tissue containing 5-HT (Noguchi *et al.* 1973, Tyce 1990, Champier *et al.* 1997). In addition, depletion or inhibition of TPH leads to reduced 5-HT levels, which is in contrast to AADC inhibition (Mohammad-Zadeh *et al.* 2008). TPH has two isoforms, TPH1, primary localized in EC cells, and TPH2, found in central and enteric neurones, which are responsible for initiating 5-HT production in the non-neuronal and neuronal tissues respectively (Walther & Bader 2003). Once formed 5-HT is rapidly packaged into vesicles by vesicular monoamine transporter (VMAT), which has two isoforms, VMAT1, found in neuroendocrine cells, and its neuronal isoform is VMAT2 (Weihe *et al.* 1994). 5-HT is released from these vesicles via exocytosis in a Ca^{2+}-dependent manner (Racke *et al.* 1995).

The bioavailability of 5-HT in tissue is dependent on both the rate of synthesis and metabolism (Tyce 1990); like many other biogenic amines, 5-HT is primarily metabolized by monoamine oxidase (MAO)

(Molinoff & Axelrod 1971). MAO via oxidative deamination metabolizes 5-HT and the end product of this process is 5-hydroxyindoleacetic acid (5-HIAA), which is excreted mainly in urine (Jonnakuty & Gragnoli 2008). Although 5-HT is metabolized rather rapidly, it can be protected from degradation by storage carried out mainly by serotonin reuptake transporter (SERT) (Bertrand & Bertrand 2010). SERT, like all monoamine transporters, is a twelve transmembrane domain spanning sodium-dependent transporter and is expressed in the CNS, GI tract, platelets, pulmonary and peripheral vasculature (Rudnick 2006). Dopamine transporter (DAT), organic cation transporter (OCT), noradrenaline transport (NET) and peripheral monoamine transporter (PMAT) also have the capacity, albeit at a lower affinity, to uptake 5-HT into cells they are expressed on (Baganz & Blakely 2012). Once in the cytoplasm, VMAT is needed to sequester 5-HT and in order to prevent degradation by mitochondrial MAO (Golan *et al.* 2011).

5-HT receptors

An imposing number of 5-HT receptors (5-HTR) mediate the diverse effects of 5-HT on a wide range of physiological functions. They have been detected in fruit flies, mollusks, round worms, rodents, rabbits, cats, dogs and humans (van den Berg *et al.* 2003). Eighteen genes encode for at least fifteen mammalian 5-HTRs that are divided into seven families (5-HTR1-7) based on signalling mechanisms (Barnes & Neumaier 2011). Alternative splicing, RNA editing, and homo- and heterodimerization propagate receptor heterogeneity (Barnes & Neumaier 2011). Furthermore, naturally occurring polymorphic variants of 5-HTR subtypes have been implicated as an additional source of biological diversity (Barnes & Neumaier 2011).

With the exception of 5-HTR3, which is a Cys-loop ligand-gated ion channel, all 5-HTRs belong to the G-protein coupled receptor (GPCR) superfamily, and like all other GPCRs, 5-HTRs activate an intracellular second messenger cascade (Derkach *et al.* 1989, Pauwels 2003). Adenylyl cyclase is negatively coupled with 5-HTR1 and 5-HTR5, thus activation of these receptors downregulate cyclic AMP (cAMP) (Pauwels 2003). In contrast to the two GPCR 5-HTRs mentioned above, the activation of 5-HTRs 4, 6 and 7 is associated with increased cAMP activity (Pauwels 2003). Activation of 5-HTR2 is associated with increased intracellular Ca^{2+} release brought on by the upregulation of inositol triphosphate and diacylglycerol pathways (Barnes & Neumaier 2011). As mentioned earlier, the only non-GPCR serotonin receptor is 5-HTR3, which is a non-selective cation channel most permeable to Ca^{2+}, Na^+ and K^+ (Derkach *et al.*

1989, Barnes & Neumaier 2011). Activation of this receptor leads to rapid depolarization of the plasma membrane (Kim & Camilleri 2000). Current literature suggests, only the 5-HTR3A subunits can form functional homopentameric channels and all other subunit subtypes have to heteropentamerize with 5-HTR3A subunits to form functional channels (Brüss *et al.* 2000, Barnes *et al.* 2009). Table 1 provides a summary of the 5-HTR families based on predominant distribution in body, as well as their expression on immune cells.

5-HT in biological functions

By virtue of ubiquitous expression of its receptors, 5-HT participates in various physiological functions in mammals. In the following sections, we briefly summarize the involvement of 5-HT in some of these functions, even though the role of 5-HT in the CNS is beyond the scope of this review, that is where we begin as it is central to all biological functions.

5-HT in the CNS

In the CNS, 5-HT is mainly produced in neurones originating from the raphe nuclei of the brainstem (Jacobs & Azmitia 1992). These serotonergic neurones are widely distributed throughout the mammalian brain, making the serotonergic system the single largest and if not the most complex efferent system of the CNS (Berger *et al.* 2009). Accordingly, the functions of 5-HT in the CNS are very broad, affecting physiology, cognition and behaviour. The rostral, dorsal and

the medial nuclei of serotonergic system diffusely innervate the CNS, and their projections participate in the regulation of temperature, appetite, sleep cycles, emesis and sexual behaviour (Mohammad-Zadeh *et al.* 2008, Berger *et al.* 2009), whereas the caudal nuclei send projections into the spinal cord and modulate nociception and motor tone (Mohammad-Zadeh *et al.* 2008, Berger *et al.* 2009). Even though only about 5% of the total bodily 5-HT is found in the CNS (Baganz & Blakely 2012), every cell in the brain is in close proximity to a serotonergic fibre and all regions of the CNS express 5-HTRs in a subtype-specific manner (Mengod *et al.* 2006). Individual neurones have been shown to express multiple 5-HTRs, which exert differential effects of 5-HT on them (Araneda & Andrade 1991, Mengod *et al.* 2006). In addition to its direct effects, the serotonergic system interacts with other important neurotransmitter systems, like the catecholaminergic system, further influencing the organism's physiology (D'Souza & Craig 2006). 5-HT has also been implicated in development of the CNS; here it acts a growth factor directing proliferation, organization and maturation of the developing brain (Riccio *et al.* 2008, Mück-šeler & Pivac 2011). Despite the plethora of CNS of functions influenced by 5-HT, the most clinically relevant aspect has been its involvement in the pathophysiology of neuropsychological disorders, such as AD, schizophrenia, depression and alcoholism (Mück-šeler & Pivac 2011). Thus, not surprisingly, many current antipsychotic, antidepressant and anxiolytic medications target components of 5-HT signalling system and are the major focus of various CNS drug development (Berger *et al.* 2009).

Table 1 Serotonergic receptors (5-HTR), their distribution as well as expression on rodent and human immune cells

Family	Distribution	Subtype	Type of immune cell expressed on
5-HTR1	CNS and CVS	A	Mast cells, Monocytes, M, NK cells, T cells, B cells
		B	DCs, T cells
		D	Unknown
		E	Monocytes, DCs
		F	Unknown
5-HTR2	CNS, PNS, GI tract, platelets and CVS	A	Monocytes, M, DCs, Eosinophils, T cells, B cells
		B	Monocytes, M, DCs
		C	M
5-HTR3	CNS, PNS, GI tract		Monocytes, DCs, T cells, B cells
5-HTR4	CNS, PNS, GI tract, CVS		Monocytes, M, DCs
5-HTR5	CNS		Unknown
5-HTR6	CNS		Unknown
5-HTR7	CNS, PNS, GI tract, CVS		Neutrophils, Monocytes, M, DCs, T cell, B cells

CNS, central nervous system; PNS (includes the enteric nervous system), peripheral nervous system; CVS, cardiovascular system; GI tract, gastrointestinal tract; M, macrophages; DCs, dendritic cells; NK cells, natural killer cells. Table adopted from (Ahern 2011, Baganz & Blakely 2012, Fidalgo *et al.* 2013).

5-HT in the GI tract

5-HT participates in the regulation of the digestive process, in some degree from the moment food enters the body, by transmitting taste information from the taste buds to the CNS (Huang *et al.* 2005), in digestion by regulating pancreatic enzyme secretion (Suzuki *et al.* 2001) and in defecation by regulating peristaltic reflex (Gershon & Tack 2007). The GI tract produces about 95% of 5-HT in body, with EC cells being the major contributor (90%) and a comparatively smaller amount coming from enteric neurones (10%) (Gershon & Tack 2007, Khan & Ghia 2010). EC cells are the best characterized endocrine cell of the largest endocrine organ of the human body, the gut, and function as sensors of gut content (Khan & Ghia 2010). Their specialized microvilli project out into the gut lumen and contain enzymes and transporters allowing them to respond to luminal stimuli directly or indirectly via mediator released by surrounding cells (Buchan 1999). EC cells release 5-HT in response to acetylcholine, neuronal stimulation, raised intraluminal pressure and low pH (Bulbring & Lin 1958, Spiller 2008). In the GI tract, 5-HT acts as a critical signalling molecule participating in intestinal secretion, sensation and peristalsis, where 5-HTR3 and 5-HTR4 are of special importance (Bülbring & Crema 1958, Gershon 1999). 5-HT-mediated activation of intrinsic sensory neurones, lying within the gut wall, results in peristalsis and secretory reflexes, whereas activation of extrinsic neurones by 5-HT leads to the sensation of pain and discomfort as well as nausea and vomiting (Bülbring & Crema 1958, Bulbring & Lin 1958, McPhee *et al.* 2006). The role of 5-HT in GI physiology and/or pathophysiology remains to be fully understood, as it exerts a confounding range of effects working through its various receptor subtypes found in the gut (Khan & Ghia 2010). However, 5-HT and its role in the gut and beyond is becoming the subject of immense interest as new discoveries are being made, such as the vital role of enteric neuronal 5-HT in the growth and maintenance of the intestinal mucosa as well as the enteric nervous system (Gross *et al.* 2012).

