ROLE OF SEROTONIN-AUTOPHAGY AXIS IN INTESTINAL INFLAMMATION

ROLE OF SEROTONIN-AUTOPHAGY AXIS IN INTESTINAL INFLAMMATION

By

SABAH HAQ, MBBS, MSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

McMaster University DOCTOR OF PHILOSOPHY (2022) Hamilton, Ontario
(Medical Sciences - Infection and Immunity)

TITLE:	Role of serotonin-autophagy axis in intestinal inflammation
AUTHOR:	Sabah Haq, MBBS, MSc
SUPERVISOR:	Dr. Waliul I. Khan
NUMBER OF PAGES:	XVI, 152

LAY ABSTRACT

Approximately 0.7% of Canadians are currently affected with inflammatory bowel disease (IBD). The gut hormone serotonin, which regulates many normal functions, is elevated in gut inflammation. Reduced serotonin levels decrease the severity of inflammation. IBD pathology has been linked to a unique cell self-eating process called autophagy. Using cell lines, mice, and samples from IBD patients, we assessed the interactions between serotonin signaling and autophagy during gut inflammation. I found that an increase in serotonin levels enhances the severity of gut inflammation by inhibiting autophagy. We also established the connection between serotonin and autophagy in the intestinal epithelial cells, and how this modulates epithelial cell function. Furthermore, we demonstrated the establishment of an altered gut microbiota upon disruption of the serotonin-autophagy axis in the epithelial cells, which subsequently influenced gut inflammation severity. Thus, we identified one of the key triggers related to the pathogenesis and severity of IBD.

ABSTRACT

Autophagy, an intracellular degradation, and recycling process is essential in maintaining cellular homeostasis. Dysregulated autophagy is linked to the pathogenesis of various diseases, including inflammatory bowel disease (IBD) which consists of Crohn's disease and ulcerative colitis. In IBD, enterochromaffin cell numbers and one of its main product serotonin (5-hydroxytryptamine; 5-HT) levels are elevated. Previously, we had shown that tryptophan hydroxylase 1 deficient (*Tph1*$^{-/-}$) mice, with reduced gut 5-HT had decreased severity of colitis. Here, we showed that gut 5-HT plays a vital role in modulating autophagy and thus regulating gut microbial composition and susceptibility to intestinal inflammation. *Tph1*$^{-/-}$ mice, had upregulated colonic autophagy via the mammalian target of rapamycin pathway (mTOR), and decreased colitis severity. *Tph1*$^{-/-}$ mice after 5-HT replenishment, and serotonin reuptake transporter deficient (*SERT*$^{-/-}$) mice, which have increased 5-HT levels, showed converse results. Deletion of intestinal epithelial cell-specific autophagy gene, Atg7, in *Tph1*$^{-/-}$ mice (DKO mice) abolished the protective effect of *Tph1* deficiency in colitis, decreased the production of antimicrobial peptide, β-defensin 1 and promoted colitogenic microbiota. Furthermore, using cecal microbial transplantation, we found that the colitic microbiota of the DKO mice contributed to the increased severity of colitis. Supporting this pathway's translational importance, we uncovered that 5-HT treatment of peripheral blood mononuclear cells from both healthy volunteers and patients with Crohn's disease inhibited autophagy via the mTOR pathway. Our results in this thesis emphasize the role of 5-HT-autophagy-microbiota axis in intestinal inflammation. Moreover, these findings suggest 5-HT as a

novel therapeutic target in intestinal inflammatory disorders such as IBD that exhibit dysregulated autophagy.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the individuals and the institution that has enabled me to reach the end of my journey as a PhD student. I have been very fortunate to work with my supervisor, Dr. Waliul I. Khan who provided me with this opportunity and excellent mentorship. Thank you Dr. Khan, for your constant guidance and positive feedback in planning experiments, analyzing the data, writing scientific documents and in overcoming the challenges. Most importantly, you have prompted me to think independently, logically and at the same time simply. For, the best teachers are the ones that make a hard topic easy to understand. Your passion and enjoyment for science has been very infectious and hopefully, I will be able to instill the same principles among my future colleagues and juniors.

I am also very grateful to my fantastic supervisory committee members, Dr. Michael Surette and Dr. Dawn Bowdish for their suggestions, helpful inputs and thoughtful discussions. Many of the roadblocks, I faced during my research were solved while discussing them with Dr. Surette and Dr. Bowdish during our meetings.

To my labmates and friends, Jensine Grondin, Yeganeh Yousefi and Suhrid Banskota, I sincerely appreciate all your help, useful insights and for always being there for me during my happy and tough times. Jensine, I will definitely miss all our discussions on life and cute baby elephant videos! Suhrid, I will always remember the enlightening debates we had about life and spirituality. A special shout of thanks to Huaqing for taking the time to teach me the lab techniques and skills that I needed to complete my experiments. I am also very thankful to all the past Khan lab members and undergrads for their support. I am very thankful to all our collaborators and funding providers, CIHR, CAG and Farncombe Family Digestive Health Research Institute for their generous support.

I am very indebted to my family, without whom nothing would have been possible. My mom and dad have always been the main source of my strength and confidence. To Mili, my mom, thank you for the daily video calls all the way from Bangladesh. To Ashraf, my dad, the scientist I have always looked up to, thank you for being the source of my inspiration. A special cheers for my 4-year-old nephew, Tahbeer, whose antics made me days during the hard pandemic times. I am also very lucky to have the support and motivation of my husband, Niaz who has taught me to become an independent person and to challenge myself. Lastly, thank you Allah for your kindness and blessings that made me capable of undertaking and completing my PhD at McMaster University.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
5-HTR	5-HT receptor
AADC	Aromatic L-alpha amino acid decarboxylase
AANAT	Aryl alkylamine N-acetyltransferase
ALDH	Aldehyde dehydrogenase
AMP	Antimicrobial peptide
AMPK	Adenosine monophosphate-activated protein kinase
APC	Antigen-presenting cell
Atg	Autophagy-related
cAMP	cyclic Adenosine mono-phosphate
CD	Crohn's disease
CgA +	Chromogranin A positive
CMA	Chaperone-mediated autophagy
DAI	Disease activity index
DC	Dendritic cell
DKO	Double knockout
DNBS	Dinitrobenzenesulfonic acid
DSS	Dextran sulphate sodium
EC	Enterochromaffin
EECs	Enteroendocrine cells

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
GCN2	General controlled non-repressed kinase
GF	Germ-free
GI	Gastrointestinal
GWAS	Genome-wide association studies
hBD	Human β-defensin
HCs	Healthy controls
HD	Human defensin
H&E	Hematoxylin and Eosin
HIF-1	Hypoxia inducible factor 1
HIOMT	Hydroxyindole-O-methyltransferase
HNP	Human neutrophil peptides
HSC70	Heat shock cognate 70 kDa protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRGM	Immunity related GTPase family M

LAMP2	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein 1A/1B-light chain 3
LPS	Lipopolysaccharide
MAO-A	Monoamine oxidase A
MAPK	Mitogen activated protein kinase
MHC II	Major histocompatibility complex II
MPO	Myeloperoxidase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEC	Necrotizing enterocolitis
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK	Natural killer
NLRP3	Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NLRP6	Nucleotide-binding oligomerization domain protein-like receptors protein 6
NOD	Nucleotide-binding oligomerization domain-containing protein
OLFR	Olfactory receptor
PAS	Phagophore assembly site
PBMCs	Peripheral blood mononuclear cells
PCoA	Principal coordinate ordination

PE	Phosphatidylethanolamine
PHA	Phytohaemagglutinin
PI3P	Phosphatidylinositol-3-phosphate
PI3K	Phosphatidylinositol-3-kinase
SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
TCR	T cell receptor
Th	T-helper
TJ	Tight junction
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF-α	Tumor necrosis factor α
Tph	Tryptophan hydroxylase
UC	Ulcerative colitis
ULK1	Unc-51 like autophagy activating kinase
VAMP8	Vesicle-associated membrane protein 8
VDR	Vitamin D receptor
Vps	Vacuolar protein sorting
WT	Wild-type

LIST OF PUBLICATIONS DURING PHD STUDIES

(*as first author or co-first author)

Haq S*, Wang H, Grondin J, Suhrid Banskota S, John K. Marshall J.K, Khan I.I, Chauhan U, Cote F, Kwon Y.H, Philpott D, Brumell J.H, Surette M, Steinberg G.R, Khan W.I. Disruption of autophagy by increased 5-HT alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease. ***Science Advances***. 2021;7(45):eabi6442.

Haq S*, **Grondin J.A***, Khan W.I. Tryptophan-derived serotonin-kynurenine balance in immune activation and intestinal inflammation. ***The FASEB Journal***. 2021;35(10):e21888.

Yousefi Y, **Haq S***, Banskota S, Kwon Y.H, Khan W.I. Trichuris muris Model: Role in Understanding Intestinal Immune Response, Inflammation and Host Defense. ***Pathogens***. 2021;10(8):925.

Banskota S, Wang H, Kwon YH, Gautam J, Gurung, P, **Haq S***, Hassan F.M.N, Bowdish D.M, Kim, J.A, Carling D, Fullerton M.D, Steinberg G.R, Khan W.I. Salicylates Ameliorate Intestinal Inflammation by Activating Macrophage AMPK. ***Inflamm Bowel Dis*** 2021;27(6):914-926

Grondin JA, Kwon Y.H, Far P.M, **Haq S***, Khan W.I. Mucins in Intestinal Mucosal Defense and Inflammation: Learning From Clinical and Experimental Studies. ***Front Immunol***. 2020;11:2054.

Haq S*, Grondin J, Banskota S, Khan W.I. Autophagy: roles in intestinal mucosal homeostasis and inflammation. ***J Biomed Sci***. 2019;26(1):19.

LIST OF PEER-REVIEWED ABSTRACTS DURING PHD STUDIES

(*as first author)

Yousefi Y, Wang H, **Haq S***, Grondin J, Surette M, Khan W.I. Enteric parasite infection-induced alteration of the gut microbiota regulates intestinal goblet cell biology and mucin production via TLR2 signaling. Canadian Digestive Disease Week, 2021.

Banskota S, Khan W.I, **Haq S***, Kwon Y.H, Singh G, Habtezion A, Brim H, Ashktorab H. Preventive effects of saffron's anti-inflammatory properties in a colitis mouse model are likely vehiculated through the gut microbiome modulation. Digestive Disease Week, 2021.

Haq S*, Wang H, Kim J, Kwon E, Banskota S, Grondin J, Hassan N, Brumell J, Philpott D, Khan W.I. Role of serotonin-autophagy axis in regulation of epithelial cell function and microbiota composition in gut. Canadian Digestive Disease Week, 2020.

Grondin J, Wang H, **Haq S***, Kwon E.Y, Surette M, Khan W.I. Protective effects of *Akkermansia muciniphila* on intestinal barrier function and inflammation. Canadian Digestive Disease Week, 2020.

Haq S*, Wang H, Kim J, Steinberg G, Brumell J, Khan W.I. Role of serotonin-autophagy axis in intestinal inflammation. Research Topics in GI Disease XVIII Meeting, Canadian Digestive Disease Week, 2019.

LIST OF AWARDS DURING PHD STUDIES

Canadian Association of Gastroenterology PhD studentship award ($40,000), 2019-2021

Farncombe studentship award ($45,000), 2018-2021

Graduate Student Publication Award, Faculty of Health Sciences, McMaster University

Internal thesis fellowship ($5000), 2020-2021

Ontario Graduate Scholarship ($15,000), 2018-2019

McMaster Medical Sciences Graduate Scholarship ($6,600), 2017-2018

Research Scholarship at McMaster University ($11,721), 2017-2018

McMaster Medical Sciences Entrance Scholarship ($1000), 2017-2018

CHAPTER 1

Literature overview

1. Autophagy

Autophagy is a catabolic process of delivering and degrading cytoplasmic constituents, organelles and infectious agents to the lysosome.[1-3] Autophagy is a highly conserved universal mechanism that participates in a range of physiological processes.[1] It is crucial in response to starvation, tissue remodeling, breakdown of cellular debris, turnover of damaged organelles, tumour suppression, regulation of immunity, and cell death.[4,5] Basal autophagy contributing to routine cell turnover is maintained in most tissues. Polyubiquitinated protein aggregates, formed during stress, aging, and disease are cleared by the autophagic process. Additionally, during infection autophagy degrades invading pathogens, suppresses the release of pro-inflammatory cytokines, participates in antigen presentation and lymphocyte development.[6] There are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA).[1,4] In macroautophagy double membrane-bound vesicles called autophagosomes are formed in the cytoplasm that engulf cytoplasmic constituents and organelles. In microautophagy the target components are directly engulfed and degraded in the lysosome. In CMA, cytosolic chaperone heat shock cognate 70kDa protein (HSC70) binds to a KFERQ-like pentapeptide motif of the target protein that promotes the translocation of the targets into the lysosomal lumen across lysosomal membranes.[4,7] The mechanisms and functions of macroautophagy has been extensively studied and linked to inflammatory bowel disease (IBD) disease pathogenesis. I am focusing on macroautophagy and it will be referred to as autophagy.

The hallmark of autophagy is the synthesis of autophagosome, a double membrane bound vesicle-containing portions of cytoplasm. Autophagosome formation occurs through three main stages: initiation, nucleation and expansion of the isolation membrane in the phagophore assembly site (PSA). The process of autophagy involves more than 30 proteins.[5] The isolation membrane is also called phagophore. Stress signals like nutrient limitation, specific cargo like defective organelles and microorganisms activate the Unc-51 like autophagy activating kinase (ULK) complex through adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR). AMPK activates autophagy while mTOR inhibits it. ULK1 complex recruits class III phosphatidylinositol-3-kinase (PI3K) complex. The PI3K converts phosphatidylinositol to phosphatidylinositol-3-phosphate (PI3P) at the phagophore marking the nucleation stage. The PI3P serves as a platform for downstream autophagy proteins to connect to the phagophore membrane, allowing it to be deformed and bent. Two ubiquitin-like enzymes, autophagy related (Atg) 7 and Atg10 activate and form the third ubiquitin like enzyme, Atg12-5-16L1 complex. The PI3P binds to the Atg12-5-16L1 complex. Simultaneously the protease Atg4 cleaves cytoplasmic microtubule-associated protein 1 light chain 3 (LC3) to LC3-I. The ubiquitin-like enzymes Atg7, Atg3 and Atg12-5-16L1 complex conjugates phosphatidylethanolamine to the LC3-I protein to form LC3-II. LC3-II is hydrophobic and is easily incorporated in the phagophore membrane elongating and closing the phagophore membrane to form a double-membrane autophagosome. In selective autophagy adaptor proteins such as p62 bind to ubiquitinated proteins and organelles and attach them to LC3-II, bringing them into the autophagosome.

p62 has been found to accumulate in autophagy deficient cells.[8] The autophagosome then fuses with the lysosome mediated by proteins like lysosome-associated membrane protein 2 (LAMP2) to form autolysosome. In the final stage, the contents are broken down by lysosomal hydrolases and released into the cytoplasm.[1,7,9-11]

Autophagy in absorptive enterocytes prevent barrier invasion by pathogens like *Salmonella*, adherent-invasive *E. coli* and opportunistic pathobiont *Enterococcus faecalis*.[12,13] Starvation induced autophagy decreases intestinal epithelial permeability that maintains the barrier.[14] Specialized epithelial cells like Paneth cells, goblet cells and enteroendocrine cells (EECs) are also regulated by autophagy. In the Paneth cells of Atg16L1 and Atg5 hypomorphic mice, aberrant granule exocytosis interferes with the release of antimicrobial peptides (AMPs). A similar change in Paneth cells in Crohn's disease (CD) patients carrying the Atg16L1 risk allele was shown.[15] In Paneth cells of intestinal epithelial cell (IEC) specific Atg7 conditional knockout ($Atg7^{\Delta IEC}$) mice, granule size is reduced and lysozyme staining is decreased.[16] In goblet cells, autophagy is critical for the formation and secretion of mucin.[17] Partial loss of autophagy in the $Atg5^{+/-}$ mice lead to goblet cell hyperplasia, disturbance in the secretory pathway and mucus layer defect.[18] Autophagy also regulates the differentiation of EECs from stem cells along with the production of EEC peptide chromogranin.[17,19] Furthermore, autophagy in the intestinal epithelium was found to reduce the severity of endotoxin-induced inflammatory response and thus contributing to the maintenance of intestinal homeostasis.[20]

Immune cells play key roles in the pathogenesis of IBD. Autophagy regulates functions of innate and adaptive immune cells such as formation of transepithelial

protrusions, antigen sampling and presentation, dendritic cell (DC) maturation, major histocompatibility complex II (MHC II) loading, T and B cell proliferation.[21-24] Autophagy has crucial roles on the transcription and degradation of pro-inflammatory cytokines like interleukin (IL)-1β and IL-18. Saitoh et al. reported increased production of lipopolysaccharide (LPS) induced IL-1β and IL-18 in Atg16L1 deficient mice macrophages.[25] This group also found that Atg16L1 deficient chimeric mice generated by transplantation of fetal liver cells into irradiated C57BL/6 (CD45.1) mice have increased susceptibility to dextran sulphate sodium (DSS) induced chemical colitis as demonstrated by acute weight loss and severe distal colon inflammation.

Autophagy and gut microbiota are important factors in the development of IBD. The composition and diversity of gut microbiota in IEC specific Atg5 conditional knockout ($Atg5^{\Delta IEC}$) and $Atg7^{\Delta IEC}$ mice have recently been shown to be highly different.[26,27] In the $Atg5^{\Delta IEC}$ mice, beneficial anti-inflammatory bacteria such as the mucin-degrading *Akkermansia muciniphila* and members of the Lachnospiraceae family are reduced. Pro-inflammatory bacteria such as *Candidatus athromitus* and potential Pasteurellaceae pathogens, on the other hand, are enhanced in the $Atg5^{\Delta IEC}$ mice. Similarly, $Atg7^{\Delta IEC}$ mice showed a larger bacterial burden, with more colitogenic bacteria such as *Prevotella* and *Bacteroides fragilis*.[26,27] However, the relationship between host autophagy and microbiota is bidirectional. Microbiota-derived metabolites have been shown to influence intestinal inflammation via the autophagy pathway. By suppressing Atg16L1, LC3-II, and p62 expression, trimethylamine N-oxide, a gut microbiota derived metabolite, boosted IEC nucleotide-binding domain, leucine-rich-containing family, pyrin

domain-containing-3 (NLRP3) inflammasome activity.[28] The mTOR signaling pathway has a key role in microbiota associated immune regulation and intestinal disease development.[29] The intestinal microbiota and its metabolites regulate various physiological functions of the host and maintain homeostasis through the mTOR pathway, which controls many cellular processes, autophagy being one of them.[29] A probiotic strain of *Bifidobacterium* attenuates various gastrointestinal (GI) disorders including chronic relapsing IBD and initiates autophagy in the IECs probably through the Atg12-5-16L1 pathway,[30] further highlighting the importance of interaction between microbiota and autophagy in IECs. Nevertheless, the upstream regulators of the autophagy-microbiota axis remain to be determined.

Recently, genome-wide association studies (GWAS) scan involving 735 CD patients and 368 controls identified an increased risk of developing CD in individuals with nonsynonymous single nucleotide polymorphism (SNP) in the Atg16L1 locus.[31] The T300A variant of the Atg16L1 gene resulted in the production of defective autophagy protein that was more prone to caspase-3 mediated degradation. This resulted in defective stress activated autophagy and xenophagy, thus establishing a state of chronic inflammation.[32] Additionally, another autophagy gene immunity related GTPase family M (IRGM) has also been linked with CD.[33,34] These two polymorphisms are responsible for dysfunctional autophagy, which propagates the inflammatory process in IBD. Autophagy prevents an exaggerated pro-inflammatory response in the gut by influencing immune response to the commensal bacteria and by regulating cytokine secretion.[35,36] Different research groups reported pharmacological inhibition of mucosal autophagy by

rapamycin or other mTOR-inhibitors resulted in reduction of intestinal inflammation in experimental models of colitis and in severe refractory CD. All these studies concluded that enhancing autophagy in the gut is protective against colitis.[37-39] Further, elaborated role of autophagy in intestinal immune response and inflammation has been discussed in the paper below.

1.1 Role of autophagy in immune activation and intestinal inflammation

AUTOPHAGY: ROLES IN INTESTINAL MUCOSAL HOMEOSTASIS AND INFLAMMATION

Published in *Journal of Biomedical Science*. Volume 26. Issue 1. Pages 1-14. 2019
https://doi.org/10.1186/s12929-019-0512-2

Sabah Haq, Jensine Grondin, Suhrid Banskota & Waliul I. Khan

In this review, we summarized how autophagy, the cell recycling process, maintains intestinal homeostasis. In the human gut, autophagy plays a vital role in many aspects of the innate and adaptive immunity. Particularly, autophagy critically regulates the intestinal epithelium. In addition, it is also involved in maintaining a healthy gut microbiota. Any perturbations in this process of autophagy results in inappropriate immune response and uncontrolled inflammation. In this review, we have also focused on autophagy and its role in the pathogenesis of IBD. This review is very relevant to my research project focused on how disruption of the autophagic process in the colon and IECs contributes to the pathogenesis of intestinal inflammation.

SH collected literature, designed and wrote the manuscript. JG edited and prepared manuscript for submission. SB edited and WK edited, revised and designed the manuscript. All authors read and approved the final manuscript.

Haq et al. Journal of Biomedical Science (2019) 26:19
https://doi.org/10.1186/s12929-019-0512-2

Journal of Biomedical Science

MOST 科技部 Ministry of Science and Technology

REVIEW Open Access

Autophagy: roles in intestinal mucosal homeostasis and inflammation

Sabah Haq[1,2], Jensine Grondin[1,2], Suhrid Banskota[1,2] and Waliul I. Khan[1,2*]

Abstract

The intestinal mucosa is a site of multiple stressors and forms the barrier between the internal and external environment. In the intestine, a complex interplay between the microbiota, epithelial barrier and the local immune system maintains homeostasis and promotes a healthy gut. One of the major cellular catabolic processes that regulate this homeostasis is autophagy. Autophagy is required to maintain anti-microbial defense, epithelial barrier integrity and mucosal immune response. Dysregulation of the autophagy process causes disruption of several aspects of the intestinal epithelium and the immune system that can lead to an inappropriate immune response and subsequent inflammation. Genome-wide association studies have found an association between several risk loci in autophagy genes and inflammatory bowel disease. The aim of the current review is to provide an update on the role of autophagy in intestinal mucosal physiology and in the control of inappropriate inflammation.

Keywords: Autophagy, Intestinal mucosa, Epithelium, Immune response, Inflammation, Microbiota

Introduction

Autophagy or "self-eating" is the catabolic process of delivering cytoplasmic constituents, organelles and infectious agents to the lysosome for degradation [1]. It is a highly conserved mechanism that takes place in all eukaryotic cells and participates in maintaining normal physiology [1]. During starvation, growth factor deficiency or high-energy demand, autophagy is induced to generate energy that supports metabolic processes [1]. Apart from starvation, autophagy is critical in responding to a diverse range of stressors namely, hypoxia, infection, endoplasmic reticulum (ER) stress, tissue remodeling, cellular debris breakdown, turnover of damaged organelles, tumor suppression, immune response, and cell death [2, 3]. Basal autophagy i.e. the baseline level of autophagy maintained as a housekeeping function in all cells, contributes to routine cell turnover and is important for the maintenance of cellular homeostasis [1, 4]. Autophagy results in the clearance of polyubiquitinated protein aggregates, which are formed during stress, aging, and disease. Autophagy also degrades invading pathogens, modulates the release of pathogen induced pro-inflammatory cytokines and participates in antigen presentation and lymphocyte development [2, 3]. Furthermore, this process is essential in maintaining intestinal barrier integrity, anti-microbial defense and mucosal immune response [5]. Even though autophagy is a survival mechanism, it can lead to cell death either in association with apoptosis or as an alternative mechanism [6]. The various roles of autophagy in regulating homeostasis and inflammation are extremely significant in the context of the intestinal mucosa, where most of the stressors are likely to converge [5]. The intestinal mucosa is constantly exposed to the gut microbiota and food particles since it is adjacent to the gut lumen. The epithelial and the resident immune cells of the mucosa are essential for defense against these antigens. Dysregulation of autophagy has been implicated in the pathogenesis of various diseases, including inflammatory bowel disease (IBD) [7]. The two major forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), are serious chronic inflammatory conditions of the human bowel [7]. In spite of decades of research, IBD pathophysiology is not fully understood. The development and course of IBD are affected by several factors, including genetic susceptibility of the host, the intestinal microbiota, other environmental factors, and the host immune system [8]. Genome-wide association studies (GWAS) revealed that polymorphisms in autophagy genes contribute to the development of IBD [9]. Therefore, understanding the role of autophagy in

* Correspondence: khanwal@mcmaster.ca
[1]Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON L8N 3Z5, Canada
[2]Department of Pathology and Molecular Medicine, McMaster University, Room 3N7, Hamilton, ON L8N 3Z5, Canada

intestinal homeostasis and pathogenesis of inflammation is important to the development of new strategies in prevention and/or treatment of intestinal inflammatory diseases.

The purpose of the review is to provide the readers with an update on the mechanisms of autophagy and present understanding on the role of autophagy in intestinal homeostasis and inflammation. The role this process plays during the inflammatory response in both patients and in experimental models will also be examined. In addition, the emerging relationship between gut microbiota and autophagy has also been discussed due to its significance in the context of gut inflammation and possible future therapies.

Mechanisms of autophagy

Autophagy comprises three major intracellular pathways in eukaryotic cells: macroautophagy, microautopahgy, and chaperone-mediated autophagy (CMA). These pathways share a common destiny of lysosomal degradation but differ mechanistically [1, 2]. In macroautophagy, double membrane-bound vesicles called autophagosomes are formed that engulf cytoplasmic constituents and organelles. The autophagosomes fuse with lysosomes, where the contents are degraded. Microautophagy, by contrast, involves direct engulfment and degradation of the target components in the lysosome. Microautophagy is important in the maintenance of organelles and membranes, as well as, cell survival during starvation. However, it is not clear whether it occurs simultaneously with macroautophagy or if it is simply a compensatory mechanism for macroautophagy to clear the excess metabolic materials [10]. In CMA, cytosolic chaperone heat shock cognate 70 kDa protein (HSC70) binds to a KFERQ-like pentapeptide motif of the target protein. HSC70 then associates with lysosome-associated membrane protein 2A (LAMP2A) resulting in its oligomerization. This promotes the translocation of the targets across lysosomal membranes into the lysosomal lumen [2]. Among the three autophagy pathways, macroautophagy has been extensively studied and linked to many intestinal physiological and pathological processes. In this review, we will focus on macroautophagy and, from this point on, this process will simply be referred to as autophagy.

The mechanisms of autophagy have been studied originally in the yeast model. Since autophagy is highly conserved, the pathways found in yeast can be translated to mammalian cells [5]. The mechanism has been summarized in Fig. 1. The central upstream regulators of autophagy are mammalian target of rapamycin complex 1 (mTORC1) and adenosine monophosphate-activated protein kinase (AMPK). AMPK activates the process of autophagy where as mTORC1 inhibits it. Nutrient sufficiency and growth factor stimulation activates mTORC1 resulting in inhibition of autophagy [11, 12]. On the other hand, starvation, growth factor withdrawal and ER stress activate AMPK that, in turn, inhibits mTORC1 and activates autophagy [11, 12]. The opposing roles of mTORC1 and AMPK in the autophagy pathway are due to phosphorylation of Unc-51 like autophagy activating kinase (ULK1) at two different sites [13]. Some of the other upstream regulators of autophagy pathway are class I phosphatidylinositol-3-kinase (PI3K), p53, death associated protein kinase and hypoxia inducible factor 1 (HIF-1) [14].

One of the hallmarks of autophagy is the formation of autophagosome, a double membrane bound vesicle containing portions of cytoplasm. Autophagosome formation occurs through initiation, nucleation and elongation of the isolation membrane in the phagophore assembly site (PAS). The origin of the isolation membrane is unclear and is thought to be derived from either the ER, the Golgi or the plasma membrane. The ULK complex composed of ULK1, autophagy-related protein 13 (Atg13), focal adhesion kinase family interacting protein of 200 kDa (FIP200) and autophagy-related protein 101 (Atg101), initiates autophagosome formation in the PAS. In the nucleation stage, the ULK1 complex recruits beclin-1, autophagy-related protein 14 (Atg14), vacuolar protein sorting 15 (Vps15) and class III phosphatidylinositol-3-kinase (PI3K) to the PAS [15]. Class III PI3K converts phosphatidylinositol to phosphatidylinositol-3-phosphate (PI3P) at the PAS. PI3P forms a platform for the binding of the downstream autophagy proteins to the isolation membrane of the forming autophagosome and allows this membrane to be deformed, bent and expanded. During the elongation stage autophagy-related protein 12–5 (Atg12–5) conjugate is activated by the enzymes autophagy-related protein 7 (Atg7) and autophagy-related protein 10 (Atg10). Atg12–5 forms a complex with autophagy-related protein 16L1 (Atg16L1) that associates with isolation membrane and functions as an ubiquitin-like enzyme. The Atg12–5-16L1 complex is required for the lipidation of the microtubule-associated protein 1 light chain 3 (LC3-I) with phosphatidylethanolamine (PE) to form LC3-II [16]. The lipidation of LC3-I to LC3-II is catalyzed by the enzymes Atg7 and Atg3 [17]. LC3-II is hydrophobic which allows for easier incorporation into, elongation of the isolation membrane, and closure of the autophagosome [17]. Selective autophagy is an autophagic process where cytoplasmic components are selected, tagged and delivered to the forming autophagosome, where as non-selective autophagy focuses on the bulk degradation of cytoplasmic components [18]. In selective autophagy, adaptor protein like p62 binds to ubiquitinated proteins and organelles, attaches them with LC3-II that helps bring them into autophagosome [17, 18]. In the final stage of its formation, the autophagosome fuses with the lysosome to form autolysosome, a process mediated by proteins like Rab7, syntaxin 17, vesicle-associated membrane protein 8

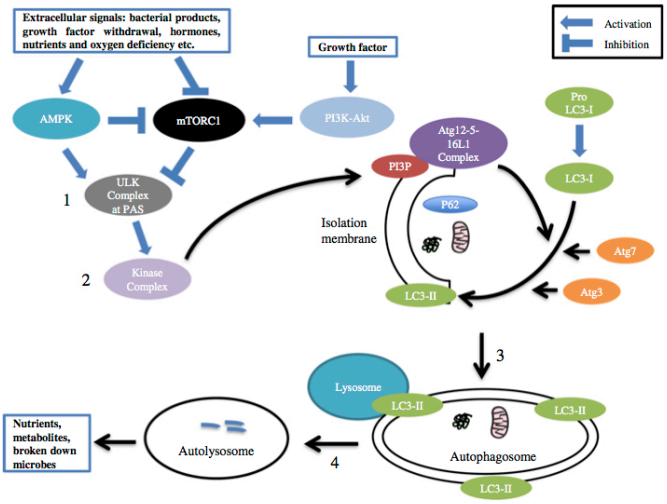


Fig. 1 Mechanisms of macroautophagy. Stress signals activate AMPK and inhibits mTORC1. mTORC1 is inhibited by AMPK and activated by growth factor induced PI3K-Akt pathway that initiates autophagy. 1. Initiation: Activated AMPK and inhibited mTORC1 initiates ULK complex. 2. Nucleation: ULK complex recruits kinase complex to the PAS that results in the production of PI3P at the isolation membrane. 3. Elongation: PI3P recruits downstream Atg proteins for elongation of the membrane. Atg12–5 forms a complex with Atg16L1 that associates with isolation membrane. The Atg12–5-16 L1 complex along with Atg7 and Atg3 are required for the formation of LC3-II from LC3-I. The LC3-II mediates the elongation and completion of the autophagosome. 4. Fusion: Autophagosome fuses with the lysosome to form the autolysosome. Lysosomal enzymes degrade the contents of the autolysosome. In selective autophagy, adaptor proteins like p62 binds to ubiquitinated proteins and organelles, attaches them with LC3-II and brings them in the autophagosome for degradation

(VAMP8) and LAMP2. The inner membrane and contents of the autolysosome are broken down by enzymes such as hydrolases and released into the cytoplasm, thus completing the process of autophagy [1, 14]. This brief overview of mammalian autophagy pathways demonstrates the complexity of the process (Fig. 1). It should be mentioned that, recent discoveries of alternative autophagic pathways such as the Atg5/Atg7 independent autophagy pathway, suggest that different autophagy pathways and autophagy-related molecules may exist in different types of cells [19]. These differences may be due to variances in stressors or in functions of specific cell types. For example, in the intestine the mechanism and molecules associated with the process of autophagy may be altered in specialized secretory epithelial cells such as Paneth, goblet and enteroendocrine (EE) cells in comparison with absorptive epithelial cells. However, further investigation is needed to elucidate this notion.

Autophagy in intestinal epithelium

The intestinal epithelium, the barrier between the lumen and intestinal mucosa, is constantly exposed to food and microbes and acts as the first line of defense against microbial invasion [5, 20]. Intestinal epithelial cells (IECs), as well as, the specialized epithelial cells such as enteroendocrine (EE) cells, goblet cells, Paneth cells, microfold (M) cells and tuft cells make up this single layer that acts as the site of digestion and absorption [3]. Basal autophagy in M cells is relatively low compared to other epithelial cells since the function of M cells is simply to transport antigens from the gut lumen through the epithelial layer to the underlying Peyer's patches. However, when mice are exposed to stresses such as cigarette smoke, autophagy is induced in both M cells and follicle associated epithelium. Even though, in the Peyer's patches of the murine ileum, autophagy levels are increased in response to cigarette smoke exposure, some of the downstream autophagy proteins does not show a significant increase compared to the air exposed control mice. It is thought that smoking results in oxidative stress that induces autophagy to repair the cell damage [21]. It can be assumed that the findings in B and T cells might be applicable to the PP since PP is in fact an aggregate of lymphoid follicles with germinal B cell center surrounded by T cell zone. Since the relationship between autophagy and M cells and tuft cells has not been explored sufficiently, future research needs to be

conducted in this area. In the intestine, a sensitive balance between tolerance and defense is required to maintain homeostasis. The conserved process of autophagy plays a critical role in maintaining this balance by regulating the functions of these different types of cells in the mucosal layer [5].

Intestinal epithelial cells

Epithelial cells are the first line of defense in host tissues against various invading gut microbiota and pathogens [22, 23]. In IECs, autophagy is activated in response to invasive bacteria, such as *Salmonella enterica*, *Enterococcus faecalis* and *Shigella*, in order to inhibit their replication, invasion and dissemination. The activation of autophagy is indicated by the presence of autophagosomes in the epithelial cells marked by LC3 puncta [23–25]. The interaction between autophagy and the toll-like receptors (TLRs) signaling pathway has also been studied in the IECs in vitro. It has been shown that IECs have high levels of autophagy that is not upregulated upon stimulation of TLR-2 or 4 or 5. However, when basal autophagy is silenced by Atg7 siRNA transfection in the IEC lines, there is decreased TLR-2, or 4 or 5 mediated interleukin 8 (IL-8) production [26]. On the contrary, Fujishima and his group have shown up-regulation of expressions of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) mRNA by lipopolysaccharide (LPS) in *Atg7* deficient murine small intestinal epithelium compared to control epithelium [27]. These contrasting findings between in vitro and in vivo systems suggest for taking caution in extrapolation of in vitro findings in relation to biological system and warrants further studies on the role of autophagy in intestinal epithelium. Furthermore, intestinal epithelial barrier integrity is regulated by autophagy [28–30]. Autophagy increases tight junction barrier function in Caco-2 IECs by enhancing the lysosomal breakdown of pore forming tight junction protein claudin-2 [28]. Similarly, autophagy activation in porcine IECs by rapamycin demonstrates a partial rescue of non-essential amino acid deprivation induced barrier dysfunction [29]. On the contrary, rapamycin mediated induction of autophagy in Caco-2 IECs has opposite effects on intestinal barrier function. Increased autophagy causes reduced transepithelial electrical resistance, enhanced paracellular permeability and disruption of zonula occludens-1 and occludin [30].

Paneth cells

Among the specialized cells of epithelial layer, the importance of autophagy in Paneth cell function has been extensively studied and has been emphasized in the context of CD. Paneth cells, located in the crypts of Lieberkuhn of the small intestine, store and secrete anti-microbial peptides (AMPs), such as lysozyme, α-defensin and phospholipase A2. The AMPs contribute to the maintenance of healthy gut microbiota [31]. Autophagy is an important regulator of Paneth cell function. *Atg16L1* and *Atg5* hypomorphic mice have abnormal granule exocytosis in Paneth cells that interferes with the secretion of AMPs and bacterial killing [32]. Autophagy gene deficient Paneth cells also show increased expression of genes involved in peroxisome proliferator-activated receptor signaling and production of leptin and adiponectin, both of which are involved in intestinal injury response. Similar changes in Paneth cells in CD patients carrying the *Atg16L1* risk allele have been reported [32]. Different groups have provided similar evidence of reduced granule size and decreased lysozyme staining in Paneth cells in IEC specific *Atg7* conditional knock out (KO) mice [33], in *Atg4B* null mice [34] and in *Atg16L1 T300A* knock in mice [35]. The secretion of lysozyme from Paneth cells during bacterial infection takes place through an autophagy-based alternative secretion pathway triggered by bacteria-induced ER stress [36]. However, a study found that mouse enteral starvation induces autophagy in Paneth cells, decreases AMP production and increases translocation of bacteria to mesenteric lymph nodes [37]. These contradictory findings may be explained as an attempt of the Paneth cells to maintain vital functions at the expense of the physiological function of autophagy. During starvation, induced autophagy results in the synthesis of new constituents. These new constituents are used for production of proteins essential for cell survival instead of proteins such as AMPs. Other factors such as a change in the microbial composition during starvation could also influence Paneth cell AMP activity [37]. The aforementioned evidence emphasizes the importance of autophagy in regulation of Paneth cell function and AMP generation, packaging and secretion [38].

Goblet cells

Goblet cells are specialized cells that are responsible for the production and preservation of the protective mucus blanket by producing high molecular weight glycoproteins known as mucins. This mucus layer is an important component of the intestinal anti-microbial systems that effectively separate the gut microbiota and intestinal epithelium and help maintain homeostasis. Up to 21 different mucin genes have been identified in humans, and the majority of their homologues have been recognized in mice and rats [39]. Among these mucin genes, MUC2 (Muc2 in mice) is the major gel forming mucin in the gut, which is the most important factor determining goblet cell morphology [40]. *Muc2* deficient mice develop spontaneous colitis and are more susceptible to dextran sulphate sodium (DSS) mediated model of colitis [41]. Additionally, in models of enteric infections, Muc2-dependent mucus production has been shown to be critical in host protection. By utilizing *Muc2* deficient mice and mice that are resistant (C57Bl/6) or susceptible

(AKR) to *Trichuris muris* infection, studies have illustrated Muc2 mucin is an important component of innate defense in this model of parasite infection [42]. An important role of Muc2 in host defense is also shown in *Citrobacter rodentium* infection (mouse model of enteropathogenic *E.Coli* (EPEC) and enterohemorrhagic *E.Coli* (EHEC)) [43]. Autophagy plays an important role in goblet cell functions. Through the production of reactive oxygen species, autophagy protein LC3 regulates the accumulation and secretion of mucin granules from colonic goblet cells. Reactive oxygen species are produced in part by LC3 positive vacuole associated nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. Thus, autophagy and NADPH oxidase activity drive reactive oxygen species production that is critical for efficient mucin formation and secretion by goblet cells [44]. In addition, mice with defective autophagy are reported to have enlarged goblet cells in the intestinal epithelial layer [35]. Wlodarska and his group [45] further reinforced the role of autophagy in the control of goblet cell function. They showed that nucleotide-binding oligomerization domain protein-like receptors protein 6 (NLRP6) inflammasome regulates goblet cell mucus secretion through the induction of autophagy [45]. *NLRP6* deficient mice exhibit defective autophagy in goblet cells and reduced mucus secretion in the large intestinal lumen. LC3-GFP tagged *NLRP6* deficient mice have no LC3-GFP signal, reduced amounts of LC3-GFP protein and accumulation of p62 in the intestinal epithelium, indicating defective autophagy. In addition, increased LC3-I/II ratio and degenerating mitochondria are also observed in the intestinal epithelium of *NLRP6* deficient mice. In order to further establish a link between impaired autophagy and goblet cell function, $Atg5^{+/-}$ mice were used to study the effects on goblet cells. Even a partial loss of autophagy function leads to goblet cell hyperplasia, perturbations in the secretory pathway and defects of the mucus layer. It was concluded from these results that inflammasome signaling is critical in maintaining a healthy goblet cell secretory function via autophagy. Further investigations in both Paneth and goblet cells is required to determine whether other mechanisms, apart from ER stress and reactive oxygen species production, are involved in autophagy-induced secretion.

Enteroendocrine cells

With enteroendocrine (EE) cells producing more than 30 different hormones, the gut is the largest endocrine organ in the body [46]. The enteric hormones such as, serotonin (5-hydroxytryptamine; 5-HT), chromogranin, cholecystokinin, secretin, glucagon-like peptide 1 and 2 (GLP-1, GLP-2), produced by EE cells are involved in a range of physiological and pathological functions [46]. Change in EE cell number and secretion patterns have been documented in patients with intestinal inflammation. There are reports of increased number of peptide YY and chromogranin A expressing cell (CgA+) density in lymphocytic colitis, as well as, increased GLP-1, GLP-2 secreting cells and increased 5-HT secreting enterochromaffin (EC) cells in IBD [46–48]. Recent evidence shows that EE cells are regulated by autophagy. CgA+ EE cells are increased in the colon of IBD patients and as well as in DSS induced mouse model of colitis. The number of CgA+ EE cells is also increased in the colonic mucosa of mice following treatment with pro-inflammatory cytokines interferon γ (IFN-γ) and TNF-α. In addition, increased levels of autophagy markers, LC3-II and Atg5, are detected in the colonic mucosa of these cytokine treated mice. In IFN-γ and TNF-α treated mice, the number of CgA+ cells are not increased following treatment with the autolysosome inhibitor, chloroquine. Furthermore, it has been shown that the increase in the number of CgA+ cells in the mucosa of DSS colitic mice is prevented following administration of chloroquine. These findings show an important role of autophagy in CgA production and differentiation of CgA+ cells during inflammation [49]. Recently, *Drosophila* guts lacking WD-40 domain of *Atg16* was found to have reduced number of mature EE cells, due to a dysfunctional Robo-Slit signaling pathway. Pre-EE cells of the *Atg16* gut mutant *Drosophila* model failed to mature into fully differentiated EE cells. The pre-EE cells containing mutant *Atg16* failed to produce enough slit (a regulator protein of EE cell fate) as evidenced by reduced mRNA and protein levels. This may have resulted in accumulation of pre-EE cells in the gut. The *Atg16 Drosophila* gut mutant also showed elevated levels of pro-inflammatory cytokines and increased susceptibility to DSS colitis. Moreover, it is evident that *Drosophila Atg16* promotes EE cell differentiation from intestinal stem cells [50]. The above stated evidence indicates a link between EE cells, autophagy and colitis. The relationship between EE hormones and intestinal inflammation has been extensively studied in experimental models of colitis. Mice are less susceptible to DSS and dinitrobenzenesulphonic acid (DNBS) induced colitis when gut 5-HT content is reduced either by using Tph enzyme inhibitor or by knocking out of the *Tph1* gene [51]. The relationship between 5-HT and gut inflammation is further supported by reports of exaggeration in trinitrobenzesulphonic acid (TNBS) colitis in serotonin reuptake transporter (*SERT*) KO mice [52]. Recently, some EE hormones have been investigated as possible regulators of autophagy. There is evidence of autophagy inhibition by 5-HT in hepatocellular carcinoma cells, in the lacrimal gland and in the rat hippocampus [53–55]. GLP-2, another EE hormone has also been shown to robustly activate the mTORC1 pathway in murine small intestine [56]. Exogenous parenteral administration of GLP-2 in C57BL/6 mice increases the phosphorylation of eukaryotic translation initiation factor 4E

(eIF4e)-binding protein 1 (4E-BP1) and S6 ribosomal protein, both of which are downstream effector proteins of the mTORC1 pathway. Pretreatment of the mice with rapamycin, an mTOR inhibitor, before administration of GLP-2 reduces levels of phosphorylated 4E-BP1 and S6 ribosomal protein. This shows that GLP-2 activates the mTORC1 pathway, which regulates autophagy. These findings suggest presence of interactions between EE hormones and autophagy process in both intestinal and extra intestinal sites. Among the various cell types in the intestinal mucosa, the role of autophagy in EE cells is underexplored within the context of intestinal inflammation. While dysregulated EE cell signaling and autophagy have been implicated in intestinal inflammation, it remains unclear whether they interact with each other in relation to intestinal pathology and pathophysiology, particularly in inflammation. Due to the vital roles of the gut hormones in the maintenance of intestinal homeostasis, it is of utmost importance to further study the relationship between EE hormones and the autophagy system.

Autophagy in intestinal immune cells

The intestinal mucosal immune system directs the appropriate immune response to a vast array of microbial and dietary challenges. The gut is one of the largest reservoirs of resident immune cells [38]. Macrophages, dendritic cells (DCs), T cells, B cells, and natural killer cells are important components of the intestinal mucosal immune system. Recent compelling evidence has revealed autophagy as a key regulator of intestinal innate and adaptive immunity [57].

Macrophages

Autophagy regulates the function of pathogen clearance in macrophages. For example, Atg7 and Atg5 expression in macrophages is essential for clearance of *Pseudomonas aeruginosa* and *Toxoplasma gondii* [58, 59]. In macrophages, TLR signaling links the components of the classical autophagy pathway and phagocytosis. The autophagy proteins LC3 and beclin-1 are rapidly recruited to the phagosome following ligand binding to TLR-2 on murine macrophages. LC3 and beclin-1 are associated with rapid fusion of the phagosome and the lysosome, leading to enhanced killing of ingested organisms like *Mycobacterium tuberculosis* [60]. However, the response of *Atg16L1* deficient mice to *C. rodentium* infection is very different compared to that of other pathogens [61]. *Atg16L1* deficient mice are resistant to *C. rodentium* infection due to a monocyte mediated heightened immune response. This illustrates an unexpected role of Atg16L1 in reducing a beneficial immune response to enteric bacterial infection. Recently, evidence showed that gut microbiota mediated type I IFN (IFN-1) signaling pathway maybe responsible for the heightened immune response [62]. The process of autophagy counteracts a spontaneous IFN-I response to gut microbiota that is beneficial in the presence of infectious and non-infectious intestinal hazards and injury. In autophagy deficient mice, the gut microbiota contributes to spontaneous IFN-I signaling resulting in enhanced resistance to *C. rodentium* infection. Conversely, inflammatory cytokines also influence autophagy. It has been reported that the Th1 type cytokines, such as IFN-γ, produced during bacterial infection activates autophagy as opposed to the inhibitory Th2 response [57]. Autophagy has crucial roles in the transcription, degradation and secretion of pro-inflammatory cytokines highlighting its importance in cytokine signaling. Macrophages from *Atg16L1* deficient mice have been documented to produce increased amounts of LPS induced IL-1β, IL-18 and TNF-α [63–65]. Fetal liver cell chimeric mice with *Atg16L1* deficient hematopoietic cells have increased susceptibility to DSS colitis as demonstrated by acute weight loss and severe distal colon inflammation suggesting a protective role of autophagy in the suppression of colitis [63]. In addition, these chimeric mice have increased levels of pro-inflammatory cytokines IL-1β and IL-18 in the sera after DSS treatment. Treatment with neutralizing antibodies for these cytokines reduce the susceptibility of *Atg16L1* deficient mice to DSS induced colitis, indicating the importance of Atg16L1 in the suppression of intestinal inflammation [63]. Cd11b + DCs and macrophages isolated from *Atg16L1 T300A* knock in mice also express increased levels of LPS induced IL-1β and decreased anti-bacterial autophagy [35]. Similarly, human macrophages isolated from patients with CD *Atg16L1 T300A* variant are reported to produce higher levels of interferon β (IFN-β) and IL-1β [66]. Both *Atg16L1* deficient chimeric and *Atg16L1 T300A* knock in mice are autophagy deficient.

Dendritic cells

Dendritic cells are one of the key components of the immune system that establish the link between innate and adaptive immunity. DCs initiate immune response by antigen uptake, presentation and activation of T cells. As demonstrated by different research groups, autophagy regulates interactions between DCs and T cells [67–69]. Using DC-IECs co-culture system, Strisciuglio and his group [67] showed that DCs form decreased transepithelial protrusions when autophagy is reduced in either DCs or epithelial cells. This leads to reduced antigen sampling and IL-10 secretion, increased DC maturation, increased T cell proliferation and production of pro-inflammatory type of DC [67]. Cooney and his group reported a decrease in bacterial trafficking, antigen presentation and T cell response to pathogens when *Atg16L1 T300A* allele is expressed in DCs [68]. Similarly, $CD4^+$ T cell activation is reduced in mice with *Atg5* deficient DCs. This is due to defective processing of cytosolic, phagocytosed and soluble antigens for major

histocompatibility complex (MHC) II loading in DCs [69]. Other antigen presenting cells (APC) such as B cells also depend on intact autophagy machinery for endogenous antigen presentation [70]. Intracellular microbes such as *M. tuberculosis* and *Listeria monocytogenes* are degraded into small peptides in the autophagolysosomes and loaded on the MHC II of APC to activate $CD4^+$ T cells [3].

T and B cells

Autophagy regulates activation and maintenance of T cells. In mice, T cell specific deletion of autophagy genes *Atg3, Atg5, Atg7, Atg16L1* or *beclin-1* results in decreased numbers of $CD4^+$, $CD8^+$ and Treg cells, defective effector and memory T cell development, and expansion of intestinal Th2 cells [71–73]. Kabat et al. [74] reported a reduction in the survival of intestinal $Foxp3^+$ regulatory T (Treg) cells when *Atg16L1* gene is deleted, which leads to severe gut inflammation in the mice [74]. Additionally, autophagy protein, Vps34, regulates the development, T cell receptor (TCR)-induced proliferation and apoptosis of T cells in the thymus and periphery. *Vps34* deficient T cells shows enhanced apoptosis due to reduced autophagic flux [75]. Other groups have shown similar dependence of $CD4^+$ and $CD8^+$ T cells on autophagy protein Atg5 and Atg7 for their development, proliferation and survival [76].

Impaired primary antibody responses to antigen immunization and defective plasma cell differentiation have been demonstrated in autophagy deficient B cells [77]. In contrast, other studies found no defects in primary antibody responses from autophagy-deficient B cells following antigen immunization [78] as well as no change in the number of mature B cells [77, 79]. The dissimilarities in these findings may be due to differences in immunization regimes, antigen and adjuvants used [57]. Secondary antibody response and survival of memory B cells, is found to be largely dependent on the process of autophagy [78, 80]. These results are comparable to memory $CD8^+$ T cell responses during viral infections where autophagy is also involved in memory cell formation during the late stages [57, 81]. The number of B cells in the intestinal lamina propria and Peyer's patches in $Atg5^{\Delta CD19}$ (Atg5 deficient B cells) mice are significantly decreased compared to the wild type. However, B cell numbers in the spleen and bone marrow are similar when compared to wild type and $Atg5^{\Delta CD19}$ mice. This observation leads us to speculate that the role of autophagy in maintaining intestinal B cell physiology is more important compared to that in the peripheral B cells, although further investigation is needed [57, 77].

