

**Characterization of the effect of the lack of chromogranin A
and pancreastatin inhibition on colonic epithelial barrier
integrity and function in male and female mice during colitis**

by

Diane Malu Tshikudi

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Department of Immunology

University of Manitoba

Winnipeg

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THESIS ABSTRACT

Ulcerative colitis (UC), an acute inflammation of the colon, is characterized by long-term care and carries an increased risk of complications, including surgery, sepsis, and cancer. Epidemiological studies have shown that UC affects more men than women, underscoring the need to consider sex as a biological variable in UC therapeutic research. Defective immune response and compromised colonic mucosa integrity and function appear central in UC etiology and pathophysiology. Growing evidence suggests that complete regeneration of the colonic mucosa in UC patients predicts long-term remission, reduced risk of complications, and improved quality of life. However, 80-85% of UC patients fail to achieve complete mucosal healing, highlighting the need to develop effective therapeutic strategies that offer complete mucosal healing for individuals with UC. Chromogranin A (CHGA), a marker for enteroendocrine cells, can undergo proteolytic cleavage and generate Pancreastatin (PST). High levels of colonic CHGA and PST in patients with UC correlate with UC severity, and mediators of inflammation and apoptosis are all known to disrupt the mucosal barrier functions. However, the effects of CHGA and PST depletion on colonic epithelial integrity and mucosal functions are not understood. Here, we characterized the influence of CHGA ablation and PST inhibition with the Pancreastatin inhibitor 8 (PSTi8) on colonic epithelial integrity, mucosal barrier functions, and healing under homeostatic and colitic conditions between sexes. Males and females CHGA knockout or C57BL/6 mice treated with PSTi8, or PBS for six days were treated with 5% dextran sodium sulfate (DSS) to induce colitis or water (control) for five days. We tested markers specific to intestinal mucosa proliferation and differentiation, epithelial cell lineages, and mucosal mediators. These studies demonstrated that female mice are protected against DSS-induced colitis due to higher healing potential, correlating with enhanced epithelial cell proliferation and differentiation and increased mucosal barrier function. Moreover, we reported distinct immune cells and cytokines spatial patterns across colon segments between sexes during experimental colitis, suggesting a link between biological sex, the compartmentalization of immune responses in the colon, and the potential susceptibility of males to colitis. However, the deletion of CHGA correlated with increased production of TGF- β , which likely reduces the abundance of hematopoietic cells, dampens mucosal inflammation and limits intestinal mucosal damage in males. However, the same process promotes a loss of intestinal integrity and mucosal barrier function in females. Similarly, PST inhibition with PSTi8 correlates with reduced epithelial cell differentiation, likely mediated by lower *Atoh1* in females and *BMP4* in males under homeostatic conditions. Furthermore, treatment with PSTi8 correlated with increased susceptibility to colitis in females while offering a protective effect in males. This research highlights notable sex-based differences in colonic mucosal integrity and barrier functions under steady state and colitic conditions. These differences were associated with variations in immune responses along the large intestine and distinct reactions to CHGA deletion and neutralization of PST between males and females.

THESIS SYNOPSIS

This thesis is a sandwich thesis, which encompasses nine chapters and two annexes. The first three chapters, **Chapter I**, **Chapter II**, and **Chapter III**, introduce the Global Literature Review, Global Hypothesis and Objectives, and Global Material Methods, respectively. Chapters IV to VII encompass four manuscripts: one under review, two under preparation, and one published. These four manuscripts are organized as such: **Chapter IV** introduces the first manuscript under preparation, which investigates the influence of sex-related differences on colonic epithelium integrity, mucosal barrier function, and mucosal healing in acute colitis using male and female C57BL/6 mice treated with dextran sulfate sodium (DSS), a well-accepted murine model of UC, to induce colitis. **Chapter V** introduces the manuscript under review in <Biology of Sex Difference> journal and describes the characterization of the immune cell and cytokine profiles along regions of the large intestine in male and female mice under colitic and control conditions. **Chapter VI** describes the second manuscript under preparation, which explores the influence of CHGA deletion on epithelium integrity, mucosal barrier function, and immune response to acute colitis in both sexes using a CHGA knockout murine model of DSS-induced acute colitis. **Chapter VII** presents the manuscript published in <International Journal of Molecular Sciences>. This Chapter investigates the effect of inhibiting pancreastatin (PST), a CHGA-derived enteroendocrine peptide, using PSTi8, a competitive inhibitor of PST, on colon mucosal integrity and healing in both sexes under homeostatic and colitic conditions using DSS-induced acute colitis mouse model. **Chapter VIII** introduces the Global discussion and conclusion, **Chapter IX** covers the Global Studies Limitations, Significance, Future Research Directions, and References. Finally, **Annex I** contains all the tables, which comprise the specifications concerning the primers and antibodies