5-HT in immune response

There is now enough evidence that demonstrates 5-HT is an important regulator of the immune system. However, as 5-HT does not cross the blood–brain barrier, peripheral sources of 5-HT represent the main suppliers of this monoamine for lymphatic tissue (Baganz & Blakely 2012). Almost all of circulating 5-HT is found in the dense granules of platelets and is released following platelet activation, in response to contact with damaged endothelium or ischaemia (Maurer-Spurej *et al.* 2004, Jonnakuty & Gragnoli 2008). Platelets cannot synthesize their own 5-HT, rather using SERT they take up 5-HT from the bloodstream, which originates from EC cells (Bertrand & Bertrand 2010). Platelet contained 5-HT is the largest source for immune cells and lymphatic tissue (Baganz & Blakely 2012). Lymphatic tissues are heavily innervated with sympathetic neurones, which along with immune cells such as mast cells, monocytes/macrophages and T cells have capacity to make 5-HT (Ahern 2011) and represent additional but smaller sources of 5-HT for the peripheral immune system.

The innate immune system is the first line of defence of the overall immune system, consisting of cells and mechanisms that defend the host from invasion by foreign organisms in a non-specific manner (Janeway *et al.* 2001). Expression of 5-HTRs has been identified on rodent and human innate immune cells, which includes neutrophils, eosinophils, monocytes, macrophages, dendritic cells (DCs), mast cells and natural killer (NK) cells (Table 1) (Ahern 2011, Baganz & Blakely 2012). Additionally, of the aforementioned innate immune cells, SERT expression has been confirmed in monocytes, macrophages, DCs and mast cells (Baganz & Blakely 2012). The expression of 5-HTRs and the ability of some of these cells to uptake 5-HT highlight the role of 5-HT in shaping innate immunity (Ahern 2011). Mast cells, basophils and platelets rapidly release 5-HT in response to injury, activation of the complement system and release of inflammatory substances, such as immunoglobulin E complexes, and platelet activating factor (PAF), resulting in chemotaxis, phagocytosis and ultimately inflammation (König *et al.* 1994, Mössner & Lesch 1998, Gordon & Barnes 2003). 5-HT directly or indirectly promotes recruitment of mast cells, eosinophils, DCs and neutrophils in acute inflammation (Boehme *et al.* 2004, Kushnir-Sukhov *et al.* 2006, Müller *et al.* 2009, Duerschmied *et al.* 2013). The chemoattractant properties of 5-HT on both human and mouse mast cells are mediated by 5-HTR1A, on human eosinophils is mediated by 5-HTR2A and on mouse DCs is mediated by 5-HTR1 and 5-HTR2 pathways (Boehme *et al.* 2004, Kushnir-Sukhov *et al.* 2006, Müller *et al.* 2009). 5-HT has been shown to alter cytokine production by DCs via 5-HTR4 and 5-HTR7, as well as to modulate the differentiation of DCs from human monocytes, the phagocytic precursor of macrophages (Ahern 2011). 5-HT stimulation increases murine peritoneal macrophages' production of pro-inflammatory cytokines, such as interleukin (IL)-1 and IL-6, in a nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB)-dependent manner and enhances their phagocytic capacity via

5-HTR1A (Freire-Garabal *et al.* 2003, Ghia *et al.* 2009). Additionally, 5-HT promotes chemotaxis of monocytes to sites of inflammation by working through its 2C receptor on alveolar macrophages (Mikulski *et al.* 2010). However, activation of 5-HTR2B and 5-HTR7 has also been shown to promote anti-inflammatory macrophage polarization (de las Casas-Engel *et al.* 2013). 5-HTR1A has also been implicated in the interaction between monocytes and NK cells, and 5-HT stimulates human NK cells to upregulate interferon-gamma (IFN-γ) production and display enhanced cytotoxicity (Baganz & Blakely 2012). 5-HT also protects these cells from oxidative damage; additionally, increased NK cell proliferation has been reported in long-term users of selective serotonin reuptake inhibitors (SSRIs; drugs that inhibit the function of SERT) (Ahern 2011).

The adaptive or specific immune system involves the destruction of pathogens via antigen-specific recognition by T and B lymphocytes; this arm of the immune system is unique to vertebrates (Janeway *et al.* 2001). DCs and macrophages are part of a special class of immune cells, called antigen-presenting cells (APCs) that are involved in priming adaptive immune cells for the generation of a specific immune response (Janeway *et al.* 2001). It has been demonstrated that, depletion of 5-HT using parachlorophenylalanine (PCPA; an irreversible inhibitor of TPH) abrogates macrophages' ability to activate T cells (Baganz & Blakely 2012). Our laboratory has established that 5-HT-stimulated DCs trigger the generation of a more inflammatory adaptive immune response, and 5-HTR7 plays a vital role in this process (Li *et al.* 2011, Kim *et al.* 2013b). In addition to influencing the activation of adaptive immune cells, 5-HT can directly affect the functions of both T and B cells through its receptors expressed on them (Table 1). In addition to its repertoire of 5-HTRs, B cells also express SERT and long-term use of SSRI in humans has been reported to increase the number of B cells (Ahern 2011). Additionally, 5-HT in conjunction with PAF can activate B cells (Matsumura *et al.* 2006). Unlike B cells, T cells do not express SERT but they do express DAT and have been reported to produce 5-HT (Ahern 2011). 5-HT has been identified as an endogenous autocrine and/or paracrine signal that promotes T-cell activation and proliferation (Leon-Ponte *et al.* 2007). This is initially mediated by 5-HTR7, phosphorylation of extracellular signal-related kinase-1 and kinase-2 (ERK1/2) and inhibitor of NF-κB in T cells (Leon-Ponte *et al.* 2007). Activation of T cells leads to increased expression of 5-HTR1B and 5-HTR2A, with the former promoting T-helper (Th) cell proliferation and the latter being involved in differentiation and function (Akiyoshi *et al.* 2006, Yin *et al.* 2006, Inoue *et al.* 2011).

It was recently suggested that, uptake of 5-HT by lymphocytes may affect the secretory functions via serotonylation, a process described as the covalent linkage of 5-HT to small intracellular GTPase, such as RhoA and Rab4, by intracellular transglutaminase leading to constitutive activation of G-protein-dependent signalling pathways (Walther *et al.* 2003, Baganz & Blakely 2012). As these GTPases are also present in lymphocytes, and other immune cells, serotonylation may help explain pathophysiological effects of enhanced intracellular 5-HT transport in various inflammatory diseases. The influence of intracellular 5-HT thus far has been described in Burkitt lymphoma cells and in the production of IL-4 by basophiles (Serafeim *et al.* 2002, Schneider *et al.* 2011). More studies investigating the role of intracellular 5-HT in immune cell functions and how this may complement or abrogate 5-HTR-mediated immune cell functions are required, as their roles in the generation of an immune response are not clearly understood.

5-HT in inflammation

The gut harbours approx. 70–80% of the immune cells found in the body (Furness *et al.* 1999). 5-HT, a potent immune modulator, has an influence not only in the gut but in systemic immunity as a whole, where platelets play an important role (Fig. 1). In the following sections, we briefly review the role of 5-HT in gut and several other forms of inflammation.

5-HT in gut inflammation

Enterochromaffin cells and immune cells are in close proximity to each other in the gut, and it is now well established that one can modulate the function of the other (Yang & Lackner 2004, Khan *et al.* 2006, Wang *et al.* 2007, Motomura *et al.* 2008, Ghia *et al.* 2009, Li *et al.* 2011, Manocha *et al.* 2012, Kim *et al.* 2013b, Shajib *et al.* 2013). It therefore follows that, EC cells have been evaluated in inflammatory conditions of the gut, such as IBD. IBD encompasses two chronic, relapsing GI inflammatory diseases of unknown origin, Crohn's disease (CD) and ulcerative colitis (UC) (Manocha & Khan 2012). Various aspects of normal 5-HT signalling, including EC cell numbers and 5-HT content, have been observed to be altered in both CD and UC (Khan & Ghia 2010). EC cells isolated from patient's with CD mucosa have a greater production of 5-HT [mediated in part by Toll-like receptor (TLR)4], and patients with UC have reduced expression of SERT, and the latter is also observed in another form of intestinal inflammation, diverticulitis (Coates *et al.* 2004, Costedio *et al.* 2008, Kidd *et al.* 2009). TPH1 mRNA levels are upregulated in patients
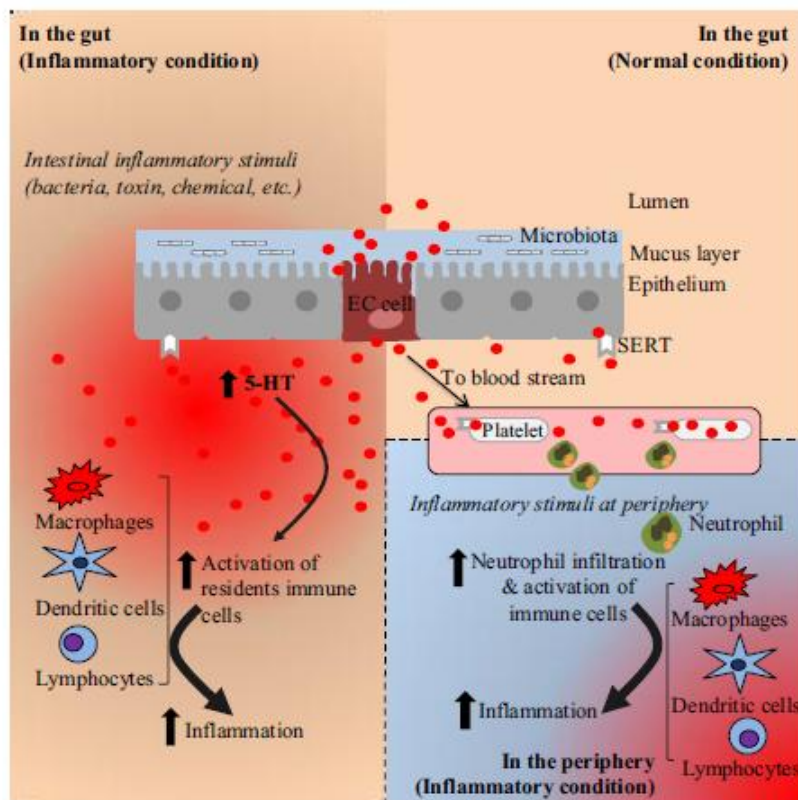
Figure 1 The role of gut-derived 5-HT in local (gut) and peripheral inflammation. 5-HT released from EC cells can act on various innate and adaptive immune cells, and under inflammatory conditions, increased 5-HT production can promote increased local (gut) inflammation. Gut-derived 5-HT can enter the blood and be stored in platelets via SERT. Once 5-HT is released by the platelets, it can participate in the pathogenesis of peripheral inflammation via its chemoattractant and immune cell activating properties. EC cells, enterochromaffin cells; Trp, tryptophan; TPH1, tryptophan hydroxylase 1; 5-HT, 5-hydroxytryptamine or serotonin; SERT, serotonin reuptake transport.