Autophagy and gut microbiota

More than 100 trillion bacteria inhabit the lower gastrointestinal tract of humans. Thus, the number of microbes in the human body exceeds the number of host cells by about 10 times. The gut microbiota is influenced by factors such as age, gender, diet and immune status [82] and the interaction between the host and these microbes is largely symbiotic. The symbiotic bacteria provide the human host with nutrients, facilitate digestion and prevent colonization of opportunistic pathogens in the gut [83]. However, under altered conditions, even the normal microbiota may induce inflammation. The gut microbiota is implicated in various gastrointestinal disorders including IBD. Clinical and animal studies have suggested that gut bacteria trigger and perpetuate chronic colitis [84]. Very recently we also demonstrated that 5-HT-microbiota axis plays an important role in gut inflammation. Higher gut mucosal 5-HT levels select for a more colitogenic microbiota, resulting in increased severity of colitis [85]. The mTOR signaling pathway has a key role in microbiota associated immune regulation and intestinal disease development [82]. Noureldein and colleagues [82] have eloquently summarized the association between microbiota and mTOR signaling. The intestinal microbiota and its metabolites regulate various physiological functions of the host and maintain homeostasis through the mTOR pathway, which controls many cellular processes, autophagy being one of them. Increased activation of AMPK in the liver, muscle and colon in germ-free mice provide evidence that gut microbiota regulates activation of AMPK in the host [82]. Both autophagy and gut microbiota are important factors in the development of CD. Recently, the composition and diversity of gut microbiota has been reported to be very different in IEC specific *Atg5* conditional KO mice [86]. Beneficial anti-inflammatory bacteria such as the mucin degrading *Akkermansia muciniphila* and members of the Lachnospiraceae family are decreased in the *Atg5* IEC specific KO mice model. On the other hand, pro-inflammatory bacteria such as *Candidatus athromitus* and potential pathogens of the Pasteurellaceae family are increased in the same *Atg5* IEC specific KO mice. To further elucidate the connection between autophagy and microbiota, it has been found that protective factors like vitamin D_3 induce autophagy in colitis model and promotes a healthier microbiota composition [87]. Vitamin D receptor (*VDR*) IEC specific KO mice are reported to have reduced amount of Atg16L1 at both the transcriptional and protein level compared to their wild type counterparts. In addition, it has been shown that Atg16L1 is required for the proper functioning of AMP producing Paneth cells that regulate the composition and diversity of gut microbiota [32]. *VDR* IEC specific KO mice with reduced Atg16L1 and defective Paneth cell have increased bacterial loads and a shift of microbial species [88]. Harmful bacteria such as *E. coli* and *Bacteroides*

are increased whereas beneficial butyrate-producing bacteria are decreased in the *VDR* IEC specific KO mice. *VDR* IEC specific KO mice also developed more severe DSS induced colitis compared to the wild type mice, implying that microbial dysbiosis may increase the susceptibility of colonic mucosa to DSS induced colitis. The administration of the short chain fatty acid, butyrate to *IL-10* KO mice increased mRNA levels of Atg16L1, suppressed IL-6 production and restored Paneth cell numbers. These findings indicate that the bidirectional relationship between the host and microbiota is regulated by a functioning autophagy system in the host intestinal epithelium [88]. So far, we discussed the effects of impaired autophagy components on the gut microbiota. However, as mentioned earlier this relationship is bidirectional, i.e. autophagy can influence gut microbiota and gut microbiota can also influence autophagy in the host. It has been reported that microbiota derived metabolites regulate intestinal inflammation by acting through the autophagy pathway. Trimethylamine N-oxide, a gut microbiota derived metabolite increased IEC nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome activity by inhibiting Atg16L1, LC3-II and p62 expression [89]. *Bifidobacteria*, a probiotic strain in the human gut microbiota initiates autophagy, probably through the Atg12–5-16 L1 pathway in IEC culture model [90] further highlighting the importance of interaction between microbiota and autophagy in IEC. Nevertheless, functional consequences of such interaction between gut bacteria and autophagy in the IEC remain to be determined. The homeostatic functions of autophagy in intestinal mucosa have been summarized in Fig. 2.

Autophagy and gut inflammation

Genome-wide association studies (GWAS) identified an increased risk of developing CD in individuals with nonsynonymous single nucleotide polymorphism (SNP) at the *Atg16L1* locus [9]. The SNP caused a threonine to alanine substitution at position 300 (T300A) of the protein Atg16L1 [9]. Nearly a decade later after this discovery, Murthy and colleagues [91] provided evidence of increased caspase-3 mediated degradation of T300A variant of Atg16L1. The caspase cleavage site was identified as the site immediately preceding the T300A polymorphism site [91]. There is a 50% reduction in Atg16L1 protein levels in glucose starved macrophages isolated from *T316A* knock in (T300A variant in mice) mice with resultant decrease in bacterial degradation by autophagy and increased expression of the pro-inflammatory cytokine IL-1β. This resulted in defective stress activated autophagy and xenophagy, thus establishing a state of chronic inflammation that predisposes individuals to CD [91]. Another autophagy gene, immunity related GTPase family M (*IRGM*), has also been linked with CD [92]. *IRGM* polymorphisms are responsible for dysfunctional autophagy which perpetuates the inflammatory process in IBD [93]. Further supporting this link is the finding that Atg16L1 and IRGM protein expression is reduced in the intestine of CD patients [94]. Autophagy has been linked in the pathogenesis of CD through nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene, a genetic risk factor for CD development. Activation of NOD2 by bacterial products like muramyl dipeptide recruits Atg16L1 to the isolation membrane and stimulates the formation of autophagosomes [68, 95]. Stimulation of NOD2 in human primary immune cells results in increased pro-inflammatory cytokine response when autophagy is inhibited [96]. In CD patients, *NOD2* and *Atg16L1* polymorphism demonstrate similar impairment of NOD2 dependent autophagy, further highlighting the importance of autophagy in CD [68, 95]. *Lrrk2*, another CD susceptibility gene, has been linked with xenophagy and Paneth cell autophagy. Deletion of *Lrrk2* results in impaired Paneth cell autophagy, which contributes to the onset of IBD [97, 98]. It is clear from the findings of GWAS that dysfunctions in autophagy genes contribute to the development of intestinal inflammation.

Several mechanisms have been proposed to explain the link between autophagy and gut inflammation. Ravindran and colleagues [99] reported enhanced intestinal inflammasome activation, increased production of IL-1β, increased Th17 response and reactive oxygen species formation in a *GCN2* (general controlled non-repressed kinase) KO mouse model. They further found that reduced autophagy in IECs and APCs in *GCN2* KO mice is responsible for intestinal inflammation [99]. The link between autophagy and inflammasome has been studied in mice models with *Atg16L1* and *Atg7* deficient macrophages [63]. Treatment of *Atg16L1* and *Atg7* deficient macrophages with LPS, a TLR-4 ligand, results in markedly elevated levels of IL-1β. However, stimulation of TLR-2 or TLR-5 does not have the same effect. The levels of IL-1β mRNA and pro-IL-1β protein are similar in the *Atg16L1* deficient and wild type macrophages. This showed that autophagy regulates IL-1β production in the post-translational stage. It has been found that LPS stimulation mediates the cleavage of caspase-1 through activation of inflammasome, which results in the release of IL-1β from pro-IL-1β. These evidence suggest that in the absence of autophagy there is excessive cytokine production through inflammasome activation [63, 100]. Autophagy is thought to regulate inflammasome activity mainly through two mechanisms; (i) it causes defects in mitophagy resulting in increased reactive oxygen species production which is linked with inflammasome hyperactivity, and (ii) it negatively regulates pro-IL-1β and other components of inflammasome pathway [101]. Autophagy prevents an exaggerated pro-inflammatory response in the gut by other mechanisms such as by influencing the immune response to commensal bacteria, by regulating

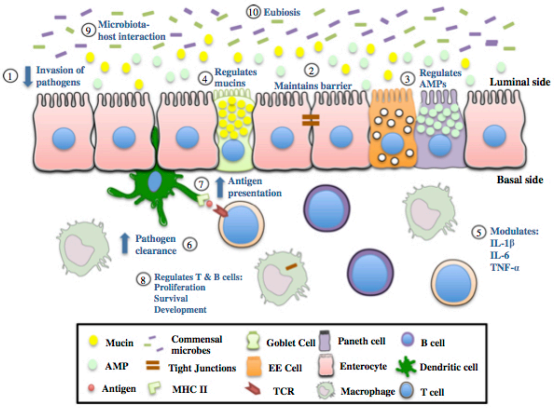


Fig. 2 Schematic representation of the role of normal autophagy in maintaining intestinal homeostasis. The intestinal mucosa includes several types of epithelial and immune cells. This schematic illustrates the importance and function of normal autophagy in the intestinal mucosa. Autophagy is critical in preventing the invasion and dissemination of pathogens (1) and maintaining barrier integrity (2). Autophagy regulates the secretion of AMPs from Paneth cells (3), the secretion of mucins from goblet cells (4) and the differentiation of EE cells in intestinal epithelial lining. Autophagy is involved in different immunological functions such as cytokine secretion (5), pathogen clearance by macrophages (6), antigen presentation by DCs (7), effector and memory T cell development (8), and secondary antibody response. The process of autophagy can regulate the interaction between the host and the gut microbiota (9). Autophagy also helps to maintain the balance between harmful and beneficial bacteria of the gut (10)

mucus secretion, by preventing accumulation of aggregates and by regulating miRNA-silencing pathways [27, 99, 102].

The in vivo role of autophagy proteins in gut inflammation has been examined in experimental models of colitis. Different autophagy deficient mice models such as *Atg16L1* deficient chimeric mice, myeloid *Atg16L1* deficient mice, colonic epithelial cell-specific *Atg7* conditional KO mice, and *Atg4B* null mice all exhibit exacerbated colitis induced by DSS [34, 63, 64, 103]. None of the autophagy deficient mice models develop spontaneous inflammation. However, severe spontaneous transmural ileitis was reported when both unfolded protein response and autophagy function are impaired in IECs [104]. On the other hand, the severity of spontaneous enterocolitis in *IL-10* KO mice decreased with increased autophagy [105, 106]. Pharmacological activation of mucosal autophagy using rapamycin or other mTOR-inhibitors results in reduction of intestinal inflammation in experimental models of colitis and in severe refractory CD. All these studies suggest that enhancing autophagy in the gut plays a protective role against experimental colitis [107–109].

Until now the mechanism by which impaired autophagy promotes a pro-inflammatory condition has mostly been explained in the context of the immune system with focus on the inflammasome. However, Grizotte-Lake and colleagues [110] recently summarized the role of autophagy in IECs in the pathogenesis of IBD [110–113]. Autophagy deficient IECs are found to be more susceptible to TNF-α induced damage and cell death. Using either murine norovirus or *Helicobacter hepaticus* or *T. gondii* infection mice models, it has been demonstrated that when autophagy is impaired in the intestinal epithelium, increased amounts of TNF-α mediates cell death. In the murine norovirus infection model, IEC specific *Atg16L1* KO mice are more susceptible to DSS induced colitis [112]. Atg16L1 protein in the IECs prevents loss of Paneth cells and TNF-α mediated necroptosis (programmed necrosis) in the intestinal epithelium of virally triggered IBD mouse model. In the same infection model, TNF-α blockage inhibits necroptosis in the IECs. Opportunistic pathogen, *H. hepaticus* triggered chronic colitis mice are recorded to have analogous findings of increased IEC apoptosis (programmed cell death) following conditional deletion of *Atg16L1* in the IECs [113]. This exaggerated IEC apoptosis is reported to be due to increased susceptibility to TNF-α and, as expected, TNF-α inhibition reduces the colitis severity in *Atg16L1* conditionally KO mice. Lastly, a *T. gondii* infection model has been employed to reach the same conclusion of increased sensitivity to TNF-α induced inflammation in autophagy deficient Paneth cells [111]. During *T. gondii* infection in a Paneth cell *Atg5* KO mouse model, there is heightened intestinal inflammation due to increased susceptibility to pro-inflammatory cytokine TNF-α. Therefore, *Atg5* expression in Paneth cells plays a

vital role in protecting against acute gastrointestinal infection. Even though in healthy state IECs can function normally without autophagy, it is in the event of an infection that autophagy in the intestinal epithelium is required to prevent cytokine induced cell death and uncontrolled inflammation. The association between TNF-α and autophagy in these three models helps to elucidate the mechanism behind anti-TNF-α therapy in IBD. It will be interesting to determine whether IBD patients with *Atg16L1* polymorphism are more responsive to anti-TNF-α therapy. More importantly, these findings may help optimize treatment protocols and maximize treatment success rates for IBD patients. Neonatal necrotizing enterocolitis (NEC) rat small intestine and NEC cell line showed increased expression of TNF-α and autophagy levels compared to the controls. TNF-α increased autophagy levels in NEC cells as evidenced by increased beclin-1 and LC3-II/I ratio. Additionally, inhibition of autophagy by wortmannin or LY294002 prevents TNF-α induced inhibition of proliferation and the promotion of apoptosis. Results also indicate that inhibition of ERK1/2 pathway significantly reduces TNF-α induced autophagy [114]. Therefore, it can be suggested that in both colitis and NEC models, TNF-α regulates apoptosis in intestinal epithelial cells via the process of autophagy. The role of autophagy in inflamed intestinal mucosa has been illustrated in Fig. 3.

Discussion and conclusion

Autophagy, a physiological process, is crucial in maintaining intestinal physiology and in controlling mucosal inflammation. Since the 1960s, the mechanisms and pathways of autophagy have been studied extensively and are still being explored today [5]. The discovery of Atg5/Atg7 independent pathway suggests that distinct autophagy pathways and molecules might be specific for different types of cells. This highly conserved process has tremendous influence on the normal functioning of the various cells of the intestine including epithelial and immune cells. Autophagy is maintained in these cell types for its support in basic functions such as maintaining pools of amino acid, as well as, removal of defective organelles [5]. Apart from these fundamental roles, autophagy regulates special functions of the intestinal mucosa. In the epithelial lining, autophagy is critical in preventing the invasion and dissemination of pathogens, maintaining barrier integrity and preserving intestinal homeostasis.

The findings of the GWAS on CD opened new avenues of research in gut autophagy in relation to pathology and

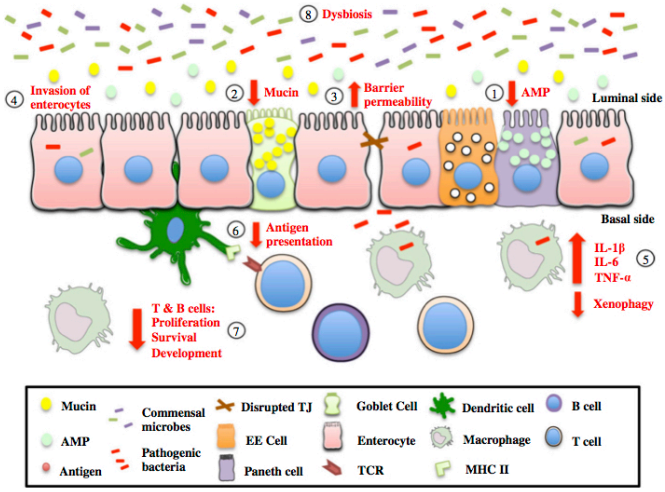


Fig. 3 Schematic representation of effect of impaired autophagy in intestinal mucosa. Defects in the autophagy system result in breakdown of mucosal homeostasis of the intestine. This diagram shows the state of the intestinal epithelium, mucosal immune system and microbiota in the context of impaired autophagy. Defective autophagy results in decreased AMP (1) and mucin (2) production, increased barrier permeability (3), increased bacterial invasion and dissemination (4), impaired cytokine production (5), reduced antigen presentation (6) and reduced T and B cell survival and development (7). Disrupted autophagy can alter the composition of bacteria within the gut, trending towards increased levels of pro-inflammatory species and decreased levels of anti-inflammatory species, thus, resulting in dysbiosis (8). Autophagy is required for the proper function of the gut and prevention of an exaggerated pro-inflammatory response

pathogenesis of gut inflammation. Evidence of autophagy regulating secretion of AMP from Paneth cells, secretion of mucins from goblet cells and differentiation of EE cells emphasizes the diverse role of autophagy in the intestinal epithelial layer. Autophagy is also involved in different immunological functions such as cytokine secretion, pathogen clearance by macrophages, antigen presentation by DCs, effector and memory T cell development and secondary antibody response [57]. The gut microbiota and autophagy have also been linked and this relationship appears to be bidirectional. It is quite clear that dysregulation of autophagy is associated with the pathophysiology of various intestinal diseases, notably IBD. GWAS have revealed a link of autophagy genes with CD, marking the beginning of a new era in intestinal inflammation research. It is now recognized that autophagy in the gut is protective against experimental colitis and that patients with CD have dysfunctional autophagy proteins suggesting that autophagy is required for the proper function of the gut and prevention of exaggerated pro-inflammatory response.

In recent years, significant progress has been made in understanding the mechanisms of autophagy and the role it plays in regulation of various key cells involved in intestinal inflammation including IECs and immune cells. Autophagy modulators such as vitamin D and rapamycin are currently being studied as potential therapeutic agents in intestinal inflammation [11]. The effectiveness of rapamycin has already been documented in a case of severe refractory CD [109]. Even though our understanding of autophagy has improved, this topic needs to be explored further before these pathways can be manipulated to treat human diseases such as IBD. As basal level of autophagy occurs within all cells, autophagy regulating drugs need further investigation as substances affecting this process may have diverse and global implications. In addition, it will be particularly important to gain further knowledge on the interactions between autophagy and various mediators like cytokines, neurotransmitters and hormones, which are upregulated during intestinal inflammation. Identification of novel regulators of autophagy in gut inflammation may trigger development of new therapeutic agents for intestinal inflammatory disorders such as IBD, and other GI disorders that display dysregulated autophagy.

Abbreviations

AMPK: Adenosine monophosphate-activated protein kinase; AMPs: Antimicrobial peptides; Atg: Autophagy-related protein; CD: Crohn's disease; CgA +: Chromogranin A positive.; CMA: Chaperone-mediated autophagy; DC: Dendritic cells; DSS: Dextran sulphate sodium; EE: Enteroendocrine; ER: Endoplasmic reticulum; FIP200: Focal adhesion kinase family interacting protein of 200 kDa; GCN2: General controlled non-repressed kinase; GWAS: Genome-wide association studies; HIF-1: Hypoxia inducible factor 1; HSC70: Heat shock cognate 70 kDa protein; IBD: Inflammatory bowel disease; IEC: Intestinal epithelial cell; IRGM: Immunity related GTPase family M; LAMP2A: Lysosome-associated membrane protein 2A; LC3: Microtubule-associated protein 1A/1B-light chain 3; LPS: Lipopolysaccharide; MHC II: Major histocompatibility complex II; mTOR: Mammalian target of rapamycin; NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen; NEC: Necrotizing enterocolitis; NLRP3: Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; NLRP6: Nucleotide-binding oligomerization domain protein-like receptors protein 6; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; PAS: Phagophore assembly site; PE: Phosphatidylethanolamine; PI3P: Phosphatidylinositol-3-phosphate; PI3K: Phosphatidylinositol-3-kinase; SNP: Single nucleotide polymorphism; TCR: T cell receptor; TJ: Tight junction; TLR: Toll-like receptor; UC: Ulcerative colitis; ULK1: Unc-51 like autophagy activating kinase; VAMP8: Vesicle-associated membrane protein 8; VDR: Vitamin D receptor; Vps: Vacuolar protein sorting

Acknowledgements

Not applicable.

Funding

This work was supported by a grant from Canadian Institute of Health Research (CIHR) to Waliul I Khan (reference number: 156262). Sabah Haq and Suhrid Banskota are recipients of Graduate Student Scholarship and Post-doctoral Fellowship Award from Farncombe Family Digestive Health Research Institute.

Availability of data and materials

Not applicable.

Authors' contributions

SH collected literature, designed and wrote the manuscript. JG edited and prepared manuscript for submission. SB edited and WK edited, revised and designed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Received: 30 November 2018 Accepted: 7 February 2019
Published online: 14 February 2019

References

1. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. Nat Rev Mol Cell Biol. 2015;16(8):461–72.
2. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. Nat Cell Biol. 2013;15(7):713–20.
3. Elshaer D, Begun J. The role of barrier function, autophagy, and cytokines in maintaining intestinal homeostasis. Semin Cell Dev Biol. 2017;61:51–9.
4. Clark SG, Graybeal LL, Bhattacharjee S, Thomas C, Bhattacharya S, Cox DN. Basal autophagy is required for promoting dendritic terminal branching in Drosophila sensory neurons. PLoS One. 2018;13(11):e0206743.
5. Randall-Demllo S, Chieppa M, Eri R. Intestinal epithelium and autophagy: partners in gut homeostasis. Front Immunol. 2013;4:301.
6. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ. 2009;16(7):966–75.
7. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448(7152):427–34.
8. Kucharzik T, Maaser C, Lugering A, Kagnoff M, Mayer L, Targan S, et al. Recent understanding of IBD pathogenesis: implications for future therapies. Inflamm Bowel Dis. 2006;12(11):1068–83.
9. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet. 2007;39(2):207–11.

10. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. Cell Mol Life Sci. 2012;69(7):1125–36.
11. Ke P, Shao BZ, Xu ZQ, Chen XW, Liu C. Intestinal autophagy and its pharmacological control in inflammatory bowel disease. Front Immunol. 2016;7:695.
12. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol. 2010;22(2):124–31.
13. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol. 2011;13(2):132–41.
14. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, et al. Mammalian autophagy: how does it work? Annu Rev Biochem. 2016;85: 685–713.
15. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol. 2013;15(7):741–50.
16. Sakoh-Nakatogawa M, Matoba K, Asai E, Kirisako H, Ishii J, Noda NN, et al. Atg12-Atg5 conjugate enhances E2 activity of Atg3 by rearranging its catalytic site. Nat Struct Mol Biol. 2013;20(4):433–9.
17. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010;221(1):3–12.
18. Lamark T, Svenning S, Johansen T. Regulation of selective autophagy: the p62/SQSTM1 paradigm. Essays Biochem. 2017;61(6):609–24.
19. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, et al. Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature. 2009;461(7264):654–8.
20. Lassen KG, Xavier RJ. Mechanisms and function of autophagy in intestinal disease. Autophagy. 2018;14(2):216–20.
21. Verschuere S, Allais L, Bracke KR, Lippens S, De Smet R, Vandenabeele P, et al. Cigarette smoke and the terminal ileum: increased autophagy in murine follicle-associated epithelium and Peyer's patches. Histochem Cell Biol. 2012;137(3):293–301.
22. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature. 2011;469(7330):323–35.
23. Benjamin JL, Sumpter R Jr, Levine B, Hooper LV. Intestinal epithelial autophagy is essential for host defense against invasive bacteria. Cell Host Microbe. 2013;13(6):723–34.
24. Leavy O. Mucosal immunology: autophagy helps man the barriers. Nat Rev Immunol. 2013;13(7):470.
25. Chang SY, Lee SN, Yang JY, Kim DW, Yoon JH, Ko HJ, et al. Autophagy controls an intrinsic host defense to bacteria by promoting epithelial cell survival: a murine model. PLoS One. 2013;8(11):e81095.
26. Li YY, Ishihara S, Aziz MM, Oka A, Kusunoki R, Tada Y, et al. Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells. Int J Mol Med. 2011;27(3):337–44.
27. Fujishima Y, Nishiumi S, Masuda A, Inoue J, Nguyen NM, Irino Y, et al. Autophagy in the intestinal epithelium reduces endotoxin-induced inflammatory responses by inhibiting NF-kappaB activation. Arch Biochem Biophys. 2011;506(2):223–35.
28. Nighot PK, Hu CA, Ma TY. Autophagy enhances intestinal epithelial tight junction barrier function by targeting claudin-2 protein degradation. J Biol Chem. 2015;290(11):7234–46.
29. Yang Y, Li W, Sun Y, Han F, Hu CA, Wu Z. Amino acid deprivation disrupts barrier function and induces protective autophagy in intestinal porcine epithelial cells. Amino Acids. 2015;47(10):2177–84.
30. Feng Y, Wang Y, Wang P, Huang Y, Wang F. Short-chain fatty acids manifest Stimulative and protective effects on intestinal barrier function through the inhibition of NLRP3 Inflammasome and autophagy. Cell Physiol Biochem. 2018;49(1):190–205.
31. Cullen TW, Schofield WB, Barry NA, Putnam EE, Rundell EA, Trent MS, et al. Gut microbiota. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. Science. 2015;347(6218):170–5.
32. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature. 2008;456(7219):259–63.
33. Wittkopf N, Gunther C, Martini E, Waldner M, Amann KU, Neurath MF, et al. Lack of intestinal epithelial atg7 affects paneth cell granule formation but does not compromise immune homeostasis in the gut. Clin Dev Immunol. 2012;2012:278059.
34. Cabrera S, Fernandez AF, Marino G, Aguirre A, Suarez MF, Espanol Y, et al. ATG4B/autophagin-1 regulates intestinal homeostasis and protects mice from experimental colitis. Autophagy. 2013;9(8):1188–200.
35. Lassen KG, Kuballa P, Conway KL, Patel KK, Becker CE, Peloquin JM, et al. Atg16L1 T300A variant decreases selective autophagy resulting in altered cytokine signaling and decreased antibacterial defense. Proc Natl Acad Sci U S A. 2014;111(21):7741–6.
36. Bel S, Pendse M, Wang Y, Li Y, Ruhn KA, Hassell B, et al. Paneth cells secrete lysozyme via secretory autophagy during bacterial infection of the intestine. Science. 2017;357(6355):1047–52.
37. Hodin CM, Lenaerts K, Grootjans J, de Haan JJ, Hadfoune M, Verheyen FK, et al. Starvation compromises Paneth cells. Am J Pathol. 2011;179(6):2885–93.
38. Baxt LA, Xavier RJ. Role of autophagy in the maintenance of intestinal homeostasis. Gastroenterology. 2015;149(3):553–62.
39. Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. Role of intestinal mucins in innate host defense mechanisms against pathogens. J Innate Immun. 2009;1(2):123–35.
40. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology. 1994;107(5):1352–63.
41. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006;131(1):117–29.
42. Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, et al. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. Gastroenterology. 2010;138(5):1763–71.
43. Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog. 2010;6(5):e1000902.
44. Patel KK, Miyoshi H, Beatty WL, Head RD, Malvin NP, Cadwell K, et al. Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. EMBO J. 2013;32(24):3130–44.
45. Wlodarska M, Thaiss CA, Nowarski R, Henao-Mejia J, Zhang JP, Brown EM, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. Cell. 2014;156(5):1045–59.
46. Gunawardene AR, Corfe BM, Staton CA. Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. Int J Exp Pathol. 2011;92(4):219–31.
47. El-Salhy M, Gundersen D, Hatlebakk JG, Hausken T. Chromogranin a cell density as a diagnostic marker for lymphocytic colitis. Dig Dis Sci. 2012; 57(12):3154–9.
48. Worthington JJ. The intestinal immunoendocrine axis: novel cross-talk between enteroendocrine cells and the immune system during infection and inflammatory disease. Biochem Soc Trans. 2015;43(4):727–33.
49. Hernandez-Trejo JA, Suarez-Perez D, Gutierrez-Martinez IZ, Fernandez-Vargas OE, Serrano C, Candelario-Martinez AA, et al. The pro-inflammatory cytokines IFNgamma/TNFalpha increase chromogranin A-positive neuroendocrine cells in the colonic epithelium. Biochem J. 2016;473(21):3805–18.
50. Nagy P, Szatmari Z, Sandor GO, Lippai M, Hegedus K, Juhasz G. Drosophila Atg16 promotes enteroendocrine cell differentiation via regulation of intestinal slit/Robo signaling. Development. 2017;144(21):3990–4001.
51. Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, et al. Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology. 2009;137(5):1649–60.
52. Bischoff SC, Mailer R, Pabst O, Weier G, Sedlik W, Li Z, et al. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. Am J Physiol Gastrointest Liver Physiol. 2009;296(3):G685–95.
53. Soll C, Jang JH, Riener MO, Moritz W, Wild PJ, Graf R, et al. Serotonin promotes tumor growth in human hepatocellular cancer. Hepatology. 2010;51(4):1244–54.
54. Imada T, Nakamura S, Hisamura R, Izuta Y, Jin K, Ito M, et al. Serotonin hormonally regulates lacrimal gland secretory function via the serotonin type 3a receptor. Sci Rep. 2017;7(1):6965.
55. Wu ZM, Zheng CH, Zhu ZH, Wu FT, Ni GL, Liang Y. SiRNA-mediated serotonin transporter knockdown in the dorsal raphe nucleus rescues single prolonged stress-induced hippocampal autophagy in rats. J Neurol Sci. 2016;360:133–40.
56. Lee J, Koehler J, Yusta B, Bahrami J, Matthews D, Rafii M, et al. Enteroendocrine-derived glucagon-like peptide-2 controls intestinal amino acid transport. Mol Metab. 2017;6(3):245–55.
57. Kabat AM, Pott J, Maloy KJ. The mucosal immune system and its regulation by autophagy. Front Immunol. 2016;7:240.

58. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe. 2008;4(5):458–69.
59. Li X, Ye Y, Zhou X, Huang C, Wu M. Atg7 enhances host defense against infection via downregulation of superoxide but upregulation of nitric oxide. J Immunol. 2015;194(3):1112–21.
60. Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007;450(7173):1253–7.
61. Marchiando AM, Ramanan D, Ding Y, Gomez LE, Hubbard-Lucey VM, Maurer K, et al. A deficiency in the autophagy gene Atg16L1 enhances resistance to enteric bacterial infection. Cell Host Microbe. 2013;14(2):216–24.
62. Martin PK, Marchiando A, Xu R, Rudensky E, Yeung F. Schuster SL, et al. Nat Microbiol: Autophagy proteins suppress protective type I interferon signalling in response to the murine gut microbiota; 2018.
63. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature. 2008;456(7219):264–8.
64. Zhang H, Zheng L, McGovern DP, Hamill AM, Ichikawa R, Kanazawa Y, et al. Myeloid ATG16L1 facilitates host-Bacteria interactions in maintaining intestinal homeostasis. J Immunol. 2017;198(5):2133–46.
65. Zhang H, Zheng L, Chen J, Fukata M, Ichikawa R, Shih DQ, et al. The protection role of Atg16l1 in CD11c(+)dendritic cells in murine colitis. Immunobiology. 2017;222(7):831–41.
66. Samie M, Lim J, Verschueren E, Baughman JM, Peng I, Wong A, et al. Selective autophagy of the adaptor TRIF regulates innate inflammatory signaling. Nat Immunol. 2018;19(3):246–54.
67. Strisciuglio C, Duijvestein M, Verhaar AP, Vos AC, van den Brink GR, Hommes DW, et al. Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions. J Crohns Colitis. 2013;7(7):534–41.
68. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med. 2010;16(1):90–7.
69. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, et al. In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity. 2010;32(2):227–39.
70. Nimmerjahn F, Milosevic S, Behrends U, Jaffee EM, Pardoll DM, Bornkamm GW, et al. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur J Immunol. 2003;33(5):1250–9.
71. Pua HH, Guo J, Komatsu M, He YW. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. J Immunol. 2009;182(7):4046–55.
72. Kovacs JR, Li C, Yang Q, Li G, Garcia IG, Ju S, et al. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. Cell Death Differ. 2012;19(1):144–52.
73. Puleston DJ, Zhang H, Powell TJ, Lipina E, Sims S, Panse I, et al. Autophagy is a critical regulator of memory CD8(+) T cell formation. elife. 2014;3.
74. Kabat AM, Harrison OJ, Riffelmacher T, Moghaddam AE, Pearson CF, Laing A, et al. The autophagy gene Atg16l1 differentially regulates Treg and TH2 cells to control intestinal inflammation. elife. 2016;5:e12444.
75. Parekh VV, Wu L, Boyd KL, Williams JA, Gaddy JA, Olivares-Villagomez D, et al. Impaired autophagy, defective T cell homeostasis, and a wasting syndrome in mice with a T cell-specific deletion of Vps34. J Immunol. 2013;190(10):5086–101.
76. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J Exp Med. 2007;204(1):25–31.
77. Conway KL, Kuballa P, Khor B, Zhang M, Shi HN, Virgin HW, et al. ATG5 regulates plasma cell differentiation. Autophagy. 2013;9(4):528–37.
78. Chen M, Hong MJ, Sun H, Wang L, Shi X, Gilbert BE, et al. Essential role for autophagy in the maintenance of immunological memory against influenza infection. Nat Med. 2014;20(5):503–10.
79. Pengo N, Scolari M, Oliva L, Milan E, Mainoldi F, Raimondi A, et al. Plasma cells require autophagy for sustainable immunoglobulin production. Nat Immunol. 2013;14(3):298–305.
80. Chen M, Kodali S, Jang A, Kuai L, Wang J. Requirement for autophagy in the long-term persistence but not initial formation of memory B cells. J Immunol. 2015;194(6):2607–15.
81. Xu X, Araki K, Li S, Han JH, Ye L, Tan WG, et al. Autophagy is essential for effector CD8(+) T cell survival and memory formation. Nat Immunol. 2014;15(12):1152–61.
82. Noureldein MH, Eid AA. Gut microbiota and mTOR signaling: insight on a new pathophysiological interaction. Microb Pathog. 2018;118:98–104.
83. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9(5):313–23.
84. Campieri M, Gionchetti P. Bacteria as the cause of ulcerative colitis. Gut. 2001;48(1):132–5.
85. Kwon YH, Denou E, Ghia JE, Rossi L, Fontes ME, Bernier SP, Shajib M, Banskota S, Collins SM, Surette MG, Khan WI. Modulation of Gut Microbiota Composition by Serotonin Signaling Influences Intestinal Immune Response and Susceptibility to Colitis. Cell Mol Gastroenterol Hepatol. 2019; pii: S2352-345X(19)30005-0. https://doi.org/10.1016/j.jcmgh.2019.01.004. [Epub ahead of print].
86. Yang L, Liu C, Zhao W, He C, Ding J, Dai R, et al. Impaired autophagy in intestinal epithelial cells alters gut microbiota and host immune responses. Appl Environ Microbiol. 2018.
87. Clark A, Mach N. Role of vitamin D in the hygiene hypothesis: the interplay between vitamin D, vitamin D receptors, gut microbiota, and immune response. Front Immunol. 2016;7:627.
88. Wu S, Zhang YG, Lu R, Xia Y, Zhou D, Petrof EO, et al. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. Gut. 2015;64(7):1082–94.
89. Yue C, Yang X, Li J, Chen X, Zhao X, Chen Y, et al. Trimethylamine N-oxide prime NLRP3 inflammasome via inhibiting ATG16L1-induced autophagy in colonic epithelial cells. Biochem Biophys Res Commun. 2017;490(2):541–51.
90. Lin R, Jiang Y, Zhao XY, Guan Y, Qian W, Fu XC, et al. Four types of Bifidobacteria trigger autophagy response in intestinal epithelial cells. J Dig Dis. 2014;15(11):597–605.
91. Murthy A, Li Y, Peng I, Reichelt M, Katakam AK, Noubade R, et al. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. Nature. 2014;506(7489):456–62.
92. Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. Autophagy. 2007;3(6):649–51.
93. Brest P, Lapaquette P, Souidi M, Lebrigand K, Cesaro A, Vouret-Craviari V, et al. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat Genet. 2011;43(3):242–5.
94. Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut. 2011;60(12):1739–53.
95. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol. 2010;11(1):55–62.
96. Plantinga TS, Crisan TO, Oosting M, van de Veerdonk FL, de Jong DJ, Philpott DJ, et al. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut. 2011;60(9):1229–35.
97. Liu TC, Naito T, Liu Z, VanDussen KL, Haritunians T, Li D, et al. LRRK2 but not ATG16L1 is associated with Paneth cell defect in Japanese Crohn's disease patients. JCI Insight. 2017;2(6):e91917.
98. Rocha JD, Schlossmacher MG, Philpott DJ. LRRK2 and Nod2 promote lysozyme sorting in Paneth cells. Nat Immunol. 2015;16(9):898–900.
99. Ravindran R, Loebbermann J, Nakaya HI, Khan N, Ma H, Gama L, et al. The amino acid sensor GCN2 controls gut inflammation by inhibiting inflammasome activation. Nature. 2016;531(7595):523–7.
100. Patel KK, Stappenbeck TS. Autophagy and intestinal homeostasis. Annu Rev Physiol. 2013;75:241–62.
101. Gardet A, Xavier RJ. Common alleles that influence autophagy and the risk for inflammatory bowel disease. Curr Opin Immunol. 2012;24(5):522–9.
102. Sibony M, Abdullah M, Greenfield L, Raju D, Wu T, Rodrigues DM, et al. Microbial disruption of autophagy alters expression of the RISC component AGO2, a critical regulator of the miRNA silencing pathway. Inflamm Bowel Dis. 2015;21(12):2778–86.
103. Tsuboi K, Nishitani M, Takakura A, Imai Y, Komatsu M, Kawashima H. Autophagy protects against colitis by the maintenance of Normal gut microflora and secretion of mucus. J Biol Chem. 2015;290(33):20511–26.
104. Adolph TE, Tomczak MF, Niederreiter L, Ko HJ, Bock J, Martinez-Naves E, et al. Paneth cells as a site of origin for intestinal inflammation. Nature. 2013;503(7475):272–6.
105. Zhao J, Dong JN, Wang HG, Zhao M, Sun J, Zhu WM, et al. Docosahexaenoic acid attenuated experimental chronic colitis in interleukin 10-deficient mice by enhancing autophagy through inhibition of the mTOR pathway. JPEN J Parenter Enteral Nutr. 2017;41(5):824–9.

106. Zhao J, Sun Y, Shi P, Dong JN, Zuo LG, Wang HG, et al. Celastrol ameliorates experimental colitis in IL-10 deficient mice via the up-regulation of autophagy. Int Immunopharmacol. 2015;26(1):221–8.
107. Macias-Ceja DC, Cosin-Roger J, Ortiz-Masia D, Salvador P, Hernandez C, Esplugues JV, et al. Stimulation of autophagy prevents intestinal mucosal inflammation and ameliorates murine colitis. Br J Pharmacol. 2017;174(15): 2501–11.
108. Hu S, Chen M, Wang Y, Wang Z, Pei Y, Fan R, et al. mTOR inhibition attenuates dextran sulfate sodium-induced colitis by suppressing T cell proliferation and balancing TH1/TH17/Treg profile. PLoS One. 2016;11(4): e0154564.
109. Massey DC, Bredin F, Parkes M. Use of sirolimus (rapamycin) to treat refractory Crohn's disease. Gut. 2008;57(9):1294–6.
110. Grizotte-Lake M, Vaishnava S. Autophagy: suicide prevention hotline for the gut epithelium. Cell Host Microbe. 2018;23(2):147–8.
111. Burger E, Araujo A, Lopez-Yglesias A, Rajala MW, Geng L, Levine B, et al. Loss of Paneth cell autophagy causes acute susceptibility to toxoplasma gondii-mediated inflammation. Cell Host Microbe. 2018;23(2):177–90 e4.
112. Matsuzawa-Ishimoto Y, Shono Y, Gomez LE, Hubbard-Lucey VM, Cammer M, Neil J, et al. Autophagy protein ATG16L1 prevents necroptosis in the intestinal epithelium. J Exp Med. 2017;214(12):3687–705.
113. Pott J, Kabat AM, Maloy KJ. Intestinal epithelial cell autophagy is required to protect against TNF-induced apoptosis during chronic colitis in mice. Cell Host Microbe. 2018;23(2):191–202 e4.
114. Yuan Y, Ding D, Zhang N, Xia Z, Wang J, Yang H, et al. TNF-alpha induces autophagy through ERK1/2 pathway to regulate apoptosis in neonatal necrotizing enterocolitis model cells IEC-6. Cell Cycle. 2018;17(11):1390–402.

2. Tryptophan and its metabolites

Tryptophan is an essential aromatic amino acid critical for normal growth and is also a precursor of various bioactive compounds including tryptamine, nicotinamide, 5-HT, melatonin, kynurenine and its metabolites.[40] Therefore, tryptophan has diverse effects in health and disease, particularly in intestinal mucosal homeostasis and pathogenesis of gut inflammatory disorders. The recommended daily dose of tryptophan for humans is 250–425 mg, however most people consume 1000 mg or more.[40] Tryptophan is released after digestion of dietary proteins and is absorbed by the IECs. The absorbed tryptophan is either delivered to the liver via the portal circulation for protein synthesis or for metabolic breakdown or delivered to the bloodstream where it circulates mainly bound to albumin or in the free form (10-20%).[41-43] The portion of the tryptophan that is not used in the liver is distributed by the circulation to other cells of the body for anabolic and catabolic functions.[42] Additionally, a small portion of the tryptophan remains in the gut lumen where it is utilized by gut microbiota or is absorbed and used by the enterochromaffin (EC) cells of the intestine.[41,44] The kynurenine and 5-HT routes are the primary mechanisms for endogenous tryptophan metabolism.[45] The vast majority of dietary tryptophan (95%) is metabolized via the kynurenine pathway. Only a small percentage of dietary tryptophan (1-2%) enters the 5-HT pathway and, as a result, contributes to 5-HT synthesis. In addition, a small percentage of ingested tryptophan (4-6%) is degraded by bacteria in the gut lumen, resulting in the production of indole and other related chemicals.[46] The metabolites of the kynurenine pathway plays various physiological roles such as fortifying immunological defenses, regulating gut motility and

maintaining functions of central nervous system (CNS).[46] 5-HT, a bioamine, is synthesized in both CNS and peripheral tissues, particularly within the GI tract.[47,48] 5-HT functions as a hormone and neurotransmitter in the body by working on a variety of receptors and as a component of intracellular signaling. In addition, 5-HT has been linked to GI function in the last 20 years, particularly in terms of motility, peristaltic activity, and intestinal secretions.[49-51] 5-HT also has a considerable impact on inflammatory pathways, as well as the development of a number of GI illnesses, such as IBD.[47,52]

2.1 EC cells

The GI tract is the largest endocrine system in terms of number of cells. It is composed of EECs located in the crypts and villi that makes up 1% of the gut epithelium cell population.[53] EECs differentiate from pluripotent stem cells at the base of the crypts regulated by the interaction between Notch signaling and basic helix-loop-helix transcription factor family.[54,55] EECs sense luminal material to produce and release hormones/signaling molecules, and modulate a number of GI and homeostatic activities. EECs either directly detect luminal contents through the microvilli or indirectly through neural or humoral pathways. In response to mechanical, chemical, or neural stimulation, EECs release their secretory products by exocytosis at the basolateral membrane. The secretory products, mostly peptides/hormones, can function locally in a paracrine way, stimulating other EECs and other cell types in the mucosa, reach distant targets through bloodstream, or act directly on nearby nerve endings.[56,57] EECs are most abundant in the proximal small intestine followed by the rectum.[58]

One of the best characterized and most common EECs are the triangular or pyramidal shaped EC cells (about 8μm in size) located in the gastric antrum, duodenum, jejunum, ileum, colon, rectum and appendix, while the number of EC cells in the large intestine remains almost contant.[58,59] These unique EC cells synthesise>90% of the body's 5-HT. Apart from 5-HT, EC cells also produce chromogranin, substance P and guanylin.[60-62] EC cells express a variety of ion channels and receptors such as transient receptor potential cation channel subfamily A member 1, taste and olfactory receptors, free fatty acid receptors, toll-like receptors (TLRs), ganglioside GM1 receptor, 5-HT receptors and somatostatin receptors.[59,63,64] EC cells act as chemosensors, mechanosensors and oxygen sensors. EC cells detect nutrient or purine induced chemical changes of the gut luminal environment. EC cells also detect changes in solute concentration, pH, oxygen level, and mechanical forces (intestinal peristalsis).[65] The secretory functions of EC cells have been demonstrated to be regulated, at least in part, by neural input.[66] It has also been shown that stimulation of β-adrenergic and pituitary adenylate cyclase-activating polypeptide receptors has stimulatory effects on 5-HT release by EC cells, while activation of γ-aminobutyric acid A and cholinergic receptors has inhibitory effects.[59]

2.2 Serotonin (5-hydroxytryptamine; 5-HT)

5-HT is a monoamine neurotransmitter and hormone mainly produced by the gut (95%).[67] EC cells are the major (90%) producer of peripheral 5-HT.[48,61] Only, about 10% of the peripheral 5-HT is synthesized and secreted by the serotonergic enteric neurons.[68]

In the EC cells 5-HT is synthesized from dietary tryptophan. Dietary tryptophan is converted to 5-hydroxy-L-trytaphan (5-HTP), catalyzed by the rate limiting enzyme tryptophan hydroxylase 1 (Tph1), an isoform of Tph.[69,70] The other isoform Tph2 is found in the brain stem and enteric neurons. Tph1 and Tph2 function independently.[69] Another enzyme aromatic L-amino acid decarboxylase further converts the 5-HTP into 5-HT.[70,71] 5-HT is then packaged and stored in the EC cells. Mechanical and chemical stimulants trigger the release of 5-HT in a calcium dependent manner.[72] The secreted 5-HT enters the surrounding IECs through the serotonin reuptake transporter (SERT).[71] Once 5-HT is taken up it is degraded by monoamine oxidase A (MAO-A) into 5-hydroxyindoleacetic acid (5-HIAA).[71] There are five families of 5-HT receptors (5-HTRs) in the gut expressed on epithelial cells, immune cells, neurons and muscle cells.[73] The 5-HT released from the EC cells participate in a range of physiological functions like motility, secretion and maintenance of the epithelial barrier.[70,71] The major providers of 5-HT for lymphatic tissue and immunological cells are EC cells via platelets. Some immune cells express the enzyme, Tph1 and thus are additional but minor sources of 5-HT for the peripheral immune system.[73] 5-HT has been shown to have a wide range of diverse effects on immune cells, often in the same cell type.[73,74] The varying effects of 5-HT on immune cells could be related to the local concentration of 5-HT and the expression of various 5-HT signaling machinery, such as Tph1, 5-HTRs and SERT, which work together to produce particular effects.

The close proximity of gut microbiota and EC cells indicates the presence of a cross talk between 5-HT and microbiota. Serum concentrations of 5-HT are reduced in

germ-free (GF) mice compared to conventionally raised mice. This was accompanied by reduced Tph1 mRNA and increased SERT mRNA expression in the proximal colon of GF mice.[75] Indigenous spore forming bacteria from mouse and human microbiota stimulate the release of 5-HT from EC cells providing a supply of 5-HT to the gut lumen, mucosa and circulating platelets.[76] Microbiota derived metabolites such as short chain fatty acids and secondary bile acids upregulate the expression of Tph1 in EC cells leading to increased synthesis and release of 5-HT.[76,77] These studies indicate that the gut microbiota regulates the 5-HT production from EC cells. Recent studies have illustrated the influence of 5-HT on the composition of gut microbiota. We discovered that the microbial composition of *Tph1*$^{+/-}$ and *Tph1*$^{-/-}$ littermates differs significantly. 5-HT directly stimulates and inhibits the growth of commensal bacteria in a species dependent manner as well as indirectly via AMPs. In summary, increased 5-HT levels promote the growth of a colitogenic microbiota that leads to increased severity of colitis.[78] Singhal et al. demonstrated a significant increase in the abundance of Firmicutes in *SERT*$^{-/-}$ (increased 5-HT in intestine) mice compared to *SERT*$^{+/+}$ mice.[79] 5-HT promotes the growth of *E. coli*, *Enterococcus faecalis*, *Rhodospirillum rubrum* and *Turicibacter sanguinis*. It is thought that 5-HT's stimulatory effect on microbial growth is due to the existence of 5-HTR on their surface, which could bind related chemicals like indole or due to the presence of a protein structurally similar to SERT.[80,81]

IBD has been associated with dysregulations of gut 5-HT signaling. The relationship between 5-HT and gut inflammation has been extensively shown in experimental models of colitis.[82-84] Our lab has demonstrated that mice were less

susceptible to DSS and dinitrobenzenesulphonic acid (DNBS) induced colitis when gut 5-HT content is significantly reduced by using Tph1 enzyme inhibitor or by knocking out the *Tph1* gene.[85] The relationship between 5-HT and gut inflammation was further supported by Gershon et al. who reported exaggeration in trinitrobenzesulphonic acid (TNBS) colitis in *SERT*$^{-/-}$ mice, which have increased 5-HT in the gut.[86] Our lab has further shown a reduction in murine colitis by inhibition of 5-HTR7.[87,88] Our lab has also found increased production of pro-inflammatory cytokines IL-1β and IL-6 by peritoneal macrophages upon treatment with 5-HT.[85] In addition, we have shown 5-HT facilitates IL-13 mediated intestinal inflammation.[89] The findings in murine models were further supported by clinical studies. In IBD patients, increase in EC cell number and 5-HT content has been documented.[44] Microscopic colitis has also been linked with consumption of SSRIs.[52] Minderhoud et al. reported an increase in mucosal Tph1 mRNA levels in the colon of CD patients who experience IBS like symptoms in remission.[90] The role of 5-HT in the regulation of immune system, gut microbiota and intestinal inflammation is described in details in the paper below.

2.3 Serotonin and its regulation of the immune system and intestinal inflammation

TRYPTOPHAN-DERIVED SEROTONIN-KYNURENINE BALANCE IN IMMUNE ACTIVATION AND INTESTINAL INFLAMMATION

Published in *The FASEB Journal*. Volume 35. Issue 10. Pages e21888. 2021
https://doi.org/10.1096/fj.202100702R

Sabah Haq, Jensine A. Grondin, Waliul I. Khan

In this review, the role of the endogenous metabolites of tryptophan in immune activation, gut microbiota regulation and intestinal inflammation has been summarized. Tryptophan is metabolized in the host via serotonin and kynurenine pathways. The review further dissects the multiple interactions between the serotonin and kynurenine pathways that have been studied and hypothesized. In my research project, I have focused on one of the metabolites, serotonin, since the EC cells of the gut are the major producers. Serotonin signaling has been extensively studied in our and other labs in the context of intestinal inflammation, particularly in IBD. From this review, I have only included in my thesis the relevant sections, figures and tables on serotonin.

SH and JAG equally reviewed the literature and wrote the manuscript. SH conceived the idea for the article. SH and JAG designed and created the figures; WIK and JAG edited and revised the manuscript. WIK supervised the project. All authors provided critical feedback and shaped the final manuscript.

Received: 25 April 2021 | Revised: 29 July 2021 | Accepted: 16 August 2021DOI:
10.1096/fj.202100702R

REVIEW

THE FASEB JOURNAL

Tryptophan-derived serotonin-kynurenine balancein immune activation and intestinal inflammation

Sabah Haq[1,2] | Jensine A. Grondin[1,2] | Waliul I. Khan[1,2,3]

[1]Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada
[2]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada
[3]Laboratory Medicine, Hamilton Health Sciences, Hamilton, Ontario, Canada

Correspondence
Waliul I. Khan, Farncombe Family Digestive Health Research Institute, McMaster University Health Sciences Centre, Room 3N7, 1280 Main Street West, Hamilton, ON, Canada.
Email: khanwal@mcmaster.ca

Funding information Gouvernement du Canada | CanadianInstitutes of Health Research (CIHR),Grant/Award Number: PJT 156262

Abstract

Endogenous tryptophan metabolism pathways lead to the production of serotonin (5-hydroxytryptamine; 5-HT), kynurenine, and several downstream metabolites which are involved in a multitude of immunological functions in both health anddisease states. Ingested tryptophan is largely shunted to the kynurenine pathway(95%) while only minor portions (1%–2%) are sequestered for 5-HT production. Though often associated with the functioning of the central nervous system, sig- nificant production of 5-HT, kynurenine and their downstream metabolites takesplace within the gut. Accumulating evidence suggests that these metabolites haveessential roles in regulating immune cell function, intestinal inflammation, aswell as in altering the production and suppression of inflammatory cytokines. Inaddition, both 5-HT and kynurenine have a considerable influence on gut micro-biota suggesting that these metabolites impact host physiology both directly andindirectly via compositional changes. It is also now evident that complex interac-tions exist between the two pathways to maintain gut homeostasis. Alterationsin 5-HT and kynurenine are implicated in the pathogenesis of many gastrointes- tinal dysfunctions, including inflammatory bowel disease. Thus, these pathwayspresent numerous potential therapeutic targets, manipulation of which may aid those suffering from gastrointestinal disorders. This review aims to update both

Sabah Haq and Jensine A. Grondin contributed equally to this work.

FASEB J. 2021;35:e21888.
https://doi.org/10.1096/fj.202100702R

the role of 5-HT and kynurenine in immune regulation and intestinal inflam-mation, and analyze the current knowledge of the relationship and interactions between 5-HT and kynurenine pathways.