used to perform qRT-PCR, flow cytometry, and immunofluorescence techniques in this thesis. **Annex II** contains the review paper published in <Nature Reviews Gastroenterology & Hepatology>, which summarizes current knowledge of the effect of biological sex on IBD, highlights research gaps, and recommends best practices in research methodology to integrate sex in IBD research. This thesis summarizes four years of research investigating the influence of sex-related differences on colonic epithelium integrity, mucosal barrier function, healing, and immune response using a murine model of DSS-induced acute colitis. Furthermore, this thesis characterizes the influence of two enteroendocrine mediators, CHGA and PST, on colonic epithelial integrity, mucosal barrier function, and mucosal healing in male and female mice in acute colitis. We demonstrate that males display increase susceptibility to colitis, which correlates with loss of epithelium integrity and mucosal barrier function. Both sexes display distinct immune cells and cytokines spatial patterns across segments of the LI during colitis. Similarly, CHGA deletion and PST inhibition with PSTi8 influence the intestinal epithelial integrity, mucosal barrier function, mucosal healing, and immune cell type profile in homeostatic and acute colitis in a sex-dependent manner.

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ABBREVIATIONS

A4	Androstenedione
ADT	Androgen deprivation therapy
ARs	Androgen receptors
ATG16L1	Autophagy proteins autophagy related 16 Like 1
Bmi1	Polycomb complex protein
Breg	Regulatory B cells
CA2	Carbonic anhydrase 2
CCR2	C-C motif chemokine receptor 2
CRP	C-reactive protein
CXCR1	C-X-C motif chemokine receptor 1
CHGA	Chromogranin A
Cld4	Claudin 4
CRAMP	Cathelin-related antimicrobial peptide
CST	Catestatin
CTSD	Cathepsin D
DAI	Disease activity index

DAPI	4', 6-diamidino-2-phenylindole
DCLK	Doublecortin-Like Kinase 1
DLL	Delta-like DLL1
DHT	Dihydrotestosterone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DSS	Dextran sulphate sodium salt
DTT	Dithiothreitol
E1	Estrone
E2	17 β -estradiol
E3	Estriol
E4	Estetrol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EICs	Extraintestinal complications (EICs)
EIMs	Extraintestinal manifestations (EIMs)
ELISA	Enzyme-linked immunoassay

ER-stress	Endoplasmic reticulum
Er- α , β	Estrogen receptor alpha, beta
EDEM2	ER degradation-enhancing alpha-mannosidase-like 2
FBS	Fetal bovine serum
FIZZ	Found in inflammatory zone 2
FOXP3	Forkhead box P3
FPR2	Formyl peptide receptors
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
(GFI)1	Growth factor-independent 1
Gp2	Glycoprotein 2
GP1R	G protein-coupled estrogen receptor 1
GWAS	Genome-wide association studies
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
Hopx	Homeodomain-only protein homeobox
HRE	Hormone-response elements
HRT	Hormone replacement therapies

IBD	Inflammatory bowel disease
IELs	Intraepithelial lymphocytes
IFN- γ	Interferon gamma
IgA, M	Immunoglobulin A, M
IL	Interleukin
ILC1, 2, 3	Innate lymphoid cell 1, 2, 3
JAK2	Janus kinase 2
Klf4	Kruppel-like factor
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
LPS	Lipopolysaccharide
Ly6	Lymphocyte antigen 6 complex
Lypd8	Ly6/Plaur domain-containing 8
MCFA	Medium-chain-fatty acids
MMP	Metalloprotease
MPO	Myeloperoxidase
Muc2	Mucin 2
NETs	Neutrophil extracellular traps