with CD in long-standing remission, who suffer from IBS-like symptoms (Minderhoud *et al.* 2007). Changes in serotonin signalling are well documented in functional GI disorder, such as IBS, and it is postulated that functional GI disorders are the result of an initial inflammatory insult on the gut that alters visceral sensitivity and/or motility (Bercik *et al.* 2005, Kim *et al.* 2013a). Altered motility causing hypoxia has been shown to induce synthesis and secretion of 5-HT by EC cells (Damen *et al.* 2013). From clinical observations, it is rather difficult to determine whether the IBD-associated changes in 5-HT signalling is the cause or effect of the immune response. However, studies have demonstrated an association between use of SSRIs with development of microscopic colitis (Khan 2013). Patients who were not previously diagnosed with IBD developed chronic diarrhoea and subsequently IBD following treatment with paroxetine for depression (Mikocka-Walus *et al.* 2006). These findings should be interpreted with a degree of caution as some of these studies did not include control groups (Mikocka-Walus *et al.* 2006). In addition, while case–control studies are able to establish an association between SSRIs and microscopic colitis, they do not establish causation. Other studies report improvements or no changes in IBD symptom scores in patients treated with SSRIs, such as paroxetine, but improvements in depression or social disability scores (Mikocka-Walus *et al.* 2006, Goodhand *et al.* 2012).

These observations suggest that more clinical studies are needed to understand the impact of SSRIs on IBD and its treatment. The more clear indication of mucosal 5-HT-driving intestinal inflammation comes from animal studies. By utilizing TPH1 knockout mice (these animal have significantly reduced production of mucosal 5-HT), in two different models of colitis [dextran sulphate sodium (DSS) and dinitrobenzenesulphonic acid (DNBS)], our laboratory has shown that 5-HT plays a key role in generation of gut inflammation (Ghia *et al.* 2009). In both models, the TPH1 knockout group had significantly reduced severity of colitis and reduced levels of colonic pro-inflammatory cytokines (Ghia *et al.* 2009). These findings were supported by the observations in SERT knockout mice, which exhibited more severe trinitrobenzenesulfonic acid (TNBS) colitis (Bischoff *et al.* 2009). We also showed that restoration of 5-HT levels in TPH1 knockout mice resulted in increased severity of DSS-induced colitis (Ghia *et al.* 2009). In addition, we have observed significantly less severe colitis in IL-13 knockout mice, which have been shown to produce significantly lower levels of mucosal 5-HT following induction of DSS colitis (Shajib *et al.* 2013). Recently, it was shown that, 5-HT through NADPH oxidase (Nox) 2-derived reactive oxygen species (ROS) increases the production of IL-6, IL-8 and monocyte chemoattractant protein-1 resulting in the initial event of inflammation, which is the adhesion of monocytes

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to colonic epithelial cells (Regmi *et al.* 2014). Further exploring the role of mucosal 5-HT in immune activation and pathogenesis of intestinal inflammation, using both acute and chronic models of DSS-induced colitis, we have shown that 5-HT stimulates 5-HTR7 on DCs to initiate innate immune mechanisms that recruits the adaptive immunity, leading to the full blown inflammation of the bowel (Li *et al.* 2011, Kim *et al.* 2013b).

Coeliac disease is another form of intestinal inflammation associated with increased EC cell numbers and mucosal 5-HT content (Challacombe *et al.* 1977, Wheeler & Challacombe 1984). Decrease in SERT expression and altered 5-HT metabolism in coeliac disease has been reported (Coleman *et al.* 2006). In stark contrast to IBD, the aetiology and pathogenesis of coeliac disease is well established; it is caused by an immune reaction to gliadin, a gluten protein found in wheat, resulting in villous atrophy and crypt hyperplasia (Manocha & Khan 2012). However, the precise role of increased 5-HT signalling observed and its immune contribution in the pathogenesis of coeliac disease requires further investigation.

The chronic low-grade inflammation that defines obesity has an intestinal component; although it is less severe than inflammation observed in IBD, it precedes significant weight gain and increased fat mass (Ding *et al.* 2010, Ding & Lund 2011, Le Beyec *et al.* 2014). Evidence supporting the association between obesity and gut inflammation is rooted in the observations of higher levels faecal inflammatory biomarkers, such as calprotectin, and endoscopic abnormalities, such as esophagitis, gastritis and ulcers in the upper GI tract of morbidly obese individuals (Csendes *et al.* 2007, Spagnuolo *et al.* 2010, Dietz *et al.* 2012, Verdam *et al.* 2013). Animal models of diet-induced obesity (DIO) also support this notion of intestinal inflammation in obesity and have implicated TLR4 signalling and intestinal macrophages in this process (de La Serre *et al.* 2010, Kim *et al.* 2012, Wang *et al.* 2013). Upregulation of mucosal 5-HT signalling was recently observed in obese individuals (Le Beyec *et al.* 2014). Additionally, models of DIO report increased TPH1 expression and 5-HT-positive EC cell numbers, which precedes weight gain, as well as downregulation of SERT (Bertrand *et al.* 2011, Le Beyec *et al.* 2014). Of note, SERT knockout mice, which are more susceptible to intestinal inflammation, are also prone to becoming obese as they as age (Chen *et al.* 2012). However, to date, studies investigating the link between 5-HT and obesity-associated intestinal inflammation are few and far between, and it is a topic that requires immediate attention as obesity in world is reaching pandemic proportion.

5-HT in other peripheral inflammation

Next to the gut, the liver is the most exposed organ to gut-derived 5-HT and it influences both glucose and lipid metabolism by the liver (Watanabe *et al.* 2014). The liver is also the primary site of 5-HT metabolism in the body, followed by the lungs (Jonnakuty & Gragnoli 2008). Although gut-derived 5-HT working via its cognate receptors 2A and 2B promotes liver regeneration, it has also been implicated liver pathophysiology (Lesurtel *et al.* 2006, 2012). 5-HT mediated activation of mammalian target of rapamycin (mTOR), and subsequent suppression of hepatic autophagy has been implicated in hepatic steatosis in a model of non-alcoholic fatty liver disease (NAFLD) (Osawa *et al.* 2011). Hepatic steatosis is the first step in the progression to inflammatory liver diseases, such as non-alcoholic steatohepatitis (NASH) (Lesurtel *et al.* 2012). Additionally, it has been suggested the excessive intracellular 5-HT degradation leading to the generation of ROS and lipid peroxides plays an important role in the pathogenesis and inflammation observed in NASH (Nocito *et al.* 2007). In viral hepatitis, 5-HT-dependent alteration of hepatic sinusoidal microcirculation results in persistence of the pathogen leading to liver cell damage and ultimately the onset of chronic hepatitis (Lang *et al.* 2008). Other organs involved in glucose and lipid metabolism where inflammatory role of 5-HT has been identified include the pancreas, where 5-HT has been shown to play a role in the onset of pancreatitis (Sonda *et al.* 2013). Additionally in white adipose tissue, it has been shown that ROS generated by 5-HT metabolism in human adipocyte promotes lipid accumulation in culture (Grès *et al.* 2013). This may ultimately lead to hypertrophy of adipocytes (5-HT is already associated with hypertrophy of cardiac cells (Villeneuve *et al.* 2009)), and hypertrophic/hypoxic adipocytes release pro-inflammatory mediators, which according to current literature are initiator of chronic low-grade systemic inflammation that defines obesity and associated disorders (Gregor & Hotamisligil 2011). 5-HT has also been shown to increase pro-inflammatory IL-1 beta and IL-8 cytokine expression in bovine adipocytes following lipopolysaccharide (LPS) stimulation (Stunes *et al.* 2011). However, the source of this 5-HT is yet to be determined, as in culture methods have additionally shown that rat adipocytes have functional serotonergic system (express TPH1, SERT and 5-HTRs) (Stunes *et al.* 2011).

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that principally attacks synovial joints, characterized by cartilage erosion and ankylosis, and is associated with increased levels of circulating 5-HT (Voog *et al.* 2004). 5-HT levels are also elevated in

affected synovial fluid, released by activated platelets, and induces synovial plasma extravasation by the release of various inflammatory mediators (Wang *et al.* 2004, Cloëz-Tayarani 2006, Seidel *et al.* 2008). In models of arthritis, it is shown that intra-articular injection of 5-HT causes joint inflammation and pain, while its depletion attenuates disease severity (Fakhfouri *et al.* 2012).

As with RA, symptomatic patients with asthma also have elevated levels of plasma 5-HT and it was found that treatment with 5-HT uptake enhancer, tianeptine, yielded clinical benefits (Lechin *et al.* 1996, 1998a,b). It has only been recently identified that platelet (gut derived), and not mast cell derived, 5-HT contributes to allergic airway inflammation (AAI) (Dürk *et al.* 2013). Furthermore, the study went on to show that 5-HT is a requirement in the bone marrow for full maturation of DCs and their Th2 priming, which characterizes allergic inflammatory response (Dürk *et al.* 2013).