KEYWORDS
immune cells, intestinal inflammation, kynurenine, microbiota, serotonin

2 SYNTHESIS AND METABOLISM OF 5-HT IN THE GUT

The generation of 5-HT is concentrated in the nervous system including the brain and enteric neurons, and the GI tract. Within the GI tract, the enterochromaffin (EC) cells of the epithelial layer produce the vast majority of the body's 5-HT.[1,2] Neuron- and EC cell-derived 5-HT represent not only locationally different sources of 5-HT but also exhibit biochemical differences in production as well.[1-3] Of the two enzymatic isoforms, tryptophan hydroxylase (Tph) 2 acts within both central and enteric neurons and controls the production of 5-HT particularly within the raphe nuclei of the hindbrain.[4-6] Tph1, in contrast, is localized largely to EC cells within the gut.[4,7] Though both enteric neurons and EC cells contribute to "gut-derived" 5-HT synthesis and secretion, 90% of this organ's 5-HT production is accounted for by the EC cells of the epithelial layer and only a minor contribution, of approximately 10% is derived from underlying enteric neurons.[7-9] Tph1 has also been found in fibroblasts, pancreatic and respiratory tissue, as well as the pineal gland, where it plays an upstream role in the production of melatonin.[3,4,10]

The process of 5-HT biosynthesis begins with the ingestion of tryptophan-containing substances. Within the gut, dietary tryptophan is taken up by enterocytes, both apically via the B^0AT1 (Solute Carrier 6A19, SLC6A19) epithelial amino acid transport system and basolaterally via the aromatic amino acid transporter TAT1 (Slc16a10) protein.[11] Within the EC cells of the gut, Tph1 catalyzes the 5-hydroxylation of this dietary-derived tryptophan to 5-hydroxytryptophan (5-HTP).[12] In EC cells, aromatic L-alpha amino acid decarboxylase (AADC), along with the cofactor, pyridoxal-5'-phosphate (the active form of vitamin B6), rapidly converts 5-HTP to 5-HT via decarboxylation (Figure 1).[11,12]

Once synthesized, 5-HT is sequestered via vesicular monoamine transporter-1 and -2 into large dense core storage vesicles and synaptic-like microvesicles located at the base of the cell.[3,11] Here, it is held until it is released into extracellular space, either apically to the gut lumen or basolaterally to the lamina propria, where it largely exerts paracrine effects on enteric neurons and can enter the bloodstream.[4,13,14] Enteroendocrine cells, of which EC cells are a subtype, act as physical and chemical sensory transducers within the gut via their apical microvilli, which extend into the lumen.[15,16] Any excess 5-HT in the EC cell not stored in the vesicles is subjected to oxidative deamination by monoamine oxidase A (MAO-A), resulting in 5-hydroxyindoleacetaldehyde and further metabolized by aldehyde dehydrogenase (ALDH)

producing 5-hydroxyindole acetic acid (5-HIAA), which is mainly excreted in the urine (Figure 1).[12,17] Upon its release from the grips of EC cells, the termination of 5-HT activity can occur in a number of ways including exerting influence on intracellular signaling by impacting one of several 5-HT receptors (5-HTRs),[1,18] having a direct impact on luminal bacteria,[19] or being subjected to reuptake by platelets, neighbouring epithelial or immune cells via serotonin reuptake transporter (SERT).[1,20,21]

Previously thought to be restricted to the pineal gland of the brain, 5-HT in the gut can also be shunted into the melatonin pathway.[22] Melatonin is known mainly as a key photo-regulated hormone involved in controlling the sleep-wake cycle/circadian rhythm and is increasingly being investigated within the context of the gut as well as for its influence on immunological processes.[22,23] Extra-pineal melatonin synthesis can occur in the EC cells of the gut where 5-HT can be directly metabolized to N-acetyl-5-hydroxytryptamine by local aryl alkylamine N-acetyltransferase (AANAT) and eventually to melatonin or N-acetyl-5-methoxytryptamine by hydroxyindole-O-methyltransferase (HIOMT) (Figure 1).[3,11,22] This extra-pineal melatonin is considered an independent reservoir from the melatonin produced in the pineal gland.[24]

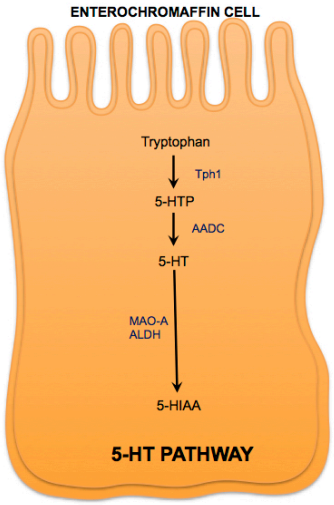


Figure 1. 5-HT pathway of tryptophan metabolism occurs mainly in the enterochromaffin cells. Key enzymes are represented in blue.

3 5-HT IN IMMUNE REGULATION

3.1 5-HT and immune cells

Several investigations over the last 20 years have revealed that 5-HT has both direct and indirect effects on immune cells. Peripheral 5-HT, produced from EC cells, is taken up by SERT and stored in the dense granules of platelets which provides the largest source of 5-HT for immune cells.[14,25] Apart from this, significantly smaller sources of 5-HT are synthesized by immune cells themselves, including mast cells, monocytes, and T cells.[26] The presence of several types of 5-HTRs and SERT on cells of innate and adaptive immunity supports the enormous influence 5-HT signaling has on the immune system. Mammalian immune cells express 7 families with 15 different subtypes of 5-HTRs (5-HTR1-7) on their plasma membrane.[15,27] All 5-HTRs belong to the GPCR superfamily except 5-HTR3 which is a Cys-loop ligand-gated ion channel.[15,27] GPCRs 5-HTR1 and 5-HTR5 couple negatively to adenylyl cyclase and downregulate cyclic adenosine mono-phosphate (cAMP) production upon activation. In contrast, stimulation of 5-HTRs 4, 6 and 7 promote cAMP activity by activation of adenylyl cyclase.[15,28] Activation of 5-HTR2 increases cytosolic Ca^{2+} by the upregulation of inositol triphosphate and diacylglycerol pathways.[15,28] Activation of 5-HTR3, which is a non-selective cation channel triggers rapid depolarization of the plasma membrane due to Ca^{2+} and Na^{+} influx and K^{+} efflux.[15,28] Furthermore, stimulation of these 5-HTRs ultimately regulate downstream signalling cascades via phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) and mitogen associated protein kinase dependent pathways.[7] The close proximity of EC cells to $CD3^{+}$ and $CD20^{+}$ lymphocytes in the lamina propria provides further evidence that 5-HT synthesized from EC cells may directly influence immune cells.[29] Table 1 summarizes the expression of 5-HTRs on the different immune cells of the body.

5-HT has both inhibitory and stimulatory effects on monocytes and macrophages depending on the dose and receptors present. Monocytes express 5-HTRs, SERT, as well as Tph1 and MAO, highlighting the influence 5-HT can have on these cells.[30] 5-HT also regulates interferon-γ (IFN-γ)-induced phagocytosis in bone marrow-derived macrophages. Notably, this effect depends on the concentration of IFN-γ. At high IFN-γ doses, 5-HT inhibits macrophage phagocytosis, whereas, at a low physiological concentration of IFN-γ, 5-HT increases this process.[31] When treated with 5-HT, mouse peritoneal macrophages exhibit increased production

of pro-inflammatory cytokines in a nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB)-dependent manner along with an upregulation of phagocytosis via 5-HTR1A in a dose-dependent manner.[32-34] 5-HT has also been shown to increase the release of superoxides from macrophages and modulate the process of macrophage polarization.[35] Interestingly, in contrast to the previously mentioned findings suggesting that 5-HT increases the production of pro-inflammatory cytokines, Casas-Engel et al. reported opposing results; in these studies, 5-HT was shown to inhibit the lipopolysaccharide-induced release of pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin (IL) 12p40. Moreover, the authors also found that 5-HT upregulates the expression of M2 polarization–associated genes through activating 5-HTR2B and 5-HTR7, and reduces the expression of M1-associated genes.[36] These contradictory findings highlight the importance of receptor subtypes in the modulation of macrophage function by 5-HT.

Natural killer (NK) cells respond to the effects of 5-HT by their surface expression of 5-HTR1A and 5-HTR2A-C.[37] In the early 1990s indirect effect of 5-HT on NK cells were identified through 5-HT mediated regulation of monocytes. 5-HT modulates the interaction between monocytes and NK cells resulting in an increase of NK cell functions including enhanced IFN-γ production and cytotoxicity.[25,38,39] In recent years, further analysis of the mechanisms of monocyte-NK cell interactions revealed that 5-HT protects NK cells from monocyte mediated apoptosis, oxidative damage and thus helps in maintaining the functions of NK cells despite the presence of inhibitory monocytes.[40] Increased concentration of serum 5-HT was linked with enhanced NK cell cytotoxicity.[37] Furthermore, major depressive disorder patients treated with selective serotonin reuptake inhibitors (SSRIs) for short term had increased NK cell cytotoxicity where as long term treatment resulted in increased NK cell proliferation.[41] 5-HT treatment of NK cell line, KHYG-1 at physiological concentrations enhanced their migratory behaviour without affecting cytotoxicity.[37] These findings suggest that the effect of 5-HT on NK cell function and behaviour is highly dependent on the concentration of 5-HT.

The differentiation of monocytes to DCs is also modulated by 5-HT.[15] Following this differentiation, 5-HT plays important roles in the functional capacity of DCs as supported by the presence of 5-HTRs in mature and immature DCs.[42] In mature DCs, activation of 5-HTR3, 5-HTR4, and 5-HTR7 enhances the release of the cytokines IL-1β, IL-6, and IL-8, and reduces the secretion of IL-12 and TNF-α.[42,43] 5-HT has also been shown to induce the production of

chemokines by DCs. Further, 5-HT treated DCs resulted in a preference for the Th2 polarization of naïve $CD4^+$ T cells.[43] Our laboratory has established a critical role of 5-HT in increasing the production of pro-inflammatory cytokine IL-12 from DCs via activation of the NF-κB pathway and sequential $CD4^+$ T cell activation in relation to generation of gut inflammation.[44]

Using a *Tph1*$^{-/-}$ mouse model which has a marked reduction in gut 5-HT, Duerschmied and colleagues demonstrated that gut-derived 5-HT promotes rolling and adhesion and, thus, impacts the recruitment of neutrophils to sites of acute inflammation.[30,45] 5-HT also plays a critical role in the migration and recruitment of other innate immune cells such as eosinophils via 5-HTR2A and mast cells via 5-HT1A to sites of acute inflammation.[46,47] Further, 5-HT promotes angiogenesis at the site of inflammation by stimulating the production of vascular endothelial growth factor in a PI3K/Akt/mTOR-dependent pathway in endothelial cells (Figure 2).[48]

In addition to its wide array of effects on innate immune cells, 5-HT also regulates the function of adaptive immune cells directly, through 5-HTRs expressed on T and B lymphocytes, and indirectly by influencing the actions of antigen-presenting cells (APCs) (Figure 2). B cells express several 5-HTRs as well as SERT.[15,30] 5-HT increases mitogen-stimulated B cell proliferation via the 5-HTR1A.[49] In this vein, long term use of SSRI in patients with major depressive disorders has been reported to increase the number of circulating B lymphocytes by ~30%.[50] Serafeim et al. demonstrated that active uptake of 5-HT via SERT drives apoptosis of Burkitt lymphoma cells, whereas the SSRIs fluoxetine, paroxetine, and citalopram blocked this action.[51] In a follow-up study, it was seen that higher doses of SSRIs trigger rapid and extensive apoptosis by inhibiting DNA synthesis. Interestingly, healthy peripheral blood mononuclear cells and tonsilar B cells are resistant to SSRI-triggered apoptosis.[52] These findings open the discussion of whether the 5-HT-altering SSRI antidepressants can be used for the targeted therapy of Burkitt lymphoma cells without affecting the function of normal B cells.

5-HT also acts as an endogenous hormone which stimulates T cell proliferation and activation via autocrine and paracrine signaling.[15] T lymphocytes express 5-HTRs along with Tph1, SERT and MAO.[30] Exogenous 5-HT induces phosphorylation of extracellular signal-regulated kinase-1 and -2 and IκBα in naive T cells, which is blocked by the 5-HTR7 antagonist SB269970 resulting in early T cell activation.[53] Additional studies in both human and mouse T cells provide further evidence that stimulation of 5-HTR1A, 5-HTR1B, 5-HTR2A, and 5-HTR3 promote T cell proliferation, differentiation, and function, demonstrating the immunostimulatory

role of 5-HT.[54-57] It should be noted, however, that despite these findings, some researchers have recorded immunosuppressive roles of 5-HT in the context of T cell biology.[58,59] It is important to acknowledge that most of the studies on T cells and 5-HT do not differentiate between the different subtypes of T cells. Thus, it can be reasoned that the contradictory findings of 5-HT-induced immune activation and suppression may be due to the differences in 5-HTRs expression by T cell subtypes.[60] This hypothesis is further supported by the fact that $CD8^+$ T cells have significantly greater expression of Tph1 and MAO-A mRNA as well as higher production of 5-HT compared to $CD4^+$ T cells isolated from the spleens of male C57BL/6 mice.[61]

TABLE 1. Family, subtype and expression of 5-HT receptors on immune cells

5-HTR family and subtypes	Immune cell type
5-HTR1A	Mast cells, eosinophils, monocytes, macrophages, NK cells, T cells, B cells
5-HTR1B	Eosinophils, macrophages, DCs, T cells
5-HTR1D	Unknown
5-HTR1E	Eosinophils, monocytes, macrophages, DCs
5-HTR1F	Unknown
5-HTR2A	Monocytes, macrophages, DCs, eosinophils, NK cells, T cells, B cells
5-HTR2B	Monocytes, macrophages, DCs, eosinophils, NK cells
5-HTR2C	Macrophages, NK cells, T cells
5-HTR3	Monocytes, macrophages, DCs, T cells, B cells
5-HTR4	Monocytes, macrophages, DCs
5-HTR5	Unknown
5-HTR6	Eosinophils
5-HTR7	Neutrophils, monocytes, macrophages, DCs, T cell, B cells

Source: The table has been adopted from Shajib et al.,[25] Wan et al.[44] and Herr et al.[47]

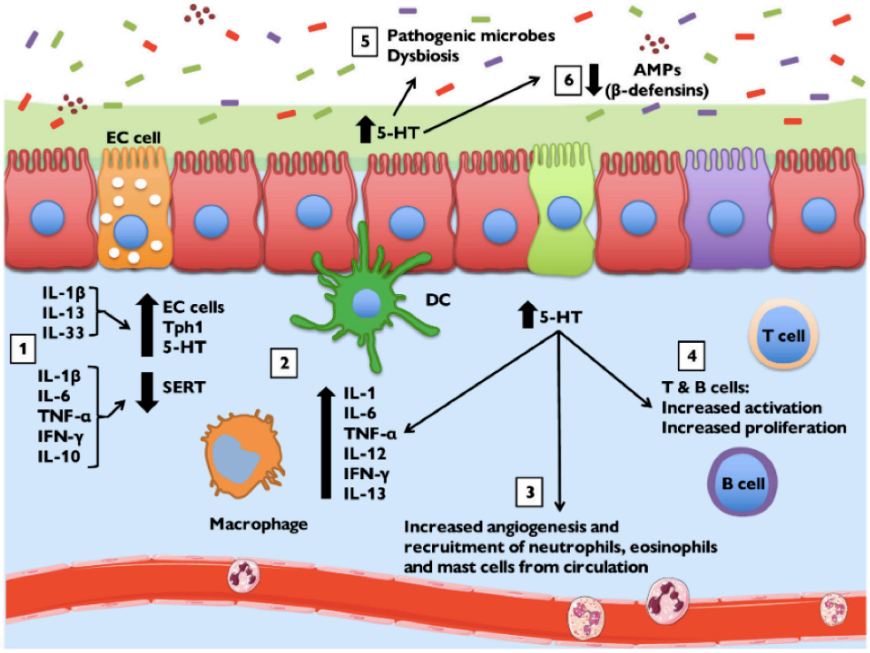


Figure 2. Schematic representation of the effect of mucosal 5-HT in the immune response and intestinal inflammation. This diagram shows the state of the mucosal immune system and microbiota in the context of increased mucosal 5-HT signaling. 1) Pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IFN-γ, and anti-inflammatory cytokine IL-10, at low levels, reduce the expression of SERT. Cytokines IL-1β, IL-33, and IL-13 enhance the secretion of 5-HT from EC cells. IL-13 increases EC cell number and Tph1 mRNA. Elevated mucosal 5-HT during intestinal inflammation leads to: 2) Increased expression of cytokines IL-1, IL-6, TNF-α, IL-12, IFN-γ, and IL-13 from activated macrophages and dendritic cells (DCs). 3) Increased angiogenesis and recruitment of neutrophils, eosinophils, and mast cells from the circulation to sites of inflammation. 4) T and B cell activation and proliferation. 5) Development of a colitogenic microbiota. 6) Decreased production of anti-microbial peptides (AMPs) such as β-defensins.

4 5-HT AND GUT MICROBIOTA

4.1 Microbiota impact on EC cells/5-HT production

In the intestinal mucosa, the close proximity between the microbiota, EC cells, and immune cells suggests the interaction between these constituents may influence the pathophysiology of intestinal inflammation.[8] Colonic EC cells express several nutrient- and

metabolite-sensing GPCRs, including those that sense microbially-derived short-chain fatty acids such as the free fatty acid receptor 2, and the olfactory receptors, OLFR78 and OLFR558.[62] In a recent study, Yano et al. demonstrated that select gut microbes and their metabolic products, including acetate, butyrate and propionate, can directly signal colonic ECs to promote Tph1 expression, modulate 5-HT biosynthesis, effect luminal and circulating 5-HT contents, and alter host physiology.[63] Short-chain fatty acids interacting on GPCR have also been shown to impact muscle contraction and motility within the gut via 5-HT signaling.[64]

Microbes may also directly modulate 5-HT production from EC cells via toll-like receptors (TLRs).[7] TLRs respond to bacterial ligands and are expressed on a variety of cells, including EC cells.[65] These receptors play an important role in innate immune surveillance. TLR2 in particular, has been shown to have a crucial role in 5-HT production in the gut.[66] Recently, we demonstrated that altering microbial composition with antibiotic administration greatly reduced EC cell number, colonic 5-HT levels, and TLR2 expression in C57BL/6 mice.[66] In alignment with these findings, Ge et al. illustrated that antibiotic-induced dysbiosis promoted decreased levels of 5-HT and Tph1 and slowed transit time and GI motility in C57BL/6 mice.[67] In our study, *TLR2*$^{-/-}$ mice revealed significantly decreased EC cell number and colonic 5-HT concentration in comparison with wild-type (WT) mice.[66] In a similar vein, expression of the intracellular bacterial sensor, nucleotide-binding oligomerization domain-containing protein 1 (NOD1), is modulated by 5-HT and TLR2, and has also been shown to inhibit SERT activity in Caco-2/TC7 cells.[68] Increased EC cell number and colonic 5-HT concentration have been reported in infection with the murine intestinal nematode, *Trichuris muris*.[69] It has also been shown that TLR2 plays an important role in mucosal 5-HT production in the gut by resident microbiota as well as by *T. muris*.[66]

Further evidence illustrating the pivotal role the gut microbiota plays in 5-HT production can be gleaned from germ-free (GF) studies. In GF mice, serum concentrations of 5-HT were reduced compared to conventionally raised mice. Furthermore, GF mice displayed reduced Tph1 and increased SERT mRNA in the proximal colon.[70] Similarly, Reigstad et al. demonstrated that conventionally raised mice and mice colonized with human fecal microbiota, despite unchanged EC cell number, had increased levels of both Tph1 protein and 5-HT concentration in the colon compared with their GF counterparts.[71] The authors propose that the microbial impact on host 5-

HT expression is directly responsible for these changes since exogenous/luminal 5-HT was not detected in the transferred microbial samples.

4.2 5-HT impact on the gut microbiota

Our laboratory has recently elucidated the influence that 5-HT has on the intestinal microbiota and colitis, illustrating 5-HT's direct impact on bacterial growth in a species-dependent manner and its indirect impact on bacterial composition via antimicrobial peptide (AMP), β-defensins (Figure 2). In this study, microbial composition differed significantly in *Tph1*$^{+/-}$ compared with *Tph1*$^{-/-}$ mice. Notably, GF mice colonized with *Tph1*$^{-/-}$ microbiota had increased abundance of mucin degrading *Akkermansia,* a touted "next-generation probiotic". We also demonstrated that the altered microbiota compositions observed play a key role in susceptibility to colitis.[19] In addition, *SERT*$^{-/-}$ mice, which have increased levels of 5-HT in the intestine,[68] exhibited significant alterations in microbial composition, particularly a significant increase in Firmicutes abundance in *SERT*$^{-/-}$ mice compared to *SERT*$^{+/+}$ mice.[72]

Intriguingly, by potentially acting as efflux pump inhibitors, SSRIs have been shown to have antibiotic effects primarily with regard to Gram-positive microorganisms, including *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Campylobacter jejuni*.[73] In addition, *in vitro* studies have indicated that 5-HT stimulates the growth of commensal strains of *Escherichia coli*, *Enterococcus faecalis,* and *Rhodospirillum rubrum* when added to the nutrient medium. It is speculated that the stimulatory effect of 5-HT on microbial growth may be due to the presence of 5-HT receptors on the surface of these microorganisms that may also bind related compounds such as indole.[74]

The 5-HT-microbiota axis is further highlighted by the multitude of microorganisms that have intimate interactions with 5-HT biosynthesis. For instance, though the enteric pathogen, *Citrobacter rodentium,* increases 5-HT release and decreases SERT immunoreactivity ten days post-infection. It has also been touted to sense 5-HT in the gut directly via membrane-bound histidine sensor kinase, CpxA.[75] Through this kinase, 5-HT is able to decrease the expression of virulence genes in this enteric pathogen.[76] Moreover, 5-HT enhances the adherence and invasion of commensal *E. coli* strains into Caco-2 cells as well as in murine gut submucosa by amplifying signals in colonic epithelial cells.[77]

The bidirectional relationship between host and microbiota is neatly reflected in the ability of *Turicibacter sanguinis,* a spore-forming gut bacteria, to uptake 5-HT via a

neurotransmitter sodium symporter-related protein structurally similar to mammalian SERT. *T. sanguinis* then is able to affect host expression of a number of metabolically relevant pathways, suggesting that host-derived 5-HT not only impacts bacteria directly but these bacteria, in turn, affect the host physiology.[78]

5 5-HT PATHWAY IN GUT INFLAMMATION

5.1 5-HT and experimental colitis

In experimental colitis models, such as trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid (DNBS), or dextran sulfate sodium (DSS), an increase in 5-HT content has been documented which reinforces similar findings in IBD patients.[79-81] Our lab has reported that mice are less susceptible to chemical- and infection-induced colitis when gut mucosal 5-HT content is significantly reduced, either by using Tph1 enzyme inhibitors or by knocking out the *Tph1* gene.[33,82] As an explanatory mechanism, we have further identified a key pro-inflammatory role of 5-HTR7 on DCs; 5-HT activates DC-expressed 5-HTR7 to initiate immune mechanisms and, thus, contributes to intestinal inflammation.[44,83] The relationship between 5-HT and gut inflammation was further supported by Gershon et al., who reported exaggeration in TNBS colitis in *SERT*$^{-/-}$ mice which have increased 5-HT in the gut.[84] Despite the above evidence, some recent studies by Spohn et al. have brought forward new insights regarding the anti-inflammatory role of 5-HT in the mucosa, specifically the protective and recuperative actions of intestinal epithelial 5-HTR4 activation in a chemical colitis model. 5-HTR4 is required for the physiological maintenance of epithelial integrity.[85] Increased epithelial proliferation, migration and resistance to oxidative stress-induced apoptosis seem to underlie the anti-inflammatory effects of 5-HTR4. Spohn et al. explained the contradictory roles of 5-HT in gut inflammation by hypothesizing that 5-HTR4- associated anti-inflammatory action is present in the basal condition whereas the 5-HTR7 pro-inflammatory effects predominate in pathological states.[2] These observations suggest that the action of mucosal 5-HT in the gut is highly dependent on the type of receptor, type of immune cells and the local environmental conditions. Further supporting this, Gershon et al. have demonstrated that in experimental colitis, the actions of mucosal and neuronal 5-HT are distinct. Interestingly, during colitis, 5-HT synthesized by Tph2 in the serotonergic neurons of the ENS was found to be anti-inflammatory and

neuroprotective, whereas mucosal 5-HT was regarded as pro-inflammatory.[86]

Cytokines, immune signaling molecules, play a critical role in gut inflammation. The relationship between 5-HT and cytokines is bidirectional. Pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IFN-γ, and low levels of the anti-inflammatory cytokine, IL-10, reduce the expression and function of SERT (Figure 2).[87-90] However, at higher concentrations, IL-10 increases the expression of SERT on epithelial cells via the PI3K signaling pathway.[87] During intestinal inflammation, both the Th1 cytokine, IL-1β, and the Th2 cytokine, IL-13, significantly enhance the secretion of 5-HT from EC cells via the activation of IL-1 and IL-13 receptors (Figure 2).[91,92] Moreover, IL-13 also increases the synthesis of mucosal 5-HT by upregulating Tph1 expression (Figure 2).[92,93] However, the effect of IL-13 and IL-1β on neuronal 5-HT is yet to be determined.

Recently, another member of the IL-1 family, an alarmin cytokine, IL-33, was found to activate the ST2 receptor on EC cells triggering calcium influx via a non-canonical phospholipase C-γ1- transient receptor potential ankyrin 1 signaling pathway to induce 5-HT release. It has also been hypothesized that the serotonergic neurons might release neuronal 5-HT by IL-33 activation regardless of their distinct synthetic mechanism.[94] As mentioned above, during inflammation, 5-HT itself also increases the secretion of pro-inflammatory cytokines like IL-1β, IL-6, and IFN-γ from macrophages, DCs and NK cells.

5.3 Clinical implications of 5-HT in GI disorders

IBD, comprised of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing inflammatory condition of the GI tract with a rising incidence worldwide.[95] In CD patients, an increase in EC cell number and 5-HT content has been documented in the intestinal mucosa, and increased mucosal 5-HT signaling has been shown to contribute to the severity of inflammation in active disease.[91,96-99] Recently, it has been reported that active CD patients have elevated Tph1 and 5-HT receptors and downregulated SERT expression in the intestinal mucosa as well as increased plasma/serum 5-HT levels.[96,100,101] However, the role of 5-HT in UC is somewhat controversial with groups reporting both increased and decreased 5-HT signaling.[102] Furthermore, changes in 5-HT signaling occur in other intestinal disorders with underlying inflammation like IBS and celiac disease.[103,104] In fact, the role of 5-HT in IBS is so well established that 5-HTR3 and 5-HTR4 targeted therapies are among approved treatments for IBS.

Studies on 5-HT-increasing SSRIs and their association with IBD have further highlighted the importance of 5-HT signaling in gut inflammation in a clinical setting. Chronic intake of SSRIs, such as sertraline increases the risk of developing microscopic colitis. Even though the mechanism is not yet known, it is postulated that SSRIs and other medications such as nonsteroidal anti-inflammatory drugs, may trigger colonic inflammation in genetically predisposed individuals or may lead to the development of more apparent GI symptoms in a previously undiagnosed patient.[105] However, other researchers have found that SSRIs can have no effect or have a beneficial/protective effect against IBD.[106-108] However, it should be noted that these studies were observational, uncontrolled and nonrandomized, and the discrepancies in these findings may be due to the small sample size and/or short follow-up periods.[108] Therefore, it is pertinent to conduct further randomized controlled trials with larger sample sizes and follow-up periods greater than 12 months to clearly understand the impact of SSRIs on gut inflammation.

6 THERAPEUTIC TARGETING OF 5-HT IN GI DISORDERS

6.1 Targeting 5-HT in GI disorders

Clinically, 5-HT targeting drugs for GI diseases are currently in use. 5-HTR3 and 5-HTR4 have been studied extensively and targeted for the treatment of nausea, vomiting, diarrhea, and constipation. 5-HTR3 antagonists, like ondansetron, exert anti-emetic effects by their action on afferent vagal nerves in the gut and in the area postrema of the medulla oblongata. These antagonists are used to treat nausea and vomiting induced by chemotherapy and radiation therapy.[109,110] Further, other 5-HTR3 antagonists such as alosetron are used to treat diarrhea and abdominal discomfort in patients with diarrhea-predominant IBS (IBS-D), though the exact mechanism of action is not known.[111,112] Similarly, 5-HTR4 agonists like tegaserod alleviate constipation and pain in IBS-C, and in chronic constipation.[113] 5-HTR4 agonists are prokinetic agents that accelerate and strengthen the peristaltic reflex, and contribute to the regulation of propulsive motility.[114,115] The prokinetic effects of 5-HTR4 agonists are mediated by their action on receptors expressed on presynaptic enteric nerve fibres and neurons.[115,116] Unfortunately, older, non-selective 5-HTR4 agonists had off-target effects on hERG potassium channels, dopamine receptors, 5-HTR1 and 5-HTR2 that resulted in

adverse cardiovascular effects and were subsequently removal from the market.[117] Increasing evidence suggests that targeting the mucosal epithelial cells with 5-HTR4 agonists helps to produce the desired clinical outcomes with greatly reduced adverse cardiovascular effects. [16,114]

With the functional classification of Tph isoforms and the evidence that gut-associated Tph1 generates more than 95% of the body's 5-HT, several research groups have probed the utilization of Tph inhibitors in the treatment of 5-HT-associated peripheral diseases. The Tph inhibitors LX-1031 and LX-1033 were the first drugs to enter clinical trials and showed promise in the treatment of non-constipating IBS.[118,119] Because of their inability to cross the intestinal barrier, the action of these drugs was restricted to Tph in the gut despite their nonselective nature.[3] With that being said, the only Tph inhibitor approved by the FDA is telotristat ethyl used to treat carcinoid syndrome diarrhoea by reducing peripheral 5-HT without affecting central 5-HT.[120] As discussed above, *Tph1*$^{-/-}$ mice and animals treated with Tph1 inhibitors are protected in several different colitis models.[33,44,82,121,122] Based on these promising results and the success in IBS clinical trials, it is clear that Tph inhibitors are promising candidates for the treatment of inflammatory disorders such as IBD.

ACKNOWLEDGEMENTS

This work was supported by grant from the Canadian Institute of Health Research (CIHR) to WIK (PJT 156262). SH was a recipient of the Canadian Association of Gastroenterology (CAG) PhD Studentship Award and Farncombe Student Award from the Farncombe Family Digestive Health Research Institute, McMaster University. JAG was a recipient of the CIHR Frederick Banting and Charles Best Canada Graduate Scholarship-Master's (CGS-M).

CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

S. Haq and J.A. Grondin equally reviewed the literature and wrote the manuscript; S. Haq conceived the idea for the article; S. Haq and J.A. Grondin designed and created the figures; W.I. Khan and J.A. Grondin edited and revised the manuscript; W. I. Khan supervised the project; all authors provided critical feedback and shaped the final manuscript.

REFERENCES

1. El-Merahbi R, Loffler M, Mayer A, Sumara G. The roles of peripheral serotonin in metabolic homeostasis. *FEBS Lett.* 2015;589(15):1728-1734.
2. Spohn SN, Mawe GM. Non-conventional features of peripheral serotonin signalling - the gut and beyond. *Nat Rev Gastroenterol Hepatol.* 2017;14(7):412-420.
3. Matthes S, Bader M. Peripheral Serotonin Synthesis as a New Drug Target. *Trends Pharmacol Sci.* 2018;39(6):560-572.
4. Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. *Biochem Pharmacol.* 2003;66(9):1673-1680.
5. Zhang X, Beaulieu JM, Sotnikova TD, Gainetdinov RR, Caron MG. Tryptophan hydroxylase-2 controls brain serotonin synthesis. *Science.* 2004;305(5681):217.
6. Gutknecht L, Waider J, Kraft S, Kriegebaum C, Holtmann B, Reif A, Schmitt A, Lesch KP. Deficiency of brain 5-HT synthesis but serotonergic neuron formation in Tph2 knockout mice. *J Neural Transm (Vienna).* 2008;115(8):1127-1132.
7. Banskota S, Ghia JE, Khan WI. Serotonin in the gut: Blessing or a curse. *Biochimie.* 2019;161:56-64.
8. Khan WI, Ghia JE. Gut hormones: emerging role in immune activation and inflammation. *Clin Exp Immunol.* 2010;161(1):19-27.
9. Erspamer V, Asero B. Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature.* 1952;169(4306):800-801.
10. Amireault P, Sibon D, Cote F. Life without peripheral serotonin: insights from tryptophan hydroxylase 1 knockout mice reveal the existence of paracrine/autocrine serotonergic networks. *ACS Chem Neurosci.* 2013;4(1):64-71.
11. Keszthelyi D, Troost FJ, Masclee AA. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. *Neurogastroenterol Motil.* 2009;21(12):1239-1249.
12. Shajib MS, Baranov A, Khan WI. Diverse Effects of Gut-Derived Serotonin in Intestinal Inflammation. *ACS Chem Neurosci.* 2017;8(5):920-931.
13. Tamir H, Gershon MD. Serotonin-storing secretory vesicles. *Ann N Y Acad Sci.* 1990;600:53-66; discussion 67.
14. Bertrand PP, Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. *Auton Neurosci.* 2010;153(1-2):47-57.
15. Shajib MS, Khan WI. The role of serotonin and its receptors in activation of immune responses and inflammation. *Acta Physiol (Oxf).* 2015;213(3):561-574.
16. Mawe GM, Hoffman JM. Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets. *Nat Rev Gastroenterol Hepatol.* 2013;10(8):473-486.
17. Boadle-Biber MC. Regulation of serotonin synthesis. *Prog Biophys Mol Biol.* 1993;60(1):1-15.
18. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. *Annu Rev Med.* 2009;60:355-366.
19. Kwon YH, Wang H, Denou E, Ghia JE, Rossi L, Fontes ME, Bernier SP, Shajib MS, Banskota S, Collins SM, Surette MG, Khan WI. Modulation of Gut Microbiota Composition by Serotonin Signaling Influences Intestinal Immune Response and Susceptibility to Colitis. *Cell Mol Gastroenterol Hepatol.* 2019;7(4):709-728.

20. Gershon MD, Ross LL. Radioisotopic studies of the binding, exchange, and distribution of 5-hydroxytryptamine synthesized from its radioactive precursor. *J Physiol.* 1966;186(2):451-476.
21. Gershon MD. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. *Aliment Pharmacol Ther.* 2004;20 Suppl 7:3-14.
22. Chen CQ, Fichna J, Bashashati M, Li YY, Storr M. Distribution, function and physiological role of melatonin in the lower gut. *World J Gastroenterol.* 2011;17(34):3888-3898.
23. Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. *Endocrine.* 2005;27(2):189-200.
24. Bubenik GA, Brown GM. Pinealectomy reduces melatonin levels in the serum but not in the gastrointestinal tract of rats. *Biol Signals.* 1997;6(1):40-44.
25. Baganz NL, Blakely RD. A dialogue between the immune system and brain, spoken in the language of serotonin. *ACS Chem Neurosci.* 2013;4(1):48-63.
26. Ahern GP. 5-HT and the immune system. *Curr Opin Pharmacol.* 2011;11(1):29-33.
27. Wan M, Ding L, Wang D, Han J, Gao P. Serotonin: A Potent Immune Cell Modulator in Autoimmune Diseases. *Front Immunol.* 2020;11:186.
28. Hannon J, Hoyer D. Molecular biology of 5-HT receptors. *Behav Brain Res.* 2008;195(1):198-213.
29. Yang GB, Lackner AA. Proximity between 5-HT secreting enteroendocrine cells and lymphocytes in the gut mucosa of rhesus macaques (Macaca mulatta) is suggestive of a role for enterochromaffin cell 5-HT in mucosal immunity. *J Neuroimmunol.* 2004;146(1-2):46-49.
30. Herr N, Bode C, Duerschmied D. The Effects of Serotonin in Immune Cells. *Front Cardiovasc Med.* 2017;4:48.
31. Sternberg EM, Wedner HJ, Leung MK, Parker CW. Effect of serotonin (5-HT) and other monoamines on murine macrophages: modulation of interferon-gamma induced phagocytosis. *J Immunol.* 1987;138(12):4360-4365.
32. Freire-Garabal M, Nunez MJ, Balboa J, Lopez-Delgado P, Gallego R, Garcia-Caballero T, Fernandez-Roel MD, Brenlla J, Rey-Mendez M. Serotonin upregulates the activity of phagocytosis through 5-HT1A receptors. *Br J Pharmacol.* 2003;139(2):457-463.
33. Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, Cote F, Mallet J, Khan WI. Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology.* 2009;137(5):1649-1660.
34. Nakamura K, Sato T, Ohashi A, Tsurui H, Hasegawa H. Role of a serotonin precursor in development of gut microvilli. *Am J Pathol.* 2008;172(2):333-344.
35. Silverman DH, Wu H, Karnovsky ML. Muramyl peptides and serotonin interact at specific binding sites on macrophages and enhance superoxide release. *Biochem Biophys Res Commun.* 1985;131(3):1160-1167.
36. de las Casas-Engel M, Dominguez-Soto A, Sierra-Filardi E, Bragado R, Nieto C, Puig-Kroger A, Samaniego R, Loza M, Corcuera MT, Gomez-Aguado F, Bustos M, Sanchez-Mateos P, Corbi AL. Serotonin skews human macrophage polarization through HTR2B and HTR7. *J Immunol.* 2013;190(5):2301-2310.
37. Zimmer P, Bloch W, Kieven M, Lovenich L, Lehmann J, Holthaus M, Theurich S, Schenk A. Serotonin Shapes the Migratory Potential of NK Cells - An in vitro Approach. *Int J Sports Med.* 2017;38(11):857-863.

38. Hellstrand K, Hermodsson S. Serotonergic 5-HT1A receptors regulate a cell contact-mediated interaction between natural killer cells and monocytes. *Scand J Immunol.* 1993;37(1):7-18.
39. Hellstrand K, Czerkinsky C, Ricksten A, Jansson B, Asea A, Kylefjord H, Hermodsson S. Role of serotonin in the regulation of interferon-gamma production by human natural killer cells. *J Interferon Res.* 1993;13(1):33-38.
40. Betten A, Dahlgren C, Hermodsson S, Hellstrand K. Serotonin protects NK cells against oxidatively induced functional inhibition and apoptosis. *J Leukoc Biol.* 2001;70(1):65-72.
41. Capellino S, Claus M, Watzl C. Regulation of natural killer cell activity by glucocorticoids, serotonin, dopamine, and epinephrine. *Cell Mol Immunol.* 2020;17(7):705-711.
42. Idzko M, Panther E, Stratz C, Muller T, Bayer H, Zissel G, Durk T, Sorichter S, Di Virgilio F, Geissler M, Fiebich B, Herouy Y, Elsner P, Norgauer J, Ferrari D. The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol.* 2004;172(10):6011-6019.
43. Muller T, Durk T, Blumenthal B, Grimm M, Cicko S, Panther E, Sorichter S, Herouy Y, Di Virgilio F, Ferrari D, Norgauer J, Idzko M. 5-hydroxytryptamine modulates migration, cytokine and chemokine release and T-cell priming capacity of dendritic cells in vitro and in vivo. *PLoS One.* 2009;4(7):e6453.
44. Li N, Ghia JE, Wang H, McClemens J, Cote F, Suehiro Y, Mallet J, Khan WI. Serotonin activates dendritic cell function in the context of gut inflammation. *Am J Pathol.* 2011;178(2):662-671.
45. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, Cifuni SM, Mauler M, Cicko S, Bader M, Idzko M, Bode C, Wagner DD. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood.* 2013;121(6):1008-1015.
46. Kang BN, Ha SG, Bahaie NS, Hosseinkhani MR, Ge XN, Blumenthal MN, Rao SP, Sriramarao P. Regulation of serotonin-induced trafficking and migration of eosinophils. *PLoS One.* 2013;8(1):e54840.
47. Kushnir-Sukhov NM, Gilfillan AM, Coleman JW, Brown JM, Bruening S, Toth M, Metcalfe DD. 5-hydroxytryptamine induces mast cell adhesion and migration. *J Immunol.* 2006;177(9):6422-6432.
48. Banskota S, Gautam J, Regmi SC, Gurung P, Park MH, Kim SJ, Nam TG, Jeong BS, Kim JA. BJ-1108, a 6-Amino-2,4,5-Trimethylpyridin-3-ol Analog, Inhibits Serotonin-Induced Angiogenesis and Tumor Growth through PI3K/NOX Pathway. *PLoS One.* 2016;11(1):e0148133.
49. Iken K, Chheng S, Fargin A, Goulet AC, Kouassi E. Serotonin upregulates mitogen-stimulated B lymphocyte proliferation through 5-HT1A receptors. *Cell Immunol.* 1995;163(1):1-9.
50. Hernandez ME, Martinez-Fong D, Perez-Tapia M, Estrada-Garcia I, Estrada-Parra S, Pavon L. Evaluation of the effect of selective serotonin-reuptake inhibitors on lymphocyte subsets in patients with a major depressive disorder. *Eur Neuropsychopharmacol.* 2010;20(2):88-95.
51. Serafeim A, Grafton G, Chamba A, Gregory CD, Blakely RD, Bowery NG, Barnes NM, Gordon J. 5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors. *Blood.* 2002;99(7):2545-2553.

52. Serafeim A, Holder MJ, Grafton G, Chamba A, Drayson MT, Luong QT, Bunce CM, Gregory CD, Barnes NM, Gordon J. Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells. *Blood.* 2003;101(8):3212-3219.
53. Leon-Ponte M, Ahern GP, O'Connell PJ. Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor. *Blood.* 2007;109(8):3139-3146.
54. Yin J, Albert RH, Tretiakova AP, Jameson BA. 5-HT(1B) receptors play a prominent role in the proliferation of T-lymphocytes. *J Neuroimmunol.* 2006;181(1-2):68-81.
55. Inoue M, Okazaki T, Kitazono T, Mizushima M, Omata M, Ozaki S. Regulation of antigen-specific CTL and Th1 cell activation through 5-Hydroxytryptamine 2A receptor. *Int Immunopharmacol.* 2011;11(1):67-73.
56. Aune TM, Golden HW, McGrath KM. Inhibitors of serotonin synthesis and antagonists of serotonin 1A receptors inhibit T lymphocyte function in vitro and cell-mediated immunity in vivo. *J Immunol.* 1994;153(2):489-498.
57. Khan NA, Poisson JP. 5-HT3 receptor-channels coupled with Na+ influx in human T cells: role in T cell activation. *J Neuroimmunol.* 1999;99(1):53-60.
58. Stefulj J, Cicin-Sain L, Schauenstein K, Jernej B. Serotonin and immune response: effect of the amine on in vitro proliferation of rat lymphocytes. *Neuroimmunomodulation.* 2001;9(2):103-108.
59. Davydova SM, Cheido MA, Gevorgyan MM, Idova GV. Effects of 5-HT2A receptor stimulation and blocking on immune response. *Bull Exp Biol Med.* 2010;150(2):219-221.
60. Wu H, Denna TH, Storkersen JN, Gerriets VA. Beyond a neurotransmitter: The role of serotonin in inflammation and immunity. *Pharmacol Res.* 2019;140:100-114.
61. Chen Y, Leon-Ponte M, Pingle SC, O'Connell PJ, Ahern GP. T lymphocytes possess the machinery for 5-HT synthesis, storage, degradation and release. *Acta Physiol (Oxf).* 2015;213(4):860-867.
62. Lund ML, Egerod KL, Engelstoft MS, Dmytriyeva O, Theodorsson E, Patel BA, Schwartz TW. Enterochromaffin 5-HT cells - A major target for GLP-1 and gut microbial metabolites. *Mol Metab.* 2018;11:70-83.
63. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell.* 2015;161(2):264-276.
64. Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T. Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *Am J Physiol Regul Integr Comp Physiol.* 2003;284(5):R1269-1276.
65. Bogunovic M, Dave SH, Tilstra JS, Chang DT, Harpaz N, Xiong H, Mayer LF, Plevy SE. Enteroendocrine cells express functional Toll-like receptors. *Am J Physiol Gastrointest Liver Physiol.* 2007;292(6):G1770-1783.
66. Wang H, Kwon YH, Dewan V, Vahedi F, Syed S, Fontes ME, Ashkar AA, Surette MG, Khan WI. TLR2 Plays a Pivotal Role in Mediating Mucosal Serotonin Production in the Gut. *J Immunol.* 2019;202(10):3041-3052.
67. Ge X, Ding C, Zhao W, Xu L, Tian H, Gong J, Zhu M, Li J, Li N. Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. *J Transl Med.* 2017;15(1):13.

68. Layunta E, Latorre E, Forcen R, Grasa L, Plaza MA, Arias M, Alcalde AI, Mesonero JE. NOD1 downregulates intestinal serotonin transporter and interacts with other pattern recognition receptors. *J Cell Physiol.* 2018;233(5):4183-4193.
69. Wang H, Steeds J, Motomura Y, Deng Y, Verma-Gandhu M, El-Sharkawy RT, McLaughlin JT, Grencis RK, Khan WI. CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection. *Gut.* 2007;56(7):949-957.
70. Sjogren K, Engdahl C, Henning P, Lerner UH, Tremaroli V, Lagerquist MK, Backhed F, Ohlsson C. The gut microbiota regulates bone mass in mice. *J Bone Miner Res.* 2012;27(6):1357-1367.
71. Reigstad CS, Salmonson CE, Rainey JF, 3rd, Szurszewski JH, Linden DR, Sonnenburg JL, Farrugia G, Kashyap PC. Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *FASEB J.* 2015;29(4):1395-1403.
72. Singhal M, Turturice BA, Manzella CR, Ranjan R, Metwally AA, Theorell J, Huang Y, Alrefai WA, Dudeja PK, Finn PW, Perkins DL, Gill RK. Serotonin Transporter Deficiency is Associated with Dysbiosis and Changes in Metabolic Function of the Mouse Intestinal Microbiome. *Sci Rep.* 2019;9(1):2138.
73. Munoz-Bellido JL, Munoz-Criado S, Garcia-Rodriguez JA. Antimicrobial activity of psychotropic drugs: selective serotonin reuptake inhibitors. *Int J Antimicrob Agents.* 2000;14(3):177-180.
74. Oleskin AV, Shenderov BA, Rogovsky VS. Role of Neurochemicals in the Interaction between the Microbiota and the Immune and the Nervous System of the Host Organism. *Probiotics Antimicrob Proteins.* 2017;9(3):215-234.
75. O'Hara JR, Skinn AC, MacNaughton WK, Sherman PM, Sharkey KA. Consequences of Citrobacter rodentium infection on enteroendocrine cells and the enteric nervous system in the mouse colon. *Cell Microbiol.* 2006;8(4):646-660.
76. Kumar A, Russell RM, Pifer R, Menezes-Garcia Z, Cuesta S, Narayanan S, MacMillan JB, Sperandio V. The Serotonin Neurotransmitter Modulates Virulence of Enteric Pathogens. *Cell Host Microbe.* 2020;28(1):41-53 e48.
77. Banskota S, Regmi SC, Gautam J, Gurung P, Lee YJ, Ku SK, Lee JH, Lee J, Chang HW, Park SJ, Kim JA. Serotonin disturbs colon epithelial tolerance of commensal E. coli by increasing NOX2-derived superoxide. *Free Radic Biol Med.* 2017;106:196-207.
78. Fung TC, Vuong HE, Luna CDG, Pronovost GN, Aleksandrova AA, Riley NG, Vavilina A, McGinn J, Rendon T, Forrest LR, Hsiao EY. Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. *Nat Microbiol.* 2019;4(12):2064-2073.
79. Oshima S, Fujimura M, Fukimiya M. Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate. *Histochem Cell Biol.* 1999;112(4):257-263.
80. Linden DR, Chen JX, Gershon MD, Sharkey KA, Mawe GM. Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol.* 2003;285(1):G207-216.
81. Khan WI, Motomura Y, Wang H, El-Sharkawy RT, Verdu EF, Verma-Gandhu M, Rollins BJ, Collins SM. Critical role of MCP-1 in the pathogenesis of experimental

colitis in the context of immune and enterochromaffin cells. *Am J Physiol Gastrointest Liver Physiol.* 2006;291(5):G803-811.

82. Kim JJ, Wang H, Terc JD, Zambrowicz B, Yang QM, Khan WI. Blocking peripheral serotonin synthesis by telotristat etiprate (LX1032/LX1606) reduces severity of both chemical- and infection-induced intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol.* 2015;309(6):G455-465.
83. Kim JJ, Bridle BW, Ghia JE, Wang H, Syed SN, Manocha MM, Rengasamy P, Shajib MS, Wan Y, Hedlund PB, Khan WI. Targeted inhibition of serotonin type 7 (5-HT7) receptor function modulates immune responses and reduces the severity of intestinal inflammation. *J Immunol.* 2013;190(9):4795-4804.
84. Bischoff SC, Mailer R, Pabst O, Weier G, Sedlik W, Li Z, Chen JJ, Murphy DL, Gershon MD. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. *Am J Physiol Gastrointest Liver Physiol.* 2009;296(3):G685-695.
85. Spohn SN, Bianco F, Scott RB, Keenan CM, Linton AA, O'Neill CH, Bonora E, Dicay M, Lavoie B, Wilcox RL, MacNaughton WK, De Giorgio R, Sharkey KA, Mawe GM. Protective Actions of Epithelial 5-Hydroxytryptamine 4 Receptors in Normal and Inflamed Colon. *Gastroenterology.* 2016;151(5):933-944 e933.
86. Gershon MD. Serotonin is a sword and a shield of the bowel: serotonin plays offense and defense. *Trans Am Clin Climatol Assoc.* 2012;123:268-280; discussion 280.
87. Latorre E, Mendoza C, Matheus N, Castro M, Grasa L, Mesonero JE, Alcalde AI. IL-10 modulates serotonin transporter activity and molecular expression in intestinal epithelial cells. *Cytokine.* 2013;61(3):778-784.
88. Barbaro MR, Di Sabatino A, Cremon C, Giuffrida P, Fiorentino M, Altimari A, Bellacosa L, Stanghellini V, Barbara G. Interferon-gamma is increased in the gut of patients with irritable bowel syndrome and modulates serotonin metabolism. *Am J Physiol Gastrointest Liver Physiol.* 2016;310(6):G439-447.
89. Zhu CB, Blakely RD, Hewlett WA. The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. *Neuropsychopharmacology.* 2006;31(10):2121-2131.
90. Kong E, Sucic S, Monje FJ, Savalli G, Diao W, Khan D, Ronovsky M, Cabatic M, Koban F, Freissmuth M, Pollak DD. STAT3 controls IL6-dependent regulation of serotonin transporter function and depression-like behavior. *Sci Rep.* 2015;5:9009.
91. Kidd M, Gustafsson BI, Drozdov I, Modlin IM. IL1beta- and LPS-induced serotonin secretion is increased in EC cells derived from Crohn's disease. *Neurogastroenterol Motil.* 2009;21(4):439-450.
92. Manocha M, Shajib MS, Rahman MM, Wang H, Rengasamy P, Bogunovic M, Jordana M, Mayer L, Khan WI. IL-13-mediated immunological control of enterochromaffin cell hyperplasia and serotonin production in the gut. *Mucosal Immunol.* 2013;6(1):146-155.
93. Shajib MS, Wang H, Kim JJ, Sunjic I, Ghia JE, Denou E, Collins M, Denburg JA, Khan WI. Interleukin 13 and serotonin: linking the immune and endocrine systems in murine models of intestinal inflammation. *PLoS One.* 2013;8(8):e72774.
94. Chen Z, Luo J, Li J, Kim G, Stewart A, Urban JF, Jr., Huang Y, Chen S, Wu LG, Chesler A, Trinchieri G, Li W, Wu C. Interleukin-33 Promotes Serotonin Release from Enterochromaffin Cells for Intestinal Homeostasis. *Immunity.* 2021;54(1):151-163 e156.

95. Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol.* 2015;12(12):720-727.
96. Shajib MS, Chauhan U, Adeeb S, Chetty Y, Armstrong D, Halder SLS, Marshall JK, Khan WI. Characterization of Serotonin Signaling Components in Patients with Inflammatory Bowel Disease. *Journal of the Canadian Association of Gastroenterology.* 2018;XX:9.
97. Bishop AE, Pietroletti R, Taat CW, Brummelkamp WH, Polak JM. Increased populations of endocrine cells in Crohn's ileitis. *Virchows Arch A Pathol Anat Histopathol.* 1987;410(5):391-396.
98. El-Salhy M, Danielsson A, Stenling R, Grimelius L. Colonic endocrine cells in inflammatory bowel disease. *J Intern Med.* 1997;242(5):413-419.
99. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology.* 2004;126(7):1657-1664.
100. Manzella CR, Jayawardena D, Pagani W, Li Y, Alrefai WA, Bauer J, Jung B, Weber CR, Gill RK. Serum Serotonin Differentiates Between Disease Activity States in Crohn's Patients. *Inflamm Bowel Dis.* 2020;26(10):1607-1618.
101. Jorandli JW, Thorsvik S, Skovdahl HK, Kornfeld B, Saeterstad S, Gustafsson BI, Sandvik AK, van Beelen Granlund A. The serotonin reuptake transporter is reduced in the epithelium of active Crohn's disease and ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol.* 2020;319(6):G761-G768.
102. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. *Cell Host Microbe.* 2018;23(6):716-724.
103. Ng QX, Soh AYS, Loke W, Lim DY, Yeo WS. The role of inflammation in irritable bowel syndrome (IBS). *J Inflamm Res.* 2018;11:345-349.
104. Coleman NS, Foley S, Dunlop SP, Wheatcroft J, Blackshaw E, Perkins AC, Singh G, Marsden CA, Holmes GK, Spiller RC. Abnormalities of serotonin metabolism and their relation to symptoms in untreated celiac disease. *Clin Gastroenterol Hepatol.* 2006;4(7):874-881.
105. Fernandez-Banares F, Esteve M, Espinos JC, Rosinach M, Forne M, Salas A, Viver JM. Drug consumption and the risk of microscopic colitis. *Am J Gastroenterol.* 2007;102(2):324-330.
106. Frolkis AD, Vallerand IA, Shaheen AA, Lowerison MW, Swain MG, Barnabe C, Patten SB, Kaplan GG. Depression increases the risk of inflammatory bowel disease, which may be mitigated by the use of antidepressants in the treatment of depression. *Gut.* 2019;68(9):1606-1612.
107. Macer BJ, Prady SL, Mikocka-Walus A. Antidepressants in Inflammatory Bowel Disease: A Systematic Review. *Inflamm Bowel Dis.* 2017;23(4):534-550.
108. Mikocka-Walus A, Hughes PA, Bampton P, Gordon A, Campaniello MA, Mavrangelos C, Stewart BJ, Esterman A, Andrews JM. Fluoxetine for Maintenance of Remission and to Improve Quality of Life in Patients with Crohn's Disease: a Pilot Randomized Placebo-Controlled Trial. *J Crohns Colitis.* 2017;11(4):509-514.
109. Gale JD. Serotonergic mediation of vomiting. *J Pediatr Gastroenterol Nutr.* 1995;21 Suppl 1:S22-28.

110. Tyers MB, Freeman AJ. Mechanism of the anti-emetic activity of 5-HT3 receptor antagonists. *Oncology.* 1992;49(4):263-268.
111. Andresen V, Montori VM, Keller J, West CP, Layer P, Camilleri M. Effects of 5-hydroxytryptamine (serotonin) type 3 antagonists on symptom relief and constipation in nonconstipated irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials. *Clin Gastroenterol Hepatol.* 2008;6(5):545-555.
112. Rahimi R, Nikfar S, Abdollahi M. Efficacy and tolerability of alosetron for the treatment of irritable bowel syndrome in women and men: a meta-analysis of eight randomized, placebo-controlled, 12-week trials. *Clin Ther.* 2008;30(5):884-901.
113. Evans BW, Clark WK, Moore DJ, Whorwell PJ. Tegaserod for the treatment of irritable bowel syndrome and chronic constipation. *Cochrane Database Syst Rev.* 2007(4):CD003960.
114. Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, Swain GM, Moses PL, Galligan JJ, Sharkey KA, Greenwood-Van Meerveld B, Mawe GM. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. *Gastroenterology.* 2012;142(4):844-854 e844.
115. Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD. Expression and function of 5-HT4 receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol.* 2005;289(6):G1148-1163.
116. Galligan JJ, Pan H, Messori E. Signalling mechanism coupled to 5-hydroxytryptamine4 receptor-mediated facilitation of fast synaptic transmission in the guinea-pig ileum myenteric plexus. *Neurogastroenterol Motil.* 2003;15(5):523-529.
117. Tack J, Camilleri M, Chang L, Chey WD, Galligan JJ, Lacy BE, Muller-Lissner S, Quigley EM, Schuurkes J, De Maeyer JH, Stanghellini V. Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther.* 2012;35(7):745-767.
118. Camilleri M. LX-1031, a tryptophan 5-hydroxylase inhibitor, and its potential in chronic diarrhea associated with increased serotonin. *Neurogastroenterol Motil.* 2011;23(3):193-200.
119. Brown PM, Drossman DA, Wood AJ, Cline GA, Frazier KS, Jackson JI, Bronner J, Freiman J, Zambrowicz B, Sands A, Gershon MD. The tryptophan hydroxylase inhibitor LX1031 shows clinical benefit in patients with nonconstipating irritable bowel syndrome. *Gastroenterology.* 2011;141(2):507-516.
120. Masab M, Saif MW. Telotristat ethyl: proof of principle and the first oral agent in the management of well-differentiated metastatic neuroendocrine tumor and carcinoid syndrome diarrhea. *Cancer Chemother Pharmacol.* 2017;80(6):1055-1062.
121. Margolis KG. A role for the serotonin reuptake transporter in the brain and intestinal features of autism spectrum disorders and developmental antidepressant exposure. *J Chem Neuroanat.* 2017;83-84:36-40.
122. Margolis KG, Stevanovic K, Li Z, Yang QM, Oravecz T, Zambrowicz B, Jhaver KG, Diacou A, Gershon MD. Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. *Gut.* 2014;63(6):928-937.

CHAPTER 2

1. Inflammatory Bowel Disease (IBD)

IBD, with two major forms, Crohn's disease (CD) and ulcerative colitis (UC) is a chronic relapsing and remitting inflammatory disorder of the GI tract.[91] In the 21st century, IBD is a global disease occurring across geographic boundaries, races, ethnicities, age and sex. The prevalence and incidence of IBD in Canada is one of the highest in the world. About 0.7% of Canadians are affected by IBD that results in substantial financial losses to the society.[92] Patients with CD or UC can present with diarrhea, abdominal pain and per rectal bleeding. Intestinal complications such as strictures, bowel obstruction, abscess and anal fistula can occur in CD where as complications like toxic megacolon, perforation and colon carcinoma are more common in UC.[93] 25-40% of IBD patients may present with extra-intestinal manifestations involving joints, muscles, skin, eyes, bones, kidneys and liver.[93,94]

Although CD and UC have many clinical and pathological features in common, evidence indicates that the two conditions have markedly distinct pathophysiological entities. In UC diffuse continuous superficial mucosal inflammation spreads proximally from the rectum to varying degrees of the colon up to the ileocecal valve. In contrast, the inflammation in CD is transmural, usually discontinuous, patchy and can involve any site of the GI tract from the mouth to the anus. However, the most commonly involved site in CD is the terminal ileum with the lesions appearing over Peyer's patches.[91,93] Endoscopically, in CD healthy mucosa may be interspersed between areas of inflamed mucosa giving the bowel the characteristic appearance of "skip lesions". Histologically, the hallmark of UC is the formation of crypt abscesses composed of transmigrated

neutrophils. On the other hand CD is characterized histologically by non-caseating granulomas consisting of macrophages, giant cells and epithelioid cells.[91] Although the exact etiology of IBD is not clear, it is considered a multifactorial disease resulting from the interactions among the following four basic components: genetic susceptibility, gut microbiota, immune system and environmental factors[95,96] (Figure I).

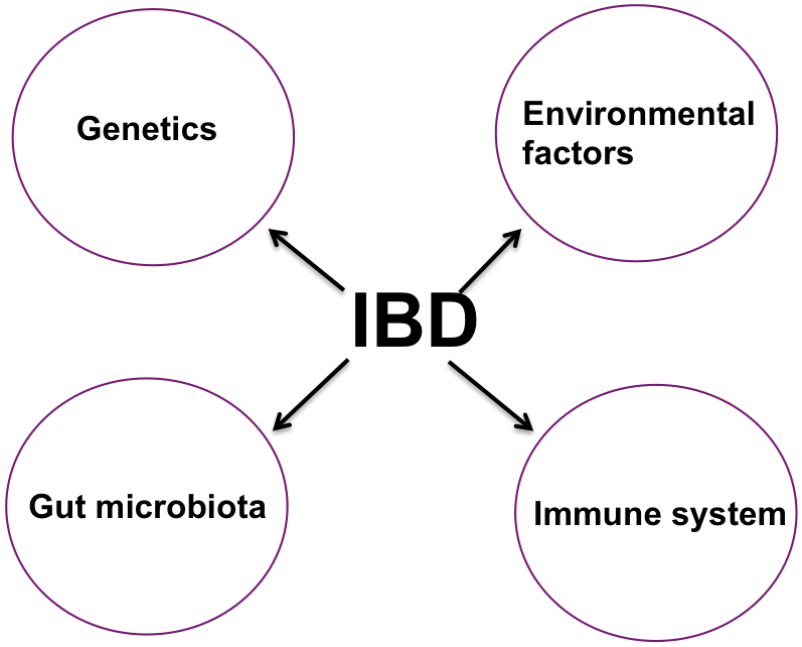


Figure I: Factors involved in the pathogenesis of IBD. The exact cause of IBD in unknown. It is thought to be a multifactorial disease resulting from interactions between host and environmental factors.

1.1 Genetics

The susceptibility and development of IBD is dependent on genetic factors. Compelling evidence from studies on the familial forms of IBD and those conducted with twins emphasize the importance of genetics as a key component in the pathology on IBD. Having a first-degree relative with IBD is an important risk factor for developing the

disease with the relative risk to siblings of patients being about 30- to 40-times higher for CD and 10- to 20-times higher for UC.[97] The rate of concordance for monozygotic twins is 30-35% and 10-15% in CD and UC respectively.[98] These data indicate that compared to UC, CD has a higher genetic load. Additionally, certain ethnic groups of European ancestry have a higher prevalence of familial IBD clustering compared to African American, Hispanic and Asian populations.[99] When multiple relatives within the same family have IBD, the risk increases by approximately 10% for each additional first degree relative affected indicating a cumulative effect of the number of family members affected. Furthermore, there is a 57-fold increase in incidence within these families compared to the general population.[99]

IBD is not inherited as a Mendelian trait, but rather may be due to an interaction of several genes identified by GWAS. Recent studies by GWAS and meta-analysis have identified about 201 IBD associated gene loci, with 137 associated with both types of IBD, 37 specific to CD and 27 specific to UC.[100] The first gene locus identified to have an association with IBD was nucleotide-binding oligomerization domain containing 2 (NOD2).[101] NOD2 gene codes for an intracellular protein receptor that recognizes muramyl dipeptide, a conserved peptidoglycan motif in the cell wall of Gram positive and Gram negative bacteria. Activation of NOD2 in myeloid and epithelial cells induces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mitogen activated protein kinase (MAPK) mediated immune responses and protects the host against luminal microbiota.[95,102] Disease-associated mutations in NOD2 gene results in defective microbial sensing and recognition leading to a loss of barrier immuno-

surveillance and induction of pro-inflammatory responses.[95,102] Autophagy gene variants such as Atg16L1, IRGM and Atg4B are linked to CD showing an indispensible role of autophagy in immune response and inflammation.[31,33,100] Autophagy has also been related to CD through NOD2 gene. When bacterial compounds like muramyl dipeptide activate NOD2, Atg16L1 is recruited to the isolation membrane and autophagosome production is stimulated. When autophagy is inhibited, NOD2 stimulation in human primary immune cells, results in an increase in pro-inflammatory cytokine response.[24,103,104] Apart from these genes, several of the IBD associated loci are involved in microbial detection, endoplasmic reticulum (ER) stress, intestinal barrier function and integration of the adaptive immune system, which are summarized in, Figure II. Although presence of multiple loci can elevate susceptibility to IBD, single genetic variants have been correlated with rare cases of very-early-onset IBD.[105] However, since majority of people with IBD-associated risk variants do not develop the disease and vice versa, it is evident that other factors as well as gene-environment interactions play a vital role in the pathophysiology of IBD.[106]

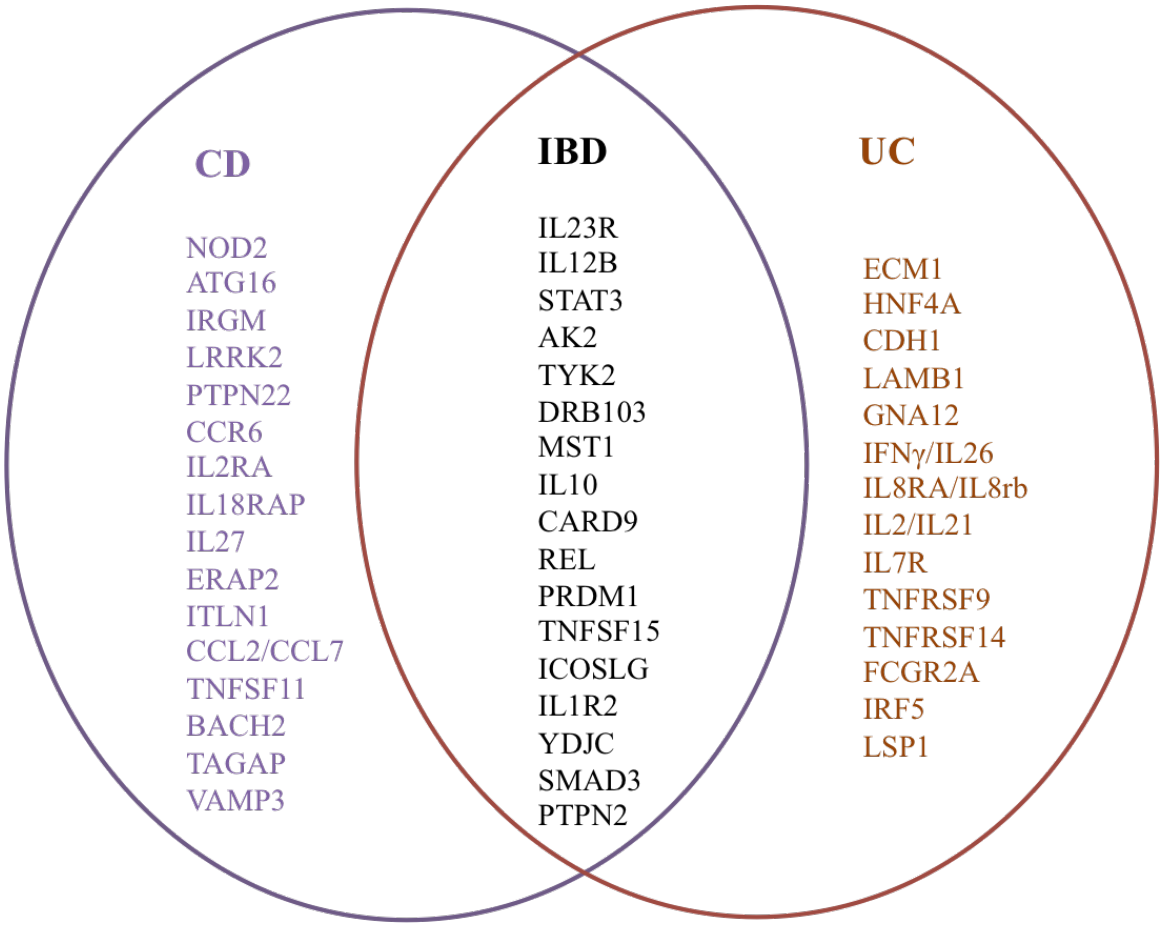


Figure II: Inflammatory bowel disease (IBD) loci associated with immune system, represented by lead gene name. Loci associated with IBD are shown in black, CD in purple and UC in brown. Adopted from Lees et al.[107]

1.2 Environmental factors

One of the basic tenets in the pathophysiology of IBD is the interaction of environmental factors with host factors. One of the most prominent theories put forward to explain how environmental exposure may interact with the immune system and contribute to the emergence of IBD is the hygiene hypothesis. This hypothesis postulates

that children exposed to hygienic and sterile environments have decreased exposure to microbes that result in inadequate education of the immune system and ultimately in the development of an abnormal immune and inflammatory response such as IBD later in life.[92,96] It has been observed that children living on a farm or rural regions, having pets and a larger family or drinking unpasteurized milk are less likely to develop IBD.[92] Epidemiological studies documenting the emergence and increased incidence of IBD in developing and newly industrialized countries highlight the effect of environmental exposure in the pathogenesis of IBD.[108,109] Additionally, increased incidence of IBD in first and second generation immigrants who moved from regions of low prevalence to high prevalence further emphasize the importance of environmental factors in IBD pathogenesis.[109] Early childhood environmental factors such as breastfeeding and exposure to antibiotics in the first year of life impact the composition of the gut microbiota, which may influence the susceptibility to inflammatory disorders including IBD. Repeated use of antibiotics in early life increases the odds of developing CD.[110,111] Intriguingly, history of infectious gastroenteritis is another risk factor linked with the incidence of childhood-onset IBD.[112] Diet is a very important environmental factor studied in the context of IBD due to its effect on the gut microbiome and intestinal barrier function. Diet high in total fat, saturated fat, polyunsaturated fatty acids and omega-6 fatty acids are linked with increased risk of IBD where as intake of high fiber, fruits and vegetables are associated with reduced risk of IBD development while the effect of meat on IBD is not clear.[109,113] Animal studies have also noted that food additives such as

sweeteners and emulsifiers are pro-inflammatory agents that contributes to the pathogenesis of IBD.[92,109]

Other environmental risk factors implicated in the development, susceptibility and severity of IBD include cigarette smoking, air pollution and psychological stress. In adults, smoking increases the risk of CD development and has deleterious effects on the disease course. Contrary to its effect on CD, UC was found to have an inverse association with smoking with studies establishing the beneficial effects of smoking on the course of UC. The mechanisms underlying the role of smoking in IBD involve the adaptive and innate immunity, gut motility, vasculature and generation of free radicals.[95,114,115] It is unclear why the effects of smoking on CD and UC are opposite in spite of sharing many similarities. Air pollution, which parallels industrialization, increases the risk of both CD and UC, possibly by altering the gut microbiota and inducing inflammatory response in the intestine.[92,116] Thus, it is evident that environmental determinants influence the development, disease course and severity by its complex interaction with the gut microbiota and the host immune response.

1.3 Gut microbiota

The gut microbiota has a strong impact in health and disease by influencing nutrient absorption, epithelial barrier function, host metabolism and development and maturation of the immune system.[117-119] Human gut microbiota includes the collection of intestinal bacteria, fungi, archaea and viruses that colonize the GI tract. In IBD, most microbiome research is focused on gut bacteria, even though fungi and viruses might be

important in the context of IBD.[120,121] The intestinal microbiota is species-rich, with each person containing at least 160 bacterial species.[122] The adult intestine harbors about 10^{14} commensal bacteria.[122] Most species belong to the phyla Firmicutes (50%), Bacteroidetes (30%), Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria, and Verrucomicrobia.[123] In the healthy state intestinal microorganisms form a symbiotic relationship with their host. In different segments of the intestinal tract the microbiota composition and density varies due to the differences in the local conditions. The microbial density in the small intestine is much lower and less stable compared to the colon as demonstrated by 10^3 cells/gram of content in the proximal small intestine and 10^{12} cells/gram of content in the distal colon.[124,125]

In IBD intestinal 'dysbiosis' occurs characterized by change in structure, diversity and function of the microbiota. In IBD there is increased penetrance of bacteria in mucosa, decreased bacterial species richness and decreased microbial community stability.[126,127] The role of gut microbiota as a causative factor in the development of intestinal inflammation is supported by observations that experimental colitis does not develop in GF mice or the intestinal inflammation is less severe when mice are treated with antibiotics.[128,129] Clinically the response of IBD patients to antibiotics and fecal diversion as well as the common sites of inflammation being areas of relative fecal stasis (terminal ileum and rectum) further supports the causative role of dysbiosis in IBD.[130] There is a shift in the balance between the anti-inflammatory commensals and the pro-inflammatory pathogenic bacteria in IBD. In IBD, bacteria with anti-inflammatory properties such as *Faecalibacterium prausnitzii* are reduced where as bacteria such as

Escherichia coli, that better tolerate inflammatory environments are increased compared to the healthy controls (HCs). In UC, even though Akkermansia which has an anti-inflammatory effect is decreased, it is unclear whether this is a cause or effect of inflammation.[130,131] During dysbiosis invasive pathogenic bacteria can grow and translocate across the mucosal barrier to activate the immune response and promote inflammation.[132]

1.4 Intestinal immune system

The intestinal immune system maintains a state of tolerance and hyporesponsiveness to the gut commensal microbiota, dietary antigens and drugs while it fights invading pathogens without leading to overwhelming immune response and intestinal inflammation.[133,134] The immune system is composed of innate and adaptive arms that are closely interlinked. The innate immune system response is non-specific, rapid and does not provide long lasting immunity. It includes anatomical barriers, pattern recognition receptors and cells such as neutrophils, DCs, monocytes, macrophages and natural killer cells.[133] The innate immune cells provide the first line of defense and also activates the second defense system, the adaptive immunity by cytokine production and antigen presentation to T cells in the gut.[135] In contrast to the innate immune system, the adaptive immune system is specific and long lasting. The antigen presenting cells (APCs) present antigens to the T-helper (Th) cells in the inductive sites such as mesenteric lymph nodes and gut associated lymphoid tissue that results in the activation and differentiation of Th cells to either Th1, Th2 or Th17 cells.[135] Th1 cells mainly secrete interferon-γ (IFN-

γ), tumor necrosis factor (TNF), IL-12, while the Th2 cells secrete IL-4, IL-5 and IL-13. Classically, the histopathological features of CD resemble Th1 mediated inflammation where as those of UC resemble Th2 mediated inflammation. However, recently the roles of Th17 and regulatory T cells have been identified in the pathogenesis of both CD and UC.[93] Additionally, deregulated amounts of immunoglobulin (Ig) G, IgA and IgM in IBD patients indicate that B cells also play a role in IBD pathogenesis.[136] The lack or dysfunction of regulatory B cells that are characterized by the production of anti-inflammatory cytokines, IL-10 and TGF-β have been documented in IBD.[137,138] Regulatory B cells reduce the severity of inflammation by directly diminishing the production of pro-inflammatory cytokines such as IL-1 and TNF-α by macrophages or by producing IgG, which neutralizes harmful agents in immune responses and tissue damage.[139]

The mucosal epithelium of the GI tract forms the first barrier between the foreign antigens and the internal host milieu.[140] In addition to being an anatomical barrier to harmful microbes, the epithelium performs the main function of absorption of nutrients and water.[141] These opposing tasks are performed by the intestinal epithelium by maintaining a dynamic interaction between its structural, molecular and immune components. However, any disruption in the structural or regulatory components of the barrier allows the flux of microbial antigens into the internal milieu and thus activates the immune system.[142-145] Thus in IBD, dysregulated epithelial barrier is suggested to play a central role in the pathogenesis since antigen translocation across the epithelium will

activate the immune system of susceptible individuals and initiate uncontrolled inflammation in the gut.[142,146]

1.4.1 Intestinal mucosal barrier

The small and large intestine are lined with non-ciliated columnar epithelial cells, which forms crypts and villi. Villi are only present in the small intestine. The epithelium consists of 80% absorptive enterocytes, 20% specialized epithelial cells such as goblet cells, EECs and Paneth cells (only in small intestine). All the four types of epithelial cells develop and differentiate from pluripotent stem cells found at the base of the crypts.[147,148] The epithelial cells are renewed every 4-5 days by developing from stem cells and proliferating in the base of the crypts followed by migration upwards towards the tip of the villi in the small bowel or to the surface of the large bowel. The only exceptions are the Paneth cells in that they remain within the crypts.[147,148]

The luminal side of the mucosal layer of the GI tract is covered by mucus gel layer that has physiological roles such as lubrication, prevention of dehydration and mechanical damage, protection against colonization by pathogenic bacteria and their toxins, protection against luminal proteases arising from bacterial and mucosal cells, and formation of a diffusion barrier for small molecules but allows for the transport of nutrients.[149] The major component of the mucus layer is the large molecular weight glycoproteins known as mucin.[149] MUC2 is the best characterized secreted mucin of the intestine.[150] In the colon the two layered mucus is composed of a clear, well-defined, attached inner layer devoid of bacteria and a less defined loose unattached outer layer

containing gut bacteria and dietary materials.[151] The inner mucus layer is maintained by continuous secretion from goblet cells.[151,152] The small intestine has a single mucus layer, unattached to the epithelium and is more permeable to bacteria.[151,152] In IBD patients especially in UC the intestinal mucus layer varies in thickness, continuity, composition as well as the mucin structure. These changes adversely affect the protective properties of the mucus layer and consequently might result in an increased vulnerability of the epithelium to bacterial invasion. In UC the mucus layer is thinner, penetrable and less continuous,[153,154] with depletion of goblet cells.[155,156] In CD, even though there is increased mucus thickness and no change in the number of goblet cells[154] there is evidence of abnormal expression and glycosylation of mucin.[156] In addition to these clinical findings, it has been seen that *Muc2*$^{-/-}$ mice spontaneously develop colitis as well as have increased severity of DSS induced colitis.[157] In a murine model of DSS induced colitis, the mucus layer is altered before the induction of any inflammation illustrating the importance of mucus layer integrity to prevent inflammation.[158] Furthermore, maintenance of the epithelial barrier by murine Muc2 is also protective against lethal infectious colitis caused by *Citrobacter rodentium* or *Salmonella enterica serovar Typhimurium*.[159,160]

The absorptive and secretory epithelial cells form a continuous and polarized monolayer that acts as the barrier between the gut lumen and lamina propria. Water and nutrients are absorbed by the epithelium through the transcellular and paracellular pathways.[161] The barrier integrity is maintained by the interconnection of IECs via junctional complexes such as tight junctions, adherens junctions and desmosomes. The

intercellular space is mainly sealed by the tight junctional complexes consisting of transmembrane proteins, claudins and occludins and peripheral membrane proteins, zonula occludin. Below the tight junctions are the adherens junctional proteins such as E-cadherin which associates with α and β-catenins.[162] Increased intestinal permeability in patients with quiescent IBD and in up to 40% of first-degree relatives of patients with CD suggest barrier defect might precede the onset of the disease.[163] In IBD expression of tight and adherens junctional proteins have been reported to be altered in response to pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-17, IL-13 and chemokines.[91,135]

The intestinal barrier is further strengthened by the production of AMPs expressed by the enterocytes of the small and large intestine and Paneth cells of the small intestine. AMPs are mostly present in the inner mucus layer. AMPs, an evolutionarily conserved innate immune mechanism defends against both commensal and pathogenic microbes by interrupting highly conserved and essential features of microbial structure and physiology, such as the cell membrane and cell wall.[164,165] Large AMPs resemble lytic enzymes or proteins that bind to specific sites of microbial macromolecules. Smaller (<100 amino acids) cationic AMPs interact with the anionic bacterial membrane and cell wall due to the electrostatic attractions. This results in the insertion of the peptides into the bacterial membrane and formation of transmembrane pores that ultimately leads to cell lysis.[165,166] Some AMPs exert their action intracellularly by inhibiting DNA and protein synthesis as well by utilizing their anti-protease activity.[167,168] One of the major types of AMPs in humans is defensins. The release of defensins is regulated by the stimulation of pattern recognition receptors. In humans the defensins are classified as α

and β based on the differences in length of peptide segments between six cysteines and the disulphide bonds between the cysteines.[165] In humans there are six types of α-defensins: human neutrophil peptides 1-4 (HNP1-4) secreted mainly by neutrophils, human defensin (HD) 5 and 6 expressed only in Paneth cells of the small intestine. In mice, there is no HNP production, instead Paneth cells express 6 α-defensins termed cryptdins which are processed into their active forms by matrix metalloproteinase-7 cleavage.[169,170] In the colon the chief AMP are β-defensins expressed by the epithelial cells. Among the six types of β-defensins, human β-defensin (hBD) 1 (mouse orthologue mDefb1) and hBD2 (mouse orthologue mDefb3) are most extensively studied. hBD1 (and mouse orthologue mDefb1) is constitutively expressed in the intestinal epithelium regardless of colitis.[171,172] hBD2, is not expressed in normal healthy colon, but is significantly induced by pro-inflammatory cytokines and microbes.[171-173] CD patients have attenuated induction of hBD2 where as UC patients have increased levels of hBD2.[172,174,175] The abnormal production of β-defensins in IBD may be associated with impaired clearance of mucosal microorganisms and alteration in the microbiota composition. This altered microbiota as discussed in the section on "Gut Microbiota" may result in exaggerated immune response in the host and lead to uncontrolled intestinal inflammation. During IBD, two critical processes, 5-HT signaling and autophagy is disturbed which have been discussed in Chapter 1.

2. Experimental models of IBD

Close to 30 different animal models of intestinal inflammation has dramatically

advanced our understanding of the pathogenesis and underlying mechanism of IBD.[176] These models are broadly classified into 3 groups: chronic T cell transfer model of intestinal inflammation, spontaneous chronic intestinal inflammation models induced by gene deletion and acute self-limiting colitis models. [176] Although no single model can fully reflect the complexity of human IBD, each one contributes vital insights into one or more main aspects of the disease, and their combined efforts have resulted in the development of a widely acknowledged set of principles for human IBD pathogenesis. In the T cell transfer model chronic intestinal inflammation develops due to the disruption of T cell homeostasis. In summary, adaptive transfer of regulatory T cell deficient naïve T cells ($CD4^{+}CD45RB^{high}$ T cells) from healthy donor mice into syngeneic immunodeficient recipient mice lacking T and B cells (SCID or $Rag1^{-/-}$) results in development of pancolitis and small intestinal inflammation after 5-10 weeks.[177] Several spontaneous genetic mouse models of intestinal inflammation have been described with $IL\text{-}10^{-/-}$ and SAMP/Yit mice being some of the most widely studied and used.[176] In chemical colitis models, acute inflammation of the colon is initiated and maintained by luminal administration of toxic chemicals such as organic solvents and acids.[176] Among the commonly used acute chemical colitis models are the DSS-induced colitis, di/trinitrobenzene sulfonic acid-induced colitis, acetic acid-induced colitis and oxazolone induced colitis models.[177] All of the models in the 3 categories have their specific uses and limitations and they are selected based on the research question. Based on our research question, we selected DSS-induced colitis model to study the role of 5-HT-autopahgy-microbiota axis in intestinal inflammation.

2.1 Dextran sulphate sodium (DSS) model of colitis

The study and understanding of IBD has advanced tremendously by the development of experimental murine models of intestinal inflammation. The animal models of inflammation are invaluable tools for the investigation of the pathophysiology and novel therapeutic strategies of IBD. Chemical models are the most commonly used experimental IBD models, inspite of having limitations, since it mimics central immunological and pathological features.[177,178] Among the chemicals used to induce intestinal inflammation, DSS, a negatively charged heparin like polysaccharide is widely used due to its simplicity, reproducibility, rapidity and controllability.[177,179] Additionally, DSS-induced model has the advantage of using mice that are immunocompetent and developmentally normal as opposed to IBD models requiring immunodeficient hosts and genetically engineered models that may have developmental abnormalities.[178]

The exact mechanism of action of DSS is not known. It is likely that upon oral administration it damages the epithelial layer of the colon, increasing the permeability. This results in exposure and dissemination of luminal antigens in the underlying mucosa and submucosa, resulting in neutrophil and macrophage activation.[177,179] The inflammation in DSS model is dependent more on the innate immunity since DSS-induced colitis develops in severe combined immunodeficient mice, where T and B cells are absent. However, when both innate and adaptive immunity are intact, the inflammation is greater.[177] By altering the duration, frequency and dose of DSS administration, either acute or chronic models of intestinal inflammation can be produced. Symptomatically, features of DSS colitis are weight loss, diarrhea and bloody stools.[178]

Morphological and pathological features of superficial ulceration, epithelial loss and neutrophil infiltration looks similar to flares of human UC.[178] Immunologically, acute DSS colitis shows a Th1/Th17 response where as chronic DSS colitis exhibits Th1/Th2 response.[180]

3. Hypothesis and aims

Autophagy is regulated by factors such as nutrient levels, growth factors, oxygen and hormones through regulators like AMPK and mTOR.[181] Recently, 5-HT has been investigated as a possible regulator of autophagy. Soll et al. provided evidence of autophagy inhibition by 5-HT in hepatocellular carcinoma cell line. The group showed that after 24 hours treatment with 5-HT there was 7 fold increased expression of p62.[182] 5-HT was reported to activate mTOR in human bronchial smooth muscle cells. 5-HT was found to have similar effects on lacrimal gland. It was seen that sustained reduction in the level of blood 5-HT in mice resulted in lacrimal gland atrophy due to increased autophagy.[183] *SERT* gene knockdown in dorsal raphe nucleus in rats increased 5-HT level, decreased hippocampal autophagy as seen by a decrease in Beclin-1 and LC3-II/LC3-I ratio.[184] Taken together, emerging evidence supports potential interactions between 5-HT and autophagy. 5-HT and autophagy are independently linked to colitis. Majority of the evidence shows that 5-HT is pro-inflammatory where as autophagy is protective against colitis. While dysregulated 5-HT signaling and autophagy have been implicated in intestinal inflammation, it remains unclear whether they interact with each other in relation to colitis.

Hypothesis: Increased 5-HT signaling inhibits autophagy in the colon and alters the gut microbiota composition and enhances the severity of gut inflammation.

Aim 1: To define the role of 5-HT-autophagy axis in intestinal inflammation.

Aim 2: To elucidate the role of 5-HT in modulation of autophagy in intestinal epithelial cells.

Aim 3: To evaluate the role of 5-HT-autophagy axis in regulation of gut microbiota composition.

CHAPTER 3

DISRUPTION OF AUTOPHAGY BY INCREASED 5-HT ALTERS GUT MICROBIOTA AND ENHANCES SUSCEPTIBILITY TO EXPERIMENTAL COLITIS AND CROHN'S DISEASE

Published in *Science Advances*. Volume 7. Issue 45. Pages eabi6442. 2021
DOI: 10.1126/sciadv.abi6442

Sabah Haq, Huaqing Wang, Jensine Grondin, Suhrid Banskota, John K. Marshall, Irfan I. Khan, Usha Chauhan, Francine Cote, Yun Han Kwon, Dana Philpott, John H. Brumell, Michael Surette, Gregory R. Steinberg, and Waliul I. Khan

In this chapter, I examined my hypothesis and my 3 aims of my project. Using *Tph1*$^{-/-}$ and *SERT*$^{-/-}$ mice, we showed that increased levels of gut 5-HT during intestinal inflammation inhibits autophagy in the whole colon resulting in aggravated colitis and vice versa. Additionally, using rapamycin we established that 5-HT mediated changes in autophagy was through the mTOR pathway (Aim 1). Next, using epithelial cells from *Tph1*$^{-/-}$ mice, double knockout mice that are deficient in Tph1 and epithelial cell specific Atg7, and human epithelial cell lines we were able to show that increased levels 5-HT in the gut also inhibited autophagy in the IECs during intestinal inflammation. Furthermore, we also demonstrated similar findings in peripheral blood mononuclear cells isolated from healthy controls and CD patients, indicating the translational significance (Aim 2). Finally, in our third aim, we found that the disruption of the 5-HT-autophagy axis in the IECs alter the gut microbial composition towards a more colitogenic phenotype which mediates the severity of inflammation (Aim 3). In this article, all the methods and results of my project have been included.

SH and WIK conceived the idea and designed the research; SH performed all experiments with help from HW, JG, SB, and YHK; SH, UC and JM recruited study participants; SH and IIK analyzed and interpreted the data; SH, and WIK wrote, edited and revised manuscript; MS, FC, DP, JHB, and GRS critically appraised the manuscript.

SCIENCE ADVANCES | RESEARCH ARTICLE

MICROBIOLOGY

Disruption of autophagy by increased 5-HT alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease

Sabah Haq[1,2], Huaqing Wang[1,2], Jensine Grondin[1,2], Suhrid Banskota[1,2], John K. Marshall[1,3], Irfan I. Khan[2,4], Usha Chauhan[3,5], Francine Cote[6], Yun Han Kwon[1,2], Dana Philpott[7], John H. Brumell[8,9], Michael Surette[1,3,10], Gregory R. Steinberg[3,10,11], Waliul I. Khan[1,2,5,11]*

Autophagy, an essential intracellular recycling process, is linked to the pathogenesis of various diseases including Crohn's disease (CD). Factors that lead to the development of impaired autophagy during intestinal inflammation remain largely unexplored. Here, we report the impact of the interaction between serotonin [5-hydroxytryptamine;(5-HT)] and autophagy in colitis in mouse and human studies. In mice, increased gut 5-HT inhibited autophagy and led to enhanced colitis susceptibility. Reciprocally, mice with reduced 5-HT exhibited up-regulated autophagy via the mammalian target of rapamycin pathway, which resulted in significantly decreased colitis. Deletion of autophagy gene, Atg7, in an epithelial-specific manner, in concert with reduced 5-HT, promoted the development of a colitogenic microbiota and abolished the protective effects conferred by reduced 5-HT. Notably, in control and patient peripheral blood mononuclear cells, we uncovered that 5-HT treatment inhibited autophagy. Our findings suggest 5-HT as a previously unidentified therapeutic target in intestinal inflammatory disorders such as CD that exhibits dysregulated autophagy.

INTRODUCTION

Crohn's disease (CD), a major form of inflammatory bowel disease (IBD), is a serious chronic inflammatory condition of the human bowel (*1*). Genome-wide association studies have identified an increased risk of developing CD in individuals with autophagy gene polymorphisms including autophagy related 16 like 1 (*ATG16L1*) and immunity-related guanosine triphosphatase family M (*IRGM*) (*2*, *3*). Autophagy is a vital conserved catabolic process that provides energy during stress and protects the cell by removing damaged organelles and infectious agents (*4*, *5*). Environmental stressors prime autophagy through the activation of 5′ adenosine monophosphate–activated protein kinase (AMPK) and the inhibition of mammalian target of rapamycin complex 1 (mTORC1) (*6*). Downstream components, Beclin-1, Atg12-5 (autophagy related 12-5) complex, LC3-II (microtubule associated protein 1 light chain 3), and Atg7 (autophagy related 7), participate in the formation of the double membrane-bound vesicle, the autophagosome (*6*). The adapter protein, p62/sequestosome 1, delivers substrates to the autophagosome for degradation. Subsequently, fusion of the autophagosome with lysosome forms the autolysosome, a site where lysosomal enzymes degrade the aforementioned substrates (*6*). Autophagy-deficient mice are highly susceptible to experimental colitis and produce more proinflammatory cytokines (*7*, *8*). Studies demonstrate that autophagy-enhancing pharmacological agents such as rapamycin and salicylates (or other mTOR-inhibiting and AMPK-activating drugs) reduce the severity of experimental colitis and refractory CD, suggesting that these substances may promote a protective role of autophagy in gut inflammation (*9*–*12*). However, therapies that inhibit mTOR/enhance autophagy such as rapamycin have many side effects including altered insulin sensitivity, nephrotoxicity, hyperlipidemia, thrombocytopenia, and diminished wound healing (*13*). Therefore, studying the process of autophagy within the context of IBD presents a unique opportunity for the development of previously unidentified therapeutic strategies for intestinal inflammatory conditions, particularly by identifying new targets to overcome defective autophagy.

In the gastrointestinal tract, the microbiota plays a fundamental role in digestion, prevention of pathogen invasion, and the development of the immune system (*14*). Disruption of the composition and function of microbiota is a risk factor for the development of IBD (*15*). The gut microbiota is influenced by signals from the host epithelium. Studies show that mice with intestinal epithelial cell (IEC)–specific *Atg7* or *Atg5* deficiency exhibit altered gut microbiota composition and diversity (*8*, *16*). These microbial changes are similar to the changes seen in CD, signifying the role of impaired autophagy in gut dysbiosis and inflammation (*16*). Furthermore, activation of the mTORC2 signaling pathway followed by inhibition of mTORC1 by resveratrol decreased the abundance of obesity-associated gut microbiota and reduced intestinal inflammation in diet-induced obese mice (*17*). Although some of the mechanisms by which dysfunctional autophagy changes the gut microbiota have been studied (*4*), little is known about the factors that lead to the development of impaired autophagy during intestinal inflammation.

Enterochromaffin (EC) cells, a subset of enteroendocrine cells, produce approximately 90% of serotonin [5-hydroxytryptamine (5-HT)] in the gut (*18*, *19*). In EC cells, the synthesis of 5-HT begins with the conversion of dietary tryptophan to 5-hydroxy-L-tryptophan (5-HTP), a process catalyzed by the rate-limiting enzyme, tryptophan

[1]Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada. [2]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada. [3]Department of Medicine, McMaster University, Hamilton, Ontario, Canada. [4]Population Health Research Institute, McMaster University, Hamilton, Ontario, Canada. [5]Hamilton Health Sciences, Hamilton, Ontario, Canada. [6]Institut Cochin, INSERM U1016, CNRS UMR 8104, Paris, France. [7]Department of Immunology, University of Toronto, Toronto, Ontario, Canada. [8]Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada. [9]Ontario and Cell Biology Program, Hospital for Sick Children, Toronto, Ontario, Canada. [10]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada. [11]Centre for Metabolism Obesity and Diabetes Research, McMaster University, Hamilton, Ontario, Canada.
*Corresponding author. Email: khanwal@mcmaster.ca

hydroxylase (Tph) (*18*). There are two isoforms of the Tph enzyme, Tph1, present mainly in EC cells, and Tph2, found in the brain stem and enteric neurons (*18*, *20*). 5-HTP is converted into 5-HT by aromatic L-amino acid decarboxylase. Apical and basolateral release of 5-HT from EC cells occurs in response to mechanical and various chemical stimulants (*20*). Once released, 5-HT is taken up by surrounding IECs and immune cells by the serotonin reuptake transporter (SERT) (*20*). In patients with active IBD, increases in EC cell numbers and 5-HT content have been documented (*21*–*23*). Recent studies on experimental models of intestinal inflammation illustrate that inhibiting 5-HT production by genetic deletion of Tph1 or by pharmacological manipulation ameliorates inflammation (*24*–*26*). We recently demonstrated the impact of 5-HT on microbial composition and subsequent effects on the susceptibility to inflammation in the intestine (*27*). Furthermore, Fung *et al.* (*28*) have also reported that 5-HT has direct effects on gut bacteria and their colonization. However, the signaling pathway by which 5-HT regulates gut microbiota remains to be determined.

Evidence that increased 5-HT mediates impaired autophagy in human hepatocellular carcinoma cells, murine lacrimal gland, and rat dorsal raphe nucleus (*29*–*31*) supports potential interactions between 5-HT and autophagy. While dysregulated 5-HT signaling, autophagy, and microbiota are implicated in intestinal inflammation, the potential interactions among these three factors in the pathogenesis of intestinal inflammation remain unclear. In this study, by using in vitro cell culture systems, experimental models of colitis, and biopsy and blood samples from patients with CD, we investigated interactions between mucosal 5-HT signaling, autophagy, and microbiota in the regulation of intestinal inflammation. Our study demonstrates that increased 5-HT in the colon inhibits autophagy, resulting in altered microbiota composition and increased severity of inflammation, and thus identifies 5-HT as a novel regulator of autophagy-microbiota axis in gut inflammation.

RESULTS

Reduced 5-HT in the gut increases autophagy in the colon and decreases the severity of intestinal inflammation

To investigate the linkage between 5-HT and autophagy in the context of intestinal inflammation, we induced acute colitis by administration of either 5% dextran sulfate sodium (DSS) for 5 days or 2.5% DSS for 7 days in drinking water to Tph1–deficient mice ($Tph1^{-/-}$ mice that have significantly reduced 5-HT in gut) and determined the level of autophagy in whole sections of colonic tissues. Similar to our previous findings (*24*), there was a significant reduction in the severity of colitis in $Tph1^{-/-}$ mice compared to $Tph1^{+/+}$ mice during both 5 and 2.5% DSS administration (Fig. 1, A and B, and fig. S1, A to E). However, the difference in severity of colitis between $Tph1^{+/+}$ and $Tph1^{-/-}$ mice was less distinct at 2.5% DSS when compared to the difference in colitis at 5% as indicated by the disease activity index (DAI) (Fig. 1A and fig. S1A). On day 5 after 5% DSS, the $Tph1^{-/-}$ mice had significantly higher levels of p-AMPKα and the autophagy markers, the lipidated form of LC3 (LC3-II), Beclin-1 and Atg12-5 conjugate, and reduced p62 and p-mTOR (S2448), in colon tissues compared to $Tph1^{+/+}$ mice (Fig. 1, C to F). Control $Tph1^{-/-}$ mice also had increased levels of autophagy proteins compared to control $Tph1^{+/+}$ group (Fig. 1, C to F). Lysosome-associated membrane protein 2 (LAMP2) is an integral membrane protein of lysosomes and autolysosomes. Since costaining of cells for LC3 and LAMP2 reflects the fusion of autophagosomes and lysosomes, it was used to measure functional autophagy (*32*). Immunofluorescence analysis revealed significantly increased $LC3B^+$ IECs and $LC3B/LAMP2^+$ double-stained IECs in the $Tph1^{-/-}$ mice regardless of DSS administration (Fig. 1, G to I). On day 7 after 2.5% DSS, the $Tph1^{-/-}$ mice had significantly increased levels of autophagy markers p-AMPKα, LC3-II, and Atg12-5 and decreased p62 and p-mTOR (S2448) in colon tissues compared to $Tph1^{+/+}$ group (fig. S1, F to I). In separate experiments, $Tph1^{+/+}$ and $Tph1^{-/-}$ mice were administered 5% DSS and euthanized, and mucosal scrapings were taken. Autophagy markers LC3-II, Beclin-1, and Atg12-5 were significantly increased in the mucosal scraping isolated from $Tph1^{-/-}$ mice compared to $Tph1^{+/+}$ counterparts (fig. S2, A and B).

To determine whether blocking 5-HT signaling in the gut can promote autophagy and alleviate the severity of inflammation, we treated colitic C57BL/6 mice orally with the Tph1 inhibitor, LX1031, on days 2 to 5 of 5% DSS administration. Treatment with LX1031 resulted in significantly reduced 5-HT level in the whole colon tissue (fig. S3A) along with decreased day 5 DAI, macroscopic scores, histological damage scores, and levels of the proinflammatory cytokine interleukin (IL)-6 (fig. S3, B to F) compared to the vehicle-treated group. LX1031 promoted autophagy in the colitic mice as indicated by the significant increase in the autophagy proteins LC3-II, Beclin-1, and Atg12-5 and by the reduction of p62 (fig. S3, G and H).

Replenishing 5-HT in $Tph1^{-/-}$ mice impairs autophagy and increases the severity of intestinal inflammation

To determine whether the increased autophagy in $Tph1^{-/-}$ mice was due to the lack of 5-HT, we restored 5-HT levels in $Tph1^{-/-}$ mice by subcutaneous injection of 5-HTP, the immediate precursor of 5-HT. As expected, 5-HTP–treated $Tph1^{-/-}$ mice had significantly increased amounts of 5-HT in the colon (Fig. 2A) compared to vehicle-treated $Tph1^{-/-}$ mice. Restoring 5-HT in the $Tph1^{-/-}$ mice increased the severity of DSS colitis (*24*) as determined by increased day 4 and day 5 DAI, macroscopic scores, histological damage scores, and levels of the proinflammatory cytokine IL-1β (Fig. 2, B to F). The increase in DSS colitis severity in $Tph1^{-/-}$ mice after restoration of 5-HT levels was associated with impaired autophagy in the colon as indicated by a reduction in LC3-II and Beclin-1 and accumulation of p62 and p-mTOR (Fig. 2, G to J). In addition, functional autophagy indicated by LC3B/LAMP2 costaining in the IECs was significantly decreased in $Tph1^{-/-}$ mice after restoration of 5-HT compared to vehicle-treated $Tph1^{-/-}$ mice (Fig. 2, K to M).

To investigate whether inducing autophagy reverses the enhanced severity of DSS colitis in $Tph1^{-/-}$ mice after 5-HT restoration, we simultaneously treated a separate group of $Tph1^{-/-}$ mice with 5-HTP and rapamycin, an mTOR-dependent autophagy inducer. Mice cotreated with rapamycin and 5-HTP had restored autophagy as indicated by the increase in LC3-II and Beclin-1 and reduction of p62 and p-mTOR (Fig. 2, G to J). In addition, functional autophagy indicated by LC3B/LAMP2 costaining in the IECs was significantly increased in mice cotreated with rapamycin and 5-HTP compared to mice receiving only 5-HTP (Fig. 2, K to M). Compared to mice receiving only 5-HTP, the rapamycin-treated group showed significant reduction in inflammation severity as indicated by reduced day 4 and day 5 DAI, macroscopic scores, histological damage scores, and expression of the proinflammatory cytokines IL-1β and IL-6 (Fig. 2, B to F).

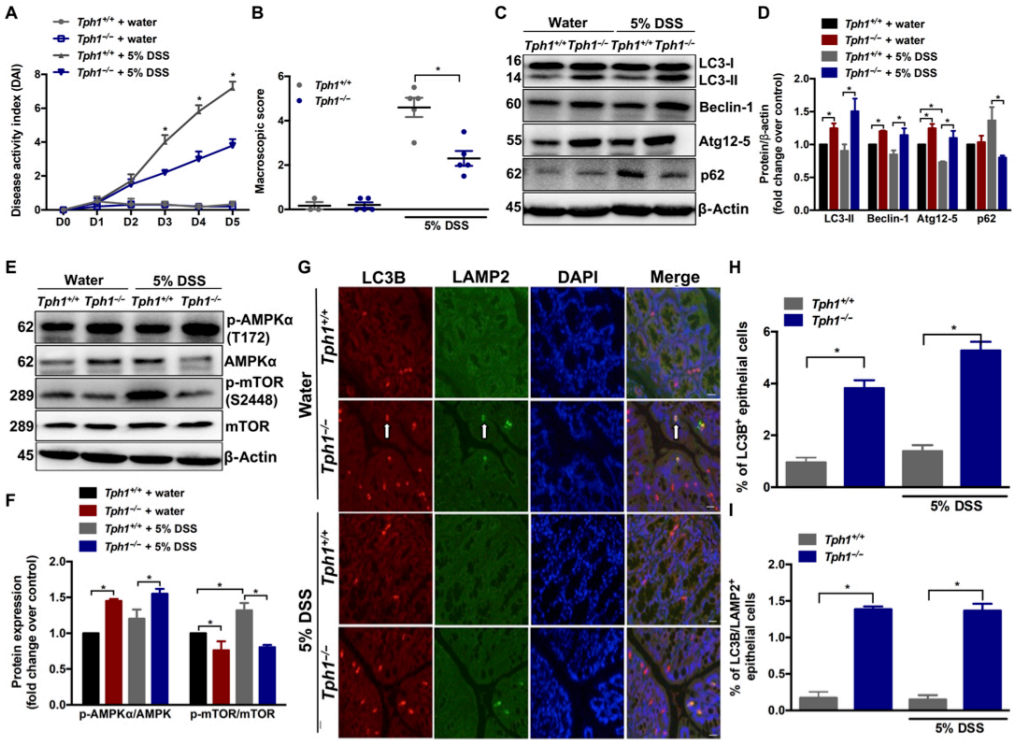


Fig. 1. Effects of reduced 5-HT on autophagy in the whole colon of mice with DSS-induced colitis. DSS (5%) was given to *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice in drinking water for 5 days to induce colitis. Control groups received autoclaved drinking water. Each group contained three to five mice. (**A**) DAI. *$P < 0.05$ between colitic *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice on days 3 to 5. (**B**) Macroscopic damage score on day 5 after DSS and in control mice. (**C**) Western blot images and (**D**) quantification of autophagy proteins LC3, Beclin-1, Atg12-5, and p62. (**E**) Western blot images and (**F**) quantification of p-AMPKα (T172) and p-mTOR (S2448). Representative Western blot with β-actin is presented as loading controls. The blot shown is representative of three to five random mice in each group. (**G**) Representative micrograph. Arrows indicate LC3B, LAMP2, and double-stained IECs. Scale bars, 100 μm. (**H**) Percentage of LC3B^{+} and (**I**) percentage of LC3B/LAMP2^{+} double-stained IECs in colonic sections immunostained for LC3B and LAMP2. Bar graph represents means ± SEM. *$P < 0.05$ for graphs (B), (D), (F), (H), and (I).