NKCC1	Na-K-2Cl cotransporter
NLRP6	NOD-like receptor pyrin family domain containing 6
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain containing 2
RANKL	Nuclear factor kappa B ligand
OCP	Oral contraceptive pills
OS9	Osteosarcoma Amplified 9
PAMPs	Pathogen-associated molecular patterns
P4	Progesterone
PBS	Phosphate buffer saline
POU2F3	POU domain, class 2, transcription factor 3
PR	Progesterone receptors
PRRs	Pathogen recognition receptors
PSC	Primary sclerosing cholangitis
PST	Pancreastatin
PSTi8	Pancreastatin inhibitor-peptide-8
RELM β	Resistin-like Molecule beta

ROR γ t	Retinoic acid–related orphan-receptor-gamma
ROS	reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
Satb	Special AT-rich sequence binding protein 1
Spib	SPI1-related protein B
STAT3	Signal transducer and activator of transcription 3
SOCS3	Suppressor of cytokine signaling 3
SOX9	SRY-Box Transcription Factor 9
SOX4	SRY-related HMG-box 4
TBP	TATA-binding protein
Tert	Telomerase reverse transcriptase
TFF3	Trefoil factor 3
TGF- β	Transforming growth factor beta
Th1, 2, 17	T helper 1, 2, 17
TJ	Tight junction
TLR	Toll-like receptor

TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF- α	Tumor necrosis factor alpha
TR	Taste receptor
TCF7	Transcription Factor 7
TRPM5	Transient Receptor Potential Cation Channel Subfamily M Member 5
UC	Ulcerative colitis
RPR	Unfolded protein response
WFDC2	WAP four-disulphide core domain protein 2
ZO1	proteins zonula Occludens

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PREFACE

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DEDICATION

Je dédie ce mémoire à ma famille. À Mes parents sans qui je ne serais pas où je suis et qui je suis et qui m'ont toujours encouragé et permis d'assouvir mes curiosités. À mon frère et mes sœurs. Merci pour votre soutien tout au long de ces 5 ans. Merci de m'avoir tendu l'oreille lorsque j'en avais besoin et taquiné lorsque nécessaire. Je vous remercie de me rappeler au monde, de me donner une vision du réel au-delà de la tour d'ivoire mental dans lequel je peux parfois m'enfermer lorsque je travail. Le fait de voir le monde à travers vous m'a offert le détachement qui m'a servi à relativiser tous les tracassés rencontrés au cours de ce doctorat.

GLOBAL INTRODUCTION

Ulcerative colitis (UC) is an acute inflammation of the colon and rectum characterized by periods of relapse, remission, and long-term care (1). UC is associated with an increased risk of intestinal surgery and deaths caused by complications such as sepsis and cancer. It is estimated to affect the quality of life of 120,000 Canadians, 10-20% of whom are children and youth under 18 years old (2–4).

Although the cause of UC remains elusive, defective immune response, compromised intestinal epithelial barrier function, and abnormal gut epithelium-microbiome interaction appear to play a central role (1). Growing evidence suggests that epithelial cells that form the gut mucosa play a crucial role in gut wall homeostasis (5,6). Furthermore, complete regeneration of the colonic mucosa in UC patients predicts long-term remission, minimizes hospitalizations, lowers the risk for surgical treatment, and improves UC patients' quality of life (6–8). In recent years, efforts have been made to develop therapies to control disease symptoms and maintain UC patients in symptomatic remission (9,10). However, despite the advances in UC therapeutics, which include aminosalicylate, corticosteroids, antimetabolites drugs (folic acid antagonist methotrexate and purine analog thiopurines), calcineurin inhibitors, anti-tumor necrosis factor (TNF)- α , anti-integrin drugs, an estimated 38% of UC patients are refractory or lose response to therapy, and 80-85% fail to achieve complete mucosal healing (9–13). These factors accentuate the need to develop effective therapeutic strategies that offer complete mucosal healing and long-term remission to advance patients' quality of life with UC (13).

Physiological differences due to biological sex are widely recognized for impacting immune responses to both self and foreign antigens (14). For example, population-based clinical and preclinical animal studies report a higher risk of developing UC in males than females. This emphasizes the need to consider sex as a biological variable when investigating therapeutic strategies (15).