The ability of gut-derived 5-HT to induce inflammation outside of the gut is probably best highlighted by its capacity to induce neutrophil recruitment by influencing endothelial cells Weibel–Palade bodies in animal models of lung inflammation, acute peritonitis, aseptic skin wounds and endotoxic shock (Duerschmied *et al.* 2013). In addition, serotonylation to activate RhoA/ROCK pathway, which subsequently downregulates efferocytosis, as defective efferocytosis has been implicated in the progression of chronic inflammatory diseases, including systemic lupus erythematosus, RA, obesity, cardiovascular disease, neurodegenerative disease, and lung diseases such as cystic fibrosis, asthma, chronic granulomatous disease, and chronic obstructive pulmonary disease (Biswas & Hoque 2013, Tanaka *et al.* 2014).

Target blocking of 5-HT to attenuate inflammation

Building on our original observation that targeted inhibition of TPH by treatment with PCPA is associated reduction in severity of DSS-induced colitis (Ghia *et al.* 2009), Margolis *et al.* has shown that reduction of mucosal 5-HT by TPH inhibitors, LP-920540 and LX1032 that do not deplete neuronal 5-HT, also reduce the severity of TNBS colitis (Ghia *et al.* 2009, Margolis *et al.* 2014). Inhibition of TPH by PCPA has been shown to reduce lung inflammation in models of allergic airway inflammation (Dürk *et al.* 2013, Bai *et al.* 2014). With regards to AAI models, 5-HTR2A antagonist, Ketanserin, has been shown to modestly downregulate inflammation as well as associated eosinophil infiltration, although the underlying mechanism is unclear (De Bie *et al.* 1998). A similar effect

has been observed with the 5-HTR2C antagonist methysergide (Lima *et al.* 2007). Ketanserin, along with another more selective 5-HTR2A antagonist ritanserin, has been shown to inhibit the production of inducible nitric oxide synthase (iNOS) in a MEK/ERK-mediated manner to curb the effects of endotoxic shock in mice (Liu *et al.* 2013). 5-HTR2A antagonist, sarpogrelate, has been shown to reduce lobular inflammation in the liver, in thioacetamide model of cirrhosis (Kim *et al.* 2013a).

5-HTR3 antagonists are the most extensively investigated group of pharmacological inhibitors of 5-HT signalling in the gut, and the list includes tropisetron, granisetron, ondansetron and ramosetron. How these antagonists produce intestinal anti-inflammatory effects remain unclear. Tropisetron and granisetron produce beneficial effects in intracolorectal model acetic acid-induced colitis by downregulating the production of inflammatory cytokines IL-1, IL-6 and tumour necrosis factor (TNF)-α (Mousavizadeh *et al.* 2009, Fakhfouri *et al.* 2010). Tropisetron has been shown to inhibit IL-2 transcription and T-cell activation; the latter is also partially achievable by ondansetron administration, and this antagonist has the ability to attenuate TNBS colitis (Vega *et al.* 2005, Mahzuni *et al.* 2012). Ondansetron and ramosetron, the latter a more potent and selective 5-HTR3 antagonist, were shown to suppress non-steroidal anti-inflammatory drug-induced intestinal lesions in a dose-dependent manner (Kato 2013). They are also capable of reducing the severity of intestinal mucositis via suppressing TNF-α expression and subsequent caspase-3/8 activation (Yasuda *et al.* 2013). Tropisetron along with another 5-HTR3 antagonist palonosetron was recently shown to be effective in curtailing NAFLD by the reduction of endotoxin translocation into the liver and subsequent hepatic steatosis and inflammation, and this anti-inflammatory effect was due to the involvement of the enteric nervous system (Haub *et al.* 2011, Ritze *et al.* 2013). Local injection of tropisetron also provided pain relief and reduced inflammation in patients with RA (Fakhfouri *et al.* 2012).

Our laboratory was the first to demonstrate that, inhibition of 5-HTR7 signalling by antagonist SB-269970 in murine models of acute and chronic DSS colitis can significantly attenuate intestinal inflammation (Kim *et al.* 2013b). It is interesting to see a recent study where inhibition of 5-HTR7 signalling appears to worsen severity of DSS colitis (Guseva *et al.* 2014). Guseva *et al.* (2014) state that the dose, route of administration and housing of animals may account for the very different results of the two studies, although they did not provide any evidence on dose, route, and housing of animals to support their conclusions. Taken together, these observations

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revealed an interesting and important area of research on targeted blocking of 5-HT signalling components and its effects on inflammation.

Conclusion

5-HT is an ancient molecule that is better known for its functions in the brain than in the periphery, even though the amount of 5-HT found in the periphery dwarfs the amount found in neurones. Not to undermine the role of 5-HT in the CNS, where it is a very important neurotransmitter, the list of physiological functions affected by peripheral 5-HT, mainly gut derived, continues to grow as our knowledge of this enteric hormone and neurotransmitter grows. This now not only includes immunity and inflammation in various disorders, but inflammation associated with ageing (Fidalgo *et al.* 2013). The influence of gut-derived 5-HT as an immune modulator reaches far beyond the gut, and the targeting of 5-HT signalling for the purposes of yielding beneficial effects in inflammatory conditions requires a better understanding of the complexity of this signalling system. In addition, there are now emerging evidences that gut microbiota plays an important role in pathogenesis of various diseases including inflammatory disorders (Penders *et al.* 2007, Cani *et al.* 2008, Sokol *et al.* 2008, Macfarlane *et al.* 2009). Due to strategic location of EC cells in the epithelial lining of the gut wall, it would also be interesting to investigate the interaction between EC cells and 5-HT with gut microbiota in the context of various inflammatory disorders. If the gut is considered the second brain, next to the CNS (Gershon 1998), then the immune system has to be considered as the mobile brain of our body and 5-HT is the common language spoken by all three brains. The ubiquitous expression of 5-HTRs and the newly defined process of serotonylation reaffirm the importance of this molecule not only as a regulator of various physiological functions but as a regulator of the individual.

Conflict of interest

None.

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—APPENDIX II—

DIVERSE EFFECTS OF GUT DERIVED SEROTONIN IN INTESTINAL

INFLAMMATION.

Diverse effects of gut derived serotonin in intestinal inflammation.

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ACS Chemical Neuroscience

Diverse Effects of Gut-Derived Serotonin in Intestinal Inflammation

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ABSTRACT: The gut is the largest producer of serotonin or 5-hydroxytryptamine (5-HT) in the human body, and 5-HT has been recognized as an important signaling molecule in the gut for decades. There are two distinct sources of enteric 5-HT. Mucosal 5-HT is predominantly produced by enterochromaffin (EC) cells of the gastrointestinal (GI) tract, and neuronal 5-HT in the gut is produced by serotonergic neurons of the enteric nervous system (ENS). The quantity of mucosal 5-HT produced vastly eclipses the amount of neuronal 5-HT in the gut. Though it is difficult to separate the functions of neuronal and mucosal 5-HT, in the normal gut both types of enteric 5-HT work synergistically playing a prominent role in the maintenance of GI functions. In inflammatory conditions of the gut, like inflammatory bowel disease (IBD) recent studies have revealed new diverse functions of enteric 5-HT. Mucosal 5-HT plays an important role in the production of pro-inflammatory mediators from immune cells, and neuronal 5-HT provides neuroprotection in the ENS. Based on searches for terms such as "5-HT", "EC cell", "ENS", and "inflammation" in pubmed.gov as well as by utilizing pertinent reviews, the current review aims to provide an update on the role of enteric 5-HT and its immune mediators in the context of intestinal inflammation.

KEYWORDS: 5-hydroxytryptamine (5-HT), enteric 5-HT, enterochromaffin (EC) cells, enteric nervous system (ENS), inflammatory bowel disease (IBD), inflammation
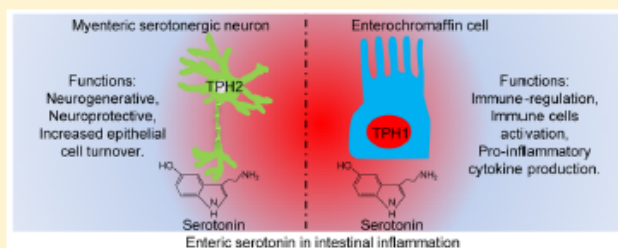
INTRODUCTION

The gastrointestinal (GI) neuroendocrine system consists of the endocrine cells in the gut mucosa and the neurons of the enteric nervous system (ENS) in the gut wall.[1] Endocrine cells of the gut, referred to as enteroendocrine cells (EECs), make it the largest endocrine organ in the human body.[2] Additionally, over two-thirds of the body's immune cells are found in the lamina propria and associated lymphoid organs of the gut.[3] The GI tract is inhabited by a complex community of microorganisms, collectively called the gut microbiota, which can influence both physiological and behavioral aspects of an individual.[4] Under normal physiological conditions, the neuroendocrine and the immune systems, as well as the gut microbiota, exist in a delicate balance among themselves, but during intestinal inflammation, such as in inflammatory bowel disease (IBD), this appears to be shifted. IBD is characterized by chronic or relapsing immune activation and inflammation in the GI tract that significantly alters GI functions.[5] There are two major forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC).[6] Given the aberrant recruitment of activated immune cells into the gut mucosa, the general consensus is that the immune system is "self-sufficient" in mitigating pathological processes in IBD, which is also evident from the current therapeutic approaches.[7] However, this assumption severely undermines the complexity of diseases like CD and UC, as well as the ENS and the enteroendocrine system and their mediators that are present in the gut.

The gut produces approximately 95% of the indoleamine serotonin or 5-hydroxytryptamine (5-HT) found within us.[8] 5-HT is a well-known neurotransmitter of the central nervous system (CNS), that is also produced by serotonergic neurons of the ENS.[9] Immune cells, such as mast cells and T cells, can also produce or have the capacity to produce 5-HT.[10] However, the amount of 5-HT produced by the enteroendocrine system overshadows all other sources of 5-HT.[2] Ninety percent of peripheral 5-HT is produced by enterochromaffin (EC) cells, a major subset of EECs.[2] This EC cell derived or mucosal 5-HT is shuttled throughout the body stored within dense granules of platelets and influences processes beyond the gut.[10] In the gut, 5-HT exerts its substantial, sometimes confounding, influence through five families of receptors expressed on various cells, including, neuron, muscle, immune, and epithelial cells.[10] Herein, we will review the aforementioned enteric 5-HT and will provide a brief overview of key immune mediators that influence enteric 5-HT in the context of intestinal inflammation.