Elevated 5-HT in SERT-deficient mice impairs autophagy and increases the severity of intestinal inflammation

To further substantiate the effect of 5-HT on autophagy in intestinal inflammation, we evaluated autophagy in mice deficient in SERT (*SERT*$^{-/-}$) after DSS administration. Deletion of SERT increases the availability of 5-HT in the bowel (*33*). *SERT*$^{-/-}$ mice had a significantly increased amount of 5-HT in the colon compared to *SERT*$^{+/+}$ mice (Fig. 3A). Intestinal inflammation was exacerbated in *SERT*$^{-/-}$ mice compared to *SERT*$^{+/+}$ mice, as demonstrated by higher day 4 and day 5 DAI, macroscopic scores, histological damage scores, and increased IL-1β and tumor necrosis factor–α (TNF-α) levels (Fig. 3, B to F). Reduction in p-AMPKα, LC3-II, Beclin-1, and Atg12-5 and accumulation of p62 and p-mTOR in *SERT*$^{-/-}$ mice compared to *SERT*$^{+/+}$ counterparts on day 5 after DSS were observed, suggesting an impairment in autophagic flux (Fig. 3, G to J). In the noncolitic group, *SERT*$^{-/-}$ mice also had decreased amounts of autophagy proteins LC3-II, Atg12-5, and p-AMPKα along with elevated p-mTOR level compared to *SERT*$^{+/+}$ mice (Fig. 3, G to J). The percentage of LC3B/LAMP2^{+} IECs was significantly reduced in both colitic and noncolitic *SERT*$^{-/-}$ mice compared to *SERT*$^{+/+}$, signifying a decrease in autophagic flux (Fig. 3, K to M). These findings further demonstrate an important role of gut 5-HT levels in influencing susceptibility to intestinal inflammation.

Reduced 5-HT in the gut up-regulates autophagy in IECs

On the basis of the importance of IECs in the pathogenesis of intestinal inflammation (*4*, *5*) and our observations regarding increased autophagy in the mucosal scrapings of *Tph1*$^{-/-}$ mice, we next investigated whether 5-HT influences autophagy in IECs. *Tph1*$^{-/-}$ and *Tph1*$^{+/+}$ mice were administered DSS and euthanized, and IECs were isolated and confirmed by E-cadherin staining (fig. S4). Western blotting and immunofluorescence analysis revealed significant

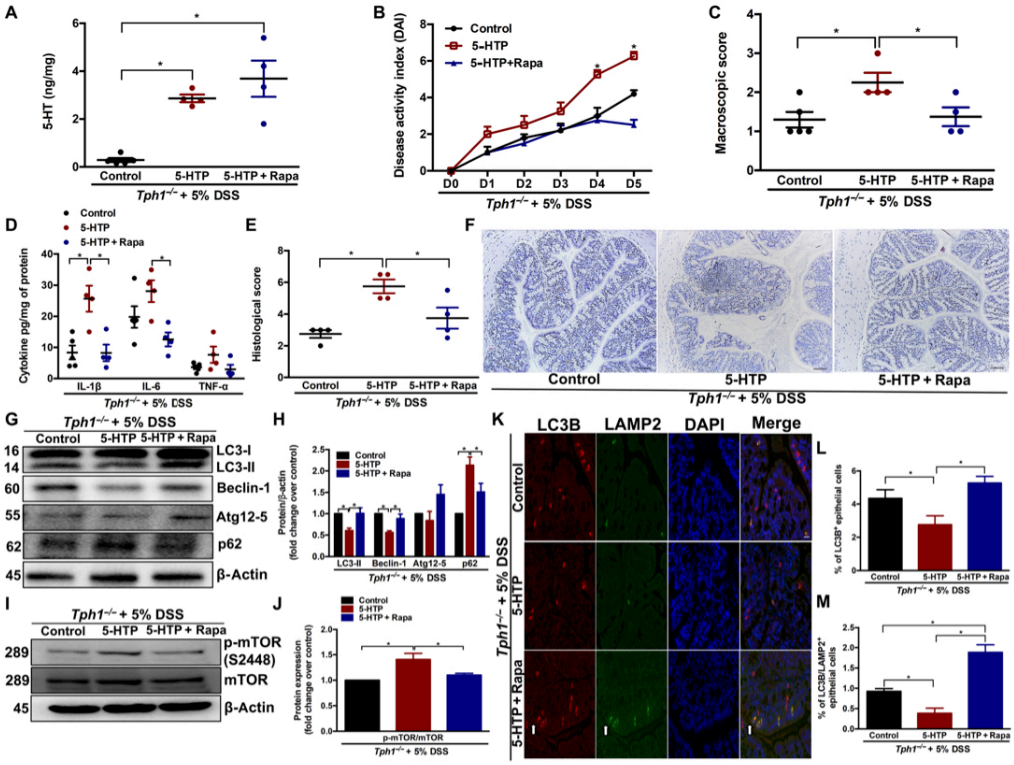


Fig. 2. Effect of 5-HTP and rapamycin treatment in *Tph1*$^{-/-}$ mice with DSS-induced colitis. DSS (5%) was given to *Tph1*$^{-/-}$ mice in drinking water for 5 days to induce colitis along with 5-HTP (50 mg/kg twice a day), 5-HTP + rapamycin (1.5 mg/kg once a day), or vehicle treatment. Each group contained four to five mice. (**A**) Colonic 5-HT levels. (**B**) DAI. *$P < 0.05$ between control and 5-HTP as well as between 5-HTP and 5-HTP + Rapa (rapamycin) on days 4 and 5. (**C**) Macroscopic damage score on day 5 after DSS. Colon tissue was collected to determine (**D**) IL-1β, IL-6, and TNF-α; (**E**) histological score; and (**F**) representative micrographs on day 5 after DSS. Scale bars, 100 μm. (**G**) Western blot images and (**H**) quantification of autophagy proteins LC3, Beclin-1, Atg12-5, and p62. (**I**) Western blot images and (**J**) quantification of p-mTOR (S2448). Representative Western blot with β-actin as loading controls. Blots are representative of three to four random mice in each group. (**K**) Representative micrograph. Arrows indicate LC3B, LAMP2, and double-stained IECs. Scale bar, 100 μm. (**L**) Percentage of LC3B$^+$ and (**M**) percentage of LC3B/LAMP2$^+$ double-stained IECs in colonic sections. Data are represented as means ± SEM. *$P < 0.05$ for graphs (A), (C), (D), (E), (H), (J), (L), and (M).

increase in LC3-II and Atg7 in IECs isolated from *Tph1*$^{-/-}$ mice on day 5 after DSS (Fig. 4, A to D).

In addition, 5-HT treatment significantly reduced LC3-II, Beclin-1, and Atg12-5 levels and enhanced expression of p62 and p-mTOR in human colonic epithelial HT-29 cells (fig. S5, A and B). 5-HT also reduced LC3-II and increased p62 expression in mouse primary IECs compared to the control (fig. S5, C and D). Furthermore, inhibition of autophagy by 5-HT treatment of HT-29 cells significantly increased the secretion of proinflammatory cytokine IL-8 compared to the control group (fig. S6C).

5-HT influences autophagy of IECs via 5-HT receptors

5-HT mediates various functions in the gut by acting on 5-HT receptors. Of the 14 subtypes of 5-HT receptors, 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptors are mainly expressed in IECs and HT-29 cell line (*34*, *35*). To determine which 5-HT receptor expressed on the enterocytes is involved in regulation of autophagy, we evaluated the effects of 5-HT$_3$, 5-HT$_4$, and 5-HT$_7$ receptor–specific antagonists (tropisetron, RS39604, and SB269970) on autophagy in HT-29 cells and in mouse primary IECs. In HT-29 cells, 5-HT–induced alteration in the autophagy proteins Atg12-5 and p62 was significantly blocked by the 5-HT$_3$ receptor antagonist tropisetron (fig. S5, A and B). 5-HT$_4$ receptor antagonist, RS39604, reversed the 5-HT–induced changes in Beclin-1, Atg12-5, and p62 in HT-29 cells (fig. S5, A and B). In HT-29 cells, 5-HT–induced reduction in the autophagy proteins LC3-II, Beclin-1, and Atg12-5 and elevation of p-mTOR and p62 were completely reversed by the 5-HT$_7$ receptor antagonist, SB269970 (fig. S5, A and B). In addition, in mouse primary IECs,

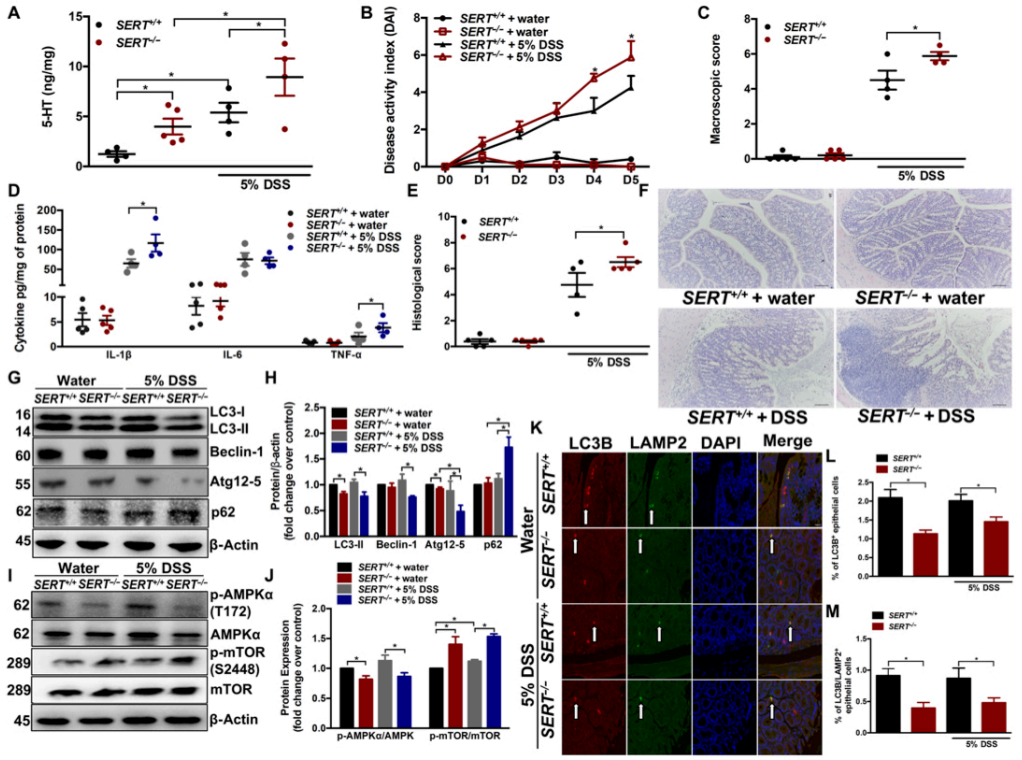


Fig. 3. Effect of elevated intestinal 5-HT on DSS-induced colitis and autophagy. DSS (5%) was given to *SERT*$^{+/+}$ and *SERT*$^{-/-}$ mice in drinking water for 5 days to induce colitis. Control groups received autoclaved drinking water. Each group contained four to five mice. (**A**) Colonic 5-HT amount. (**B**) DAI. *$P < 0.05$ between colitic *SERT*$^{+/+}$ and *SERT*$^{-/-}$ on days 4 and 5. (**C**) Macroscopic damage score on day 5 after DSS and in control mice. Colon tissue was collected to determine (**D**) IL-1β, IL-6, and TNF-α; (**E**) histological score; and (**F**) representative micrographs on day 5 after DSS. Scale bars, 100 μm. (**G**) Western blot images and (**H**) quantification of autophagy proteins LC3, Beclin-1, Atg12-5, and p62. (**I**) Western blot images and (**J**) quantification of p-AMPKα (T172) and p-mTOR (S2448). Representative Western blot with β-actin as loading controls. Data are representative of three to four random mice in each group. (**K**) Representative micrograph. Arrows indicate LC3B, LAMP2, and double-stained IECs. Scale bar, 100 μm. (**L**) Percentage of LC3B^{+} and (**M**) percentage of LC3B/LAMP2^{+} double-stained IECs in colonic sections. Data are represented as means ± SEM. *$P < 0.05$ for graphs (A), (C), (D), (E), (H), (J), (L), and (M).

5-HT–induced alteration in the autophagy proteins LC3-II and p62 was significantly blocked by the 5-HT$_3$ receptor antagonist tropisetron, 5-HT$_4$ receptor antagonist RS39604, and 5-HT$_7$ receptor antagonist SB269970 (fig. S5, C and D). These findings indicate that although 5-HT impairs autophagy via all three 5-HT receptors expressed on IECs, 5-HT$_7$ receptor might be playing a major role. Together, these findings suggest an important role of 5-HT and its receptors in the regulation of autophagy in IECs.

Disruption of the autophagy gene, Atg7, in IECs abolishes the protective effects of reduced 5-HT in *Tph1*$^{-/-}$ mice during DSS-induced colitis

Atg7, an E1-like enzyme, is a critical factor for Atg12 and LC3 conjugation and autophagosome formation and is thus essential for autophagy (*8*). To determine whether reduced severity of inflammation in the intestines of *Tph1*$^{-/-}$ mice arises from up-regulated autophagy in IECs, double-knockout (DKO) mice deficient in both Tph1 and IEC-specific Atg7 were generated (fig. S7). Deletion of Atg7 from IECs was confirmed by a marked reduction in immunofluorescence staining of LC3B^{+} IECs isolated from the colon of *Atg7*$^{\Delta IEC}$ mice (fig. S8A). The level of LC3-II was markedly reduced in the DKO mice compared to *Tph1*$^{-/-}$ and increased in comparison to *Atg7*$^{\Delta IEC}$ mice (fig. S8, B and C). In addition, there was no difference between the 5-HT levels in the colon of the naïve *Atg7*$^{\Delta IEC}$ mice and the *Atg7*$^{fl/fl}$ mice (fig. S9).

Acute colitis was induced in *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$, and *Atg7*$^{fl/fl}$ mice by oral administration of DSS. Both the *Tph1*$^{-/-}$ and DKO mice had reduced 5-HT levels in the colon (Fig. 4E). As

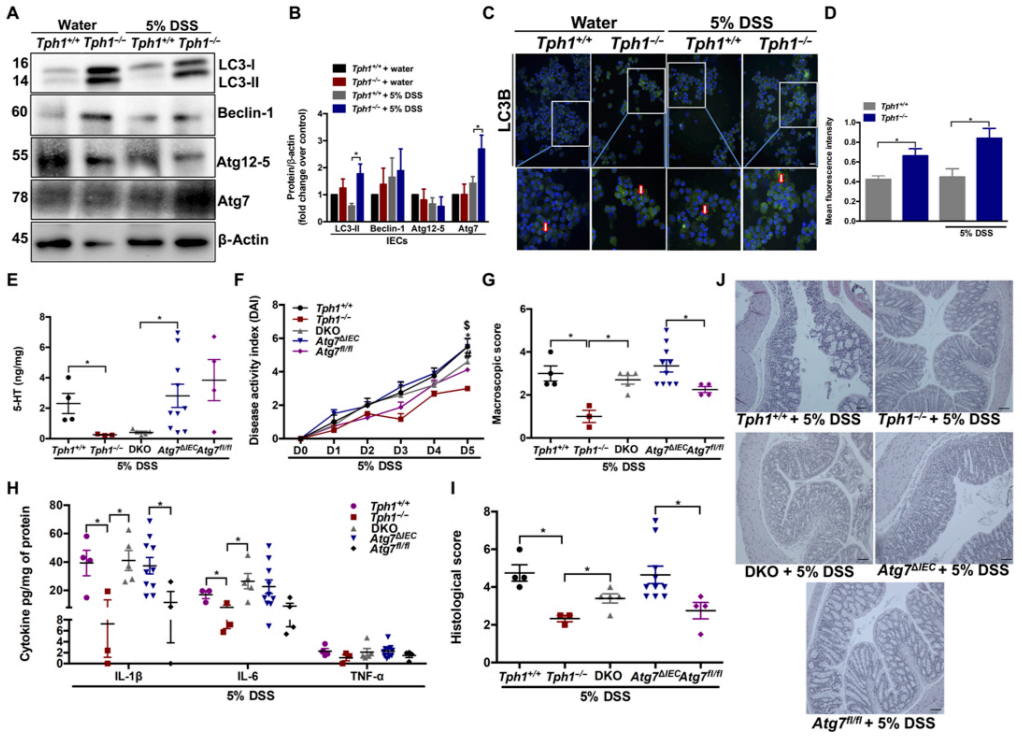


Fig. 4. Impact of 5-HT on autophagy in IECs and the effects of impaired IEC 5-HT–autophagy axis on colitis. DSS (5%) was given to *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice in drinking water for 5 days to induce colitis. Control groups received autoclaved drinking water. Each group contained three to four mice. IECs were isolated from the colon. (**A**) Western blot images and (**B**) quantification of autophagy proteins LC3, Beclin-1, Atg12-5, and Atg7 in the IECs. Representative Western blot with β-actin as loading controls. Data are representative of three to four random mice in each group. (**C**) Representative micrograph. Arrows indicate LC3B^{+} puncta in the IECs. Scale bar, 100 μm. (**D**) Mean fluorescence intensity of LC3B staining in isolated IECs. DSS (5%) was given to *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$, and *Atg7*$^{fl/fl}$ mice in drinking water for 5 days to induce colitis. Each group contained 3 to 10 mice. (**E**) Colonic 5-HT amount. (**F**) DAI. $$P < 0.05$ between *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$, *$P < 0.05$ between *Atg7*$^{\Delta IEC}$ and *Atg7*$^{fl/fl}$, and #$P < 0.05$ between DKO and *Tph1*$^{-/-}$. (**G**) Macroscopic damage score on day 5 after DSS. Colon tissue was collected to determine (**H**) IL-1β, IL-6, and TNF-α; (**I**) histological score; and (**J**) representative micrographs on day 5 after DSS. Scale bars, 100 μm. Data are represented as means ± SEM. *$P < 0.05$ for graphs (B), (D), (E), (G), (H), and (I).

demonstrated previously, *Atg7*$^{\Delta IEC}$ mice exhibited exacerbated inflammation compared to the *Atg7*$^{fl/fl}$ mice (*8*), reflected by elevation in several parameters of inflammation such as DAI, macroscopic scores, histological damage scores, and proinflammatory cytokine IL-1β (Fig. 4, F to J). The protective effect of reduced 5-HT in the *Tph1*$^{-/-}$ mice was abrogated when mice lacked the autophagy protein, Atg7, in the IECs, demonstrated by an increase in day 5 DAI, macroscopic scores, histological damage scores, and proinflammatory cytokines IL-1β and IL-6 in DKO mice compared to *Tph1*$^{-/-}$ mice on day 5 after DSS (Fig. 4, F to J).

Tph1 and IEC-specific Atg7 DKO mice exhibit altered gut microbiota composition

Because the severity of DSS-induced colitis was markedly increased in DKO mice compared to *Tph1*$^{-/-}$ mice, we hypothesized that the disruption of autophagy in IECs abolished the protective effects in colitis in *Tph1*$^{-/-}$ mice by modulating gut microbial composition via influencing antimicrobial peptide production from IECs. IECs constitutively produce antimicrobial peptide, β-defensin-1 (mDefb1) (*36*). We observed that DSS-receiving *Tph1*$^{-/-}$ mice had increased mDefb1 mRNA expression and protein levels compared with colitic DKO mice, whereas colitic DKO and *Atg7*$^{\Delta IEC}$ mice had similar levels of mDefb1 (Fig. 5, A to C). To examine the gut microbiota, we compared cecal bacterial profiles by 16*S* ribosomal RNA (rRNA) sequencing of *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$, and *Atg7*$^{fl/fl}$ mice after DSS administration. For each group, the microbial composition was dominated by Firmicutes, Proteobacteria, and Bacteroidetes (fig. S10A). The five groups of mice separated into distinct clusters shown by the visualization of Bray-Curtis diversity by principal coordinate ordination (PCoA) (Fig. 5D). In all pairings, the differences between the groups were significant with a false discovery rate of <5%. Mouse genotype significantly accounted for 54% of the variation, while 46%

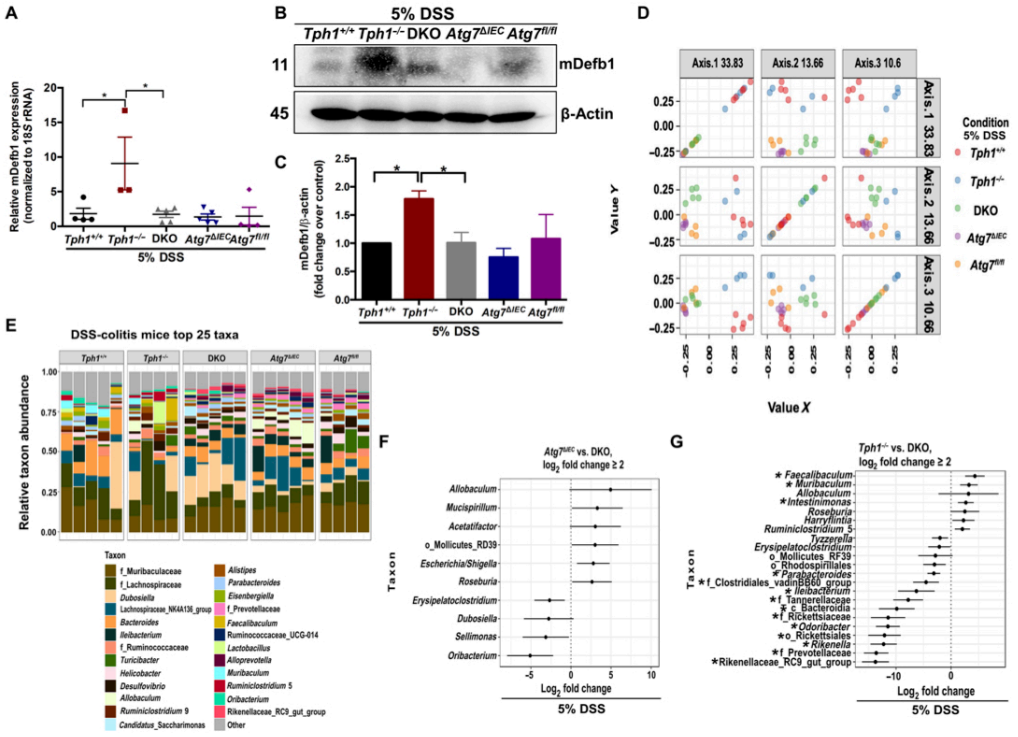


Fig. 5. Effect of impaired IEC 5-HT–autophagy axis on gut microbial composition during DSS colitis. DSS (5%) was given to *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$, and *Atg7*$^{fl/fl}$ mice in drinking water for 5 days to induce colitis. Each group contained four to five mice. (**A**) Quantification of mDefb1 mRNA in the colonic tissue. (**B**) Western blot images and (**C**) quantification of mDefb1 protein in the colonic tissue. *$P < 0.05$ for graphs (A) and (C). 16*S* rRNA sequencing of cecal content of all groups was performed. (**D**) PCoA of Bray-Curtis dissimilarity. (**E**) Taxonomic comparison of gut microbiota in the five experimental groups. (**F**) With DKO as the baseline, all taxa in the *Atg7*$^{\Delta IEC}$ group for which one $\log_2$ fold change is ≥2. (**G**) With DKO as the baseline, all taxa in the *Tph1*$^{-/-}$ group for which one $\log_2$ fold change is ≥2. *$P < 0.01$ for graph (G).

of the variation was due to residual factors that were not significant. Taxonomic comparisons revealed greater similarity between the DKO and *Atg7*$^{\Delta IEC}$ mice and more dissimilarity between DKO and *Tph1*$^{-/-}$ mice (Fig. 5E and fig. S10B). Despite deficiencies in both Tph1 and IEC Atg7, the microbial composition of DKO mice was not an aggregate of *Tph1*$^{-/-}$ and *Atg7*$^{\Delta IEC}$ mice but showed higher similarity to *Atg7*$^{\Delta IEC}$ mice. The graphs in Fig. 5 (F and G) and table S2 illustrate that 16 bacterial taxa in *Tph1*$^{-/-}$ mice and no bacterial taxa in *Atg7*$^{\Delta IEC}$ mice were significantly different from those of DKO mice. In *Tph1*$^{-/-}$ mice, beneficial bacteria such as *Faecalibaculum* were higher, while colitogenic bacteria such as *Parabacteroides* and *Rikenella* were decreased compared to DKO mice (Fig. 5G).

Transfer of colitogenic gut microbiota from DKO mice increases the severity of DSS-induced colitis in antibiotic-treated naïve mice

To further determine whether the altered microbiota in the DKO mice influences the severity of intestinal inflammation, we transferred the cecal content of DSS-receiving *Tph1*$^{-/-}$ or DKO or *Atg7*$^{\Delta IEC}$ mice to antibiotic-treated C57BL/6 mice, followed by administration of DSS to induce colitis (Fig. 6A). The microbiota profile of the DSS-treated donor and their recipient mice was analyzed by 16*S* rRNA sequencing of fecal samples to determine how closely the recipients resemble their donors. Bray-Curtis analysis, Jaccard plot, taxonomic composition, and the mean relative abundance demonstrate that among the three pairs of donors and recipients, the *Tph1*$^{-/-}$ recipients are closest to the *Tph1*$^{-/-}$ donors (fig. S11, A to D). The clustering of the DKO donors and recipients and *Atg7*$^{\Delta IEC}$ donors and recipients is apparent in axis 1 of the Jaccard plot (fig. S11B). The microbiota of the DKO and *Atg7*$^{\Delta IEC}$ recipients grouped together, whereas the *Tph1*$^{-/-}$ recipients formed distinct separate clusters (fig. S11, A and B). The recipient mice microbiota had a significant proportion of the donor amplicon sequence variants (ASVs) present, indicated by 75.2, 61.2, and 59.4% of ASVs for *Tph1*$^{-/-}$, DKO, and *Atg7*$^{\Delta IEC}$ donors, respectively (fig. S11E).

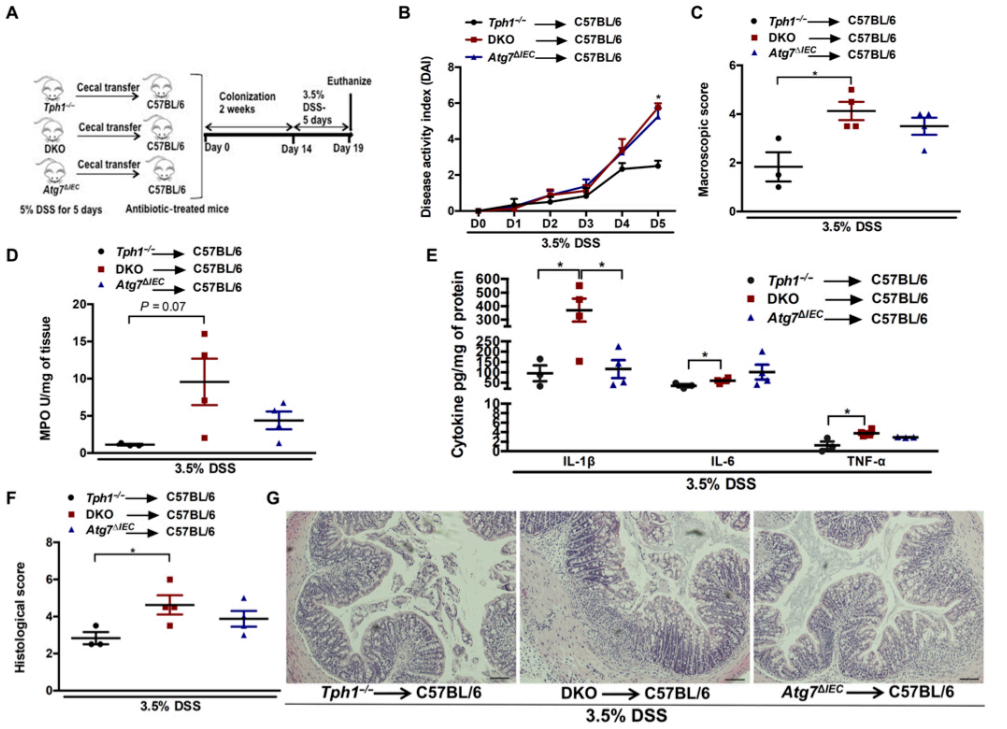


Fig. 6. Assessment of severity of colitis following adoptive transfer of DSS-treated microbiota to antibiotic-treated C57BL/6 mice. (**A**) Schematic of cecal microbiota transfer followed by induction of colitis with 3.5% DSS. Each group had three to four mice. (**B**) DAI. *P < 0.05 between C57BL/6 mice receiving DKO microbiota and C57BL/6 mice receiving *Tph1*$^{-/-}$ microbiota. (**C**) Macroscopic damage score on day 5 after DSS. Colon tissue was collected to determine (**D**) MPO activity; (**E**) IL-1β, IL-6, and TNF-α; (**F**) histological score; and (**G**) representative micrographs on day 5 after DSS. Scale bars, 100 μm. Data are represented as means ± SEM. *P < 0.05 for graphs (C), (D), (E), and (F).

Higher levels of day 5 DAI, macroscopic and histological damage scores, myeloperoxidase (MPO) activity, and proinflammatory cytokines IL-1β, IL-6, and TNF-α were observed in antibiotic-treated mice colonized with microbiota from DKO mice as compared to those colonized with microbiota from *Tph1*$^{-/-}$ mice (Fig. 6, B to G). There was no significant difference in colitis severity between the mice receiving microbiota from DKO mice and mice receiving microbiota from *Atg7*$^{\Delta IEC}$ mice except for IL-1β levels. These results suggest that deletion of this autophagy-related gene Atg7 in *Tph1*$^{-/-}$ mice promotes the development of a colitogenic microbiota in DKO mice that contributes to the differences in severity of inflammation. The comparison of donor and recipient microbiota revealed that the more colitic DKO and *Atg7*$^{\Delta IEC}$ donor and recipient groups had significantly higher levels of bacteria belonging to the genera *Dubosiella* and *Turicibacter* compared to the less colitic *Tph1*$^{-/-}$ donor and recipient (figs. S12 and S13). However, some members of Lachnospiraceae were increased, while others were reduced in the more colitic groups (figs. S12 and S13).

Naïve DKO mice have altered gut microbiota composition that mediates the increased severity of DSS-induced colitis

To further investigate whether the microbial changes seen in DKO mice were due to deficiency of the 5-HT–autophagy axis in the IECs, we analyzed the expression of the antimicrobial peptide mDefb1 in the naïve state and the naïve microbiota of *Tph1*$^{-/-}$, DKO, and *Atg7*$^{\Delta IEC}$ mice. We observed that naïve *Tph1*$^{-/-}$ mice had increased mDefb1 mRNA and protein levels compared with naïve DKO mice, whereas naïve DKO and *Atg7*$^{\Delta IEC}$ mice had similar levels of mDefb1 (Fig. 7, A to C). 16*S* rRNA sequencing revealed that the naïve DKO mice were separated into distinct clusters compared to *Tph1*$^{-/-}$ and *Atg7*$^{\Delta IEC}$ groups, as shown by the PCoA and taxonomic composition of the gut microbiota (Fig. 7, D and E, and fig. S14A). Genetic differences were found to be a significant driver of variation, with 63% of the variation attributable to genotype. In all pairings, the differences between the groups are significant with a false discovery rate of <5%. Seven bacterial taxa in the *Tph1*$^{-/-}$ mice and 26 taxa in the *Atg7*$^{\Delta IEC}$ mice were significantly different from those in the DKO

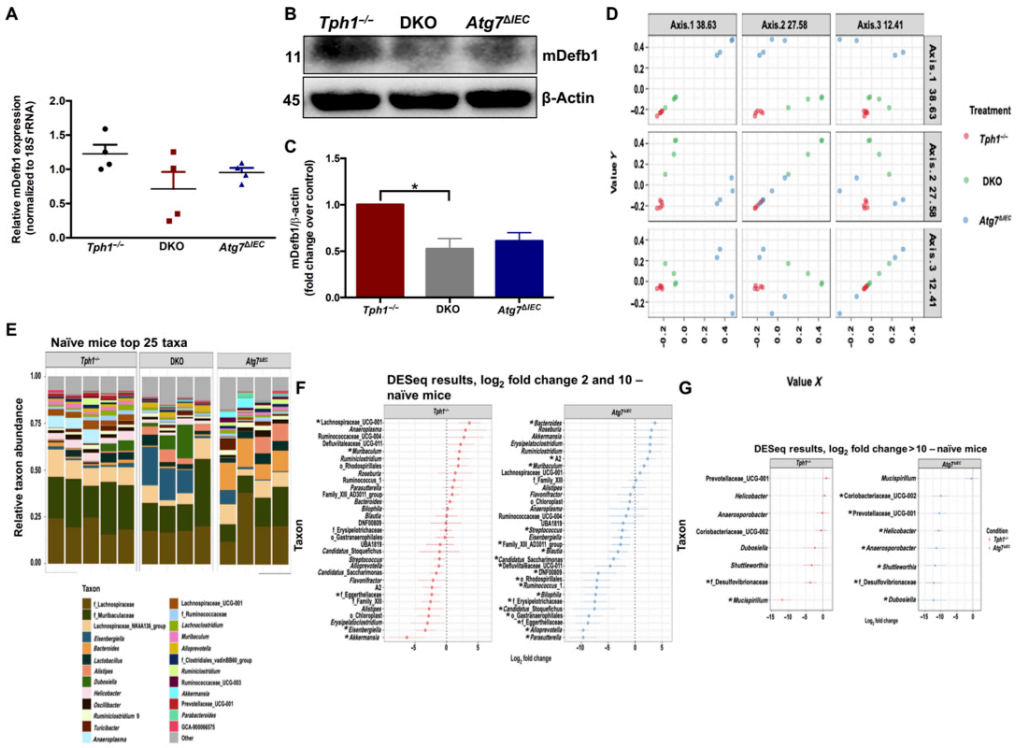


Fig. 7. Effect of impaired IEC 5-HT–autophagy axis on gut microbial composition of naïve mice. (**A**) Quantification of mDefb1 mRNA in the colonic tissue. (**B**) Western blot images and (**C**) quantification of mDefb1 protein in the colonic tissue. *$P < 0.05$ for graph (C). 16*S* rRNA sequencing was performed on the cecal content from naïve *Tph1*$^{-/-}$, DKO, and *Atg7*$^{\Delta IEC}$ mice. Each group had four to five mice. (**D**) PCoA of Bray-Curtis dissimilarity. (**E**) Taxonomic composition of the gut microbiota. (**F**) With DKO as the baseline, all taxa in the *Tph1*$^{-/-}$ and *Atg7*$^{\Delta IEC}$ groups for which one $\log_2$ fold change is 2 to 10. (**G**) With DKO as the baseline, all taxa in the *Tph1*$^{-/-}$ and *Atg7*$^{\Delta IEC}$ groups for which one $\log_2$ fold change is >10. *$P < 0.01$ for graphs (F) and (G).

mice (Fig. 7, F and G, and table S3). *Tph1*$^{-/-}$ mice had increased amounts of Lachnospiraceae and reduced levels of *Mucispirillum* and Desulfovibrionaceae compared to DKO mice (Fig. 7, F and G).

Following transplantation of naïve microbiota, mice receiving microbiota from DKO mice showed elevated parameters of intestinal inflammation such as day 3 to day 5 DAI, macroscopic and histological damage scores, and proinflammatory cytokines IL-1β, IL-6, and TNF-α compared to those receiving *Tph1*$^{-/-}$ microbiota (Fig. 8, B, C, and E to G). There was no difference in colitis severity between the groups receiving microbiota from DKO and *Atg7*$^{\Delta IEC}$ mice except for IL-1β levels. These findings further suggest that the change in microbiota caused by disruption of the 5-HT–autophagy axis in the IECs mediates the severity of inflammation.

5-HT treatment of human peripheral blood mononuclear cells inhibits autophagy and is reversed by rapamycin

To substantiate our findings in experimental models, we next sought to investigate whether 5-HT plays a role in autophagy in human individuals with or without CD using biopsy and blood samples. In the initial cohort, colonic biopsy samples were obtained from eight healthy controls (HCs) and eight patients with active CD. LC3B^{+} IECs were significantly lower in the inflamed tissue of patients with CD compared to the noninflamed tissue of the HCs (Fig. 9, A and B). Four to five HCs and active CD colonic biopsy samples were randomly selected from the initial cohort to measure the key CD risk factors IRGM and ATG16L1, which are critical in the autophagy pathway (*3*). IRGM mRNA expression was significantly reduced in patients with active CD compared to HCs (Fig. 9C).

In another cohort, blood samples were collected from 10 HCs and 10 patients with active CD indicated by symptoms, endoscopy, and high C-reactive protein (CRP) and fecal calprotectin levels (table S1). To determine the effect of 5-HT signaling in the regulation of autophagy in the gut, we used peripheral blood mononuclear cells (PBMCs) isolated from whole blood, since abnormalities in these cells are often reflective of disorders within the gut (*37*). The higher plasma 5-HT in patients with CD (Fig. 9E) was associated with

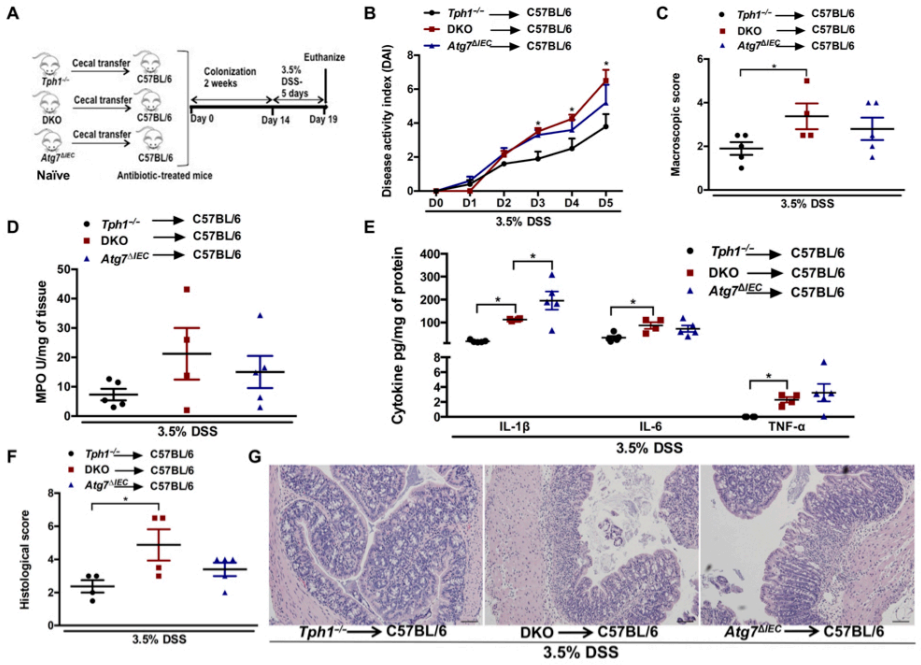


Fig. 8. Assessment of severity of colitis following adoptive transfer of naïve microbiota to antibiotic-treated C57BL/6 mice. (**A**) Schematic of cecal microbiota transfer followed by induction of colitis with 3.5% DSS. Each group had four to five mice. (**B**) DAI. *$P < 0.05$ between C57BL/6 mice receiving DKO microbiota and C57BL/6 mice receiving *Tph1*$^{-/-}$ microbiota. (**C**) Macroscopic damage score on day 5 after DSS. Colon tissue was collected to determine (**D**) MPO activity; (**E**) IL-1β, IL-6, and TNF-α; (**F**) histological score; and (**G**) representative micrographs on day 5 after DSS. Scale bars, 100 μm. Data are represented as means ± SEM. *$P < 0.05$ for graphs (C), (D), (E), and (F).

impaired autophagy in PBMCs, determined by significantly reduced LC3-II, Beclin-1, and Atg12-5 and accumulated p62 compared to HCs (Fig. 9, F and G). In addition, there was a significant negative correlation between levels of plasma 5-HT and autophagic flux in the PBMC denoted by LC3-II/β-actin levels (Fig. 9H). Compared to the control, treatment of PBMCs with 5-HT resulted in the down-regulation of autophagy proteins LC3-II, Beclin-1, and Atg12-5 and accumulation of p62 in both HCs and patients with CD. This was reversed by cotreatment with rapamycin (Fig. 9, I to L). These findings indicate that 5-HT–mediated impairment of autophagy in both patients with CD and HCs is through the activation of the mTOR signaling pathway.

DISCUSSION

IBD is a chronic inflammatory condition of the human bowel currently affecting several million people worldwide (*1*). Although the exact etiology of IBD is unknown, studies provide evidence that dysregulated immune responses, genetic factors, gut microbiota, and environmental factors contribute to the pathogenesis of IBD (*38*). 5-HT is a key enteric signaling molecule, and changes in gut 5-HT signaling are observed in IBD and in experimental colitis (*21*, *23*, *39*, *40*). Evidence suggests that autophagy, the body's process of clearing out old and damaged cells, is disrupted in IBD (*38*). Polymorphisms in autophagy genes result in impaired stress activation of autophagy, establishing a state of chronic inflammation that predisposes individuals to CD (*41*). Although changes in 5-HT, microbiota, and autophagy are associated with IBD, it remained to be determined whether a tripartite relationship exists among these factors. As illustrated in Fig. 10, in the current study, we find that increased 5-HT signaling impairs AMPKα/mTOR-dependent autophagy in the colon, which results in up-regulation of inflammation severity by increasing the production of proinflammatory cytokines and by selecting for a colitogenic microbiota.

To investigate the impact of 5-HT on autophagy, we studied *Tph1*$^{-/-}$ and *SERT*$^{-/-}$ mice, with different levels of 5-HT in the gut. *Tph1*$^{-/-}$ mice with low levels of 5-HT (*24*) had decreased severity of colitis and elevated autophagic flux, while *SERT*$^{-/-}$ mice, with elevated levels of 5-HT, had reduced autophagy and increased severity of colitis. Apart from the colon, it is also reported that *SERT* gene knockdown in the dorsal raphe nucleus in rats increased 5-HT level and impaired hippocampal autophagy (*31*). We did not observe

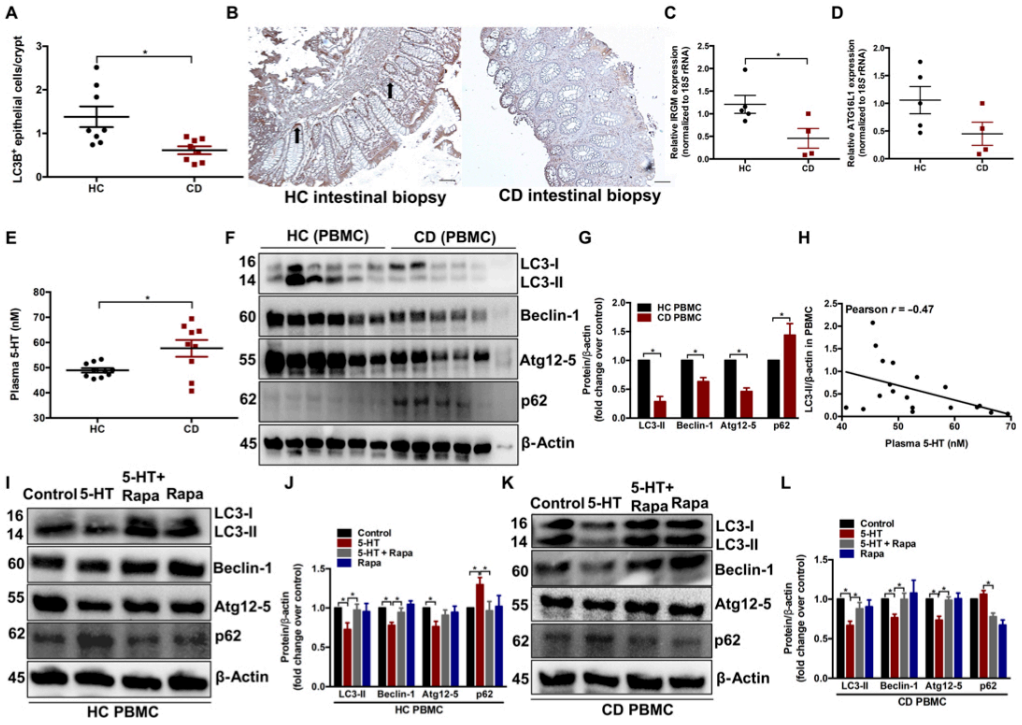


Fig. 9. Effect of 5-HT on autophagy in HCs and patients with CD. (**A**) Number of LC3B-expressing IECs per crypts in colonic sections in cohort 1 HCs ($n = 8$) and patients with CD ($n = 8$). (**B**) Representative micrograph. Arrows indicate $LC3B^+$ IECs. Scale bars, 100 μm. (**C**) Quantification of *IRGM* and (**D**) *ATG16L1* mRNA expression in the colonic mucosal biopsies from four to five randomly selected HCs and patients with active CD in cohort 1. (**E**) Comparison of plasma 5-HT concentration between HCs ($n = 10$) and patients with CD ($n = 10$) in cohort 2. (**F**) Western blot images and (**G**) quantification of autophagy proteins (LC3, Beclin-1, Atg12-5, and p62) performed on protein extracts obtained from PBMCs of cohort 2. Each lane represents an individual participant, and the bar graph represents means ± SEM. (**H**) Significant negative correlation between plasma 5-HT and PBMC LC3-II levels. PBMCs from either HCs or CD in cohort 2 were treated in the presence of phytohemagglutinin (PHA; 5 μg/ml) with vehicle, 5-HT (10 μM), or rapamycin (50 nM) or cotreated with 5-HT and rapamycin for 6 hours, and the extracted protein was analyzed for expression of LC3, Beclin-1, Atg12-5, and p62. (**I** and **J**) Western blot images and quantification graphs in HCs and (**K** and **L**) Western blot images and quantification graphs in CD. Representative Western blot with β-actin as loading controls. Blots shown are representative of 10 HCs and 10 patients with CD from cohort 2. Bar graph represents means ± SEM. *$P < 0.05$ for graphs (A), (C), (E), (G), (J) and (L).

differences in autophagy markers between the wild-type colitic and noncolitic mice. Cabrera and colleagues (*7*) reported up-regulated autophagy in DSS colitis as a protective mechanism by which cells adapt to different types of stressors, whereas Ortiz-Masia *et al.* (*42*) observed reduced autophagy in association with colitis. The disparities between these observations might be due to the differences in DSS concentration and duration in these studies. Our findings suggest that an increase in 5-HT in DSS colitis decelerates autophagy that contributes to disease severity. Blocking 5-HT signaling by the administration of Tph1 inhibitor released this brake, promoted autophagy, and alleviated the severity of colitis. Furthermore, our data indicate that increased 5-HT levels before inflammation inhibit autophagy and make the host more susceptible to colitis.

To further understand the role of 5-HT in autophagy in the context of intestinal inflammation, we restored 5-HT level in $Tph1^{-/-}$ mice and observed up-regulated severity of DSS-induced colitis along with impairment in autophagy. Notably, in $Tph1^{-/-}$ mice, the elevation in the severity of intestinal inflammation attributed to the restoration of 5-HT was attenuated by the mTOR inhibitor, rapamycin. Rapamycin also regulates autophagy-independent cellular processes that can result in immunosuppression (*13*, *43*) and alleviation of colitis. Thus, although other mechanisms cannot be ruled out, our results in Fig. 2 strongly suggest that 5-HT–induced impairment of autophagy in the inflamed colon is reversed by rapamycin-mediated rescue of autophagy. These findings were further confirmed by the loss of the protective action of reduced 5-HT in the $Tph1^{-/-}$ mice when

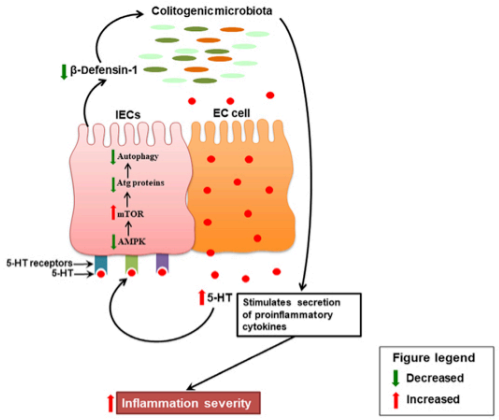


Fig. 10. Schematic representation of the data. During colitis, increased mucosal 5-HT secreted from the EC cells in the colon binds to 5-HT_3, 5-HT_4, and 5-HT_7 receptors on the IECs and inhibits AMPK. Inhibition of AMPK subsequently activates mTOR. Activation of mTOR inhibits formation of autophagy (Atg) proteins that leads to impairment in autophagy in the IECs. This leads to the reduced production of the antimicrobial peptide, β-defensin-1, by the IECs, which alters the composition of the normal gut microbiota. The colitogenic microbiota stimulates the secretion of proinflammatory cytokines (IL-8, IL-1β, IL-6, and TNF-α) from IECs and immune cells and, ultimately, exacerbates the severity of inflammation.

the autophagy protein Atg7 was deficient in the IECs. This suggests that 5-HT impairs mTOR-dependent autophagy in the colon of DSS-treated mice and increases the severity of intestinal inflammation. In addition, we observed that increased 5-HT in the colon reduced the expression of p-AMPKα, a known upstream regulator of mTOR (*44*). Similar to our findings, Guragain *et al.* (*45*) reported that 5-HT treatment of colon epithelial cells induced dephosphorylation of AMPKα in a dose-dependent manner. This suggests that increased 5-HT inhibits phosphorylation of AMPKα, leading to activation of mTOR, since it is known that AMPK regulates mTORC1 signaling (*6*, *44*). Although the role of AMPK in autophagy has been reported before (*6*), this is the first demonstration that 5-HT is an important regulator of the interaction between AMPK and autophagy.

IECs form the barrier between the gut lumen and mucosa and act as the first line of defense against invading microbes and potential pathogens (*46*). Functional autophagy in the IECs is required to maintain homeostasis and prevent intestinal inflammation (*4*, *5*). The presence of 5-HT receptors on IECs (*18*), as well as their proximity to 5-HT–secreting EC cells, suggests that 5-HT plays a critical role in maintaining IEC homeostasis. Our study demonstrates that during DSS-induced colitis, reduced 5-HT in the gut promotes autophagy in the IECs. To further study the 5-HT–autophagy axis in the IECs, we generated DKO mice that were deficient in Tph1 globally and in Atg7 in the IECs. The protective effect of reduced 5-HT in the *Tph1*$^{-/-}$ mice against DSS-induced colitis was abrogated when the *Tph1*$^{-/-}$ mice were deficient in autophagy in the IECs. Recently, we demonstrated that blocking 5-HT_7 receptor signaling with an antagonist or genetic deletion of this receptor reduced the severity of chemical colitis and increased antimicrobial peptide mDefb1 and mDefb3 productions (*27*, *47*). Another study demonstrated that 5-HT_4 receptor stimulation via enema administration had a protective effect in experimental colitis but was not protective when delivered by intraperitoneal injection (*48*). This protective effect was associated with enhanced proliferation of IECs and increase in gut motility. These findings suggest that 5-HT may incite divergent actions on different cell types in the context of colitis. Our findings suggest that 5-HT–mediated disruption of autophagy in the IECs is arbitrated by 5-HT_3, 5-HT_4, and 5-HT_7 receptor, with 5-HT_7 receptor playing a more potent role. It is unclear how the activation of the G protein–coupled receptors 5-HT_4 and 5-HT_7 and the ligand-gated ion channel 5-HT_3 receptors is linked with the stimulation of the mTOR pathway. A possible mechanism for the 5-HT_4 and 5-HT_7 receptors to activate mTOR is through the elevation of the second messenger cyclic AMP (*49*) and subsequent inhibition of AMPK. Activation of the 5-HT_3 receptor results in the influx of extracellular calcium ions that mobilizes and increases the concentration of intracellular calcium (*49*). The regulation of mTOR-dependent autophagy by intracellular calcium remains controversial (*50*). Therefore, further studies are deserved to elucidate the role of 5-HT receptors expressed on IECs in the regulation of autophagy in CD. In addition to receptor sensing, 5-HT can alter gene expression through serotonylation of histone H3 (*51*). While little is known on the direct evidence of autophagy regulation via serotonylation, recently, it has been demonstrated that 5-HT treatment of human colon cancer cells activates mTORC1 through serotonylation (*52*). Exploring the role of serotonylation in autophagy in gut inflammation will further enhance our understanding on the interaction between 5-HT signaling and autophagy.