Chromogranin A (CHGA), a marker for enteroendocrine cells, can undergo proteolytic cleavage to generate biologically activated peptides such as Pancreastatin (PST). CHGA and PST are highly expressed in blood, colonic tissues, and feces in patients with UC (16,17). They significantly correlate with UC severity and markers of disease activity, such as higher pro-inflammatory and pro-apoptotic mediators and reduced anti-inflammatory molecules, all known to disrupt the mucosal barrier function (17). In a chemically induced-colitis model mediated by dextran sulphate sodium (DSS), our laboratory reported that lack of CHGA in male mice reduced proinflammatory cytokines and increased anti-inflammatory and mucosal healing markers (17). Interestingly, elevated PST was also associated with a decreased mucosal healing process in human colonic epithelial cell lines (18).

Moreover, our preliminary data indicate that PSTi8 reduces inflammation and disease activity in animal models of colitis. However, little is known about the effect of CHGA and PST on colonic epithelial barrier function and gut mucosal integrity. Given all these factors, I hypothesize that CHGA and PST disrupt gut epithelial cells and the epithelial barrier function, impairing mucosa healing in male and female mice with colitis. I also propose investigating PSTi8 as a potential treatment for mucosa repair.

CHAPTER I

GLOBAL LITERATURE REVIEW

1.1. The large intestine anatomy and physiology

The large intestine is a component of the gastrointestinal (GI) tract and consists of the appendix, caecum, colon, and rectum (19). In humans, the colon is subdivided into ascending, transverse, descending, and sigmoid regions, which are segmented in pouches called Haustra (20).

The large intestine, also interchangeably referred to as the colon, has three primary functions: the absorption of water and electrolytes, the production and recovery of vitamins from microbiota-mediated luminal contents fermentation, and the formation, storage, and elimination of feces (21,22). Like most of the GI tract, the large intestine consists of several tissue layers, including the serosa, muscularis propria, submucosa, and mucosa, and they serve multiple specialized functions (19,23). The serosa, a smooth membrane composed of a thin and loose layer of connective tissue overlaying a single sheet of mesothelium, protects the large intestine's outer surface (19). The muscularis, consisting mainly of smooth muscle cells, plays an essential role in GI motor activity (19). The submucosa is a dense network of connective tissues and cells such as fibroblasts, lymphocytes, eosinophils, macrophages, plasma cells, and mast cells (23). Further, to serve as a scaffold for the enteric nervous system and blood and lymphatic vessels, the submucosa also supports the absorptive function of the mucosa (19,23,24).

The colonic mucosa comprises the muscularis mucosae, lamina propria, and epithelium. The mucosal layer is involved in the large intestine's absorptive, secretive, and protective barrier

functions (25). While every layer of the large intestine is essential for proper functioning, the importance of the colonic mucosa structure and functions has recently gained much attention. Growing evidence suggests that epithelial cells play a crucial role in gut wall homeostasis by regulating mucosa regeneration (5,6). Recent studies have associated disrupted mucosal structure and function with both acute and chronic gut inflammation, contributing to the onset and progression of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC) (26). In contrast to CD, inflammation in UC is mainly restricted to the mucosal layer of the large intestine. In recent years, studies have shown that complete colonic mucosal regeneration, also known as healing, which is defined as the absence of both macroscopic (endoscopic) and microscopic (histological) mucosal ulceration in UC patients, can predict long-term remission, minimized hospitalizations, lowered the risk for surgical treatment, and overall, improves patients' quality of life (6–8,27,28). Therefore, advancements in understanding UC's large intestine mucosal dysfunctions are essential in improving UC diagnosis and treatment.

1.2. Ulcerative colitis

UC, a GI disorder, is a chronic and life-long acute mucosal inflammation of the rectum and colon characterized by fluctuating periods of relapse associated with mucosal erosion followed by periods of remission (29). Most prevalent in industrialized countries, the incidence rate of patients with UC is rising worldwide, with rates ranging from 0.15 (Philippines) to 57.9 (Faroe Islands) cases per 100.000 persons/year, potentially associated with a worldwide increased westernization, becoming an emerging global health problem (30). The onset of UC has a bimodal age distribution

with a predominant peak between 20 and 30 years and a second and smaller peak between 50 and 80 years (29,31). Yet, UC can occur at any age and is accompanied by clinical symptoms such as diarrhea, rectal bleeding, abdominal pain, and weight loss. The histological features of UC include lymphoplasmacytic infiltration, mucin depletion, and crypt distortion (29).