TRYPTOPHAN AND ENTERIC 5-HT

Tryptophan (Trp) is the least abundant of all the essential amino acids.[11] It is the building block for necessary proteins
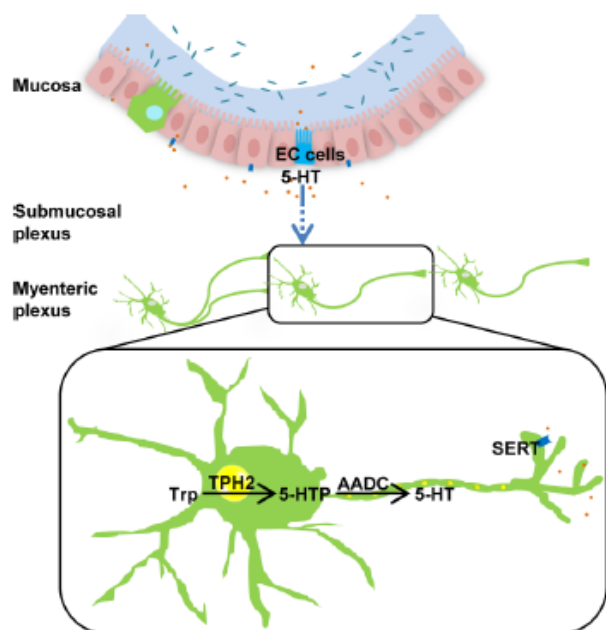
Figure 1. Schematic representation of the enteric nervous system (ENS) derived 5-HT. EC cells act as sensors of gut content and release 5-HT in response to luminal stimuli to communicate with surrounding cells and the ENS. Mucosal 5-HT activates interneurons via intrinsic primary afferent neurons, 5-HT is also a transmitter produced by myenteric interneurons. Serotonergic neurons of the myenteric plexus preferentially but not exclusively project on to each other, and TPH2 is the rate-limiting enzyme in the production of neuronal 5-HT in the ENS (inset), which plays an important role in gut motility. EC cells, enterochromaffin cells; Trp, tryptophan; TPH2, tryptophan hydroxylase 2; 5-HTP, 5-hydroxytryptophan; ADDC, aromatic amino acid decarboxylase; 5-HT, 5-hydroxytryptamine; SERT, selective serotonin reuptake transporter.

and the only source of substrate for several important molecules, including 5-HT. In mammals, the two major catabolic routes of peripheral Trp are the kynurenine (Kyn) and 5-HT pathway.[11] The Kyn pathway utilizes the majority of peripheral Trp and its downstream metabolites 3-hydroxykynurenine and quinolinic acid have been associated with inflammation and immune activation.[12−14]

The first step in the biosynthesis of 5-HT is the conversion of Trp to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH).[10] In a second enzymatic step, aromatic amino acid decarboxylase (AADC) rapidly converts 5-HTP into 5-HT.[9] There are two isoforms of TPH, TPH1, and TPH2.[10] TPH1 is predominantly found in EC cells, and TPH2 is found in all serotonergic neurons (Figures 1 and 2).[10] Therefore, TPH1 deficiency (TPH1KO) significantly reduces gut 5-HT content compared to control but does not affect neuronal 5-HT production,[15] whereas deficiency of TPH2 (TPH2KO) eliminates nearly all neuronal 5-HT but minimally influences the total gut 5-HT content, given the enormous quantities of 5-HT produced by EC cells.[15] 5-HT is a positively charged molecule at physiological pH and requires active transport to cross cell wall barriers, which is mainly performed by the selective serotonin reuptake transporter (SERT).[16] Once transported into the cell (mainly enterocytes and vascular endothelial cells[17]), 5-HT, via oxidative deamination, is primarily metabolized by monoamine oxidase (MAO) A and is expelled from the body as 5-hydroxyindoleacetic acid (5-HIAA) in urine.[10] It is important to note here that platelets express SERT but not TPH, thus they do not produce 5-HT but can take up enteric 5-HT while circulating the gut.[18−20] Both neuronal and mucosal 5-HT play pivotal roles in GI functions, and the bioavailability of 5-HT is tightly regulated.[10] This is evident from the presence of SERT on nerve terminals that release 5-HT and by the expression of SERT on every epithelial cell lining the luminal surface of the gut.[21] Given the importance of SERT in 5-HT signaling machinery and in intestinal inflammation, we describe the 5-HT transporter in greater details below.
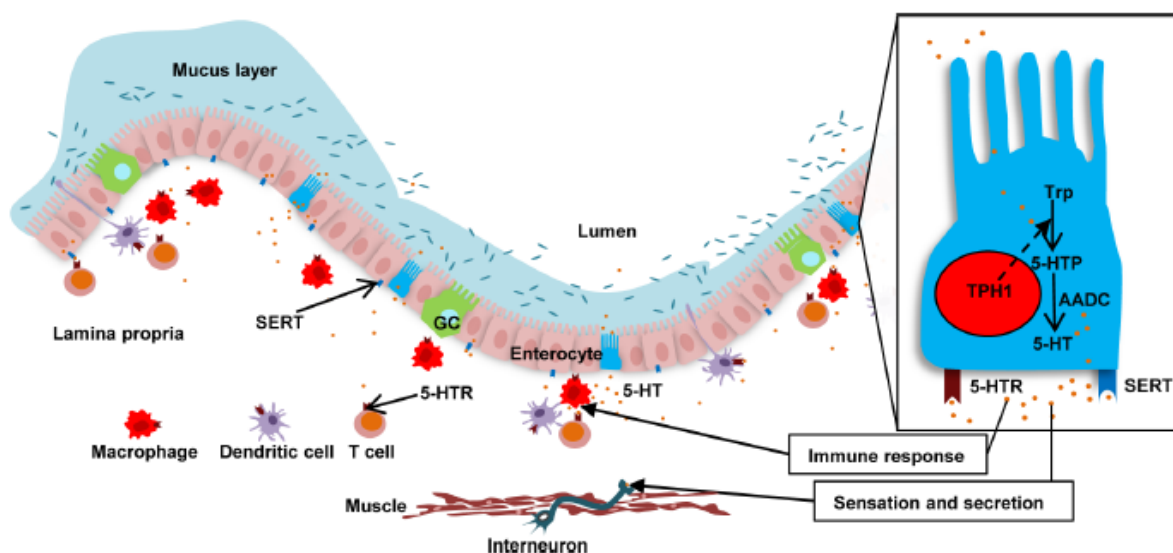
Figure 2. Schematic representation of mucosal 5-HT production and functions. EC cells of the gastrointestinal (GI) tract are the main source of mucosal 5-HT, which participates in various GI functions and in the generation of the immune response in intestinal inflammation. TPH1, found within EC cells, is the rate-limiting enzyme in the production of mucosal 5-HT (inset). EC cells, enterochromaffin cells; Trp, tryptophan; TPH1, tryptophan hydroxylase 1; 5-HTP, 5-hydroxytryptophan; ADDC, aromatic amino acid decarboxylase; 5-HT, 5-hydroxytryptamine; SERT, selective serotonin reuptake transporter; 5-HTR, 5-HT receptor; GC, Goblet cell.

5-HT RECEPTORS IN THE GI TRACT

With the exception of 5-HT receptor (5-HTR) families 5 and 6, all other families of 5-HTRs (1–4 and 7) are found in the gut.[10] These receptors demonstrate a bewildering biological diversity caused by alternative splicing, RNA editing, and homo- and heterodimerization, as well as naturally occurring polymorphic variations.[10] 5-HTR1, 2, 4, and 7, are part of the G-protein coupled receptor (GPCR) superfamily and signal via activation of intracellular secondary messenger cascade.[10] 5-HTR1 activation down-regulates cyclic AMP (cAMP), as adenylyl cyclase is negatively coupled to this receptor. 5-HTR2 activation increases intracellular Ca^{2+} release due to the up-regulation of inositol triphosphate and diacylglycerol pathways.[10] In contrast to 5-HTR1, activation of 5-HTRs 4 and 7 are associated with increased cAMP activity.[10] Unlike the other 5-HTRs, 5-HTR3 is a Cys-loop ligand-gated ion channel, most permeable to cations, such as Ca^{2+}, Na^+, and K^+, and activation of 5-HTR3 causes rapid depolarization of the plasma membrane.[22–24]

5-HTRs and Nonimmune Cells. Numerous cells of the GI tract express 5-HTRs, such as intrinsic and extrinsic afferent neurons, interneurons, inhibitory and excitatory motor neurons, interstitial cells of Cajal (ICC), smooth muscle cells, EECs, goblet cells, and enterocytes.[21,25] Once released 5-HT performs a multitude of functions, including as a paracrine mediator that transduces information from EC to intrinsic primary afferent neurons, which express 5-HTR1A, 3, 4, and 7, and to adjacent cells of the mucosa and submucosa.[26] It also plays an important role in GI motility predominantly via 5-HTR1B, 3, 4, and 7, which requires the coordinated activity of several cell types including nerves, smooth muscle cells, and ICC.[26–29] ICC are specialized cells of the GI tract essential for normal motility and express 5-HTR2B, 3, and 4.[26] 5-HT, through 5-HTR2B, plays an important role in the proliferation of ICC and network density.[29,30] 5-HT stimulates cholinergic neurons via 5-HTR2B, 3, and 4 to release acetylcholine, resulting in smooth muscle contraction, and via 5-HTR1A, 1D, and 4 on inhibitory nitrergic neurons to release nitric oxide, which results in smooth muscle relaxation.[31] 5-HT activates reflex-mediated secretory response via 5-HTR1P, 3, and 4 on neurons and via 5-HTR2 on enterocytes.[32–34]

5-HTRs and Immune Cells. Almost all human and rodent immune cells express 5-HTRs.[10] During acute inflammation, 5-HT promotes recruitment of innate immune cells, such as mast cells, eosinophils, immature dendritic cells (DCs), and monocytes. This chemotactic influence of 5-HT is mediated by 5-HTR1A on both human and mouse mast cells, by 5-HTR2A on human eosinophils, by 5-HTR1B and 2A on mouse DCs following lipopolysaccharide (LPS) stimulation, and by 5-HTR2C on mouse alveolar macrophages, which release CCL2 inducing monocyte migration.[35–38] Additionally, 5-HT, along with other platelet-derived chemotactic factors, recruits neutrophils, which express 5-HTR7, to sites of acute inflammation.[39,40]