Disruption of the balanced composition of gut microbiota is associated with IBD. Clinical and animal studies suggest that changed gut microbiota structure and function trigger and perpetuate chronic intestinal inflammation (*53*). Both the process of autophagy and 5-HT signaling are implicated in the regulation of gut microbiota (*8*, *27*, *54*). Despite these findings, it is currently unknown whether increased 5-HT influences autophagy in the IECs and results in altered production of the antimicrobial peptide mDefb1 and thus contributes to dysbiosis. In this study, we observed that, in the context of colitis, the protective effects associated with *Tph1*$^{-/-}$ microbiota are lost when the IECs have impaired autophagy. These findings also coincided with reduced expression of mDefb1 when the IECs were deficient in autophagy. During colitis in *Tph1*$^{-/-}$ mice, beneficial bacteria such as *Faecalibaculum* (*55*) were higher, while bacteria associated with inflammation such as *Parabacteroides* and *Rikenella* (*15*) were decreased compared to DKO mice. Under the naïve condition, *Tph1*$^{-/-}$ mice had elevated levels of protective bacteria such as Lachnospiraceae and reduced levels of colitogenic bacteria such as *Mucispirillum* and Desulfovibrionaceae compared to DKO mice (*56*, *57*). Moreover, *Dubosiella* and *Turicibacter* that are increased during colitis (*58*, *59*) were significantly higher in the more colitic DKO and *Atg7*$^{\Delta IEC}$ donors and recipients. Furthermore, the transfer of colitic or naïve DKO microbiota to antibiotic-treated mice was associated with increased inflammation severity. Together, these findings identify the 5-HT–autophagy axis in the IECs as a previously unknown key regulator of gut microbiota during intestinal inflammation.

An increase in plasma/serum 5-HT levels is observed in patients with IBD, and the use of selective 5-HT reuptake inhibitors is associated with microscopic colitis (*21*, *23*, *39*, *40*, *60*, *61*). In addition, dysfunction in the autophagic process is implicated in the

development of intestinal inflammation in patients with IBD (*5*). Previous studies demonstrated reduced expression of the autophagy proteins in the inflamed intestinal mucosa of patients with CD (*42*, *62*). In this study, we observed impaired autophagy in the colonic biopsy samples and PBMCs along with elevated plasma 5-HT levels in patients with active CD compared with HCs. The inhibitory role of 5-HT in autophagy was further supported by 5-HT–mediated down-regulation of autophagy in PBMCs isolated from both HCs and patients with CD, which was reversed by treatment with the autophagy enhancer, rapamycin. These findings reflect the translational significance of the 5-HT–autophagy axis in CD. However, since autophagy gene polymorphisms have been associated with CD, an important area for future research is investigating the effect of 5-HT on autophagy in CD patients with and without autophagy gene polymorphisms.

This study is the first to report the important role of the 5-HT–autophagy axis in IECs and its contribution to the regulation of microbiota in relation to susceptibility to intestinal inflammation. Our findings suggest that blocking 5-HT signaling may promote autophagy and alleviate the severity of intestinal inflammation. Given recent reports of impaired autophagy in patients with CD (*2*, *42*) and the established changes in 5-HT production in IBD (*20*–*22*), this research sheds light on 5-HT as a novel regulator of autophagy in gut inflammation. These findings may ultimately lead to the discovery of novel therapeutic strategies in intestinal inflammatory conditions such as IBD and other health disorders that exhibit dysregulated autophagy.

MATERIALS AND METHODS

Animals

All mice were 8 to 12 weeks old and on a C57BL/6 background. Both male and female mice were used. Breeding pairs of *Tph1*$^{-/-}$ mice and *Tph1*$^{+/+}$ littermates on a C57BL/6 background were obtained from the Université Pierre et Marie Curie, France (*63*). These mice express normal amounts of 5-HT in the brain and show no observed differences in food intake or body weight as compared with *Tph1*$^{+/+}$ mice. *SERT*$^{+/+}$ and *SERT*$^{-/-}$ as well as *Atg7*$^{fl/fl}$ and *Atg7*$^{fl/fl}$ villin-Cre (*Atg7*$^{\Delta IEC}$) mice, generated as described previously (*64*, *65*), were provided by G.R.S. (McMaster University, ON) and D.P. (University of Toronto, ON), respectively. *Tph1*$^{-/-}$ mice were crossed with *Atg7*$^{\Delta IEC}$ mice to generate *Tph1*$^{-/-}$*Atg7*$^{flox/flox}$*villin-Cre* DKO (fig. S7). C57BL/6 mice were purchased from Charles River Laboratories in Massachusetts, USA. All mice were housed under specific pathogen–free conditions in the central animal facility and were approved by the McMaster University Animal Care Committee and conducted according to Canadian Council guidelines for animal research.

Experimental design

DSS (molecular mass, 40 kDa; catalog no. 02160110, MP Biomedicals Inc.) was added to autoclaved drinking water at 5% (w/v) for 5 days to induce acute colitis. The control mice received autoclaved drinking water. In a separate experiment, *Tph1*$^{-/-}$ mice were injected subcutaneously with 5-HTP (50 mg/kg) twice daily for 8 days beginning 3 days before induction of 5% DSS colitis, whereas the control *Tph1*$^{-/-}$ mice received saline as vehicle. Another group of *Tph1*$^{-/-}$ mice received a similar dosage and duration of 5-HTP along with rapamycin at a dose of 1.5 mg/kg per day for 5 days starting with DSS administration. All interventions were performed during the light cycle.

Adoptive microbiota transfer

C57BL/6 mice received a cocktail of broad-spectrum antibiotics neomycin, ampicillin, vancomycin, and metronidazole (all 0.5 g/liter) in sterile drinking water for 10 days. After 10 days, 200 μl of cecal contents from naïve or DSS-treated *Tph1*$^{-/-}$ or DKO or *Atg7*$^{\Delta IEC}$ mice was gavaged to three groups of C57BL/6 mice for 3 days. Following 2 weeks of colonization, 3.5% (w/v) DSS was added to the drinking water for 5 days to induce colitis.

Drugs and reagents

5-HT (catalog no. H9523, Sigma-Aldrich), 5-HTP (catalog no. H9772, Sigma-Aldrich), rapamycin (catalog no. 37094, Sigma-Aldrich), and phytohemagglutinin (PHA; catalog no. L1668, Sigma-Aldrich) were prepared according to the manufacturer's instructions.

Isolation of murine IECs

Murine colon epithelial cells were isolated according to the protocol by Gracz *et al.* (*66*). A total of 1×10^6 IECs (100 μl) were plated on slides using cytospin. IECs were stained with rabbit monoclonal anti-LC3A/B antibody [1:200; catalog no. 12741, Cell Signaling Technology (CST)] or with rabbit monoclonal anti–E-cadherin antibody (1:200; catalog no. 3195, CST) according to the CST protocol. The remaining cell pellet was homogenized and used for Western blot analysis.

Isolation of PBMCs from human whole blood and human colonic biopsy collection

The study was approved by the Hamilton Integrated Research Ethics Board (Project# 12-239) (Clinicaltrials.gov #NCT01650311). All biopsy and blood samples were obtained at McMaster University Medical Centre from consenting adults. The exclusion criteria for HCs for both cohorts included history of gastrointestinal complaints, use of drugs such as antidepressants, steroids, nonsteroidal anti-inflammatory drugs, and biologics. Any participant from the HC or CD groups was excluded if he/she was considered unfit by the gastroenterologist. The first cohort included eight HCs and eight patients with CD who donated colonic biopsy samples for immunohistochemistry. In the second cohort, 10 HCs and 10 patients with CD donated 30 ml of whole blood. One milliliter of blood was used to measure plasma 5-HT levels by enzyme-linked immunosorbent assay (ELISA). The remaining 29 ml was used to isolate PBMCs according to the STEMCELL Technologies protocol using Lymphoprep (STEMCELL Technologies). Isolated PBMCs were cultured in RPMI media and 1% penicillin/streptomycin. The PBMCs were treated in the presence of PHA (5 μg/ml) with 5-HT (10 μM), rapamycin (50 nM), or both for 6 hours at 37°C at 5% CO_2. The cell lysates were collected for the determination of levels of autophagy proteins by Western blotting.

Evaluation of inflammation and autophagy proteins

Colitis was assessed using DAI, macroscopic score, histological score of hematoxylin and eosin–stained colonic sections, and MPO activity based on established systems (*25*). DAI is a combined score of weight loss, stool consistency, and fecal bleeding as previously published (*25*). This scoring system is defined as weight loss: 0, no loss; 1, 1 to 5%; 2, 5 to 10%; 3, 10 to 20%; 4, >20%; stool: 0, normal; 2, loose stool; 4, diarrhea; and bleeding: 0, no blood; 2, Hemoccult positive (Hemoccult II; Beckman Coulter); 4, gross blood (blood around anus). DAI was measured on all 5 days of DSS administration. Levels of colonic tissue proinflammatory cytokines IL-1β, IL-6, and TNF-α

were determined by ELISA. Autophagy proteins were analyzed by Western blot and immunofluorescence staining of proteins extracted either from whole colon, mucosa, or IECs.

Western blotting

Murine colonic tissue was homogenized, and total protein was extracted using radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail and phosphatase inhibitor. Total protein concentration of homogenized tissue was determined using the DC Protein Assay Kit (catalog no. 5000111, Bio-Rad). Equal amounts of protein from each group were loaded and separated by 7 to 20% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using Trans-Blot Turbo transfer system (Bio-Rad) as per the manufacturer's instructions. Membranes were blocked with 5% bovine serum albumin in tris-buffered saline–Tween 20 (TBST) for 1 hour at room temperature (r.t). Membranes were incubated overnight at 4°C with primary antibodies against LC3 (1:1000; catalog no. 12741, CST), Beclin-1 (1:1000; catalog no. 3495, CST), Atg12-5 (1:1000; catalog no. 4180, CST), Atg7 (1:1000; catalog no. 8558, CST), p62 (1:1000; catalog no. 5114, CST), p-AMPKα (T172) (1:1000; catalog no. 2535, CST), AMPKα (1:1000; catalog no. 5831, CST), p-mTOR (S2448) (1:1000; catalog no. 5536, CST), mTOR (1:1000; catalog no. 2983, CST), mDefb1 (1:500; catalog no. PA575666, Thermo Fisher Scientific), and β-actin (1:1000; catalog no. 4970, CST). Membranes were washed three times with TBST and incubated with anti-rabbit horseradish peroxidase–linked antibody (1:5000; catalog no. 7074, CST) for 1 hour at r.t. Proteins were treated with Clarity Max Western enhanced chemiluminescence substrate (catalog no. 1705062, Bio-Rad) and exposed to a luminescent image analyzer (ChemiDoc Touch Imaging System) to visualize the proteins. Densitometric analysis was performed on Western blots with ImageJ software (version 1.51), normalized to total β-actin.

Immunohistochemistry/immunofluorescence

Formalin-fixed, paraffin-embedded human mucosal biopsies were stained for LC3B. Colonic tissue sections were deparaffinized with CitriSolv (catalog no. 04355121, Thermo Fisher Scientific) and rehydrated in graded concentrations of ethanol. Sections were subjected to heat-induced epitope retrieval, blocked with 3% bovine serum albumin, and incubated with a polyclonal rabbit anti-LC3B (1:200; catalog no. 48394, Abcam) antibody for 1 hour at r.t. Sections were washed with phosphate-buffered saline/0.5% Tween 20 and incubated with EnVision (horseradish peroxidase–coupled anti-rabbit secondary reagent; DakoCytomation, catalog no. K4003, Dako) for 30 min. Sections were developed using 3,3′-diaminobenzidine solution (SIGMAFAST, catalog no. 079K8208, Sigma-Aldrich) and counterstained with Mayer's hematoxylin solution (catalog no. MHS1, Sigma-Aldrich). Sections were visualized using a Nikon Eclipse 80i microscope (Nikon Instruments Inc.). The number of $LC3B^+$ cells per 10 glands was counted in four different areas for each section.

For detecting autophagosome and lysosome fusion in the murine colonic tissue sections, double immunofluorescence staining for LC3B and LAMP2 was performed. Colon tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin sections of 4 μm were cut, mounted, dewaxed, and rehydrated. Antigen retrieval was performed on paraffin sections before immunostaining by heating the slides in 0.01 M citrate buffer (pH 6.0) for 30 min. Sections were blocked with 5% normal goat serum (Sigma-Aldrich) and incubated with primary antibodies overnight at 4° C., followed by secondary antibodies for 1 hour at r.t. Primary antibodies were polyclonal rabbit anti-LC3B (1:100; catalog no. 48394, Abcam) and monoclonal rat anti-LAMP2 (1:100; catalog no. B4247, LSBio); secondary antibodies were Alexa Fluor 594–conjugated goat anti-rabbit immunoglobulin G (IgG) (1:100; catalog no. A11037, Invitrogen) for LC3 staining and Alexa 488–conjugated goat anti-rat IgG (1:100; catalog no. A11006, Invitrogen) for LAMP2 staining. After washing, sections were mounted with Fluoroshield mounting medium with DAPI (4′,6-diamidino-2-phenylindole; catalog no. 104139, Abcam) for nuclear counterstaining. Immunostaining was examined, and images were captured using a fluorescence microscope (Nikon Eclipse 80i) with 40× objective lens. All pictures were taken with a Nikon digital camera attached to the microscope using a NIS-Elements Basic Research imaging software. The percentage of $LC3B/LAMP2^+$ epithelial cells was determined by counting the number of LC3/LAMP2 coexpressed epithelial cells and total epithelial cells in three to four different areas for each section.

Enzyme-linked immunosorbent assay

5-HT levels were measured using commercially available ELISA kits (catalog no. IM1749, Beckman Coulter) following its extraction from murine colonic tissue by established protocol (*27*) or in human plasma. For intestinal cytokine measurement, colonic tissues were homogenized in TBS containing a protease inhibitor mixture (catalog no. P8340, Sigma-Aldrich), centrifuged for 5 min at 3300*g*, and the resulting supernatants used for measuring cytokine levels (IL-1β, catalog no. SMLB00C; IL-6, catalog no. SM6000B; TNF-α, catalog no. SMTA00B) according to the manufacturer's instructions (Quantikine Murine, R&D Systems). Total protein levels were quantified in the homogenates using the DC Protein Assay Kit.

Quantitative real-time polymerase chain reaction

Total RNA from human intestinal mucosal biopsy or mouse colon tissue was extracted using TRIzol reagent (catalog no. 15596026, Thermo Fisher Scientific). Total RNA was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), followed by complementary DNA (cDNA) preparation from 1 μg of total RNA using the iScript cDNA Synthesis Kit (catalog no. 1708891, Bio-Rad). Quantitative real-time polymerase chain reaction (qRT-PCR) assay was completed using SsoFast EvaGreen SYBR Green PCR Master Mix (catalog no. 1725201, Bio-Rad) and CFX96 real-time PCR system (Bio-Rad). Commercially available primers for ATG16L1 and IRGM were used (Bio-Rad assay IDs qHsaCED0042534 and qHsaCED0004388). The primer used for mDefb1 has been previously reported (*27*). The reference gene selected was human or mouse 18*S* rRNA as described previously (*21*, *27*). The data were analyzed according to the $2^{-\Delta\Delta CT}$ method and expressed as relative abundances (means ± SEM).

Microbiome profiling and analysis

DNA was extracted from the cecal samples of mice, and the 16*S* rRNA variable 3 (V3) gene region was amplified as previously described (*67*). The forward and reverse primer sequences used for the amplification of the V3 gene region have been specified in tables S4 and S5. Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100 base pairs. Sequence variants were then resolved from the trimmed raw reads using DADA2, an accurate sample inference pipeline from 16*S* amplicon data (*68*). DNA sequence reads were filtered and trimmed on the basis of the

quality of the reads for each Illumina run separately, and error rates were learned and sequence variants were determined by DADA2. Sequence variant tables were merged to combine all information from separate Illumina runs. Bimeras were removed, Ribosomal Database Project classifier was used to assign taxonomy using the SILVA reference database version 1.3.2, and analysis was conducted on DESeq2 R package and using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software) (*69*, *70*). The ASV table was filtered to remove taxa whose mean abundance was 10 or less, samples with a sampling depth of <2500, and those reads not assigned to bacteria or archaea. After filtering, the dataset had 3,473,842 reads in 479 ASVs across 36 samples. The sample read depth ranged from 54,329 to 137,507. To measure beta diversity, we used Bray-Curtis distances based on taxon relative abundance.

Statistical analysis

Three to 10 biological replicates were used for every experiment. Results are represented as means ± SEM. Unpaired Student's *t* test (two-tailed) or one-way analysis of variance, followed by Newman-Keuls multiple comparison post hoc test or Pearson correlation coefficient test, was performed to analyze data using GraphPad Prism version 6 (GraphPad Software). $P < 0.05$ was considered statistically significant. For microbial analysis, statistically significant differences in beta diversity were examined using permutational multivariate analysis of variance. DESeq2 was used to determine significantly different taxa between mice groups, where the difference was considered significant, if $P < 0.01$ after adjustment for multiple testing via Benjamini-Hochberg multiple testing adjustment procedure (*70*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abi6442

View/request a protocol for this paper from *Bio-protocol*.

REFERENCES AND NOTES

1. R. Caruso, B. C. Lo, G. Nunez, Host-microbiota interactions in inflammatory bowel disease. *Nat. Rev. Immunol.* **20**, 411–426 (2020).
2. J. Hampe, A. Franke, P. Rosenstiel, A. Till, M. Teuber, K. Huse, M. Albrecht, G. Mayr, F. M. De La Vega, J. Briggs, S. Gunther, N. J. Prescott, C. M. Onnie, R. Hasler, B. Sipos, U. R. Folsch, T. Lengauer, M. Platzer, C. G. Mathew, M. Krawczak, S. Schreiber, A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* **39**, 207–211 (2007).
3. D. C. Massey, M. Parkes, Genome–wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy* **3**, 649–651 (2007).
4. A. Larabi, N. Barnich, H. T. T. Nguyen, New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy* **16**, 38–51 (2020).
5. S. Haq, J. Grondin, S. Banskota, W. I. Khan, Autophagy: Roles in intestinal mucosal homeostasis and inflammation. *J. Biomed. Sci.* **26**, 19 (2019).
6. Z. Yang, D. J. Klionsky, Mammalian autophagy: Core molecular machinery and signaling regulation. *Curr. Opin. Cell Biol.* **22**, 124–131 (2010).
7. S. Cabrera, A. F. Fernandez, G. Marino, A. Aguirre, M. F. Suarez, Y. Espanol, J. A. Vega, R. Laura, A. Fueyo, M. S. Fernandez-Garcia, J. M. Freije, G. Kroemer, C. Lopez-Otin, ATG4B/autophagin-1 regulates intestinal homeostasis and protects mice from experimental colitis. *Autophagy* **9**, 1188–1200 (2013).
8. K. Tsuboi, M. Nishitani, A. Takakura, Y. Imai, M. Komatsu, H. Kawashima, Autophagy protects against colitis by the maintenance of normal gut microflora and secretion of mucus. *J. Biol. Chem.* **290**, 20511–20526 (2015).
9. D. C. Macias-Ceja, J. Cosin-Roger, D. Ortiz-Masia, P. Salvador, C. Hernandez, J. V. Esplugues, S. Calatayud, M. D. Barrachina, Stimulation of autophagy prevents intestinal mucosal inflammation and ameliorates murine colitis. *Br. J. Pharmacol.* **174**, 2501–2511 (2017).
10. S. Hu, M. Chen, Y. Wang, Z. Wang, Y. Pei, R. Fan, X. Liu, L. Wang, J. Zhou, S. Zheng, T. Zhang, Y. Lin, M. Zhang, R. Tao, J. Zhong, mTOR inhibition attenuates dextran sulfate sodium-induced colitis by suppressing T cell proliferation and balancing Th_1/Th_{17}/Treg profile. *PLOS ONE* **11**, e0154564 (2016).
11. D. C. Massey, F. Bredin, M. Parkes, Use of sirolimus (rapamycin) to treat refractory Crohn's disease. *Gut* **57**, 1294–1296 (2008).
12. S. Banskota, H. Wang, Y. H. Kwon, J. Gautam, P. Gurung, S. Haq, F. M. N. Hassan, D. M. Bowdish, J. A. Kim, D. Carling, M. D. Fullerton, G. R. Steinberg, W. I. Khan, Salicylates ameliorate intestinal inflammation by activating macrophage AMPK. *Inflamm. Bowel Dis.* **27**, 914–926 (2021).
13. J. Li, S. G. Kim, J. Blenis, Rapamycin: One drug, many effects. *Cell Metab.* **19**, 373–379 (2014).
14. A. L. Kau, P. P. Ahern, N. W. Griffin, A. L. Goodman, J. I. Gordon, Human nutrition, the gut microbiome and the immune system. *Nature* **474**, 327–336 (2011).
15. F. Imhann, A. Vich Vila, M. J. Bonder, J. Fu, D. Gevers, M. C. Visschedijk, L. M. Spekhorst, R. Alberts, L. Franke, H. M. van Dullemen, R. W. F. Ter Steege, C. Huttenhower, G. Dijkstra, R. J. Xavier, E. A. M. Festen, C. Wijmenga, A. Zhernakova, R. K. Weersma, Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut* **67**, 108–119 (2018).
16. L. Yang, C. Liu, W. Zhao, C. He, J. Ding, R. Dai, K. Xu, L. Xiao, L. Luo, S. Liu, W. Li, H. Meng, Impaired autophagy in intestinal epithelial cells alters gut microbiota and host immune responses. *Appl. Environ. Microbiol.* **84**, e00880-18 (2018).
17. M. J. Jung, J. Lee, N. R. Shin, M. S. Kim, D. W. Hyun, J. H. Yun, P. S. Kim, T. W. Whon, J. W. Bae, Chronic repression of mTOR complex 2 induces changes in the gut microbiota of diet-induced obese mice. *Sci. Rep.* **6**, 30887 (2016).
18. S. N. Spohn, G. M. Mawe, Non-conventional features of peripheral serotonin signalling - The gut and beyond. *Nat. Rev. Gastroenterol. Hepatol.* **14**, 412–420 (2017).
19. S. Banskota, J. E. Ghia, W. I. Khan, Serotonin in the gut: Blessing or a curse. *Biochimie* **161**, 56–64 (2019).
20. M. S. Shajib, A. Baranov, W. I. Khan, Diverse effects of gut-derived serotonin in intestinal inflammation. *ACS Chem. Neurosci.* **8**, 920–931 (2017).
21. M. S. Shajib, U. Chauhan, S. Adeeb, Y. Chetty, D. Armstrong, S. L. S. Halder, J. K. Marshall, W. I. Khan, Characterization of serotonin signaling components in patients with inflammatory bowel disease. *J. Can. Assoc. Gastroenterol.* **2**, 132–140 (2019).
22. M. Kidd, B. I. Gustafsson, I. Drozdov, I. M. Modlin, IL1β- and LPS-induced serotonin secretion is increased in EC cells derived from Crohn's disease. *Neurogastroenterol. Motil.* **21**, 439–450 (2009).
23. M. Manocha, W. I. Khan, Serotonin and GI disorders: An update on clinical and experimental studies. *Clin. Transl. Gastroenterol.* **3**, e13 (2012).
24. J. E. Ghia, N. Li, H. Wang, M. Collins, Y. Deng, R. T. El-Sharkawy, F. Cote, J. Mallet, W. I. Khan, Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology* **137**, 1649–1660 (2009).
25. J. J. Kim, H. Wang, J. D. Terc, B. Zambrowicz, Q. M. Yang, W. I. Khan, Blocking peripheral serotonin synthesis by telotristat etiprate (LX1032/LX1606) reduces severity of both chemical- and infection-induced intestinal inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **309**, G455–G465 (2015).
26. K. G. Margolis, K. Stevanovic, Z. Li, Q. M. Yang, T. Oravecz, B. Zambrowicz, K. G. Jhaver, A. Diacou, M. D. Gershon, Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. *Gut* **63**, 928–937 (2014).
27. Y. H. Kwon, H. Wang, E. Denou, J. E. Ghia, L. Rossi, M. E. Fontes, S. P. Bernier, M. S. Shajib, S. Banskota, S. M. Collins, M. G. Surette, W. I. Khan, Modulation of gut microbiota composition by serotonin signaling influences intestinal immune response and susceptibility to colitis. *Cell. Mol. Gastroenterol. Hepatol.* **7**, 709–728 (2019).
28. T. C. Fung, H. E. Vuong, C. D. G. Luna, G. N. Pronovost, A. A. Aleksandrova, N. G. Riley, A. Vavilina, J. McGinn, T. Rendon, L. R. Forrest, E. Y. Hsiao, Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. *Nat. Microbiol.* **4**, 2064–2073 (2019).
29. C. Soll, J. H. Jang, M. O. Riener, W. Moritz, P. J. Wild, R. Graf, P. A. Clavien, Serotonin promotes tumor growth in human hepatocellular cancer. *Hepatology* **51**, 1244–1254 (2010).
30. T. Imada, S. Nakamura, R. Hisamura, Y. Izuta, K. Jin, M. Ito, N. Kitamura, K. F. Tanaka, M. Mimura, I. Shibuya, K. Tsubota, Serotonin hormonally regulates lacrimal gland secretory function via the serotonin type 3a receptor. *Sci. Rep.* **7**, 6965 (2017).
31. Z. M. Wu, C. H. Zheng, Z. H. Zhu, F. T. Wu, G. L. Ni, Y. Liang, SiRNA-mediated serotonin transporter knockdown in the dorsal raphe nucleus rescues single prolonged stress-induced hippocampal autophagy in rats. *J. Neurol. Sci.* **360**, 133–140 (2016).
32. M. N. Sharifi, E. E. Mowers, L. E. Drake, K. F. Macleod, Measuring autophagy in stressed cells. *Methods Mol. Biol.* **1292**, 129–150 (2015).
33. S. C. Bischoff, R. Mailer, O. Pabst, G. Weier, W. Sedlik, Z. Li, J. J. Chen, D. L. Murphy, M. D. Gershon, Role of serotonin in intestinal inflammation: Knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296**, G685–G695 (2009).
34. S. C. Regmi, S. Y. Park, S. K. Ku, J. A. Kim, Serotonin regulates innate immune responses of colon epithelial cells through Nox2-derived reactive oxygen species. *Free Radic. Biol. Med.* **69**, 377–389 (2014).

35. G. M. Mawe, J. M. Hoffman, Serotonin signalling in the gut-functions, dysfunctions and therapeutic targets. *Nat. Rev. Gastroenterol. Hepatol.* **10**, 473–486 (2013).
36. R. L. Gallo, L. V. Hooper, Epithelial antimicrobial defence of the skin and intestine. *Nat. Rev. Immunol.* **12**, 503–516 (2012).
37. T. Matsuura, G. A. West, K. R. Youngman, J. S. Klein, C. Fiocchi, Immune activation genes in inflammatory bowel disease. *Gastroenterology* **104**, 448–458 (1993).
38. R. J. Xavier, D. K. Podolsky, Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427–434 (2007).
39. M. D. Coates, C. R. Mahoney, D. R. Linden, J. E. Sampson, J. Chen, H. Blaszyk, M. D. Crowell, K. A. Sharkey, M. D. Gershon, G. M. Mawe, P. L. Moses, Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* **126**, 1657–1664 (2004).
40. S. J. MacEachern, C. M. Keenan, E. Papakonstantinou, K. A. Sharkey, B. A. Patel, Alterations in melatonin and 5-HT signalling in the colonic mucosa of mice with dextran-sodium sulfate-induced colitis. *Br. J. Pharmacol.* **175**, 1535–1547 (2018).
41. A. Murthy, Y. Li, I. Peng, M. Reichelt, A. K. Katakam, R. Noubade, M. Roose-Girma, J. DeVoss, L. Diehl, R. R. Graham, M. van Lookeren Campagne, A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature* **506**, 456–462 (2014).
42. D. Ortiz-Masia, J. Cosin-Roger, S. Calatayud, C. Hernandez, R. Alos, J. Hinojosa, N. Apostolova, A. Alvarez, M. D. Barrachina, Hypoxic macrophages impair autophagy in epithelial cells through Wnt1: Relevance in IBD. *Mucosal Immunol.* **7**, 929–938 (2014).
43. S. N. Sehgal, Sirolimus: Its discovery, biological properties, and mechanism of action. *Transplant. Proc.* **35**, S7–S14 (2003).
44. D. M. Gwinn, D. B. Shackelford, D. F. Egan, M. M. Mihaylova, A. Mery, D. S. Vasquez, B. E. Turk, R. J. Shaw, AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* **30**, 214–226 (2008).
45. D. Guragain, P. Gurung, J. H. Chang, N. Katila, H. W. Chang, B. S. Jeong, D. Y. Choi, J. A. Kim, AMPK is essential for IL-10 expression and for maintaining balance between inflammatory and cytoprotective signaling. *Biochim Biophys Acta Gen Subj* **1864**, 129631 (2020).
46. J. L. Benjamin, R. Sumpter Jr., B. Levine, L. V. Hooper, Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe* **13**, 723–734 (2013).
47. J. J. Kim, B. W. Bridle, J. E. Ghia, H. Wang, S. N. Syed, M. M. Manocha, P. Rengasamy, M. S. Shajib, Y. Wan, P. B. Hedlund, W. I. Khan, Targeted inhibition of serotonin type 7 (5-HT7) receptor function modulates immune responses and reduces the severity of intestinal inflammation. *J. Immunol.* **190**, 4795–4804 (2013).
48. S. N. Spohn, F. Bianco, R. B. Scott, C. M. Keenan, A. A. Linton, C. H. O'Neill, E. Bonora, M. Dicay, B. Lavoie, R. L. Wilcox, W. K. MacNaughton, R. De Giorgio, K. A. Sharkey, G. M. Mawe, Protective actions of epithelial 5-hydroxytryptamine 4 receptors in normal and inflamed colon. *Gastroenterology* **151**, 933–944.e3 (2016).
49. J. Hannon, D. Hoyer, Molecular biology of 5-HT receptors. *Behav. Brain Res.* **195**, 198–213 (2008).
50. Y. X. Hu, X. S. Han, Q. Jing, Ca(2+) ion and autophagy. *Adv. Exp. Med. Biol.* **1206**, 151–166 (2019).
51. L. A. Farrelly, R. E. Thompson, S. Zhao, A. E. Lepack, Y. Lyu, N. V. Bhanu, B. Zhang, Y. E. Loh, A. Ramakrishnan, K. C. Vadodaria, K. J. Heard, G. Erikson, T. Nakadai, R. M. Bastle, B. J. Lukasak, H. Zebroski III, N. Alenina, M. Bader, O. Berton, R. G. Roeder, H. Molina, F. H. Gage, L. Shen, B. A. Garcia, H. Li, T. W. Muir, I. Maze, Histone serotonylation is a permissive modification that enhances TFIID binding to H3K4me3. *Nature* **567**, 535–539 (2019).
52. D. Ye, H. Xu, H. Xia, C. Zhang, Q. Tang, F. Bi, Targeting SERT promotes tryptophan metabolism: Mechanisms and implications in colon cancer treatment. *J. Exp. Clin. Cancer Res.* **40**, 173 (2021).
53. M. Campieri, P. Gionchetti, Bacteria as the cause of ulcerative colitis. *Gut* **48**, 132–135 (2001).
54. S. Lavoie, K. L. Conway, K. G. Lassen, H. B. Jijon, H. Pan, E. Chun, M. Michaud, J. K. Lang, C. A. Gallini Comeau, J. M. Dreyfuss, J. N. Glickman, H. Vlamakis, A. Ananthakrishnan, A. Kostic, W. S. Garrett, R. J. Xavier, The Crohn's disease polymorphism, ATG16L1 T300A, alters the gut microbiota and enhances the local Th1/Th17 response. *eLife* **8**, e39982 (2019).
55. Y. Liu, X. Wang, Q. Chen, L. Luo, M. Ma, B. Xiao, L. Zeng, *Camellia sinensis* and *Litsea coreana* ameliorate intestinal inflammation and modulate gut microbiota in dextran sulfate sodium-induced colitis mice. *Mol. Nutr. Food Res.* **64**, e1900943 (2020).
56. X. Gao, Q. Cao, Y. Cheng, D. Zhao, Z. Wang, H. Yang, Q. Wu, L. You, Y. Wang, Y. Lin, X. Li, Y. Wang, J. S. Bian, D. Sun, L. Kong, L. Birnbaumer, Y. Yang, Chronic stress promotes colitis by disturbing the gut microbiota and triggering immune system response. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E2960–E2969 (2018).
57. M. G. Rooks, P. Veiga, L. H. Wardwell-Scott, T. Tickle, N. Segata, M. Michaud, C. A. Gallini, C. Beal, J. E. van Hylckama-Vlieg, S. A. Ballal, X. C. Morgan, J. N. Glickman, D. Gevers, C. Huttenhower, W. S. Garrett, Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J.* **8**, 1403–1417 (2014).
58. K. Sheng, G. Zhang, M. Sun, S. He, X. Kong, J. Wang, F. Zhu, X. Zha, Y. Wang, Grape seed proanthocyanidin extract ameliorates dextran sulfate sodium-induced colitis through intestinal barrier improvement, oxidative stress reduction, and inflammatory cytokines and gut microbiota modulation. *Food Funct.* **11**, 7817–7829 (2020).
59. L. Shang, H. Liu, H. Yu, M. Chen, T. Yang, X. Zeng, S. Qiao, Core altered microorganisms in colitis mouse model: A comprehensive time-point and fecal microbiota transplantation analysis. *Antibiotics* **10**, 643 (2021).
60. C. R. Manzella, D. Jayawardena, W. Pagani, Y. Li, W. A. Alrefai, J. Bauer, B. Jung, C. R. Weber, R. K. Gill, Serum serotonin differentiates between disease activity states in Crohn's patients. *Inflamm. Bowel Dis.* **26**, 1607–1618 (2020).
61. F. Fernandez-Banares, M. Esteve, J. C. Espinos, M. Rosinach, M. Forne, A. Salas, J. M. Viver, Drug consumption and the risk of microscopic colitis. *Am. J. Gastroenterol.* **102**, 324–330 (2007).
62. C. W. Lees, J. C. Barrett, M. Parkes, J. Satsangi, New IBD genetics: Common pathways with other diseases. *Gut* **60**, 1739–1753 (2011).
63. F. Cote, E. Thevenot, C. Fligny, Y. Fromes, M. Darmon, M. A. Ripoche, E. Bayard, N. Hanoun, F. Saurini, P. Lechat, L. Dandolo, M. Hamon, J. Mallet, G. Vodjdani, Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13525–13530 (2003).
64. D. Bengel, D. L. Murphy, A. M. Andrews, C. H. Wichems, D. Feltner, A. Heils, R. Mossner, H. Westphal, K. P. Lesch, Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("ecstasy") in serotonin transporter-deficient mice. *Mol. Pharmacol.* **53**, 649–655 (1998).
65. M. T. Sorbara, E. G. Foerster, J. Tsalikis, M. Abdel-Nour, J. Mangiapane, I. Sirluck-Schroeder, I. Tattoli, R. van Dalen, D. E. Isenman, J. R. Rohde, S. E. Girardin, D. J. Philpott, Complement C3 drives autophagy-dependent restriction of cyto-invasive bacteria. *Cell Host Microbe* **23**, 644–652.e5 (2018).
66. A. D. Gracz, B. J. Puthoff, S. T. Magness, Identification, isolation, and culture of intestinal epithelial stem cells from murine intestine. *Methods Mol. Biol.* **879**, 89–107 (2012).
67. F. J. Whelan, C. P. Verschoor, J. C. Stearns, L. Rossi, K. Luinstra, M. Loeb, M. Smieja, J. Johnstone, M. G. Surette, D. M. Bowdish, The loss of topography in the microbial communities of the upper respiratory tract in the elderly. *Ann. Am. Thorac. Soc.* **11**, 513–521 (2014).
68. B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. Johnson, S. P. Holmes, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
69. C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glockner, The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590–D596 (2013).
70. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
71. Y. Cao, J. Cai, S. Zhang, N. Yuan, X. Li, Y. Fang, L. Song, M. Shang, S. Liu, W. Zhao, S. Hu, J. Wang, Loss of autophagy leads to failure in megakaryopoiesis, megakaryocyte differentiation, and thrombopoiesis in mice. *Exp. Hematol.* **43**, 488–494 (2015).

Acknowledgments: We thank N. Pai and S. Shajib for assistance in recruiting patients with CD. We also thank J. J. Kim, X. Wang, J. C. Szamosi, F. M. Nazmul Hassan, and P. M. Far for support in this study. **Funding:** This work was supported by grants from the following: Canadian Institutes of Health Research (CIHR) to W.I.K. (PJT 156262); Canadian Association of Gastroenterology (CAG) doctoral fellowship and Farncombe Student Award to S.H.; and Diabetes Canada Investigator Award, a Tier 1 Canada Research Chair, and the J. Bruce Duncan Chair in Metabolic Diseases to G.R.S. G.R.S. is a cofounder of Espervita Therapeutics and received research funding from Espervita Therapeutics, Esperion Therapeutics, Poxel Pharmaceuticals, and Novo Nordisk. **Author contributions:** S.H. and W.I.K. conceived the idea and designed the research. S.H. performed all experiments with help from H.W., J.G., S.B., and Y.H.K. S.H., U.C., and J.K.M. recruited study participants. S.H. and I.I.K. analyzed and interpreted the data. S.H. and W.I.K. wrote, edited, and revised the manuscript. M.S., F.C., D.P., J.H.B., and G.R.S. critically appraised the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 23 March 2021
Accepted 16 September 2021
Published 5 November 2021
10.1126/sciadv.abi6442

Science Advances
AAAS

Supplementary Materials for

Disruption of autophagy by increased 5-HT alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease

Sabah Haq, Huaqing Wang, Jensine Grondin, Suhrid Banskota, John K. Marshall, Irfan I. Khan, Usha Chauhan, Francine Cote, Yun Han Kwon, Dana Philpott, John H. Brumell, Michael Surette, Gregory R. Steinberg, Waliul I. Khan*

*Corresponding author. Email: khanwal@mcmaster.ca

Published 5 November 2021, *Sci. Adv.* **7**, eabi6442 (2021)
DOI: 10.1126/sciadv.abi6442

This PDF file includes:

Supplementary Method

Drugs and reagents

LX1031 (Tph1 inhibitor; Cat#ab269814; abcam), Tropisetron (5-HT_3 receptor antagonist; Cat#2459; Tocris Bioscience), RS39604 (5-HT_4 receptor antagonist; Cat#0991; Tocris Bioscience), SB269970 (5-HT_7 receptor antagonist; Cat#1612; Tocris Bioscience) were prepared according to the manufacturer's instructions.

Experimental design

Dextran sulphate sodium (DSS; molecular mass 40 kDa; Cat#02160110; MP Biomedicals Inc) was added to autoclaved drinking water at 2.5% weight/volume for 7 days to induce acute colitis. In another experiment, mice were treated orally with vehicle or LX1031 at a dose of 135 mg/kg/day for 4 days starting 2 days after 5% DSS administration. 5% DSS was administered for 5 days.

Cell Culture

HT-29 cells (human colorectal adenocarcinoma, epithelial-like, ATCC HTB-38TM) were grown in Dulbecco modified Eagle medium/F12 (1:1) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, modified Eagle medium, HEPES buffer (pH 7.5) and 1% penicillin and streptomycin in a humidified 5% CO_2 incubator at 37^{0}C. At ~80% confluence, HT-29 cells were

plated in a 6-well culture plate at a density of 5 X 10^5 cells/ml. Following overnight cell attachment, cells were pretreated with tropisetron (1 μM) or RS39604 (1 μM) or SB269970 (1 μM) for 1 hour followed by 5-HT (10 μM) treatment for 6 hours. Autophagy proteins LC3, Beclin-1, Atg12-5, p62, and p-mTOR were measured in the cell lysate by western blotting. IECs were isolated from mouse colon according to the Gracz et al. protocol described in the main text (*66*). IECs were plated in a 6-well culture plate at a density of 5 X 10^5 cells/ml and pretreated with tropisetron (1 μM) or RS39604 (1 μM) or SB269970 (1 μM) for 1 hour followed by 5-HT (10 μM) treatment for 6 hours. Autophagy proteins LC3 and p62 were measured in the cell lysate by western blotting.

In a separate experiment, HT-29 cells were plated in a 12-well culture plate at a density of 5 X 10^5 cells/ml. Following overnight cell attachment, cells were treated with either 5-HT (10^{-7} M) or serum free medium alone for 24 hours. The supernatant was used for measurement of human IL-8 by ELISA (Cat#D8000C; R&D Systems) and autophagy proteins LC3-I and II was measured in the cell lysate by western blotting.

Genotyping of the double knock-out mice

The PCR conditions and primers used for the *Atg7fl/fl* pair 1 and pair 2 alleles were described previously *(71)*. The *vilin-Cre* was detected following the protocol by Jackson laboratory (Stock # 004586). PCR for the *Tph1* allele was performed as follows: 95^{0}C for 3 mins, 30 cycles of 94^{0}C for 30 secs, 55^{0}C for 30 secs and 72^{0}C for 1 min 20 secs followed by 72^{0}C for 5 mins. After gel electrophoresis, the wild-type (WT) band of *Tph1* was detected at 314 bp and the mutant band at 350 bp. The following primers were used: *Tph1*-F:

GCTATGCTAGGCAGTGTCTCA; *Tph1*-R for the WT allele:

CTGGAAGATTTTCAGCACTTTTATGAGTCCTCCGACTTCATTCTCCAAGG; and *Tph1*-R

for the mutant allele: CGATTAAGTTGGGTAACGCCAGGGT.

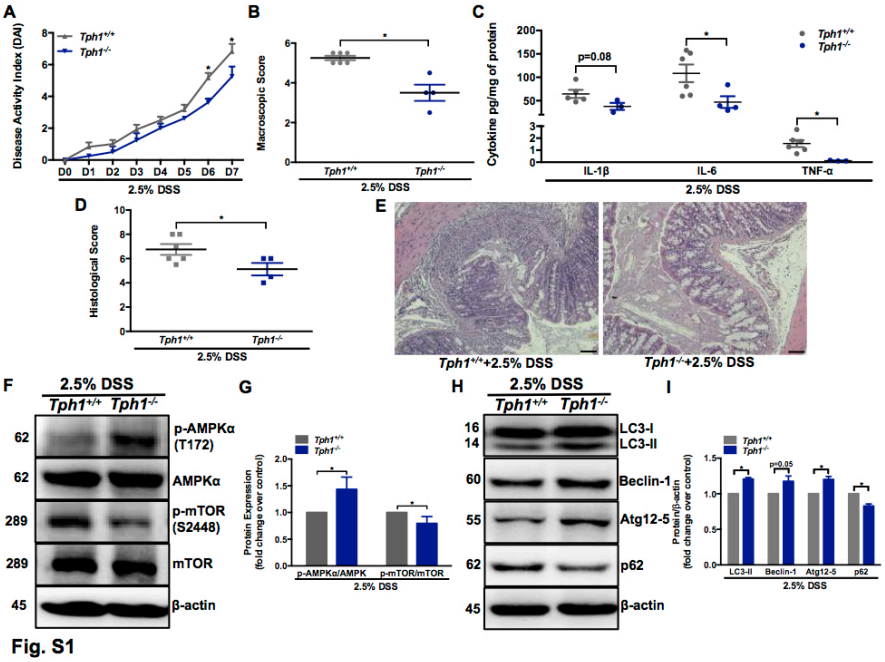


Fig. S1

Fig. S1: Effect of reduced intestinal 5-HT on 2.5% DSS-induced colitis and autophagy.

2.5% DSS was given to *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice in drinking water for 7 days to induce colitis. Each group contained 4-6 mice. (**A**) DAI. *p<0.05 between colitic *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ on days 6 and 7. (**B**) Macroscopic damage score on day 7 post-DSS. Colon tissue was collected to determine: (**C**) IL-1β, IL-6 and TNF-α. (**D**) Histological score and (**E**) Representative micrographs on day 5 post-DSS (scale bar represents 100 μm). (**F**) Western blot images and (**G**) Quantification of autophagy proteins, LC3, Beclin-1, Atg12-5 and p62. (**H**) Western blot images and (**I**) Quantification of p-AMPKα (T172) and p-mTOR (S2448). Representative western blot with β-actin as loading controls. Data are representative of 4 random mice in each group. Data are represented as mean ± S.E.M. *p<0.05.

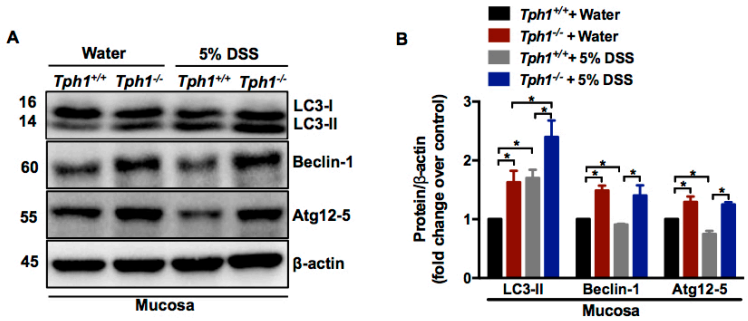


Fig. S2

Fig. S2: Effect of reduced 5-HT on mucosal autophagy in mice with DSS-induced colitis.

5% DSS was given to *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice in drinking water for 5 days to induce colitis. Control mice received water for 5 days. Mucosa was isolated from the colon of mice. Each group contained 3-4 mice. Tissue protein extracts obtained from the mucosa of *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice with and without colitis were analyzed by western blotting. (**A**) Western blot images and (**B**) Quantification of autophagy proteins, LC3, Beclin-1 and Atg12-5 in the mucosa. Representative western blot with β-actin is presented as loading controls. Blots are representative of 3-4 random mice in each group. Data are represented as mean ± S.E.M. *p<0.05.

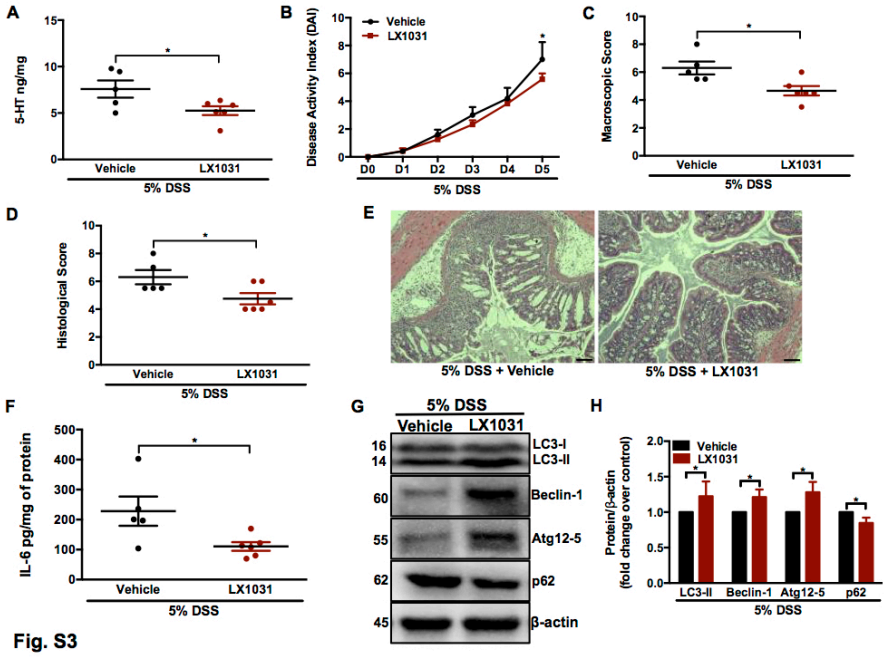


Fig. S3

Fig. S3: Effect of blocking gut mucosal 5-HT on severity of colitis and autophagy.

5% DSS was given to C57BL/6 mice in drinking water for 5 days to induce colitis. Colitic mice were treated orally with vehicle or LX1031 at a dose of 135 mg/kg/day for 4 days starting 2 days after DSS administration. Each group contained 5-6 mice. (**A**) Colonic 5-HT levels. (**B**) DAI. *p<0.05 between colitic vehicle and LX1031 treated groups on day 5. (**C**) Macroscopic damage score on day 5 post-DSS. (**D**) Histological score and (**E**) Representative micrographs on day 5 post-DSS (scale bar represents 100 μm). Colon tissue was collected to determine: (**F**) IL-6. (**G**) Western blot images and (**H**) Quantification of autophagy proteins, LC3, Beclin-1, Atg12-5 and p62. Representative western blot with β-actin as loading controls. Data are representative of 5 random mice in each group. Data are represented as mean ± S.E.M. *p<0.05.

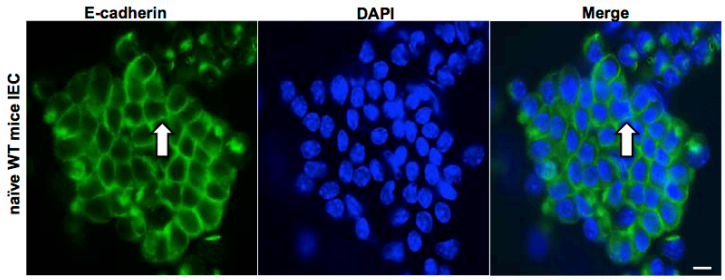


Fig. S4

Fig. S4: E-cadherin staining of isolated colon epithelial cells.

Colon epithelial cells were isolated from naïve C57BL/6 mice and immunostained with E-cadherin according to the protocol described in the methods and materials section of the main text. Representative images of colon sections and arrows indicate E-cadherin expressing IECs. Scale bar represents 100 μm. 85-90% of the isolated cells were E-cadherin positive as determined by counting all the E-cadherin positive cells.

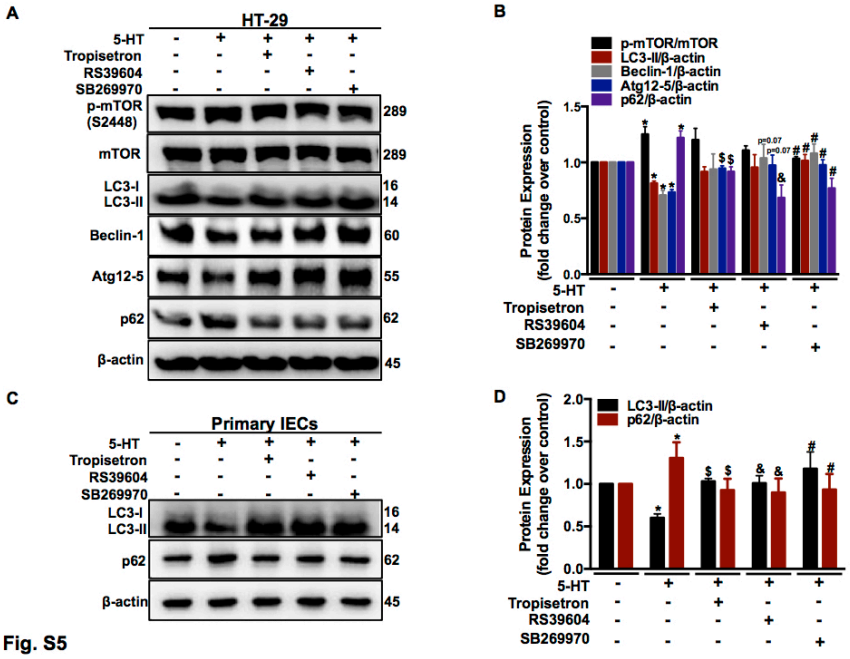


Fig. S5

Fig. S5: 5-HT and 5-HT$_3$, 5-HT$_4$ and 5-HT$_7$ receptor antagonist treatments of human colon epithelial carcinoma HT-29 cells and mouse primary intestinal epithelial cells.

HT-29 cells or mouse primary IECs were pretreated with Tropisetron (1 μM), RS39604 (1 μM), SB269970 (1 μM) for 1 hour followed by 5-HT (10 μM) treatment for 6 hours and the extracted protein was analyzed for expression of LC3, Beclin-1, Atg12-5, p62 and p-mTOR. (**A**) Western blot images and (**B**) Quantification graphs of HT-29 cells. (**C**) Western blot images and (**D**) Quantification graphs of mouse primary IECs. Data shown are representative of three independent experiments. $^{*}p<0.05$ compared to vehicle treated control group. $^{\$,\&,\#}p<0.05$ compared to 5-HT treated group.

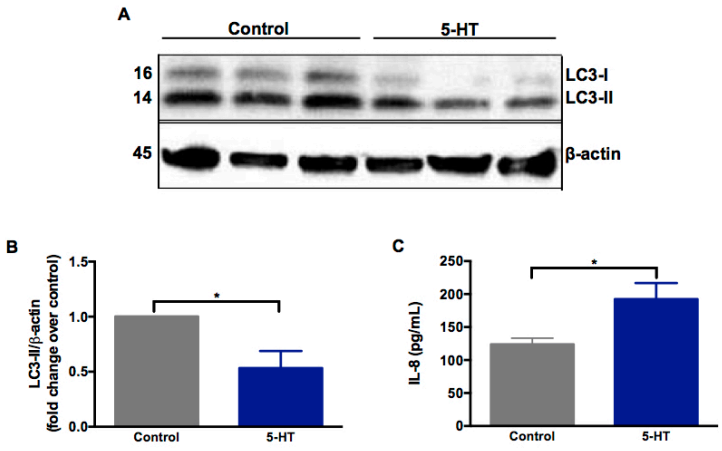


Fig. S6

Fig. S6: 5-HT treatment of human colon epithelial carcinoma HT-29 cells reduces LC3-II and increases IL-8 levels.