In UC, inflammation is mainly restricted in the large intestine, where the disease commonly starts in the rectum and progresses toward part or the entire large intestine (32,33). The phenotype of UC at diagnosis is primarily stratified into proctitis (rectum), proctosigmoiditis (rectum to the sigmoid colon), left-side colitis (rectum to splenic flexure), pancolitis (entire colon), and cecal patch (cecum) depending on the extent and location of the inflammation (33). As a systemic and incurable disorder, longstanding UC is associated with an increased risk for colectomy, toxic megacolon, and colorectal cancer (34,35). Consequently, factors such as the early onset of UC, fluctuating disease course, and lifelong medical care significantly diminish UC patients' quality of life and impose a significant burden on the healthcare system (13,31,36).

Despite extensive studies, the mechanisms that mediate UC etiology remain unclear. Factors such as host genetic susceptibility, environmental factors, abnormal gut epithelium-microbiome interaction, compromised intestinal epithelial barrier function, and impaired immune response appear to play a central role in the pathology of UC. Therefore, a better understanding of UC etiology and the natural history of the disease are critical to propose preventive and therapeutic strategies to remediate UC pathogenesis and pathophysiology (31).

1.3. Chemically inducible dextran sodium sulphate (DSS) mouse model of UC

Numerous animal models have been developed over the past years to study the underlying mechanisms driving the pathogenesis of IBD (37). These IBD models can be categorized based on the method of colitis induction into spontaneous, genetically modified, adoptive transfer, and chemically inducible models of colitis (38). One of the chemically inducible animal models, dextran sodium sulfate (DSS)-induced colitis, is the most widely utilized model due to its rapidity, simplicity, reproducibility, and controllability (39). Oral administration of DSS to mice through drinking water at various concentrations and frequencies can lead to the development of acute, chronic, or relapsing models of intestinal inflammation that mimic many clinical, histological, and immunological features of UC. Clinical manifestations of DSS-induced acute colitis include diarrhea, bloody stools, weight loss, short colon length, anemia, and, in severe cases, death. Histological changes associated with this condition include mucosal ulceration, loss of goblet cells, mucin depletion, crypt damage, submucosal edema, infiltration of inflammatory cells (neutrophils and mononuclear cells), and crypt abscesses (39–41). The clinical manifestations of DSS-induced chronic colitis do not correspond with the degree of inflammation or histological characteristics observed in the large intestine. However, these manifestations are linked to several histological features, including mucosal infiltration by mononuclear leukocytes, lymphocytosis, transmural inflammation, disorganization of the crypt architecture, re-epithelialization of the distal colon, and dysplasia resulting from colitis (39).

DSS effectiveness in inducing colitis is affected by several factors, including DSS molecular weight, concentrations, duration and frequency of administration, mice's genetic background, sex,

and the gut microbial environment (38). DSS, a water-soluble polyanionic sulfate polysaccharide with a molecular weight ranging between 5-1400 kDa, has a distinctive effect in the induction of colitis. Thus, mice treated with DSS 5kDa develop a mild form of colitis associated with patchy lesions, which predominantly affect the cecum and proximal colon. In contrast, DSS 36-50 kDa causes a severe form of colitis throughout the large intestine, exhibiting more severe histological features in the mid and distal colon. Meanwhile, the higher molecular weight DSS of 500 kDa does not induce colitis, likely because its large size prevents it from penetrating the mucosa (42). Moreover, studies suggest that the number of sulfate functional groups per molecule of DSS critically influences DSS-mediated colitis induction. Thus, DSS molecules with sulfur content lower than 16.6% are associated with reduced colitis induction and may be less abundant in low molecular weight DSS molecules, which helps explain mild colitis (42). In addition to the molecular weight, DSS-mediated colitis severity correlates with DSS concentrations. Thus, colonic mucosal injury is generally induced by continuous administration of 2-5% DSS (36-50 kDa) for 4 to 9 days, whereas continuous or cyclic exposure to low concentrations of DSS in susceptible mouse strains, such as C3H/HeJ, NOD/LtJ, and C57BL/6J, to induce chronic colitis (39,41). Moreover, the mucosal injury induced by DSS differs anatomically between murine strains with different genetic backgrounds. For example, the DBA/2J murine model has increased susceptibility to DSS-mediated inflammation at the cecum but is resistant in the colon, contrasting with C57BL/6J mouse strong cecal resistance and enhanced susceptibility to DSS-mediated inflammation, potentially due to either difference in mice strains ability to withstand the mucosal damage through the healing process or limit intestinal inflammation (43).