Monocytes are the circulating precursors of macrophages and DCs and their differentiation depends on the environmental stimuli.[41] Human blood monocytes express 5-HTR1E, 2A, 3, 4, and 7.[42] Stimulation of these cells with 5-HT reduces tumor necrosis factor (TNF)-α production in a dose-dependent manner via 5-HTR4.[42] However, once activated with LPS, 5-HT stimulated monocytes increase their secretion of interleukin (IL)-1β, IL-6, and IL-8 via the combined actions of 5-HTR3, 4, and 7, as well as IL-12p40 through 5-HTR7.[42] IL-12p40, is a chemoattractant for macrophages and DCs.[42] 5-HT can stimulate the differentiation of DCs from human monocytes.[43] DCs express various 5-HTRs throughout their maturation process.[43] In mature DCs, 5-HT via 5-HTR4 and 7 regulates cytokine production, through nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB).[43,44] Furthermore, 5-HTR7 signaling is required for morphological and migratory modulation of DCs, which involves CDC42 and chemokine receptor CCR7, respectively.[45] Macrophages also differentiate from monocytes.[41] 5-HT, via 5-HTR1A, enhances the phagocytic capacity of peritoneal macrophages and significantly up-regulates IL-1β and IL-6 production in an NF-κB dependent manner.[46–48] Stimulation of 5-HTR1A on monocytes up-regulates their interaction with natural killer (NK) cells.[49] 5-HT protects NK cells from oxidative damage and promotes proliferation.[10] In addition to that, 5-HT via 5-HTR2B and 5-HTR7 promotes anti-inflammatory macrophage polarization.[50]

5-HT enhances the ability of macrophages to activate T cells and via 5-HTR7 on DCs to trigger a more pro-inflammatory T cell response.[44,49,51] Additionally, 5-HT through the repertoire of 5-HTRs expressed on T and B cells can directly influence their functions. 5-HT promotes T cell activation and proliferation, by 5-HTR7 mediated phosphorylation of extracellular signal-related kinase-1 and -2 and inhibitor of NF-κB.[52–55] This leads to the up-regulation of 5-HTR1B and 2A, where the 1B promotes T helper (Th) cell proliferation and 2A promotes differentiation and function.[52–55] 5-HT, through 5-HTR1A, also participates in B cell proliferation.[56]

ENTERIC 5-HT AND INTESTINAL INFLAMMATION

In the normal gut, neuronal and mucosal 5-HT work synergistically to regulate a variety of intestinal functions including epithelial barrier function, the transport of fluid and electrolytes, and the secretion of mucins, as well as motility.[9] The ENS is the independent, local nervous system of the GI tract. This intricate network of millions of neurons is organized into two major layers, the myenteric and the submucosal plexus, which are embedded in the gut wall.[57] Neurons of the ENS do not innervate the lumen but rather rely on EC cells that act as sensors of gut content.[2,58] The microvilli of EC cells project out into the lumen and contain enzymes and transporters; this allows them to transduce physiochemical signals from the lumen into biochemical endocrine signals and communicate with surrounding cells and the ENS primarily by discharging 5-HT.[10,59] EC cells release 5-HT both apically into the lumen and basolaterally into the lamina propria (Figure 2).[59] Enteric 5-HT either directly or indirectly participates in the regulation of GI functions, from transmitting taste information, which in the case of noxious stimuli, like geranyl and cytotoxic chemotherapy, can induce nausea and vomiting,[60,61] to regulating pancreatic enzyme secretion and peristaltic reflex.[59,62,63] Neuronal 5-HT in the normal gut plays a more prominent role in motility.[15] By utilizing both TPH1KO and TPH2KO mice, Li et al. demonstrated that intestinal transit and colonic motility were significantly reduced in TPH2KO mice, but not in TPH1KO mice.[15]

In intestinal inflammation, abnormalities associated with infiltration of activated immune cells are observed in both the ENS and enteroendocrine system. These changes, be it structural or functional, interrupt normal GI functions and manifest as persistent symptoms of IBD, such as diarrhea, cramping, and pain due to compromised barrier function,

abnormal secretion, altered patterns of motility, and visceral hypersensitivity.[64]

ENS and Its 5-HT. Structural changes in the ENS, such as axonal degradation, ganglia and nerve bundle hypertrophy or hyperplasia, glial cell hyperplasia, near total depletion of ICC, and increased expression of the major histocompatibility complex class II antigens, have been observed in association with IBD.[65−69] In CD patients, these abnormalities are observed in both inflamed and noninflamed sections of the bowel.[69] The severe and extensive necrosis of gut axons observed in CD differentiates this disease from other forms of IBD.[69] Furthermore, hyperinnervation has been suggested as a pro-inflammatory factor that may predate inflammation in IBD and may also contribute to the recurrence of CD following resection.[70,71]

"Cause−effect" relationships are difficult to establish from clinical observations. Therefore, animal models are utilized to better understand colitis pathogenesis and to test new therapeutic strategies. Similar to human IBD, structural ENS abnormalities, such as axonal hypertrophy and reduction of myenteric neuronal cells, begin rapidly at the onset of chemical models of colitis, such as dinitrobenzene- and trinitrobenzene-sulfonic acid (DNBS, TNBS)-induced colitis; and these changes persist long after the resolution of inflammation.[72−74] DNBS- and TNBS-induced colitis resemble human CD and are characterized by a prominent Th1/17 immune response marked by increased production of TNF-α, interferon (IFN)-γ, IL-12, and IL-17.[72,73,75,76] Additionally in the TNBS model, following the neurotoxic insult, rapid regeneration of axons from the surviving neurons and increased neuronal density have been observed.[70,72] The latter has also been observed in the dextran sulfate sodium (DSS)-induced model of colitis.[70] Typified by a Th1/Th17 immune response, DSS-induced colitis is additionally marked by a significant increase in Th2 cytokine IL-13 but not IL-4 and bears resemblance to the superficial inflammation and atypical Th2 immune profile associated with UC.[48,75−78] In rodents infected with *Trichinella spiralis* (*T. spiralis*), another model of gut inflammation, significantly reduced myenteric nitric oxide synthase immunoreactive neurons and macrophage-mediated transient reduction in the number of ICC have been observed.[79−81] These changes have been suggested as the underlying cause of altered motility. Notably, IL-13 plays a prominent role in the *T. spiralis*-infection model as well.[79,82,83] In myenteric ganglionitis, both in humans and the *T. spiralis* model, the onset of neuronal destruction is associated with infiltration of the myenteric ganglia by eosinophils, neutrophils, mast cells, plasma cells, and T cells.[74,80,84,85] Eosinophils and T cells have also been shown to specifically target myenteric ganglia, in TNBS-induced colitis.[86] The increased presence of immune cells during ganglionitis is suggestive of neuropathology.[85] Boher et al. demonstrated pharmacological inhibition of neutrophil recruitment, and subsequent reduction in caspase-3-dependent loss of myenteric neurons can improve colonic motility in DNBS-induced colitis.[87]

Considering the indiscriminate nature of neuronal destruction observed in IBD, it can be postulated that serotonergic neurons of the ENS are not immune to this insult. 5-HT in the ENS is produced by approximately 2% of the myenteric neurons.[88] These serotonergic neurons are found only in the myenteric plexus and preferentially synapse onto one another (Figure 1).[88−90] Neuronal 5-HT is neurogenerative in the normal gut. It was demonstrated that TPH2KO mice, but not

TPH1KO mice, had a significantly lower number total, as well as dopaminergic, neurons in the ENS compared to wild-type (WT) littermates.[91] Additionally, 5-HT in vitro promotes the development and survival of enteric neurons, including dopaminergic neurons, via 5-HTR4.[91,92] 5-HT2B may also be involved in this process,[93] as 5-HT through this receptor acts on cells of neuronal lineage to stimulate the growth of late-developing enteric neurons.[93] The neurogenerative and protective properties described above are common to all neuronal 5-HT. 5-HT was identified as the first neurotransmitter to promote adult neurogenesis in the rodent brain.[94] 5-HT regulates granule cell neurogenesis in the hippocampus of both adult and fetal rats and is neuroprotective in the hippocampus, as well as in cerebral ischemia.[94−96] Neuroprotection is hypothesized to be an important factor in reducing the severity of IBD.[71] It was reported that TPH2KO mice had increased severity of DSS-induced colitis in comparison with WT mice.[9] This was associated with significant up-regulation of pro-inflammatory cytokine, IL-1β, IL-6, and TNF-α production and increased mortality in these mice.[9] It is difficult to discern from these observations, whether the increased severity of DSS-induced colitis in TPH2KO mice is due to changes in intestinal motility or due to impaired neuroprotection resulting from reduced neuronal 5-HT.[9] Nevertheless, these findings highlight neurogenerative and potentially protective properties of neuronal 5-HT in intestinal inflammation.[97,98] In addition to promoting enteric neuronal survival and connectivity, the proliferative role of 5-HT on ICC described earlier may play an important role in restoring network density and recovery of function.[30] Furthermore, ENS derived 5-HT, via 5-HTR2A, on submucosal cholinergic neurons and their muscarinic innervations to epithelial effectors promote growth as well as turnover of the intestinal epithelium.[97] These observations by Gross et al.[97] are not in the context of inflammation, but they highlight the potential role of neuronal 5-HT in intestinal epithelial wound healing. Damage and impairment of the intestinal surface barrier are common in the course of IBD and may increase exposure to toxic and immunogenic factors leading to inflammation and the uncontrolled immune response observed in IBD.[99] It is also possible that neuronal 5-HT can influence the production of other neurotransmitters, which subsequently can modulate colitis. Collectively, these studies put forth strong evidence supporting the role of neuronal 5-HT in the development and function of the gut, as well as a protective or helpful role in recovery following inflammatory damage. However, more studies are required to further understand the role of neuronal 5-HT in inflammation as well as the effects of inflammation on serotonergic neurons of the ENS and their functions, as aberrant neurogenesis or epithelial cell turnover and growth due to altered ENS 5-HT signaling may lead to hyperinnervation that may play a role in the recurrence of IBD or may lead to colitis-associated colorectal cancer.