HT-29 cells were treated with 5-HT (10^{-7} M) for 24 hours. (**A** to **B**) Western blot images and quantification of autophagy protein LC3-I and II with β-actin as loading controls. (**C**) IL-8 levels in culture supernatant. Bar graph represents mean ± S.E.M. *$p<0.05$.

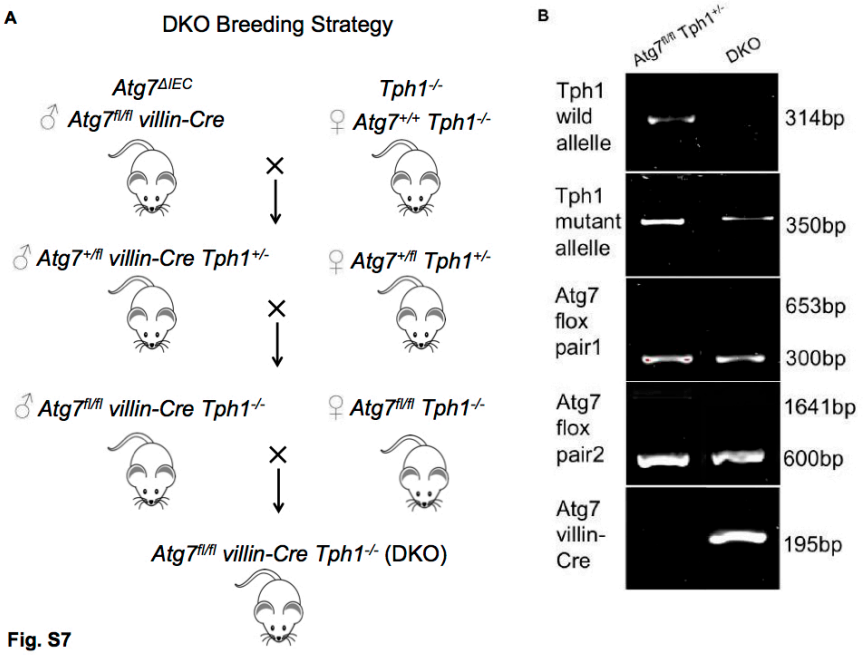


Fig. S7

Fig. S7: DKO breeding protocol.

(**A**) *Atg7$^{flox/flox}$villin-Cre Tph1$^{-/-}$* DKO breeding program. The breeding started with the parent mice of *Atg7$^{\Delta IEC}$* (*Atg7$^{fl/fl}$villin-Cre*) and *Tph1$^{-/-}$* (*Atg7$^{+/+}$ Tph1$^{-/-}$*). The offspring were genotyped and inbred to produce heterozygotes *Atg7$^{+/fl}$villin-Cre Tph1$^{+/-}$* and *Atg7$^{+/fl}$ Tph1$^{+/-}$*. The second-generation offspring mice were inbred to produce *Atg7$^{fl/fl}$ Tph1$^{-/-}$* and DKO *Atg7$^{fl/fl}$villin-Cre Tph1$^{-/-}$*. These third-generation offspring were inbred to produce stable DKO mouse line. (**B**) PCR genotyping analysis of the Tph1 and Atg7 loci in genomic DNA of mice.

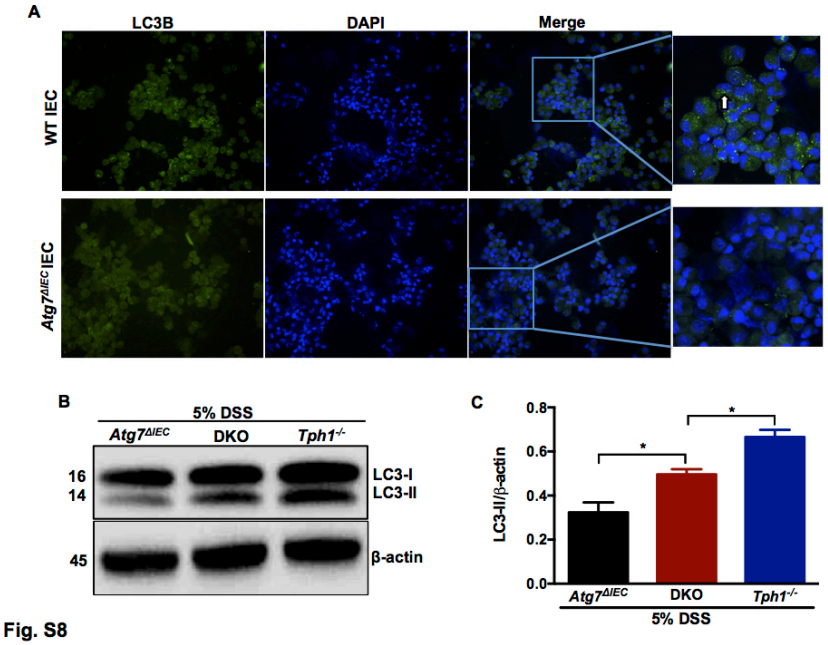


Fig. S8

Fig. S8: Detection of LC3 proteins.

(**A**) Representative images of colonic sections. Colon epithelial cells were isolated from naïve C57BL/6 and *Atg7*$^{\Delta IEC}$ mice followed by immunostaining with LC3B. Arrows indicate LC3B+ve IECs. *Atg7*$^{\Delta IEC}$ did not express any LC3B+ve IECs. (**B**) Western blot images and (**C**) Quantification of autophagy protein LC3-I and II. Representative western blot with β-actin is presented as loading controls. Blots are representative of 3 random mice in each group. Bar graph represents mean ± S.E.M. *$p<0.05$.

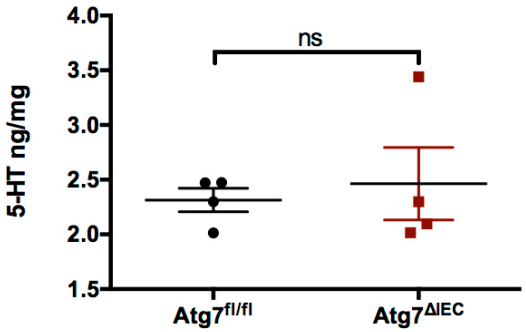


Fig. S9

Fig. S9: Measurement of colonic 5-HT levels in autophagy deficient mice.

The amount of 5-HT in the colon tissue of naïve *Atg7*$^{\Delta IEC}$ mice and in the naïve *Atg7*$^{fl/fl}$ mice was measured by ELISA. Each group contained 4 mice. Bar graph represents mean ± S.E.M. ns = not significant.

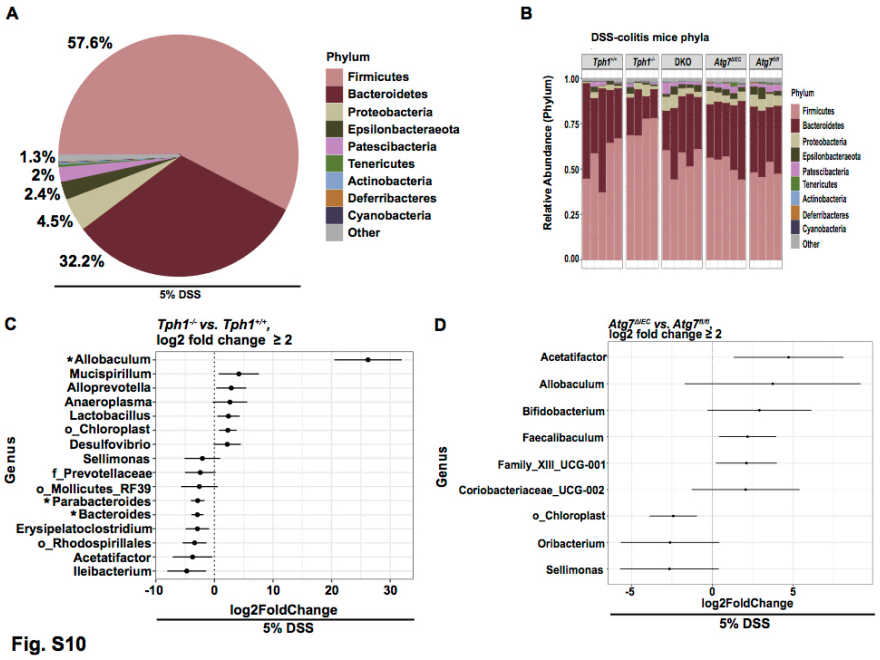
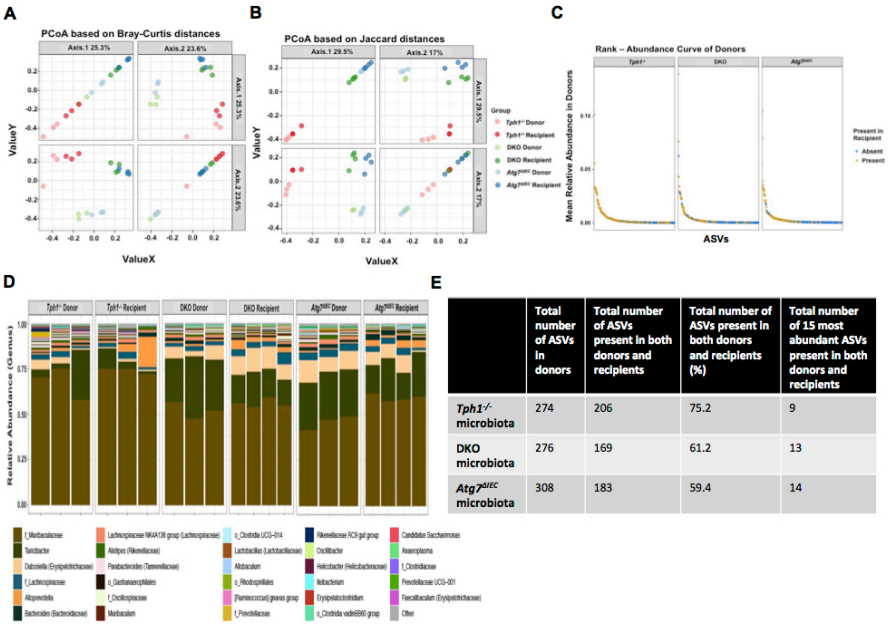


Fig. S10

Fig. S10: Effect of impaired IEC 5-HT-autophagy axis on gut microbiota composition during colitis.

5% DSS was given to *Tph1*$^{+/+}$, *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$ and *Atg7*$^{fl/fl}$ mice in drinking water for 5 days to induce colitis. Each group contained 4-5 mice. 16S rRNA sequencing of cecal content of all groups was performed. (**A**) Pie chart showing the relative abundance of each type of bacteria in all 5 groups at the phylum level. (**B**) Taxonomic summaries at the phylum level. (**C**) All genera in the *Tph1*$^{-/-}$ group for which one log2 fold change is ≥2 with *Tph1*$^{+/+}$ as the baseline. (**D**) All genera in the *Atg7*$^{\Delta IEC}$ group for which one log2 fold change is ≥2 with *Atg7*$^{fl/fl}$ as the baseline.



	Total number of ASVs in donors	Total number of ASVs present in both donors and recipients	Total number of ASVs present in both donors and recipients (%)	Total number of 15 most abundant ASVs present in both donors and recipients
Tph1$^{-/-}$ microbiota	274	206	75.2	9
DKO microbiota	276	169	61.2	13
Atg7$^{\Delta IEC}$ microbiota	308	183	59.4	14

Fig. S11

Fig. S11: Microbial composition of donor (DSS treated) and recipient mice following FMT.

Fecal samples from *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$ donors receiving 5% DSS and there corresponding recipients after 2 weeks of colonization were analyzed by 16S rRNA sequencing. Each group contained 3-4 mice. There was a total of 1,811,745 reads, with sample depths ranging from 52,280 to 122,673. The mean number of reads per sample was 90,587. Data was filtered by removing taxa whose mean abundance was 10 or less, and those reads not assigned to bacteria or archaea. (**A**) PCoA of Bray-Curtis dissimilarity. (**B**) PCoA of abundance Jaccard index. (**C**) Amplicon sequence variants (ASVs) are plotted by mean abundance within each set of donors. Golden dots indicate that an ASV is also present in at least one recipient mouse of the same type. (**D**) Taxonomic summaries at the genus level. (**E**) ASV count analysis of microbiota transplant experiment. The calculations were based on the proportion of the number of ASVs that matched between the donor and recipient groups over the total donor ASVs.

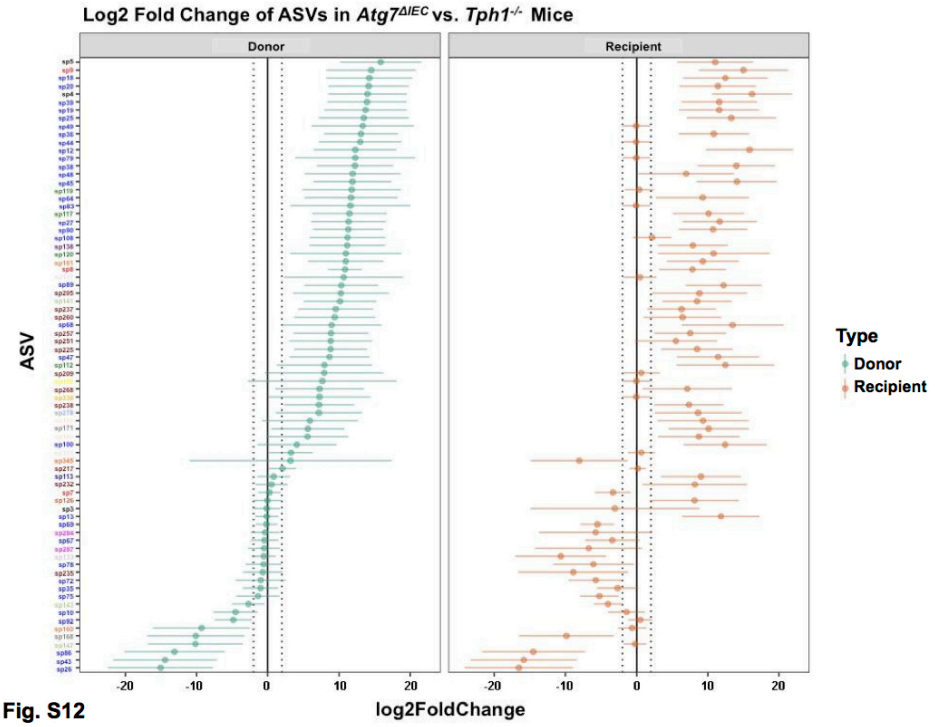


Fig. S12

Fig. S12: Microbial composition of donor (DSS treated) and recipient mice following FMT.

With *Tph1*$^{-/-}$ donor or recipient as the baseline, all ASVs in the *Atg7*$^{\Delta IEC}$ donor or recipient group for which one log2 fold change is ≥2. The following color codes indicate the ASVs: Turicibacter – Black; Dubosiella – Red; f_Muribaculaceae – Blue; Bacteroides (Bacteroidaceae) – Green; [Ruminococcus] gnavus group – Purple; Alistipes (Rikenellaceae) – Orange; Parabacteroides (Tannerellaceae) – Grey; f_Lachnospiraceae – Brown; Lachnospiraceae NK4A136 group (Lachnospiraceae) – Light Green; Anaeroplasma – Yellow; f_Oscillospiraceae – Golden; Erysipelatoclostridium – Light Blue; o_Gastranaerophilales – Cream; Allobaculum – Dark Grey; o_Clostridia vadinBB60 group – Dark orange; Lactobacillus (Lactobacillaceae) – Dark Blue; Muribaculum – Brick Red; o_Clostridia UCG-014 – Pink.

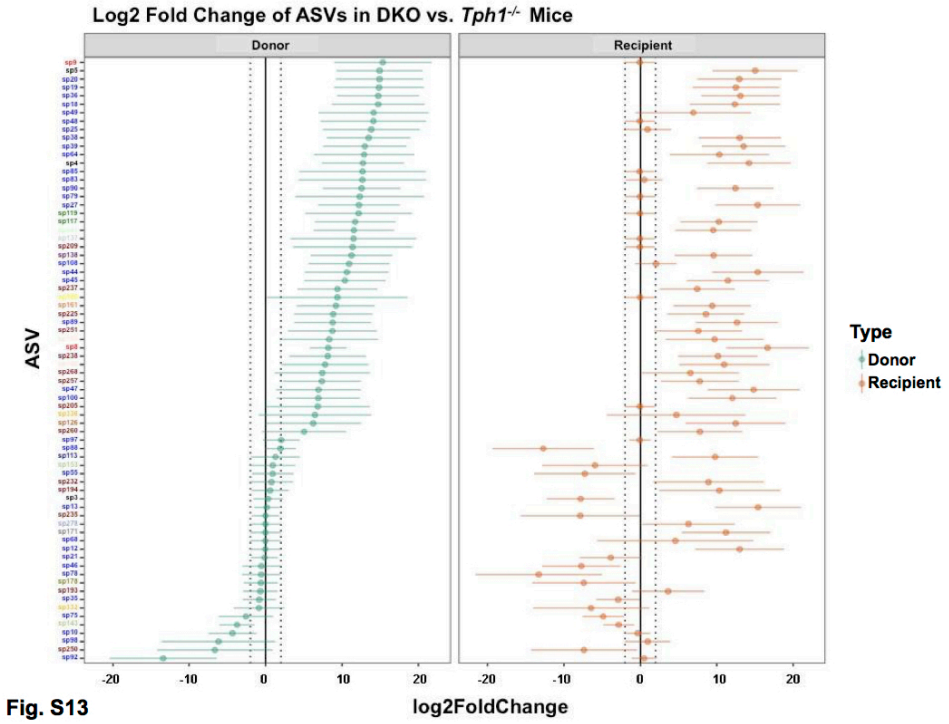


Fig. S13

Fig. S13: Microbial composition of donor (DSS treated) and recipient mice following FMT.

With *Tph1*$^{-/-}$ donor or recipient as the baseline, all ASVs in the DKO donor or recipient group for which one log2 fold change is ≥2. The following color codes indicate the ASVs: Turicibacter – Black; Dubosiella – Red; f_Muribaculaceae – Blue; Bacteroides (Bacteroidaceae) – Green; [Ruminococcus] gnavus group – Purple; Alistipes (Rikenellaceae) – Orange; Parabacteroides (Tannerellaceae) – Grey; f_Lachnospiraceae – Brown; Lachnospiraceae NK4A136 group (Lachnospiraceae) – Light Green; Anaeroplasma – Yellow; f_Oscillospiraceae – Golden; Erysipelatoclostridium – Light Blue; o_Gastranaerophilales – Cream; Allobaculum – Dark Grey; Lactobacillus (Lactobacillaceae) – Dark Blue; Muribaculum – Brick Red; f_Clostridiaceae – Asparagus.

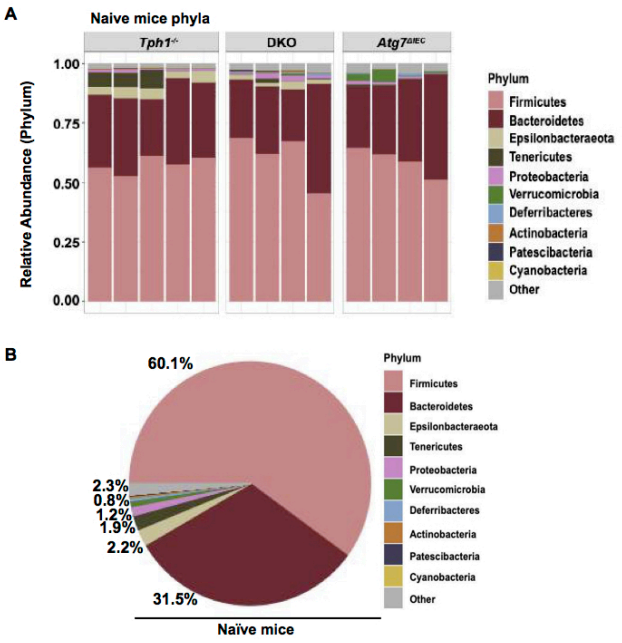


Fig. S14

Fig. S14: Effect of impaired IEC 5-HT-autophagy axis on gut microbiota composition of naïve mice.

16S rRNA sequencing of cecal content from naive *Tph1*$^{-/-}$, DKO, *Atg7*$^{\Delta IEC}$ mice was performed. Each group contained 4-5 mice. (**A**) Taxonomic summaries at the phylum level. (**B**) Pie chart showing the relative abundance of each type of bacteria in all 3 groups at the phylum level.

Table S1: Demographic data of HC and CD patients. Cohort 1: investigation of LC3B, IRGM and ATG16L1 expression in intestinal biopsies. Cohort 2: investigation of plasma 5-HT concentration and autophagy proteins in PBMCs. *n/a represents not applicable.

Cohort 1	**Healthy control (HC)**	**Crohn's Disease (CD)**
Number of participants	8	8
Mean age (range) in years	63.1 (50-76)	37.5 (24-68)
% Female	50	50
Assessment by endoscopy and biopsy	*n/a	Active inflammation
Cohort 2	**Healthy control (HC)**	**Crohn's Disease (CD)**
Number of participants	10	10
Mean age (range) in years	26.7 (19-36)	32.7 (18-58)
% Female	90	70
Mean disease duration (range) in years	n/a	12.4 (0.5-38)
% of participants with high CRP (high > 5.1mg/L)	n/a	70
% of participants with high Fecal calprotectin (high > 50μg/g)	n/a	70
Assessment by endoscopy and biopsy	n/a	Active inflammation

Table S2: DESeq2 table for microbial composition analysis of DSS treated mice. DESeq2 analysis used to determine significantly different genera between *Tph1*$^{-/-}$ and DKO mice, and between *Atg7*$^{\Delta IEC}$ and DKO mice, where the difference is significant, if p<0.01 after adjustment for multiple testing via Benjamini-Hochberg multiple testing adjustment procedure.

ASV	Base Mean	log2 Fold Change	lfcSE	stat	p value	p adjusted	Baseline	Condition	Genus
sp2	4060.37779	-6.293219	1.69692464	-3.7086025	0.00020841	0.001103898	DKO	*Tph1*$^{-/-}$	*Ileibacterium*
sp5	12500.6337	1.47909001	0.34441294	4.29452509	1.75E-05	0.000120956	DKO	*Tph1*$^{-/-}$	*f_Lachnospiraceae*
sp8	7183.08635	-1.9362726	0.52486734	-3.6890705	0.00022508	0.001103898	DKO	*Tph1*$^{-/-}$	*Bacteroides*
sp18	1521.53837	-13.596306	1.14144002	-11.911538	1.03E-32	7.83E-31	DKO	*Tph1*$^{-/-}$	*f_Prevotellaceae*
sp22	1288.87845	4.33593334	0.90417037	4.79548265	1.62E-06	1.23E-05	DKO	*Tph1*$^{-/-}$	*Faecalibaculum*
sp45	850.866652	-13.7315	1.25156776	-10.97144	5.24E-28	1.99E-26	DKO	*Tph1*$^{-/-}$	*Rikenellaceae_RC9_gut_group*
sp46	1504.41523	-3.1242811	0.5915247	-5.2817425	1.28E-07	1.08E-06	DKO	*Tph1*$^{-/-}$	*Parabacteroides*
sp53	511.490191	-12.09604	1.50706307	-8.0262335	1.01E-15	1.53E-14	DKO	*Tph1*$^{-/-}$	*o_Rickettsiales*
sp57	1029.78737	3.2564455	0.81619558	3.98978574	6.61E-05	0.000418842	DKO	*Tph1*$^{-/-}$	*Muribaculum*
sp72	355.58696	-11.429329	1.59472126	-7.1669761	7.67E-13	9.71E-12	DKO	*Tph1*$^{-/-}$	*f_Rickettsiaceae*
sp95	364.1383	-12.24909	1.22070134	-10.034469	1.08E-23	2.04E-22	DKO	*Tph1*$^{-/-}$	*Rikenella*
sp112	317.082451	-11.471674	1.11080382	-10.327363	5.30E-25	1.34E-23	DKO	*Tph1*$^{-/-}$	*Odoribacter*
sp139	125.248681	-9.862823	1.65786953	-5.9490948	2.70E-09	2.93E-08	DKO	*Tph1*$^{-/-}$	*c_Bacteroidia*
sp161	343.130562	-4.542743	1.19654583	-3.7965475	0.00014673	0.000857779	DKO	*Tph1*$^{-/-}$	*f_Clostridiales_vadinBB60_group*
sp166	148.68628	2.69632501	0.73251513	3.68091376	0.0002324	0.001103898	DKO	*Tph1*$^{-/-}$	*Intestinimonas*
sp379	11.8726475	-7.7781503	1.38399471	-5.6200723	1.91E-08	1.81E-07	DKO	*Tph1*$^{-/-}$	*f_Tannerellaceae*

Table S3: DESeq2 table for microbial composition analysis of naive mice. DESeq2 analysis used to determine significantly different genera between *Tph1*$^{-/-}$ and DKO mice, and between *Atg7*$^{\Delta IEC}$ and DKO mice, where the difference is significant, if p<0.01 after adjustment for multiple testing via Benjamini-Hochberg multiple testing adjustment procedure.

ASV	Base Mean	log2 Fold Change	lfcSE	stat	p value	p adjusted	Baseline	Condition	Genus
sp2	4546.04038	-3.3775504	0.84212595	-4.0107426	6.05E-05	0.0009075	DKO	*Tph1*$^{-/-}$	*Eisenbergiella*
sp30	1243.33491	3.62069941	0.89955987	4.02496769	5.70E-05	0.0009075	DKO	*Tph1*$^{-/-}$	*Lachnospiraceae_UCG-001*
sp31	646.283267	-6.3159803	1.44653798	-4.3662734	1.26E-05	0.00032017	DKO	*Tph1*$^{-/-}$	*Akkermansia*
sp46	1053.46736	2.03920093	0.38887003	5.24391389	1.57E-07	5.97E-06	DKO	*Tph1*$^{-/-}$	*Muribaculum*
sp69	345.246602	-11.8729	1.36833373	-8.6769038	4.07E-18	3.09E-16	DKO	*Tph1*$^{-/-}$	*Mucispirillum*
sp146	256.323214	-3.6603939	0.92413875	-3.9608705	7.47E-05	0.0009075	DKO	*Tph1*$^{-/-}$	*f_Desulfovibrionaceae*
sp357	25.6094646	-2.3507906	0.5975755	-3.9338804	8.36E-05	0.0009075	DKO	*Tph1*$^{-/-}$	*f_Eggerthellaceae*
sp8	3697.54969	3.70897612	0.41490596	8.93931755	3.92E-19	7.44E-18	DKO	*Atg7*$^{\Delta IEC}$	*Bacteroides*
sp12	1907.10763	-10.672009	0.7381303	-14.458164	2.23E-47	8.46E-46	DKO	*Atg7*$^{\Delta IEC}$	*Helicobacter*
sp23	2716.44403	-12.315796	1.95849441	-6.2883998	3.21E-10	1.63E-09	DKO	*Atg7*$^{\Delta IEC}$	*Dubosiella*
sp27	1187.2435	-9.6265826	0.54802119	-17.566077	4.48E-69	3.41E-67	DKO	*Atg7*$^{\Delta IEC}$	*Alloprevotella*
sp46	1053.46736	1.68709108	0.40978994	4.11696558	3.84E-05	0.00015356	DKO	*Atg7*$^{\Delta IEC}$	*Muribaculum*
sp48	645.406299	-10.529227	1.10944406	-9.4905432	2.30E-21	5.82E-20	DKO	*Atg7*$^{\Delta IEC}$	*Prevotellaceae_UCG-001*
sp76	574.788943	1.5157401	0.48315713	3.13715767	0.00170594	0.00563703	DKO	*Atg7*$^{\Delta IEC}$	*Parabacteroides*
sp78	277.518457	-11.431127	1.40121839	-8.1579912	3.41E-16	4.31E-15	DKO	*Atg7*$^{\Delta IEC}$	*Anaerosporobacter*
sp135	198.715867	-11.849196	1.7084857	-6.9354959	4.05E-12	3.08E-11	DKO	*Atg7*$^{\Delta IEC}$	*Shuttleworthia*
sp136	177.099706	-3.956906	0.59966241	-6.598556	4.15E-11	2.63E-10	DKO	*Atg7*$^{\Delta IEC}$	*Candidatus_Saccharimonas*
sp146	256.323214	-12.261337	1.40783077	-8.7093826	3.06E-18	4.64E-17	DKO	*Atg7*$^{\Delta IEC}$	*f_Desulfovibrionaceae*
sp159	93.5269332	-10.036348	1.36968668	-7.3274771	2.35E-13	2.14E-12	DKO	*Atg7*$^{\Delta IEC}$	*Coriobacteriaceae_UCG-002*
sp160	260.258504	2.34578358	0.78247727	2.99789359	0.00271853	0.00794646	DKO	*Atg7*$^{\Delta IEC}$	*A2*
sp170	147.327267	-3.1692048	0.94008659	-3.3711839	0.00074846	0.00258559	DKO	*Atg7*$^{\Delta IEC}$	*Blautia*
sp179	128.639108	-9.6354235	1.31679275	-7.3173424	2.53E-13	2.14E-12	DKO	*Atg7*$^{\Delta IEC}$	*Parasutterella*
sp223	45.8417755	-2.3333761	0.51649657	-4.5176991	6.25E-06	2.64E-05	DKO	*Atg7*$^{\Delta IEC}$	*Streptococcus*
sp268	18.3825062	-7.3282116	1.12277161	-6.5268943	6.71E-11	3.65E-10	DKO	*Atg7*$^{\Delta IEC}$	*Bilophila*
sp290	35.123911	-7.0628847	1.31693973	-5.363104	8.18E-08	3.66E-07	DKO	*Atg7*$^{\Delta IEC}$	*o_Rhodospirillales*
sp291	36.9919368	-8.5770107	1.29872009	-6.6042027	4.00E-11	2.63E-10	DKO	*Atg7*$^{\Delta IEC}$	*o_Gastranaerophilales*
sp299	26.260971	-7.1417282	1.31759476	-5.4202767	5.95E-08	2.83E-07	DKO	*Atg7*$^{\Delta IEC}$	*Ruminococcus_1*
sp354	18.249815	-7.4741424	1.14074347	-6.5519923	5.68E-11	3.32E-10	DKO	*Atg7*$^{\Delta IEC}$	*f_Erysipelotrichaceae*
sp357	25.6094646	-8.7595207	1.18429572	-7.3963965	1.40E-13	1.52E-12	DKO	*Atg7*$^{\Delta IEC}$	*f_Eggerthellaceae*
sp370	7.99998457	-4.618761	1.49257631	-3.094489	0.00197152	0.00617882	DKO	*Atg7*$^{\Delta IEC}$	*Defluviitaleaceae_UCG-011*
sp409	11.716816	-6.8300445	1.66455175	-4.1032335	4.07E-05	0.00015482	DKO	*Atg7*$^{\Delta IEC}$	*DNF00809*
sp434	13.80677	-7.5288094	1.99799863	-3.7681755	0.00016445	0.00059514	DKO	*Atg7*$^{\Delta IEC}$	*Candidatus_Stoquefichus*
sp493	6.77387068	-2.6900964	0.87186768	-3.0854411	0.00203251	0.00617882	DKO	*Atg7*$^{\Delta IEC}$	*Family_XIII_AD3011_group*

Table S4: Primer sequence for 16S rRNA V3 region.

Primer	Sequence
V3_Forward	CCTACGGGAGGCAGCAG
V3_Reverse	ATTACCGCGGCTGCTGG

CHAPTER 4

Discussion

1. Implications of our findings

IBD, classified as CD and UC, is a chronic intestinal inflammation that develops due to interaction among genetic susceptibility of the host, impaired immune system, environmental factors and microbial dysbiosis.[91] In IBD genetics play a major role in its development as demonstrated by family and twin studies. The prevalence of IBD was significantly higher among relatives of IBD patients.[185] GWAS have identified more than 240 susceptibility loci of IBD including autophagy related genes such as Atg16L1[31] and IRGM.[33,186] Nearly a decade later, Murthy et al.[32] exhibited that the SNP in Atg16L1 resulted in increased caspase 3 mediated cleavage of T300A variant of Atg16L1. This resulted in impaired stress activated autophagy, thus establishing a state of chronic inflammation that predisposes individuals to CD.[32] We have seen a marked reduction in LC3B+ IECs and *IRGM* transcripts in the colonic mucosa of CD patients with active inflammation. Comparably, Atg16L1 and IRGM protein expression is reported to be reduced in the intestine of individuals with CD.[107] However, data indicate that the percentage of heritability explained by genetic variants is only 13.1% in CD, and 8.2% in UC.[186,187] Therefore, exploring the role of other factors such as the gut endocrine system is crucial in understanding the pathogenesis of IBD. In the present study we tested the hypothesis that increased 5-HT impairs autophagy in the colon, particularly in the colonic epithelial cells, resulting in microbial dysbiosis and ultimately contributing to pathogenesis of colitis.

An emerging contributing factor in IBD is the role of the gut endocrine system. In

particular, many studies have established that IBD patients and mice with colitis in experimental models have increased EC cell number and mucosal 5-HT content.[52,188-191] Microscopic colitis has been linked with consumption of SSRIs where SSRIs decrease the expression of SERT.[192] Our group has demonstrated that CD patients have increased levels of plasma 5-HT, upregulated expression of Tph1, 5-HTR3 and 5-HTR7 and reduced expression of SERT mRNA in the colon.[193] In accordance with previous findings our present study displays elevated plasma 5-HT level in CD patients with active inflammation compared to HCs. In our study we demonstrated that an increase in plasma 5-HT in patients with active CD was associated with impaired autophagy in peripheral blood mononuclear cells (PBMCs) isolated from whole blood indicated by significantly reduced autophagy proteins, LC3-II, Beclin-1 and Atg12-5 and accumulated p62 compared to HCs. In this cohort, even though we did not determine the association between disease activity and impaired 5-HT-autophagy axis, the findings of impaired autophagy in active CD patients with elevated plasma 5-HT does indicate that disruption of the 5-HT-autophagy axis may play a role in patient's experience of IBD associated symptoms. In addition, we observed a reduction in autophagy markers in PBMCs from both patients with active CD and HCs following direct treatment with 5-HT. Soll et al.[182] demonstrated analogous finding of reduced expression of LC3B and 7-fold increased expression of p62 after 24 hours treatment with 5-HT in liver cancer cells. In contrast, Niture and colleagues[194] reported that under physiological conditions, 5-HT increases autophagy in liver cancer cells through the activation of Notch signaling pathway. The differences in dose, duration of treatment and cell lines between the two groups may

explain the disparities in their data. mTOR is a key regulator of autophagy and essential regulator of cell metabolism and growth.[195] Activation of mTORC1 by nutrient sufficiency and growth factor stimulation inhibits autophagy.[7,196] Rapamycin is an autophagy inducing drug that mediates its effect through the inhibition of the mTOR pathway.[197] We investigated whether inhibition of mTOR by rapamycin could overcome the 5-HT induced impaired autophagy. We found that addition of rapamycin to 5-HT treated PBMCs reversed the effect of 5-HT on autophagy in HCs and active CD patients indicating that 5-HT might possibly be inhibiting autophagy through the mTOR pathway. However, future studies should be undertaken to address some of the limitations of our study such as sample size and determining how 5-HT modulates autophagy in patients with and without autophagy polymorphisms.

Using two mice models (*Tph1*$^{-/-}$ *and SERT*$^{-/-}$) with different levels of 5-HT in the gut, our findings clearly showed that 5-HT is an important negative regulator in the upstream end of mTOR dependent autophagy pathway. As Tph1 is the rate limiting enzyme of 5-HT synthesis,[69,70] mice deficient in Tph1 have markedly reduced levels of 5-HT in the gut.[85] *SERT*$^{-/-}$ mice, in contrast, have significantly elevated level of 5-HT in the gut compared to wild-type (WT) mice, since 5-HT is unable to be transported back into the surrounding epithelial cells.[71] As reported previously,[85] we observed significantly reduced severity of intestinal inflammation in *Tph1*$^{-/-}$ mice compared with WT controls. The hallmark of autophagy is the formation of double membrane bound vesicles containing portions of cytoplasm, called autophagosome.[7] LC3B is essential for autophagosome formation and used as a marker of autophagy.[7,11] Lipidation of LC3-I

with phosphatidylethanolamine to form hydrophobic LC3-II is catalyzed by ubiquitin like enzymes Atg12-5-16L1 complex, Atg7 and Atg3.[7,17] LC3-II incorporates into and elongates the isolation membrane resulting in closure of autophagosome. The level of adapter protein p62 rises in response to inhibition of selective autophagy because its degradation is dependent on autophagy. p62 can therefore be used as marker to study autophagic flux.[198] In the colitic mice groups, we observed a significant rise in LC3-II along with depletion of p62 in the *Tph1*$^{-/-}$ mice compared to WT mice. In the noncolitic groups there was comparable findings of increased autophagy in *Tph1*$^{-/-}$ mice. In addition, the autophagy regulator p-mTOR was markedly reduced in *Tph1*$^{-/-}$ mice, further indicating that 5-HT inhibits autophagy through the mTOR pathway. To further investigate whether 5-HT activates p-mTOR and inhibits autophagy, 5-HT restored *Tph1*$^{-/-}$ mice were given rapamycin. Indeed, rapamycin treatment attenuated intestinal inflammation as shown previously[37] along with reversal of the impaired autophagy. These findings corroborate our human PBMC data that shows the inhibitory effect of increased 5-HT on autophagy in the context of colitis. *SERT*$^{-/-}$ mice, model of elevated 5-HT, showed increased severity of DSS-induced colitis. These findings were correlated with a marked reduction in the process of autophagic flux. In addition, we also observed downregulated autophagy in the *SERT*$^{-/-}$ mice without colitis. However, this did not result in the development of spontaneous colitis. Previously, different research groups have established that autophagy deficient mice models do not develop spontaneous colitis.[17,25,199,200] It can be speculated that impaired autophagy in the intestine predisposes the *SERT*$^{-/-}$ mice to increased severity of colitis, after colitis develops by the

administration of an external agent such as DSS. As with the intestine, *SERT* gene knockdown in dorsal raphe nucleus in rats increased 5-HT level and impaired hippocampal autophagy as seen by a decrease in Beclin-1 and LC3-II/LC3-I ratio.[184] These results further strengthen the notion that during colitis, increased 5-HT impairs autophagy in the gut and contribute to increased severity of inflammation. Our findings further validate various studies that have established that functional autophagy is protective against intestinal inflammation by preventing an exaggerated pro-inflammatory response in the gut.

The first line of defense in the gut against invading gut microbiota and pathogens are the IECs.[201,202] Due to the close proximity of the EC cells to the adjacent IECs as well as the presence of 5-HTR on IECs,[64] it is likely that 5-HT plays a critical role in maintaining IEC homeostasis. Our study, to our knowledge, for the first time demonstrates that during DSS-induced colitis, reduced 5-HT in the gut increases autophagy in the IECs. This was indicated by the increased level of autophagy markers in the intestinal mucosa and in the IECs isolated from *Tph1*$^{-/-}$ mice compared to the WT control. These results were similar to that found in the whole colon. The importance of autophagy in the maintenance of homeostasis and the prevention of uncontrolled inflammation was confirmed when *Atg7*$^{\Delta IEC}$ mice exhibited increased severity of colitis compared to *Atg7*$^{fl/fl}$ mice. Similar to our findings, Tsubio and his group[27] demonstrated that *Atg7*$^{\Delta IEC}$ mice have exacerbated DSS colitis due to decreased expression of AMPs and mucins, expansion of abnormal gut microbiota and bacterial invasion into the colonic epithelium. Once the elevated autophagy in the IECs of *Tph1*$^{-/-}$ mice was reduced by

knocking out Atg7 gene, the protective effects of reduced 5-HT in the gut was absent. The severity of colitis of DKO mice was markedly increased compared to the *Tph1*$^{-/-}$ mice, while resembling the colitis severity of *Atg7*$^{\Delta IEC}$. Our findings in the DKO mice thus demonstrate that the interaction of 5-HT and autophagy in the IECs regulate colitis.

The gut microbiota is linked with many GI disorders including IBD. Alteration in the gut microbiota structure and function might trigger and perpetuate chronic colitis.[203] Recently, both the process of autophagy and 5-HT signaling has been implicated in the regulation of gut microbiota.[27,78,204,205] Our lab has shown that *Tph1*$^{+/-}$ and *Tph1*$^{-/-}$ naïve littermates have a significant difference in microbial composition. Adoptive transfer of microbiota from *Tph1*$^{-/-}$ to *Tph1*$^{+/-}$ littermates and vice versa alters severity of colitis, with microbiota from *Tph1*$^{-/-}$ mice mediating protective effects.[78] In the present study the PCoA plot in axis 3 of Figure 5D of chapter 3 shows a significant difference in β-diversity of microbial composition between *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice. Of note, Allobaculum is significantly increased in colitic *Tph1*$^{-/-}$ compared to *Tph1*$^{+/+}$ mice. Allobaculum is regarded as anti-inflammatory following the observation that it is decreased in DSS colitis but is increased after administration of probiotics or therapeutic traditional medicine.[206,207] In addition, to *Tph1*$^{-/-}$ mice, *SERT*$^{-/-}$ mice with increased levels of 5-HT in the intestine, and the gut lumen[81] have a markedly changed microbial composition compared to *SERT*$^{+/+}$ mice.[79] 5-HT directly stimulates or inhibits the growth of microbiota in a species dependent manner possibly via 5-HTRs or SERT like transporters on the surface of microbes or indirectly by AMP production[78,79,81] Dysfunctional autophagy is implicated in the development of intestinal dysbiosis. After

DSS administration, the β-diversity of *Atg7*$^{\Delta IEC}$ mice was significantly different compared to *Atg7*$^{fl/fl}$ group. Previously, *Atg7*$^{\Delta IEC}$ showed altered microbiota composition, with an increase in colitogenic microbiota that contributed to severe colitis. Autophagy deficient mouse models such as *Atg7*$^{\Delta IEC}$, *Atg5*$^{\Delta IEC}$ and *Atg16L1*^{T300A} knock-in mice exhibit an increase in bacterial burden, reduction in diversity, increase in pro-inflammatory bacteria such as *Bacteroides fragilis* and decrease in anti-inflammatory bacteria such as *Akkermansia muciniphila* and ultimately influences host immune response.[27,204,205] The microbial changes observed in autophagy deficient models reflect those in IBD patients.[208] It is evident that increased 5-HT and impaired autophagy promotes a colitogenic microbiota that plays a crucial role in the development, severity and perpetuation of intestinal inflammation. In spite of these findings, there was no evidence whether increased 5-HT impairs autophagy in the IECs and results in dysbiosis. It is apparent from our study that the protective microbiota in colitic and naive *Tph1*$^{-/-}$ mice is lost when the Atg7 gene is also deficient in IECs. The microbiota of colitic DKO mice is very similar to that of colitic *Atg7*$^{\Delta IEC}$ mice in spite of also being deficient in *Tph1* gene. This might be because the *Atg7*$^{\Delta IEC}$ mice already have disrupted autophagy in the IECs, when the *Tph1* gene is knocked out and thus the 5-HT-autophagy axis regulating the gut microbiota is disturbed. Even though the taxonomic summaries showed no significant differences, the microbiota of the *Atg7*$^{fl/fl}$ mice separated into distinct cluster from the microbiota of *Atg7*$^{\Delta IEC}$ mice as seen by the PCoA plots in spite of having similar levels of gut 5-HT. This indicates that when autophagy is disrupted in the IECs, the composition of the gut microbiota is altered irrespective of the levels of gut 5-HT. These results signify

that the development of the protective microbiota in mice with very reduced amounts of 5-HT in the gut might be due to elevated levels of autophagy in the IECs. One of the mechanisms by which 5-HT-autophagy axis in the IECs might be regulating the development of a colitogenic microbiota is through the production and secretion of the basal AMP, mDefb1. We found that not only in the colitic, but also in the naïve DKO mice, the levels of mDefb1 are reduced compared to *Tph1*$^{-/-}$ group and is similar to *Atg7*$^{\Delta IEC}$ mice that supported our hypothesis. For further proving that the dysbiotic microbiota is mediating the change in colitis in the DKO mice we performed cecal adoptive transfer experiment from both the colitic and naïve DKO mice to antibiotic-treated mice. The transfer of microbiota from colitic and naïve DKO mice resulted in more severe colitis compared to mice receiving microbiota from *Tph1*$^{-/-}$ group. These findings indicate that the impairment in the 5-HT-autophagy axis in the IECs alter the gut microbial composition and influence the severity of intestinal inflammation.

2. Future directions of our finings

5-HTR and AMPK signaling in autophagy:

In our present study, we observed that 5-HT–mediated disruption of autophagy in the IECs is arbitrated by 5-HTR3, 5-HTR4, and 5-HTR7, with 5-HTR7 playing a more dominant role. 5-HT mediated reduction of autophagy markers and elevation of negative regulator p-mTOR in the HT-29 and primary mouse IECs cells were reversed when the above mentioned 5-HTR were blocked by specific antagonists. However, it is not clear how the inhibition of these 5-HTRs result in activation of autophagy via the mTOR

pathway. Even more perplexing, is that 5-HTR4 and 5-HTR7 are G protein-coupled receptor where as 5-HTR3 is a ligand gated ion channel.[44] In our animal models, we found that mice with increased 5-HT in the gut have reduced levels of p-AMPKα while the opposite is true for mice with low levels of gut 5-HT. These findings suggest AMPK might be the upstream regulator of the 5-HT-autophagy axis in the IECs. AMPK is a heterotrimeric serine/threonine kinase with a catalytic α-subunit and regulatory β- and γ-subunits that create an evolutionary conserved complex. AMPK induces autophagy and regulates cellular metabolism to maintain energy homeostasis.[209] AMPK is activated by low levels of cellular energy (ATP) or high AMP/ATP ratio and it slows down energy-intensive processes in part via inactivating mTOR which is also a negative regulator of autophagy.[209] There are mounting evidence that the second messenger cAMP regulates metabolism, growth and differentiation via its action on AMPK.[210] There are evidences of cAMP either activating or inhibiting AMPK and autophagy depending on the tissue, cell line and the condition being studied.[210,211] From these discussion, it can be hypothesized that the increase of the second messenger cAMP[212] and subsequent inhibition of AMPK may be one way for 5-HTR4 and 5-HTR7 to activate mTOR. However, a different second messenger system might be playing a role in the 5-HTR3-autophagy axis. The activation of 5-HTR3 causes an inflow of extracellular calcium ions, which mobilises and raises intracellular calcium concentration.[212] The role of intracellular calcium in mTOR-dependent autophagy is still debated.[213] As a result, future research studies into link connecting 5-HTRs expressed on IECs and mTOR in the regulation of autophagy in IBD is needed.

Role of 5-HT-autophagy axis in immune cells:

One of the mechanisms by which 5-HT regulates intestinal inflammation is through its action on mucosal innate immune cells. The presence of 5-HTRs, SERT and Tph1 in the immune cells such as macrophages and DCs indicate the dependence of the immune cells on 5-HT signaling. The diverse action of 5-HT in the regulation of immune cell function has been discussed in Chapter 1. Specifically, our lab has shown that DCs from DSS-induced *Tph1*$^{-/-}$ mice generates less pro-inflammatory cytokine IL-12 than those isolated from *Tph1*$^{+/+}$ mice, which was consistent with the severity of colitis. *Tph1*$^{-/-}$ DC cytokine production was restored after *in vitro* 5-HT stimulation, and adoptive transfer of 5-HT pulsed DCs into *Tph1*$^{-/-}$ mice up-regulated colitis. In addition, CD4^{+} T cells primed by *Tph1*$^{-/-}$ DCs produce lower amounts of IL-17 and interferon-g.[214] Additionally, we have also found that stimulation of 5-HTR7 on DCs, increase the production of pro-inflammatory cytokines via the NF-κB pathway.[87] 5-HT also enhances the infiltration of macrophages and their cytokine production during colitis.[85] As discussed in Chapter 1 it is established that autophagy has critical role in innate immune cell function such as cytokine production, antigen presentation, and phagocytosis.[17] Different groups have reported highly elevated production and secretion of pro-inflammatory cytokines by DCs and macrophages isolated from mice and humans with autophagy impairment.[17] Based on the following evidence of: 5-HT is a pro-inflammatory signaling molecule for innate immune cells; autophagy is protective against inflammation through its action on innate immune cells, and our findings in this thesis on the inhibitory role of 5-HT on autophagy in IECs during colitis, it is imperative to further investigate

whether increased 5-HT levels has similar inhibitory effects on autophagy in mucosal innate immune cells that leads to increased severity of intestinal inflammation.

3. Autophagy and 5-HT-autophagy axis in the management of intestinal inflammation

A case report and a retrospective case review indicated that the mTOR inhibitor, rapamycin, is a good candidate for the treatment of refractory adult and paediatric IBD unresponsive to conventional therapy. Massey et al.[215] reported that in a 37 years old woman with severe CD, refractory to standard medical therapies, the use of oral rapamycin at a dose of 4mg/day for 6 months in addition to 6 weekly infliximab infusions resulted in a striking and sustained improvement in CD symptoms and complications indicated by a significant decrease of the Harvey–Bradshaw index from 13 to 3. Her symptomatic improvements were associated with endoscopic remission and a marked reduction and normalisation of markers of inflammation such as C-reactive protein (decreased from 79 to 2), albumin and platelet count. However, since the patient was concomitantly on infliximab it is unclear whether infliximab augmented the effects of rapamycin. Nevertheless, upon administration of rapamycin, the efficacy of infliximab was not only recaptured but also maintained for 3 months since the rapamycin was discontinued. Similar to this case report, in a retrospective case review series it was demonstrated that rapamycin was moderately effective as an adjuvant in children with refractory IBD.[216] This was a small study where medical records of paediatric IBD patients between 2006 and 2012 were reviewed retrospectively to evaluate clinical

response, remission and mucosal healing following administration of rapamycin as an adjuvant therapy. In this study, from the records of 11 UC and 3 CD patients analyzed, it was seen that 5 (45%) UC patients attained clinical remission, 2 (18%) UC patients achieved clinical response and the remaining 4 (36%) did not respond. All CD patients showed clinical remission. However, only 5 UC and 2 CD patients achieved mucosal healing. Due to the small sample size, the decline in rate of colectomy in rapamycin responders failed to reach statistical significance. Another limitation of the study was the simultaneous use of other immunomodulatory drugs in the patients. Therefore, the findings from this study should be interpreted with caution. It is imperative to conduct well-designed randomized placebo-controlled study with larger sample size and long-term outcome goals to establish the efficacy of rapamycin as an adjuvant or alternative treatment in children and adults with refractory IBD. However, a major caveat in the clinical application of autophagy modulating drugs is that these drugs are not selective for autophagy pathway and has multiple targets. Future research is needed to develop highly specific autophagy modulators that are disrupted during IBD.

To date, there are no clinical studies to determine the efficacy of blocking mucosal 5-HT in IBD either by using a specific Tph1 inhibitor or by using specific 5-HTR antagonists. Oral Tph inhibitors LX-1031 and LX-1033 have been found to be effective in non-constipating IBS without any major side effects.[217,218] In addition, the Tph inhibitor telotristat ethyl is currently Food and Drug administration approved for treating carcinoid syndrome diarrhoea.[219] Based on these promising clinical trials, it is justifiable to investigate the effects of mucosal 5-HT modulators as a potential therapy for IBD. From

our findings it is evident that 5-HT is a regulator of autophagy in IECs. Modulating levels of 5-HT may also be another means of targeting dysfunctional autophagy in IBD. Future studies will have to address the methods of targeting the disrupted 5-HT-autophagy axis in the colon, particularly in the IECs with an aim to develop novel therapeutics for IBD.

4. Concluding statement

IBD is a global disease with its incidence increasing worldwide.[220] Even though many studies have unraveled various important factors in the pathogenesis of IBD, it still remains incurable.[93] It is evident that both dysfunctional 5-HT signaling and autophagy are associated with IBD. In IBD the link between the two has not been studied before us. From our investigations, it is clear that elevated 5-HT levels, which occur in IBD inhibits and disrupts autophagy with resultant increase in severity of colitis. One of the mechanisms by which the disturbed 5-HT-autophagy axis in the gut contributes to IBD pathology is through the alteration of the microbiota. Our study highlights the vital role of the host immune-endocrine interactions with the gut microbiota in IBD, which underscores the multifactorial nature of IBD pathogenesis. However, further studies are needed to gather more information and build upon these findings with an aim to facilitate the development of new improved therapeutics in intestinal disorders with dysregulated autophagy including IBD.