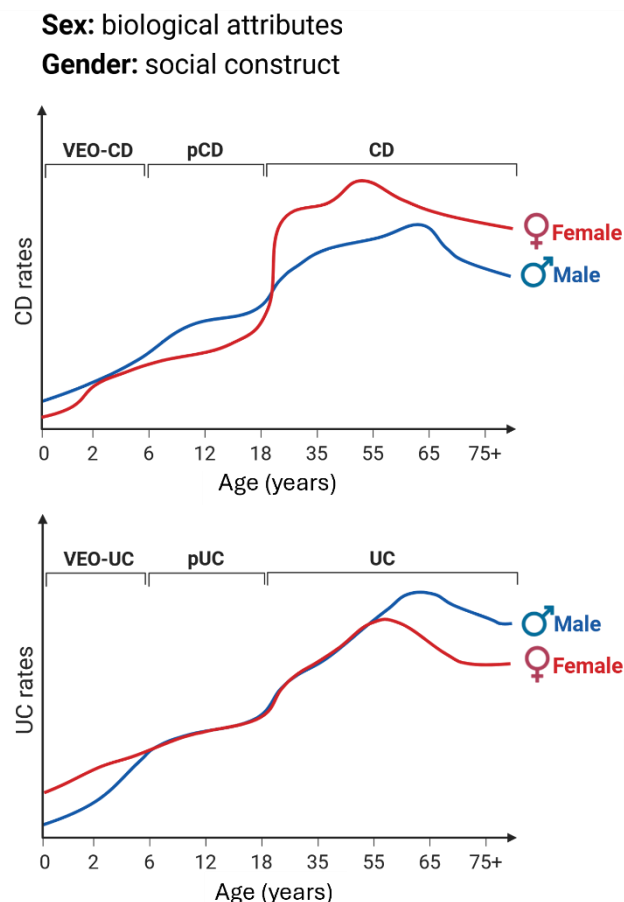
Although the mechanism by which DSS-induced colitis occurs remains poorly understood, studies have reported that DSS complexed with medium-chain fatty acids (MCFAs), which are highly abundant in the colonic lumen, is absorbed and partially metabolized by colonic epithelial cells (44). The complex DSS-MCFAs form nanovesicles that fuse with the membranes of epithelial colonocyte cells. This fusion allows the transport of DSS into the cell cytoplasm, where it might interfere with reverse transcriptase and ribonuclease activities. Such interference could disrupt critical cellular functions, leading to intestinal epithelial cell cycle arrest and apoptosis (44–46). Moreover, MCFAs have been used to promote epithelial permeability to hydrophilic molecules as they can induce the redistribution of tight junction (TJ) proteins zonula Occludens (ZO)-1 and occluding (47). The MCFAs-DSS complex might alter the TJ mucosal barrier functions and promote DSS transport within the colonic mucosa (44). In agreement with this view, studies reported that DSS increased the colonic epithelium paracellular permeability 1-2 hours following administration and accumulated within the nuclei of various cells within the lamina propria (48).

Overall, the mainstay view suggests that DSS harms the epithelial barrier by reducing the number of proliferating epithelial cells through cell cycle arrest and disrupting the mucosal barrier functions. This enables the infiltration of microbiota into the lamina propria, subsequently leading to the recruitment and activation of neutrophils, macrophages, and T cells during the acute phase while also causing dysplasia and the formation of colorectal adenocarcinoma in chronic conditions (46,48,49).

1.4. Sex-based difference in ulcerative colitis (See review in Annex II for additional information)

Many studies have demonstrated the importance of sex-based differences in the pathogenesis of immune-mediated disorders, and UC is no exception (14) (**Figure 1**). Despite being conflicting, several clinical studies report sex-related differences in the incidence, presentation, disease progression, response to treatment, and complications among UC patients. Overall, in Westernized countries, men are 20% more likely to develop UC than women, albeit the sex ratio in UC prevalence depends on age and geographic location (50). Thus, a population-based study that included 16 Westernized countries and 478 million people showed that girls aged 5 to 9 had a 22% higher risk of being diagnosed with UC compared to boys at matching age. Throughout an individual's reproductive years, the incidence rate of UC was similar between men and women but shifted during middle age (age group 40-44 years), as the rate of women diagnosed with UC decreased significantly compared to men (50). However, a similar study conducted in Asia-Pacific populations with 567.8 million people reported that the incidence rate of UC was 20-42% higher in men than women ($P<0.05$) across all ages (15). These epidemiological studies, in line with several other studies, suggest that biological sex is a variable risk factor for UC.