EC Cells and Its 5-HT. Scattered throughout the GI mucosa, EECs account for less than 1% of gut epithelial cells but comprise the largest endocrine organ of the human body.[58] 5-HT producing EC cells of the small and large intestine are a particularly well-studied type of EEC, and the highest density of EC cells are in the terminal ileum and rectum of the human GI tract.[100,101] EC cells in the gut mucosa are in contact with or in very close proximity to immune cells, and changes in EC cell numbers, as well as mucosal 5-HT content, have been associated with IBD.[2] Inflammation associated alterations at the

upstream end of the mucosal 5-HT signaling are not always observed in clinical studies, such as changes in EC cell numbers and TPH1 expression, but all studies have reported decreased mucosal SERT expression.[59] SERT expression is also significantly lower in the inflamed mucosa than the healing mucosa of IBD patients.[102]

Mucosal 5-HT signaling has also been studied in various experimental models of intestinal inflammation, such as the TNBS, DNBS, DSS, *T. spiralis*, and *Trichuris muris* (*T. muris*) model to name a few. In all these animal models, increase in EC cell numbers and mucosal 5-HT content and decreased SERT expression were observed, implicating an up-regulated mucosal 5-HT signaling.[48,102−108] Additionally, in TNBS-induced ileitis, an increase in EC cell numbers and 5-HT release were observed in noninflamed regions of the colon.[109] However, there are differences in 5-HT signaling in response to different pathogens. In *Citrobacter rodentium* infection, EC cell numbers remained unchanged but 5-HT release was up-regulated and mucosal SERT expression was down-regulated.[110] Furthermore, a decrease in EC cell numbers was reported in the duodenal mucosa of patients with giardiasis.[111] This study also suggested that 5-HT release and SERT function may be impaired in these patients.[111]

Recent studies have identified 5-HT as a key pro-inflammatory molecule in intestinal inflammation in experimental models. We demonstrated this in a seminal study from our laboratory.[112] By inducing DSS and DNBS colitis in THP1KO mice, which have significantly reduced mucosal 5-HT content, we observed a significant down-regulation in the severity of colitis and delayed onset in TPH1KO mice compared to WT mice.[112] This was accompanied by a reduction in pro-inflammatory cytokine, TNF-α, IL-1β, and IL-6 production.[112] We additionally observed that pharmacological regulation of mucosal 5-HT could regulate the severity of colitis.[112] Margolis et al.[113] showed a TPH inhibitor that reduces mucosal 5-HT without affecting neuronal 5-HT down-regulated the severity of TNBS-induced colitis. We have made similar observations in the DSS and *T. muris*-infection induced models of intestinal inflammation.[114] The pro-inflammatory role of mucosal 5-HT was further confirmed by our observations in IL-13KO mice.[48] Compared to WT mice, these mice had a significantly lower number of EC cells and lower mucosal 5-HT content, which was accompanied by reduced production of pro-inflammatory cytokines and down-regulation of DSS-induced colitis severity.[48] By treating IL-13KO mice with recombinant IL-13 and 5-HTP, we identified that increased production of mucosal 5-HT was the regulating factor in the difference observed in the severity of colitis.[48] Reduced mucosal 5-HT has been associated with reduced rolling of leukocytes, which is the first step in the recruitment of immune cells to sites of inflammation.[115,116] In DSS-induced colitis, we have observed significantly lower F4/80+ macrophage infiltration in both TPH1KO and IL-13KO mice in comparison with their respective WT littermates.[48,112] The onset of intestinal inflammation is marked by the adhesion of monocytes to colonic epithelial cells; 5-HT facilitates this process via NADPH oxidase (NOX) 2 derived reactive oxygen species.[117] Furthermore, we have shown that DCs isolated from TPH1KO mice had reduced ability in conditioning T cells to produce IFN-γ and IL-17.[44,51]

Notably, an anti-inflammatory role of mucosal 5-HT in experimental colitis was recently identified.[118] Spohn et al.[118] were able to reduce the severity of TNBS and DSS-induced colitis via intraluminal administration of 5-HTR4 agonist, tegaserod. They also identified that mucosal 5-HT, via 5-HTR4, increases epithelial cell proliferation, migration, and resistance to oxidative stress-induced apoptosis, subsequently aiding in the recovery from active colitis.[118] Findings of this study add to the protective role played by mucosal 5-HT, via 5-HTR4, which induces flushing of the bowl by increased mucosal secretion particularly in response to toxins such as cholera toxin.[119] Taken together, these findings highlight the versatility of this ancient molecule, potentially fueling more investigation into enteric 5-HT.

SERT. As evident from the body of evidence presented above, 5-HT signaling and its SERT-mediated termination are potentially involved in the pathophysiology of IBD. SERT, a member of the Na$^+$/Cl$^-$ neurotransmitter transporter family, is a critical component in 5-HT signaling.[23] In the healthy gut, SERT expression varies along the GI tract, with the highest expression observed in the ileum and the lowest in the colon.[120] SERT transcription in the intestinal epithelium differs from that of neurons suggesting tissue-specific regulation of transcription of this protein[121] Bischoff et al.[122] demonstrated the importance of SERT in intestinal inflammation. In TNBS-induced colitis, SERT-KO mice, which have significantly higher levels of mucosal and neuronal 5-HT, exhibited increased severity of colitis compared to WT mice.[122] Additionally, the severity of colitis associated with IL-10 deficiency (model of spontaneous colitis) is enhanced when the IL-10 deficiency is combined with a SERT deficiency.[123] These findings complemented our findings in TPH1KO mice. Furthermore, SERT-KO mice exhibit alternating bouts of diarrhea and constipation, resembling the symptoms of functional GI disorder, such as irritable bowel syndrome (IBS).[124] IBS is associated altered 5-HT signaling, and IBS-like symptoms are highly prevalent among IBD patients.[21,71]

Enteric 5-HT and Functional Changes Associated with Inflammation. Altered bowel movements and abdominal pain are common symptoms of functional GI disorders, for example, IBS, and IBS-like symptoms frequently precede clinical manifestation of IBD or coincide with periods of remission.[125−128] Inflammation induced structural changes lead to symptoms associated with functional abnormalities in IBD patients. IBS-like symptoms are reported by nearly one-third of the UC patients, and more than half of the CD patients in clinical remission, which is a prevalence rate that is higher than the general population.[71] IBS itself has been described as a low-grade residual inflammation caused by immune and non-immune cell mediators, including 5-HT, that influences the function of enteric and sensory afferent nerves and epithelial tight junctions[71,125,126,129,130] IBS symptoms can develop following an episode of acute infectious gastro-enteritis.[131] Following *Campylobacter jejuni* gastroenteritis, increased number of T cells and 5-HT expressing EC cells were observed in rectal biopsies of patients.[132,133,135] Increased EC cell numbers have also been identified as an important predictor of postinfectious IBS (PI-IBS) development.[136] In animal models of PI-IBS, we have observed an increase in EC cell numbers and 5-HT content in mice following infection with *T. muris*, which is driven by CD4+ T cells, and our findings were supported by Wheatcroft et al. in the *T. spiralis* model.[106,134,135] Wheatcroft et al. additionally reported a down-regulation of SERT expression in the small intestine of *T. spiralis*-infected animals, which has also been observed in the small intestine of IBS-D patients.[106,136] The role of 5-HT in

IBS-D is further supported by the therapeutic efficacy of 5-HTR3 antagonists.[137] In summary, up-regulation of mucosal 5-HT signaling provides a plausible explanation for IBS-like symptoms in IBD patients.

■ IMMUNE MEDIATORS AND ENTERIC 5-HT IN INTESTINAL INFLAMMATION

Inflammation in IBD is marked by the aberrant production of immune mediators, including various cytokines. Cytokines in IBD are being studied extensively. In comparison, studies investigating the effects of these cytokines in the regulation of enteric 5-HT signaling are lacking. Current literature suggests pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFN-γ, as well as anti-inflammatory cytokine IL-10, effect 5-HT signaling by influencing the expression and function of SERT. TNF-α, IL-6, IFN-γ, and low levels of IL-10 down-regulate epithelial SERT expression and function.[138−142] However, at higher concentrations, IL-10 up-regulates epithelial SERT expression and activity via IL-10 receptor−phosphatidylinositol 3 kinase signaling pathway.[140] Neuronal SERT function is up-regulated by TNF-α and IL-1β and is down-regulated by IL-10 and IL-6.[140,142] At the upstream end of 5-HT signaling, IL-1β enhances 5-HT secretion by colonic EC cells isolated from CD patients,[143] and we have demonstrated that IL-13 has a positive influence on the upstream end of mucosal 5-HT signaling.[144] IL-13, a pleiotropic cytokine produced mainly Th2 cells, is an important cytokine effective in UC and in fistulizing CD.[145] In the DSS and the *T. muris*-infection induced models of intestinal inflammation, up-regulated IL-13 production by CD4+ T cells was accompanied by increased colonic EC numbers and 5-HT amount.[48,77,135] Additionally, by using *T. muris* infection resistant BALB/C mice and susceptible AKR mice, which generate Th2 and Th1 immune response, respectively, we have shown that a Th2 immune profile was more capable of generating EC cell hyperplasia and increasing 5-HT content.[134]

Similar observations were made in the *T. spiralis* model and intraepithelial NK cells were the source of IL-13.[82,106] We have since described the presence of IL-13 receptor-α1 on murine colonic EC cells and a model of human EC cells (BON cells).[135,144] IL-13 also up-regulates TPH1 mRNA and protein expression, culminating in increased 5-HT content, which mediates intestinal inflammation (Figure 3).[48,144] However, the influence of these up-regulated IL-13 levels on the ENS and neuronal 5-HT remains to be understood. IL-13 is a potent regulator of eosinophil and mast cell functions,[146] and as mentioned earlier, these immune cells are prominently associated with neuronal destruction in the ENS during intestinal inflammation.