CHAPTER 5

References

1. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol.* 2015;16(8):461-472.
2. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol.* 2010;221(1):3-12.
3. Antoni L, Nuding S, Wehkamp J, Stange EF. Intestinal barrier in inflammatory bowel disease. *World J Gastroenterol.* 2014;20(5):1165-1179.
4. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol.* 2013;15(7):713-720.
5. Elshaer D, Begun J. The role of barrier function, autophagy, and cytokines in maintaining intestinal homeostasis. *Semin Cell Dev Biol.* 2017;61:51-59.
6. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med.* 2013;368(7):651-662.
7. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol.* 2010;22(2):124-131.
8. Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell.* 2009;137(6):1001-1004.
9. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, Menzies FM, Rubinsztein DC. Mammalian Autophagy: How Does It Work? *Annu Rev Biochem.* 2016;85:685-713.
10. Huang J, Brumell JH. Bacteria-autophagy interplay: a battle for survival. *Nat Rev Microbiol.* 2014;12(2):101-114.
11. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* 2008;132(1):27-42.
12. Conway KL, Kuballa P, Song JH, Patel KK, Castoreno AB, Yilmaz OH, Jijon HB, Zhang M, Aldrich LN, Villablanca EJ, Peloquin JM, Goel G, Lee IA, Mizoguchi E, Shi HN, Bhan AK, Shaw SY, Schreiber SL, Virgin HW, Shamji AF, Stappenbeck TS, Reinecker HC, Xavier RJ. Atg16l1 is required for autophagy in intestinal epithelial cells and protection of mice from Salmonella infection. *Gastroenterology.* 2013;145(6):1347-1357.
13. Mimouna S, Bazin M, Mograbi B, Darfeuille-Michaud A, Brest P, Hofman P, Vouret-Craviari V. HIF1A regulates xenophagic degradation of adherent and invasive Escherichia coli (AIEC). *Autophagy.* 2014;10(12):2333-2345.
14. Nighot PK, Hu CA, Ma TY. Autophagy enhances intestinal epithelial tight junction barrier function by targeting claudin-2 protein degradation. *J Biol Chem.* 2015;290(11):7234-7246.
15. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman BP, Li E, Mizushima N, Stappenbeck TS, Virgin HWt. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature.* 2008;456(7219):259-263.
16. Wittkopf N, Gunther C, Martini E, Waldner M, Amann KU, Neurath MF, Becker C. Lack of intestinal epithelial atg7 affects paneth cell granule formation but does

not compromise immune homeostasis in the gut. *Clin Dev Immunol.* 2012;2012:278059.
17. Haq S, Grondin J, Banskota S, Khan WI. Autophagy: roles in intestinal mucosal homeostasis and inflammation. *J Biomed Sci.* 2019;26(1):19.
18. Wlodarska M, Thaiss CA, Nowarski R, Henao-Mejia J, Zhang JP, Brown EM, Frankel G, Levy M, Katz MN, Philbrick WM, Elinav E, Finlay BB, Flavell RA. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell.* 2014;156(5):1045-1059.
19. Nagy P, Szatmari Z, Sandor GO, Lippai M, Hegedus K, Juhasz G. Drosophila Atg16 promotes enteroendocrine cell differentiation via regulation of intestinal Slit/Robo signaling. *Development.* 2017;144(21):3990-4001.
20. Fujishima Y, Nishiumi S, Masuda A, Inoue J, Nguyen NM, Irino Y, Komatsu M, Tanaka K, Kutsumi H, Azuma T, Yoshida M. Autophagy in the intestinal epithelium reduces endotoxin-induced inflammatory responses by inhibiting NF-kappaB activation. *Arch Biochem Biophys.* 2011;506(2):223-235.
21. Strisciuglio C, Duijvestein M, Verhaar AP, Vos AC, van den Brink GR, Hommes DW, Wildenberg ME. Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions. *J Crohns Colitis.* 2013;7(7):534-541.
22. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, Mizushima N, Grinstein S, Iwasaki A. In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity.* 2010;32(2):227-239.
23. Nimmerjahn F, Milosevic S, Behrends U, Jaffee EM, Pardoll DM, Bornkamm GW, Mautner J. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur J Immunol.* 2003;33(5):1250-1259.
24. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, Ferguson DJ, Campbell BJ, Jewell D, Simmons A. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med.* 2010;16(1):90-97.
25. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M, Tanaka K, Kawai T, Tsujimura T, Takeuchi O, Yoshimori T, Akira S. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature.* 2008;456(7219):264-268.
26. Yang L, Liu C, Zhao W, He C, Ding J, Dai R, Xu K, Xiao L, Luo L, Liu S, Li W, Meng H. Impaired autophagy in intestinal epithelial cells alters gut microbiota and host immune responses. *Appl Environ Microbiol.* 2018.
27. Tsuboi K, Nishitani M, Takakura A, Imai Y, Komatsu M, Kawashima H. Autophagy Protects against Colitis by the Maintenance of Normal Gut Microflora and Secretion of Mucus. *J Biol Chem.* 2015;290(33):20511-20526.
28. Yue C, Yang X, Li J, Chen X, Zhao X, Chen Y, Wen Y. Trimethylamine N-oxide prime NLRP3 inflammasome via inhibiting ATG16L1-induced autophagy in colonic epithelial cells. *Biochem Biophys Res Commun.* 2017;490(2):541-551.
29. Noureldein MH, Eid AA. Gut microbiota and mTOR signaling: Insight on a new pathophysiological interaction. *Microb Pathog.* 2018;118:98-104.

30. Lin R, Jiang Y, Zhao XY, Guan Y, Qian W, Fu XC, Ren HY, Hou XH. Four types of Bifidobacteria trigger autophagy response in intestinal epithelial cells. *J Dig Dis.* 2014;15(11):597-605.
31. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Gunther S, Prescott NJ, Onnie CM, Hasler R, Sipos B, Folsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet.* 2007;39(2):207-211.
32. Murthy A, Li Y, Peng I, Reichelt M, Katakam AK, Noubade R, Roose-Girma M, DeVoss J, Diehl L, Graham RR, van Lookeren Campagne M. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature.* 2014;506(7489):456-462.
33. Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy.* 2007;3(6):649-651.
34. McCarroll SA, Huett A, Kuballa P, Chilewski SD, Landry A, Goyette P, Zody MC, Hall JL, Brant SR, Cho JH, Duerr RH, Silverberg MS, Taylor KD, Rioux JD, Altshuler D, Daly MJ, Xavier RJ. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat Genet.* 2008;40(9):1107-1112.
35. Jones SA, Mills KH, Harris J. Autophagy and inflammatory diseases. *Immunol Cell Biol.* 2013;91(3):250-258.
36. Ravindran R, Loebbermann J, Nakaya HI, Khan N, Ma H, Gama L, Machiah DK, Lawson B, Hakimpour P, Wang YC, Li S, Sharma P, Kaufman RJ, Martinez J, Pulendran B. The amino acid sensor GCN2 controls gut inflammation by inhibiting inflammasome activation. *Nature.* 2016;531(7595):523-527.
37. Macias-Ceja DC, Cosin-Roger J, Ortiz-Masia D, Salvador P, Hernandez C, Esplugues JV, Calatayud S, Barrachina MD. Stimulation of autophagy prevents intestinal mucosal inflammation and ameliorates murine colitis. *Br J Pharmacol.* 2017;174(15):2501-2511.
38. Hu S, Chen M, Wang Y, Wang Z, Pei Y, Fan R, Liu X, Wang L, Zhou J, Zheng S, Zhang T, Lin Y, Zhang M, Tao R, Zhong J. mTOR Inhibition Attenuates Dextran Sulfate Sodium-Induced Colitis by Suppressing T Cell Proliferation and Balancing TH1/TH17/Treg Profile. *PLoS One.* 2016;11(4):e0154564.
39. Bhonde MR, Gupte RD, Dadarkar SD, Jadhav MG, Tannu AA, Bhatt P, Bhatia DR, Desai NK, Deore V, Yewalkar N, Vishwakarma RA, Sharma S, Kumar S, Dagia NM. A novel mTOR inhibitor is efficacious in a murine model of colitis. *Am J Physiol Gastrointest Liver Physiol.* 2008;295(6):G1237-1245.
40. Friedman M. Analysis, nutrition, and health benefits of tryptophan. *Int J Tryptophan Res.* 2018;11:1178646918802282.
41. Hyland NP, Cavanaugh CR, Hornby PJ. Emerging effects of tryptophan pathway metabolites and intestinal microbiota on metabolism and intestinal function. *Amino Acids.* 2022;54:57-70.

42. Moffett JR, Namboodiri MA. Tryptophan and the immune response. *Immunol Cell Biol.* 2003;81(4):247-265.
43. Gao K, Mu CL, Farzi A, Zhu WY. Tryptophan metabolism: a link between the gut microbiota and brain. *Adv Nutr.* 2020;11(3):709-723.
44. Haq S, Grondin JA, Khan WI. Tryptophan-derived serotonin-kynurenine balance in immune activation and intestinal inflammation. *FASEB J.* 2021;35(10):e21888.
45. Miura H, Ozaki N, Sawada M, Isobe K, Ohta T, Nagatsu T. A link between stress and depression: shifts in the balance between the kynurenine and serotonin pathways of tryptophan metabolism and the etiology and pathophysiology of depression. *Stress.* 2008;11(3):198-209.
46. Keszthelyi D, Troost FJ, Masclee AA. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. *Neurogastroenterol Motil.* 2009;21(12):1239-1249.
47. El-Merahbi R, Loffler M, Mayer A, Sumara G. The roles of peripheral serotonin in metabolic homeostasis. *FEBS Lett.* 2015;589(15):1728-1734.
48. Spohn SN, Mawe GM. Non-conventional features of peripheral serotonin signalling - the gut and beyond. *Nat Rev Gastroenterol Hepatol.* 2017;14(7):412-420.
49. Keating DJ, Spencer NJ. What is the role of endogenous gut serotonin in the control of gastrointestinal motility? *Pharmacol Res.* 2019;140:50-55.
50. Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. *Neurogastroenterol Motil.* 2007;19 Suppl 2:25-31.
51. Gershon MD. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment Pharmacol Ther.* 1999;13 Suppl 2:15-30.
52. Manocha M, Khan WI. Serotonin and GI Disorders: An Update on Clinical and Experimental Studies. *Clin Transl Gastroenterol.* 2012;3:e13.
53. Sternini C, Anselmi L, Rozengurt E. Enteroendocrine cells: a site of 'taste'in gastrointestinal chemosensing. *Curr Opin Endocrinol Diabetes Obes.* 2008;15(1):73-78.
54. Li HJ, Ray SK, Singh NK, Johnston B, Leiter AB. Basic helix-loop-helix transcription factors and enteroendocrine cell differentiation. *Diabetes Obes Metab.* 2011;13:5-12.
55. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science.* 1999;284(5415):770-776.
56. Gribble FM, Reimann F. Enteroendocrine cells: chemosensors in the intestinal epithelium. *Annu Rev Physiol.* 2016;78:277-299.
57. Psichas A, Reimann F, Gribble FM. Gut chemosensing mechanisms. *J Clin Investig.* 2015;125(3):908-917.
58. Gunawardene AR, Corfe BM, Staton CA. Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. *Int J Exp Pathol.* 2011;92(4):219-231.

59. Modlin IM, Kidd M, Pfragner R, Eick GN, Champaneria MC. The functional characterization of normal and neoplastic human enterochromaffin cells. *J Clin Endocrinol Metab.* 2006;91(6):2340-2348.
60. Heitz P, Polak JM, Timson CM, Pearse AG. Enterochromaffin cells as the endocrine source of gastrointestinal substance P. *Histochemistry.* 1976;49(4):343-347.
61. Khan WI, Ghia JE. Gut hormones: emerging role in immune activation and inflammation. *Clin Exp Immunol.* 2010;161(1):19-27.
62. Cetin Y, Kuhn M, Kulaksiz H, Adermann K, Bargsten G, Grube D, Forssmann WG. Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. *Proc Natl Acad Sci U S A.* 1994;91(8):2935-2939.
63. Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B. The gut as a sensory organ. *Nat Rev Gastroenterol Hepatol.* 2013;10(12):729-740.
64. Mawe GM, Hoffman JM. Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets. *Nat Rev Gastroenterol Hepatol.* 2013;10(8):473-486.
65. Linan-Rico A, Ochoa-Cortes F, Beyder A, Soghomonyan S, Zuleta-Alarcon A, Coppola V, Christofi FL. Mechanosensory signaling in enterochromaffin cells and 5-HT release: potential implications for gut inflammation. *Front Neurosci.* 2016;10:564.
66. Ferrara A, Zinner MJ, Jaffe BM. Intraluminal release of serotonin, substance P, and gastrin in the canine small intestine. *Dig Dis Sci.* 1987;32(3):289-294.
67. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology.* 2007;132(1):397-414.
68. Gershon MD. Serotonin is a sword and a shield of the bowel: serotonin plays offense and defense. *Trans Am Clin Climatol Assoc.* 2012;123:268-280; discussion 280.
69. Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. *Biochem Pharmacol.* 2003;66(9):1673-1680.
70. Shajib MS, Baranov A, Khan WI. Diverse Effects of Gut-Derived Serotonin in Intestinal Inflammation. *ACS Chem Neurosci.* 2017;8(5):920-931.
71. Bertrand PP, Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. *Auton Neurosci.* 2010;153(1-2):47-57.
72. Racke K, Reimann A, Schworer H, Kilbinger H. Regulation of 5-HT release from enterochromaffin cells. *Behav Brain Res.* 1996;73(1-2):83-87.
73. Shajib MS, Khan WI. The role of serotonin and its receptors in activation of immune responses and inflammation. *Acta Physiol (Oxf).* 2015;213(3):561-574.
74. Ahern GP. 5-HT and the immune system. *Curr Opin Pharmacol.* 2011;11(1):29-33.
75. Sjogren K, Engdahl C, Henning P, Lerner UH, Tremaroli V, Lagerquist MK, Backhed F, Ohlsson C. The gut microbiota regulates bone mass in mice. *J Bone Miner Res.* 2012;27(6):1357-1367.

76. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell.* 2015;161(2):264-276.
77. Reigstad CS, Salmonson CE, Rainey JF, 3rd, Szurszewski JH, Linden DR, Sonnenburg JL, Farrugia G, Kashyap PC. Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *FASEB J.* 2015;29(4):1395-1403.
78. Kwon YH, Wang H, Denou E, Ghia JE, Rossi L, Fontes ME, Bernier SP, Shajib MS, Banskota S, Collins SM, Surette MG, Khan WI. Modulation of Gut Microbiota Composition by Serotonin Signaling Influences Intestinal Immune Response and Susceptibility to Colitis. *Cell Mol Gastroenterol Hepatol.* 2019;7(4):709-728.
79. Singhal M, Turturice BA, Manzella CR, Ranjan R, Metwally AA, Theorell J, Huang Y, Alrefai WA, Dudeja PK, Finn PW, Perkins DL, Gill RK. Serotonin Transporter Deficiency is Associated with Dysbiosis and Changes in Metabolic Function of the Mouse Intestinal Microbiome. *Sci Rep.* 2019;9(1):2138.
80. Oleskin AV, Shenderov BA, Rogovsky VS. Role of Neurochemicals in the Interaction between the Microbiota and the Immune and the Nervous System of the Host Organism. *Probiotics Antimicrob Proteins.* 2017;9(3):215-234.
81. Fung TC, Vuong HE, Luna CDG, Pronovost GN, Aleksandrova AA, Riley NG, Vavilina A, McGinn J, Rendon T, Forrest LR, Hsiao EY. Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. *Nat Microbiol.* 2019;4(12):2064-2073.
82. Oshima S, Fujimura M, Fukimiya M. Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate. *Histochem Cell Biol.* 1999;112(4):257-263.
83. Linden DR, Chen JX, Gershon MD, Sharkey KA, Mawe GM. Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol.* 2003;285(1):G207-216.
84. Khan WI, Motomura Y, Wang H, El-Sharkawy RT, Verdu EF, Verma-Gandhu M, Rollins BJ, Collins SM. Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. *Am J Physiol Gastrointest Liver Physiol.* 2006;291(5):G803-811.
85. Ghia JE, Li N, Wang H, Collins M, Deng Y, El-Sharkawy RT, Cote F, Mallet J, Khan WI. Serotonin has a key role in pathogenesis of experimental colitis. *Gastroenterology.* 2009;137(5):1649-1660.
86. Bischoff SC, Mailer R, Pabst O, Weier G, Sedlik W, Li Z, Chen JJ, Murphy DL, Gershon MD. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice. *Am J Physiol Gastrointest Liver Physiol.* 2009;296(3):G685-695.
87. Kim JJ, Bridle BW, Ghia JE, Wang H, Syed SN, Manocha MM, Rengasamy P, Shajib MS, Wan Y, Hedlund PB, Khan WI. Targeted inhibition of serotonin type 7 (5-HT7) receptor function modulates immune responses and reduces the severity of intestinal inflammation. *J Immunol.* 2013;190(9):4795-4804.

88. Kim JJ, Wang H, Terc JD, Zambrowicz B, Yang QM, Khan WI. Blocking peripheral serotonin synthesis by telotristat etiprate (LX1032/LX1606) reduces severity of both chemical- and infection-induced intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol.* 2015;309(6):G455-465.
89. Shajib MS, Wang H, Kim JJ, Sunjic I, Ghia JE, Denou E, Collins M, Denburg JA, Khan WI. Interleukin 13 and serotonin: linking the immune and endocrine systems in murine models of intestinal inflammation. *PLoS One.* 2013;8(8):e72774.
90. Minderhoud IM, Oldenburg B, Schipper ME, ter Linde JJ, Samsom M. Serotonin synthesis and uptake in symptomatic patients with Crohn's disease in remission. *Clin Gastroenterol Hepatol.* 2007;5(6):714-720.
91. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature.* 2007;448(7152):427-434.
92. Kaplan GG, Bernstein CN, Coward S, Bitton A, Murthy SK, Nguyen GC, Lee K, Cooke-Lauder J, Benchimol E. The Impact of Inflammatory Bowel Disease in Canada 2018: Epidemiology. *J Can Assoc Gastroenterol.* 2019;2(Supplement_1):S6–S16.
93. Bouma G, Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol.* 2003;3:521–533.
94. Levine JS, Burakoff R. Extraintestinal manifestations of inflammatory bowel disease. *Gastroenterol Hepatol (N Y).* 2011;7(4):235-241.
95. Zhang YZ, Li YY. Inflammatory bowel disease: pathogenesis. *World J Gastroenterol.* 2014;20(1):91-99.
96. de Souza H, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* 2016;13:13–27.
97. Strisciuglio C, Giugliano F, Martinelli M, Cenni S, Greco L, Staiano A, Miele E. Impact of environmental and familial factors in a cohort of pediatric patients with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr.* 2017;64(4):569-574.
98. Khor B, Gardet A, Xavier R. Genetics and pathogenesis of inflammatory bowel disease. *Nature.* 2011;474:307–317.
99. Santos MP, Gomes C, Torres J. Familial and ethnic risk in inflammatory bowel disease. *Ann Gastroenterol.* 2018;31(1):14.
100. Liu J, van Sommeren S, Huang Hea. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet.* 2015;47:979–986.
101. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.* 2001;411(6837):603-606.
102. Kucharzik T, Maaser C, Lugering A, Kagnoff M, Mayer L, Targan S, Domschke W. Recent understanding of IBD pathogenesis: implications for future therapies. *Inflamm Bowel Dis.* 2006;12(11):1068-1083.
103. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, Yuan L, Soares F, Chea E, Le Bourhis L, Boneca IG, Allaoui A, Jones NL, Nunez

G, Girardin SE, Philpott DJ. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol.* 2010;11(1):55-62.

104. Plantinga TS, Crisan TO, Oosting M, van de Veerdonk FL, de Jong DJ, Philpott DJ, van der Meer JW, Girardin SE, Joosten LA, Netea MG. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. *Gut.* 2011;60(9):1229-1235.
105. Uhlig HH, Schwerd T, Koletzko S, Shah N, Kammermeier J, Elkadri A, Ouahed J, Wilson DC, Travis SP, Turner D, Klein C, Snapper SP, Muise AM. The diagnostic approach to monogenic very early onset inflammatory bowel disease. *Gastroenterology.* 2014;147(5):990-1007.
106. Ye BD, McGovern DP. Genetic variation in IBD: progress, clues to pathogenesis and possible clinical utility. *Expert Rev Clin Immunol.* 2016;12(10):1091-1107.
107. Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. *Gut.* 2011;60(12):1739-1753.
108. Ng SC. Emerging leadership lecture: Inflammatory bowel disease in Asia: Emergence of a "Western" disease. *J Gastroenterol Hepatol.* 2015;30(3):440–445.
109. Gu P, Feagins LA. Dining With Inflammatory Bowel Disease: A Review of the Literature on Diet in the Pathogenesis and Management of IBD. *Inflamm Bowel Dis.* 2020;26(2):181–191.
110. Virta L, Auvinen A, Helenius H, Huovinen P, Kolho KL. Association of Repeated Exposure to Antibiotics With the Development of Pediatric Crohn's Disease—A Nationwide, Register-based Finnish Case-Control Study. *Am J Epidemiol.* 2012;175(8):775–784.
111. Ungaro R, Bernstein CN, Gearry R, Hviid A, Kolho KL, Kronman MP, Shaw S, Van Kruiningen H, Colombel JF, Atreja A. Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. *Official journal of the American College of Gastroenterology| ACG.* 2014;109(11):1728-1738.
112. Porter CK, Tribble DR, Aliaga PA, Halvorson HA, Riddle MS. Infectious gastroenteritis and risk of developing inflammatory bowel disease. *Gastroenterology.* 2008;135(3):781-786.
113. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Official journal of the American College of Gastroenterology| ACG.* 2011;106(4):563-573.
114. Birrenbach T, Böcker U. Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. *Inflamm Bowel Dis.* 2004;10(6):848-859.
115. Cosnes J. What is the link between the use of tobacco and IBD? *Inflamm Bowel Dis.* 2008;14(suppl_2):S14-15.
116. Kish L, Hotte N, Kaplan GG, Vincent R, Tso R, Gänzle M, Rioux KP, Thiesen A, Barkema HW, Wine E, Madsen KL. Environmental particulate matter induces

murine intestinal inflammatory responses and alters the gut microbiome. *PloS One.* 2013;8(4):e62220.
117. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature.* 2011;474(7351):327-336.
118. Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet.* 2003;361(9356):512-519.
119. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol.* 2009;9(5):313-323.
120. Norman JM, Handley SA, Baldridge MT, Droit L, Liu CY, Keller BC, Kambal A, Monaco CL, Zhao G, Fleshner P, Stappenbeck TS, McGovern DPB, Keshavarzian A, Mutlu EA, Sauk J, Gevers D, Xavier RJ, Wang D, Parkes M, Virgin HW. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell.* 2015;160(3):447-460.
121. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, Cohen D, Liguori G, Bourrier A, Nion-Larmurier I, Cosnes J, Seksik P, Langella P, Skurnik D, Richard ML, Beaugerie L. Fungal microbiota dysbiosis in IBD. *Gut.* 2017;66(6):1039-1048.
122. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A.* 2011;108 Suppl 1:4659-4665.
123. Juge N, Tailford L, Owen CD. Sialidases from gut bacteria: a mini-review. *Biochem Soc Trans.* 2016;44(1):166-175.
124. Hollister EB, Gao C, Versalovic J. Compositional and functional features of the gastrointestinal microbiome and their effects on human health. *Gastroenterology.* 2014;146(6):1449-1458.
125. Sommer F, Bäckhed F. The gut microbiota—masters of host development and physiology. *Nat Rev Microbiol.* 2013;11(4):227-238.
126. Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol.* 2012;9(10):599-608.
127. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut.* 2011;60(5):631-637.
128. Kiesler P, Fuss IJ, Strober W. Experimental models of inflammatory bowel diseases. *Cell Mol Gastroenterol Hepatol.* 2015;1(2):154-170.
129. Gkouskou K, Deligianni C, Tsatsanis C, Eliopoulos AG. The gut microbiota in mouse models of inflammatory bowel disease. *Front Cell Infect Microbiol.* 2014;4:28.
130. Ni J, Wu GD, Albenberg L, Tomov VT. Gut microbiota and IBD: causation or correlation? *Nat Rev Gastroenterol Hepatol.* 2017;14(10):573-584.
131. Pittayanon R, Lau JT, Leontiadis GI, Tse F, Yuan Y, Surette M, Moayyedi P. Differences in gut microbiota in patients with vs without inflammatory bowel diseases: a systematic review. *Gastroenterology.* 2020;158(4):930-946.
132. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut.* 2004;53(1):1-4.

133. Cader MZ, Kaser A. Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut.* 2013;62(11):1653-1664.
134. De Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* 2016;13(1):13-27.
135. Choy MC, Visvanathan K, De Cruz P. An overview of the innate and adaptive immune system in inflammatory bowel disease. *Inflamm Bowel Dis.* 2017;23(1):2-13.
136. Silva FA, Rodrigues BL, Ayrizono MD, Leal RF. The immunological basis of inflammatory bowel disease. *Gastroenterol Res Pract.* 2016.
137. Oka A, Ishihara S, Mishima Y, Tada Y, Kusunoki R, Fukuba N, Yuki T, Kawashima K, Matsumoto S, Kinoshita Y. Role of regulatory B cells in chronic intestinal inflammation: association with pathogenesis of Crohn's disease. *Inflamm Bowel Dis.* 2014;20(2):315-328.
138. Wang X, Zhu Y, Zhang M, Wang H, Jiang Y, Gao P. Ulcerative colitis is characterized by a decrease in regulatory B cells. *J Crohns Colitis.* 2016;10(10):1212-1223.
139. Mizoguchi A, Bhan AK. A case for regulatory B cells. *J Immunol.* 2006;176(2):705-710.
140. Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Vallance BA. The intestinal epithelium: central coordinator of mucosal immunity. *Trends Immunol.* 2018;39(9):677-696.
141. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol.* 2014;14(3):141-153.
142. Nalle SC, Turner JR. Intestinal barrier loss as a critical pathogenic link between inflammatory bowel disease and graft-versus-host disease. *Mucosal Immunol.* 2015;8(4):720-730.
143. Kucharzik T, Walsh SV, Chen J, Parkos CA, Nusrat A. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *Am J Pathol.* 2001;159(6).
144. Somsouk M, Estes JD, Deleage C, Dunham RM, Albright R, Inadomi JM, Martin JN, Deeks SG, McCune JM, Hunt PW. Gut epithelial barrier and systemic inflammation during chronic HIV infection. *AIDS.* 2015;29(1):43.
145. Leffler DA, Dennis M, Hyett B, E K, D S, Kelly CP. Etiologies and predictors of diagnosis in nonresponsive celiac disease. *Clin Gastroenterol Hepatol.* 2007;5(4):445-450.
146. Merga Y, Campbell BJ, Rhodes JM. Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy. *Dig Dis.* 2014;32(4):475-483.
147. Van Der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol.* 2009;71:241-260.
148. Vidrich A, Buzan JM, De La Rue SA, Cohn SA. Physiology of gastrointestinal stem cells. *Physiology of the Gastrointestinal Tract* 4ed. Amsterdam: Elsevier Inc; 2006:307-344.
149. Kim JJ, Khan WI. Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens (Basel, Switzerland).* 2013;2(1):55-70.

150. Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. *Front Genet.* 2015;6:81.
151. Grondin JA, Kwon YH, Far PM, Haq S, Khan WI. Mucins in intestinal mucosal defense and inflammation: learning from clinical and experimental studies. *Front Immunol.* 2020;11:2054.
152. Hansson GC. Role of mucus layers in gut infection and inflammation. *Curr Opin Microbiol.* 2012;15(1):57-62.
153. Birchenough GM, Johansson ME, Gustafsson JK, Bergstrom JH, Hansson GC. New developments in goblet cell mucus secretion and function. *Mucosal Immunol.* 2015;8(4):712-719.
154. Pullan RD, Thomas GA, Rhodes M, Newcombe RG, Williams GT, Allen A, Rhodes J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut.* 1994;35(3):353-359.
155. Gersemann M, Becker S, Kubler I, Koslowski M, Wang G, Herrlinger KR, Griger J, Fritz P, Fellermann K, Schwab M, Wehkamp J, Stange EF. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation.* 2009;77(1):84-94.
156. Sicard JF, Le Bihan G, Vogeleer P, Jacques M, Harel J. Interactions of Intestinal Bacteria with Components of the Intestinal Mucus. *Front Cell Infect Microbiol.* 2017;7:387.
157. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Buller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology.* 2006;131(1):117-129.
158. Wrzosek L, Miquel S, Noordine ML, Bouet S, Joncquel Chevalier-Curt M, Robert V, Philippe C, Bridonneau C, Cherbuy C, Robbe-Masselot C, Langella P, Thomas M. Bacteroides thetaiotaomicron and Faecalibacterium prausnitzii influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biol.* 2013;11:61.
159. Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, Ryz N, Huang T, Velcich A, Finlay BB, Chadee K, Vallance BA. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathogens.* 2010;6(5):e1000902.
160. Zarepour M, Bhullar K, Montero M, Ma C, Huang T, Velcich A, Xia L, Vallance BA. The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella enterica serovar Typhimurium colitis. *Infect Immun.* 2013;81(10):3672-3683.
161. Snoeck V, Goddeeris B, Cox E. The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes Infect.* 2005;7(7-8):997-1004.
162. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol.* 2009;9(11):799-809.
163. Martini E, Krug S, Siegmund B, Neurath M, Becker C. The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease. *Cell Mol Gastroenterol Hepatol.* 2017;4(1):33-46.

164. Elphick DA, Mahida YR. Paneth cells: their role in innate immunity and inflammatory disease. *Gut.* 2005;54(12):1802-1809.
165. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* 2003;3(9):710-720.
166. Otte JM, Kiehne K, Herzig KH. Antimicrobial peptides in innate immunity of the human intestine. *J Gastroenterol.* 2003;38(8):717-726.
167. Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J.* 2009;276(22):6483-6496.
168. Couto MA, Harwig SS, Lehrer RI. Selective inhibition of microbial serine proteases by eNAP-2, an antimicrobial peptide from equine neutrophils. *Infect Immun.* 1993;61(7):2991-2994.
169. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol.* 2011;9(5):356-368.
170. Eisenhauer PB, Lehrer RI. Mouse neutrophils lack defensins. *Infect Immun.* 1992;60(8):3446-3447.
171. O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF. Expression and regulation of the human β-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol.* 1999;163(12):6718-6724.
172. Wehkamp J, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B, Duchrow M, Wohlschläger C, Feller AC, Stange EF. Human β-defensin 2 but not β-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *Eur J Gastroenterol Hepatol.* 2002;14(7):745-752.
173. Rahman A, Fahlgren A, Sundstedt C, Hammarström S, Danielsson Å, Hammarström ML. Chronic colitis induces expression of β-defensins in murine intestinal epithelial cells. *Clin Exp Immunol.* 2011;163(1):123-130.
174. Zilbauer M, Jenke A, Wenzel G, Postberg J, Heusch A, Phillips AD, Noble-Jamieson G, Torrente F, Salvestrini C, Heuschkel R, Wirth S. Expression of human beta-defensins in children with chronic inflammatory bowel disease. *PLoS One.* 2010;5(10):e15389.
175. Fahlgren A, Hammarström S, Danielsson Å, Hammarström ML. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol.* 2003;131(1):90-101.
176. Grisham MB. Do different animal models of IBD serve different purposes? *Inflamm Bowel Dis.* 2008;14(suppl_2):S132-133.
177. Kiesler P, Fuss IJ, Strober W. Experimental Models of Inflammatory Bowel Diseases. *Cell Mol Gastroenterol Hepatol.* 2015;1(2):154-170.
178. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, Neurath MF. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* 2017;12(7):1295-1309.
179. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc.* 2007;2(3):541-546.
180. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis.* 2009;15(3):341-352.

181. Russell RC, Yuan HX, Guan KL. Autophagy regulation by nutrient signaling. *Cell Res.* 2014;24(1):42-57.
182. Soll C, Jang JH, Riener MO, Moritz W, Wild PJ, Graf R, Clavien PA. Serotonin promotes tumor growth in human hepatocellular cancer. *Hepatology.* 2010;51(4):1244-1254.
183. Imada T, Nakamura S, Hisamura R, Izuta Y, Jin K, Ito M, Kitamura N, Tanaka KF, Mimura M, Shibuya I, Tsubota K. Serotonin hormonally regulates lacrimal gland secretory function via the serotonin type 3a receptor. *Sci Rep.* 2017;7(1):6965.
184. Wu ZM, Zheng CH, Zhu ZH, Wu FT, Ni GL, Liang Y. SiRNA-mediated serotonin transporter knockdown in the dorsal raphe nucleus rescues single prolonged stress-induced hippocampal autophagy in rats. *J Neurol Sci.* 2016;360:133-140.
185. Nunes T, Fiorino G, Danese S, Sans M. Familial aggregation in inflammatory bowel disease: is it genes or environment? *World J Gastroenterol.* 2011;17(22):2715-2722.
186. Zeng Z, Mukherjee A, Zhang H. From Genetics to Epigenetics, Roles of Epigenetics in Inflammatory Bowel Disease. *Front Genet.* 2019;10:1017.
187. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra HJ, Yamazaki K, Yang SK, International Multiple Sclerosis Genetics C, International IBDGC, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly MJ, Kubo M, Anderson CA, Weersma RK. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet.* 2015;47(9):979-986.
188. Ahonen A, Kyosola K, Penttila O. Enterochromaffin cells in macrophages in ulcerative colitis and irritable colon. *Ann Clin Res.* 1976;8(1):1-7.
189. Belai A, Boulos PB, Robson T, Burnstock G. Neurochemical coding in the small intestine of patients with Crohn's disease. *Gut.* 1997;40(6):767-774.
190. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology.* 2004;126(7):1657-1664.
191. Bishop AE, Pietroletti R, Taat CW, Brummelkamp WH, Polak JM. Increased populations of endocrine cells in Crohn's ileitis. *Virchows Arch A Pathol Anat Histopathol.* 1987;410(5):391-396.
192. Mawe GM, Coates MD, Moses PL. Review article: intestinal serotonin signalling in irritable bowel syndrome. *Aliment Pharmacol Ther.* 2006;23(8):1067-1076.
193. Shajib MS, Chauhan U, Adeeb S, Chetty Y, Armstrong D, Halder SLS, Marshall JK, Khan WI. Characterization of Serotonin Signaling Components in Patients

with Inflammatory Bowel Disease. *J Can Assoc Gastroenterol.* 2019;2(3):132-140.
194. Niture S, Gyamfi MA, Kedir H, Arthur E, Ressom H, Deep G, Kumar D. Serotonin induced hepatic steatosis is associated with modulation of autophagy and notch signaling pathway. *Cell Commun Signal.* 2018;16(1):78.
195. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006;124(3):471-484.
196. Ke P, Shao BZ, Xu ZQ, Chen XW, Liu C. Intestinal Autophagy and Its Pharmacological Control in Inflammatory Bowel Disease. *Front Immunol.* 2016;7:695.
197. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab.* 2014;19(3):373-379.
198. Bjorkoy G, Lamark T, Pankiv S, Overvatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. *Methods Enzymol.* 2009;452:181-197.
199. Cabrera S, Fernandez AF, Marino G, Aguirre A, Suarez MF, Espanol Y, Vega JA, Laura R, Fueyo A, Fernandez-Garcia MS, Freije JM, Kroemer G, Lopez-Otin C. ATG4B/autophagin-1 regulates intestinal homeostasis and protects mice from experimental colitis. *Autophagy.* 2013;9(8):1188-1200.
200. Zhang H, Zheng L, McGovern DP, Hamill AM, Ichikawa R, Kanazawa Y, Luu J, Kumagai K, Cilluffo M, Fukata M, Targan SR, Underhill DM, Zhang X, Shih DQ. Myeloid ATG16L1 Facilitates Host-Bacteria Interactions in Maintaining Intestinal Homeostasis. *J Immunol.* 2017;198(5):2133-2146.
201. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature.* 2011;469(7330):323-335.
202. Benjamin JL, Sumpter R, Jr., Levine B, Hooper LV. Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe.* 2013;13(6):723-734.
203. Campieri M, Gionchetti P. Bacteria as the cause of ulcerative colitis. *Gut.* 2001;48(1):132-135.
204. Yang L, Liu C, Zhao W, He C, Ding J, Dai R, Xu K, Xiao L, Luo L, Liu S, Li W, Meng H. Impaired Autophagy in Intestinal Epithelial Cells Alters Gut Microbiota and Host Immune Responses. *Appl Environ Microbiol.* 2018;84(18).
205. Lavoie S, Conway KL, Lassen KG, Jijon HB, Pan H, Chun E, Michaud M, Lang JK, Gallini Comeau CA, Dreyfuss JM, Glickman JN, Vlamakis H, Ananthakrishnan A, Kostic A, Garrett WS, Xavier RJ. The Crohn's disease polymorphism, ATG16L1 T300A, alters the gut microbiota and enhances the local Th1/Th17 response. *Elife.* 2019;8.
206. Seo SH, Unno T, Park SE, Kim EJ, Lee YM, Na CS, Son HS. Korean Traditional Medicine (Jakyakgamcho-tang) Ameliorates Colitis by Regulating Gut Microbiota. *Metabolites.* 2019;9(10).
207. Mendes MCS, Paulino DS, Brambilla SR, Camargo JA, Persinoti GF, Carvalheira JBC. Microbiota modification by probiotic supplementation reduces colitis associated colon cancer in mice. *World J Gastroenterol.* 2018;24(18):1995-2008.

208. Larabi A, Barnich N, Nguyen HTT. New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy.* 2019:1-14.
209. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 2005;1(1):15-25.
210. Aslam M, Ladilov Y. Emerging Role of cAMP/AMPK Signaling. *Cells.* 2022;11(2):308.
211. Grisan F, Iannucci LF, Surdo NC, Gerbino A, Zanin S, Di Benedetto G, Pozzan T, Lefkimmiatis K. PKA compartmentalization links cAMP signaling and autophagy. *Cell Death Differ.* 2021;28(8):2436-2449.
212. Hannon J, Hoyer D. Molecular biology of 5-HT receptors. *Behav Brain Res.* 2008;195(1):198-213.
213. Hu YX, Han XS, Jing Q. Ca(2+) Ion and Autophagy. *Adv Exp Med Biol.* 2019;1206:151-166.
214. Li N, Ghia JE, Wang H, McClemens J, Cote F, Suehiro Y, Mallet J, Khan WI. Serotonin activates dendritic cell function in the context of gut inflammation. *Am J Pathol.* 2011;178(2):662-671.
215. Massey DC, Bredin F, Parkes M. Use of sirolimus (rapamycin) to treat refractory Crohn's disease. *Gut.* 2008;57(9):1294-1296.
216. Mutalib M, Borrelli O, Blackstock S, Kiparissi F, Elawad M, Shah N, Lindley K. The use of sirolimus (rapamycin) in the management of refractory inflammatory bowel disease in children. *J Crohns Colitis.* 2014;8(12):1730-1734.
217. Camilleri M. LX-1031, a tryptophan 5-hydroxylase inhibitor, and its potential in chronic diarrhea associated with increased serotonin. *Neurogastroenterol Motil.* 2011;23(3):193-200.
218. Brown PM, Drossman DA, Wood AJ, Cline GA, Frazier KS, Jackson JI, Bronner J, Freiman J, Zambrowicz B, Sands A, Gershon MD. The tryptophan hydroxylase inhibitor LX1031 shows clinical benefit in patients with nonconstipating irritable bowel syndrome. *Gastroenterology.* 2011;141(2):507-516.
219. Masab M, Saif MW. Telotristat ethyl: proof of principle and the first oral agent in the management of well-differentiated metastatic neuroendocrine tumor and carcinoid syndrome diarrhea. *Cancer Chemother Pharmacol.* 2017;80(6):1055-1062.
220. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet.* 2018;390(10114):2769-2778.

Appendix

Permission to use published articles

Autophagy: roles in intestinal mucosal homeostasis and inflammation

Author: Sabah Haq et al
Publication: Journal of Biomedical Science
Publisher: Springer Nature
Date: Feb 14, 2019

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Jan 05, 2022

This Agreement between Sabah Haq ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	5222641111996
License date	Jan 05, 2022
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	THE FASEB JOURNAL
Licensed Content Title	Tryptophan−derived serotonin−kynurenine balance in immune activation and intestinal inflammation
Licensed Content Author	Sabah Haq, Jensine A. Grondin, Waliul I. Khan
Licensed Content Date	Sep 2, 2021
Licensed Content Volume	35
Licensed Content Issue	10
Licensed Content Pages	22

Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title	ROLE OF SEROTONIN-AUTOPHAGY AXIS IN INTESTINAL INFLAMMATION
Institution name	McMaster University
Expected presentation date	Apr 2022
Requestor Location	Sabah Haq Apartment 1012 1001 Main Street West Hamilton, Ontario, ON L8S1A9 Canada Attn: Sabah Haq
Publisher Tax ID	EU826007151
Total	0.00 CAD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that

you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto
- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS

OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes

all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library
http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Manuscript Title: Disruption of autophagy by increased 5-HT alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease

License

***Science* Journals License to Publish**

I. Requirement of Acceptance:

The following Grant of License ("License") must be signed and returned to the American Association for the Advancement of Science ("AAAS") before a manuscript can be accepted for publication in *Science* or one of its sister journals (*Science Advances, Science Immunology, Science Robotics, Science Signaling, Science Translational Medicine*).

By signing this License, you represent and warrant that you have the authority and all necessary rights to execute this License. For example, if your institution places limitations on publishing agreements or asserts its own right to distribute or provide access to the works of its faculty, you must obtain a waiver from your institution that releases you from such restrictions so that you can sign this License. After publication of your manuscript by AAAS, your institution may exercise all rights to use the Work (as defined below) as are retained by Authors in section III of this License.

If the copyright in the contribution is owned by your employer, your employer or an authorized representative must sign this form.

Should AAAS decide not to publish your manuscript, upon final written notification to you that AAAS will not publish your manuscript, this License shall be null and void. AAAS retains complete discretion as to whether to publish any manuscript, and in what form.

II. Rights of Publication
(https://www.science.org/journals)

In consideration of publication by AAAS of the manuscript currently titled Disruption of autophagy by increased 5-HT alters gut microbiota and enhances susceptibility to experimental colitis and Crohn's disease (abi6442) (the "Work") and authored by Sabah Haq, et. al. ("You"), You hereby grant to AAAS the sole and exclusive, irrevocable right to publish, reproduce, distribute, transmit, display, store, translate, create derivative works from and otherwise use the Work, for any purpose, including commercial purpose, in any form, manner, format, or medium, whether now known or hereafter developed, throughout the world and in any language, for the entire duration of any such right and any renewal or extension thereof and to permit/sublicense others to do any or all of the foregoing as well.

With regard to associated supplemental materials, data, audio and/or video files that You have submitted for publication with Your manuscript, You hereby grant to AAAS the non-exclusive right to publish, reproduce, distribute, transmit, display, store, translate, create derivative works from and otherwise use these supplemental materials in any form, manner, format, or medium, whether now known or hereafter developed, throughout the world and in any language, for the entire duration of any such right and any renewal or extension thereof and to permit/sublicense others to do any or all of the foregoing as well.

You retain copyright, subject to the rights You grant to AAAS above, and all rights not expressly granted in this License. No rights in patents or trademarks or other intellectual property rights other than as described above are transferred to AAAS in this License.

You also authorize AAAS, but AAAS undertakes no obligation to, at its own expense, enforce the rights granted under this license on Your behalf against third parties whom AAAS believes to be infringing the copyright in the Work.

III. Author Rights

For the purposes of this License the "Final Published Version" shall be defined as the final, copyedited and proofed version of the Work that is published by AAAS.

For the purposes of this License the "Accepted Version"shall be defined asthe version of the Work that is accepted for publication by AAAS including changes resulting from peer review but prior to AAAS's copy editing and production.

Some but not all of AAAS's journals make the Final Published Version available under the author's choice of Creative Commons license. Any such options will be made available to You after acceptance and may be subject to an Article Processing Charge ("APC").

A. Except as provided for in Sections III.C and III.D,after publication of the Work by AAAS, You and Your co-authors retain the non-exclusive right to use the Work in the following ways without further permission:

1. Reprint the Final Published Version in print collections of Author's own writings.

2. Reprint the Final Published Version in print format for inclusion in a thesis or dissertation that the Author writes.

3. Present the Final Published Version orally.

4. Reproduce the Final Published Version for use in courses the Author is teaching. (If the Author is employed by an academic institution, that institution may also reproduce it for course teaching.)

5. Distribute photocopies or a PDF of the Final Published Version to colleagues for non-commercial purposes only (providing that recipients are informed that they may not further distribute or copy it).

6. Post a copy of the Accepted Version on the Author's personal website or in their Institution's archival database repository, provided a hyperlink to the Final Published Version on the *Science* website is included and provided the Accepted Version is marked with the following notice: "This is the author's version of the work. It is posted here by permission of the AAAS for personal use, not for redistribution. The definitive version was published in Science Advances 7, (2021-11-05), doi: 10.1126/sciadv.abi6442".

7. Reuse figures and tables created by the Author in future works the Author writes.

8. The Author's employer may use the Accepted Version for non-commercial research purposes. ("Non-commercial research purposes" is defined as research undertaken for purposes other than (a) a profit motive, (b) for commercial exploitation, (c) monetary gain derived from the outcome of the research, (d) research undertaken on behalf of a commercial entity, or (e) other similar purposes.)

9. Author may use or authorize use of Supporting Online Material associated with the Work for any purpose and in any format.

B. Authors of Research Articles, Reports, Reviews, Research Resources or Technical Comments arising out of NIH grants awarded no earlier than 2 May 2005 or who are required by their funding agencies to make their research results publicly available may do so under the following conditions:

The Author may cause the posting, no sooner than 6 months after final publication of the Work by AAAS, of the Accepted Version in their funding body's archive or designated repository provided it includes a hyperlink to the final published version on the Science website and the full reference citation and provided that further use of the posted article copy is restricted to non-commercial research purposes ("Non-commercial research purposes" is defined as research undertaken for purposes other than (a) a profit motive, (b) for commercial exploitation, (c) monetary gain derived from the outcome of the research, (d) research

undertaken on behalf of a commercial entity, or (e) other similar purposes). This policy does not apply to article types that are not specifically mentioned above. The Author must ensure that the Accepted Version is not released on their funding body's archive or [illegible] 6 months after its final publication date in a *Science* journal.

C. If the option to publish the Work under the Creative Commons Attribution 4.0 International Public License (CC BY) was provided by AAAS after acceptance of Your manuscript and You selected such option and paid any applicable APC: After publication of the Work by AAAS, You and Your co-authors may make all uses of the article permitted by the CC BY license, subject to all conditions of that license.

D. If the option to publish the Work under the Creative Commons Attribution-Non Commercial 4.0 International Public License (CC BY-NC) was provided by AAAS after acceptance and You selected such option and paid any applicable APC: After publication of the Work by AAAS, You and Your co-authors may make all uses of the article permitted by the CC BY-NC license (subject to all conditions of that license), plus, if applicable, the uses set forth in III.B. above as well as the following commercial uses:

1. Use the work or a portion of the work as part of a larger work that You create (e.g. use figures in new journal articles or book chapters, use excerpts in new articles, book chapters, blog posts, use the entire work in a collection of Your work), under the following conditions:

a. credit must be given to its first publication in the appropriate volume of the relevant *Science* journal;
b. the author of the larger work must be the same author of the Work published hereunder;
c. republication of the Work or any substantially similar version of the Work in another journal is not permitted.

2. Use and permit others to use the Supplemental Materials for any purpose provided credit is given to its first publication in the appropriate issue of the relevant *Science* journal.

IV. User Access and Rights (applicable only to Works published under a Creative Commons License)

Where AAAS publishes the Final Published Version under a Creative Commons License, AAAS will make the Final Published Version freely accessible online without barriers or embargo. Upon publication, AAAS will also submit the Final Published Versionto PMC/UKPMC for immediate release.

AAAS shall publish the Work under the Creative Commons License selected by the Contributing Author after acceptance. Use of the Final Published Version by Yourself and others shall be subject to the terms of the Creative Commons License that is selected:

1) Creative Commons Attribution 4.0 International Public License (CC BY): This license allows users to copy, redistribute, remix, transform, and build upon a work, in any format or medium, for any purpose including commercial purpose, on a perpetual basis provided they credit the work and the authors. Users must explain any changes that were made from the original and may not suggest the authors endorse the use. The resultant work must be made available under the same terms, and must include a link to the CC BY 4.0 International License.

The full legal code of the Creative Commons Attribution 4.0 International Public License may be found at **https://creativecommons.org/licenses/by/4.0/legalcode (https://creativecommons.org/licenses/by/4.0/legalcode)**

2) Creative Commons Attribution-Non Commercial 4.0 International Public License (CC BY-NC): This license allows users to copy, redistribute, remix, transform, and build upon a work in any format or medium, but only for non-commercial purposes and not for commercial advantage or monetary compensation. These rights are granted on a perpetual basis although users must credit the work and the authors. Users must explain any changes that were made from the original and may not suggest the authors endorse the use. The resultant work must include a link to the CC BY-NC 4.0 International Public License.

The full legal code of the Creative Commons Attribution-Non Commercial 4.0 International Public License may be found at **http://creativecommons.org/licenses/by-nc/4.0/legalcode (http://creativecommons.org/licenses/by-nc/4.0/legalcode)**.

V. Additional Author Representations

You warrant and represent that:

- the Work is original;
- all the facts contained therein are true and accurate;
- the Work does not contain any libelous statements;
- the Work has not been published elsewhere;
- the Work does not infringe upon any copyright, proprietary, or personal right of any third party.

You agree to indemnify, defend and hold harmless AAAS against any claims in respect of the warranties above.

VI. Third Party Content IMPORTANT: If the Work contains any material that is owned or controlled by a third party, You must obtain permission for its use and submit written evidence of that permission to Your Editor and You must clearly acknowledge the source and copyright holder of that material within the text of the Work.

☐ This work was created under U.S. Government Contract

Grant Number:

The AAAS recognizes the U.S. Government's non-exclusive rights to use the Work for non-commercial, governmental purposes where such rights are established in the grant or contract.

Addendum to the License to Publish for Research Funded by cOAlition S Organizations*

THIS ADDENDUM hereby modifies and supplements the License to Publish only with regard to research papers funded by cOAlition S Organizations that have implemented an Open Access policy that aligns with Plan S.

This Addendum does not apply to Works that are not funded by cOAlition S Organizations.

For Works funded by cOAlition S Organizations that have implemented an Open Access policy that aligns with Plan S ("cOAlition S Funded Work(s)"), this Addendum and the License to Publish, taken together, allocate all rights under copyright with respect to all versions of the cOAlition S Funded Work. The parties agree that wherever there is any conflict between this Addendum and the License to Publish, the provisions of this Addendum shall govern and control and the License to Publish shall be construed accordingly.

AAAS licenses back the following rights to the Author in the version of the cOAlition S Funded Work that has been peer-reviewed and accepted for publication, (the "Accepted Version") but not the final, copyedited and proofed version published by AAAS (the "Final Published Version"):

The right to self-archive and distribute the Accepted Version under either a CC BY 4.0 license or a CC BY-ND license, including on the Author's personal website, in the Author's company/institutional repository or archive, and in not for profit subject-based repositories such as PubMed Central, without embargo but only following publication of the Final Published Version.

AAAS' license back of the foregoing rights to the Author under this Addendum does not extend to any material in the Accepted Version that is owned by a third party. The Author is solely responsible for obtaining rights from any such owner for use of that material in connection with the Author's exercise of rights licensed back under this Addendum.

The Author may not replace the Accepted Version with the Final Published Version. The Accepted Version posted must contain a legend as follows: "This is the accepted version of the following article: FULL CITATION, which has been published in final form at [Link to final article]."

Author rights with regard to the Final Published Version remain as stated in the License to Publish.

A list of cOAlition S Organizations is maintained by cOAlition S at* ***https://www.coalition-s.org/plan-s-funders-implementation/ (https://www.coalition-s.org/plan-s-funders-implementation/)*

✔ I Agree

***Signature:** Sabah Haq