5-HT has variable effects on the cytokines mentioned above. 5-HT up-regulates IFN-γ production by human NK cells, as well as by T cells, the latter via its effects on DCs.[44,147] 5-HT increases the secretion of IL-1β and IL-6 by mature and immature DCs, respectively. In addition to that, co-stimulation with 5-HT and IL-3 reduces IL-6 secretion by human peripheral basophils.[148] Furthermore, 5-HTR2A agonist (R)-DOI blocks TNF-α mediated systemic inflammation, which includes suppression of circulating IL-6 levels.[149] 5-HTR3 antagonist, tropisetron, and granisetron have been shown to reduce acetic acid-induced colitis severity by down-regulating the production of pro-inflammatory cytokines IL-1, IL-6, and TNF-α.[150] By suppressing TNF-α expression, ondansetron and ramosetron can also curb the severity of intestinal mucositis.[150] 5-HT is an important factor in regulating Th17/T regulatory cell balance in autoimmune arthritis.[151] In intestinal inflammation, we have observed reduced IL-17 production in association with decreased 5-HT levels in IL-13KO mice.[48] 5-HT also enhances the ability of DCs to induce IL-17 production by T cells, and reduced peripheral 5-HT is associated with increased IL-10 production.[44,114] Stimulation of human peripheral basophils with IL-3 and 5-HT blocks IL-13
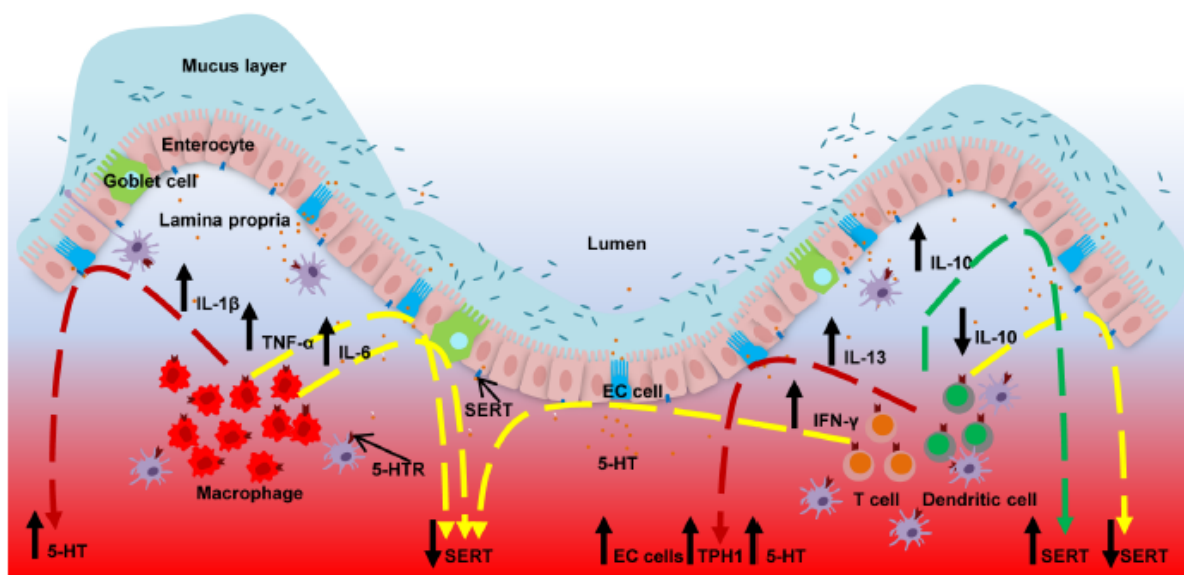
Figure 3. Influence of immune mediators on mucosal 5-HT signaling in intestinal inflammation. Evidence from clinical and animal studies indicates that immune mediators have diverse effects on mucosal 5-HT signaling. Increase in TNF-α, IL-6, and IFN-γ down-regulate epithelial SERT expression. High and low levels of IL-10 increase and decrease SERT activity, respectively. At the upstream end of 5-HT signaling, IL-1β and IL-13 upregulate 5-HT release by EC cells. IL-13, additionally, upregulates EC cell numbers and TPH1 mRNA expression. TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IL-13, interleukin-13; EC cells, enterochromaffin cells; TPH1, tryptophan hydroxylase 1; 5-HT, 5-hydroxytryptamine; SERT, selective serotonin reuptake transporter; 5-HTR, 5-HT receptor.
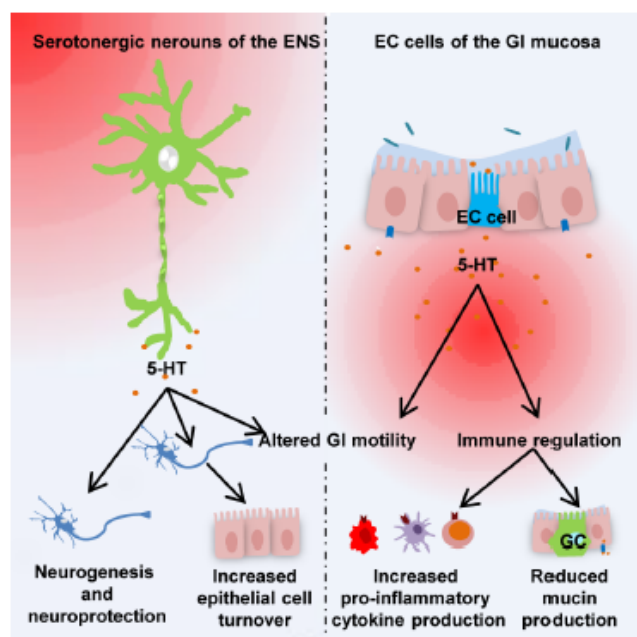
Figure 4. Diverse effects of enteric 5-HT. The influence of the two enteric 5-HT depots are different. In intestinal inflammation, EC cell derived mucosal 5-HT acts a pro-inflammatory mediator by regulating immune cell activation leading to increased pro-inflammatory cytokine and reduced mucin production. 5-HT in the ENS is produced by myenteric serotonergic neurons and is an important factor in enteric neurogenesis as well as neuroprotection. Additionally, via submucosal cholinergic neurons, neuronal 5-HT participates in epithelial cell turnover. Evidence from clinical and animal studies indicate enteric 5-HT plays an important role in altered GI motility observed in intestinal inflammation. GI, gastrointestinal; EC cells, enterochromaffin cells; ENS, enteric nervous system; 5-HT, 5-hydoxytryptamine; GC, Goblet cell.

release,[148] and unpublished data from our laboratory indicates that 5-HT in a dose-dependent manner inhibits the secretion of IL-13 by CD25− CD4+ allogeneic splenocytes.

GUT MICROBIOTA AND ENTERIC 5-HT

The gut is colonized after birth with a complex community of bacteria; for every human cell, there are 1.3 times as many bacterial cells.[152] The intestinal microbiota and host−microbiota interactions influence a number of physiological processes both within and beyond the gut. In the gut, microbial byproducts participate in GI functions, either directly by influencing the ENS or indirectly by stimulating receptors on EECs, including EC cells.[153] Specific gut microbes by producing short-chain fatty acids, such as acetate and butyrate, can up-regulate TPH1 expression in EC cells and subsequent production of 5-HT.[154,155] Yano et al.[154] reported that modulation of 5-HT by gut microbiota is both inducible and reversible and can occur at any point. Though these observations are not in the context of inflammation, they open avenues for therapeutic exploration. Regulation of enteric 5-HT production via specific microbes or microbial metabolites could potentially aid in the management of IBD by modulating the immune response. However, more concrete evidence from studies in intestinal inflammation is required.

It will be also important to explore the effect of enteric 5-HT on gut microbiota composition in relation to the regulation of colitis. As previously mentioned, we have observed that

inhibition of TPH1 in the *T. muris*-infection model is associated with increased IL-10 production.[114] IL-10 plays an important role in intestinal goblet cell hyperplasia and mucin production, as well as in the preservation of the mucus barrier in intestinal inflammation.[156,157] Though the underlying mechanisms are yet to be determined, it is possible that 5-HT mediated regulation of IL-10 production and, in turn, mucin may contribute to intestinal inflammation associated changes in gut microbiota.

CONCLUDING REMARKS

Since its discovery over six decades ago in the gut and until now, the role of enteric 5-HT has been ever expanding. Though many more questions remain to be answered, it is now generally accepted that the influence of enteric 5-HT goes beyond the gut. Within the GI tract, the synthesis and release of 5-HT can be influenced by various factors, including gut microbes. In the normal gut, it is best known for its actions in GI motility, sensation, and secretion. However, immune-mediated changes in the inflamed gut, as well as to various elements of normal 5-HT signaling, give rise to the dichotomous functions of the same molecule (Figure 4). It remains to be determined what specific role key components, such as receptors, play in mitigating the heterogeneous influences of enteric 5-HT in intestinal inflammation. In this review, we have highlighted the diverse roles of enteric 5-HT in intestinal inflammation. This presents an interesting opportunity for a potentially safe and effective treatment of IBD by targeting a single molecule, leading to not just the resolution of inflammation but the restoration of gut health.

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M.S.S. performed the literature search, wrote the article, and prepared the figures with assistance from A.B. W.I.K. supervised the review and performed the final editing of the manuscript.

ABBREVIATIONS

5-HT, 5-hydroxytryptamine; TPH, tryptophan hydroxylase; GI, gastrointestinal; ENS, enteric nervous system; EECs, enteroendocrine cells; EC cells, enterochromaffin cells; SERT, selective serotonin reuptake transporter; 5-HTR, 5-HT receptor; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome;

CNS, central nervous system; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TPH1KO, tryptophan hydroxylase 1 knockout; TPH2KO, tryptophan hydroxylase 2 knockout; IL-13KO, interleukin-13 knockout; WT, wild-type; SERT-KO, serotonin reuptake transporter knockout; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; DCs, dendritic cells; DNBS, dinitrobenzenesulfonic acid; TNBS, trinitrobenzenesulfonic acid; DSS, dextran sulfate sodium

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