Sex Difference in IBD Epidemiology and outcome



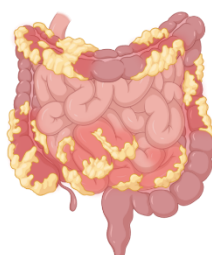
inflammatory bowel diseases (IBD)



pIBD:
 ↑ anemia, infection (F vs M)
 ↑ mood disorders (F vs M)
 ↓ malnutrition (F vs M)

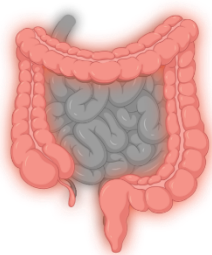
IBD:
 ↑ arthritis (F vs M)
 ↓ osteoporosis (F vs M)
 ↑ tx intolerance (F vs M)

Crohn disease (CD)



VEO-CD:
 ↓ hospitalization (F vs M)
pCD:
 ↓ ileal disease (F vs M)
 ↑ colonic disease (F vs M)
 ↑ proctitis (F vs M)
 ↑ severity/EIM (F vs M)

ulcerative colitis (UC)



VEO-UC:
 ↓ outpatient visits (F vs M)
 ↑ colectomy (F vs M)

Figure 1: **Sex differences in inflammatory bowel disease (IBD) epidemiology across age groups.** Females (F) and males (M) show significant differences in Crohn disease (CD) and ulcerative colitis (UC) incidence and outcomes across ages from very-early (VEO) and pediatric (P) to adult CD and UC onset (Figure extracted from Review (51)) .

A few clinical studies have shown no significant differences in UC disease activity, inflammatory burden, and disease progression between sexes (52–54). Some studies suggest that men face a higher risk of more severe disease associated with extensive colitis. However, this finding conflicts with others that demonstrated higher remission rates for men while women experienced increased disease activity with UC (52,55,56).

UC is commonly associated with extraintestinal symptoms, including extraintestinal manifestations (EIMs) and complications (EICs). EIMs commonly occur at distant sites and are inflammatory disorders involving multiple organs, such as the musculoskeletal, dermatologic, oral, hepatopancreatobiliary, ocular, metabolic, and renal systems (57). Affecting 6-47% of patients with UC, EIMs can result from the underlying active disease, peripheral arthritis, or erythema nodosum, an inflammation of subcutaneous fat tissue, are perfect examples (34,58). Conversely, EIMs such as primary sclerosing cholangitis (PSC) (Bile duct) and ankylosing spondylitis (Spine joint) can also occur independently from UC disease activity (15). Research shows more consistent findings regarding sex-related differences in EIMs. Most studies report an overall higher prevalence of EIMS, including erythema nodosum, pyoderma gangrenosum, rheumatoid arthritis, and psoriatic arthritis in women compared to men with UC, who experienced PSC more frequently (15,59). By contrast, although limited and conflicting, some studies described a higher incidence of complications in male patients with UC as defined by the higher risk for surgery such as proctocolectomy and colectomy, which consists of the removal of the colon and rectum (15,60). Complications in UC can be either disease-driven, including severe GI hemorrhage, toxic megacolon, perforation, stenosis, and cancer, or they can result from treatment complications, infections, and hematopathology disorder (61). Surgical interventions are necessary for 15-18.9%

of patients with UC 10 years post-diagnosis. They are primarily indicated for patients with chronic symptoms that are medically uncontrolled, high-grade dysplasia associated with an increased risk for cancer, or emergencies due to severe colitis, megacolon, or perforation (34,35). Thereby, population-based cohort studies involving UC patients from the USA reported that men were twice as likely as women to undergo a colectomy (60). However, the etiopathology of UC-mediated extraintestinal disorders and the influence of sex bias in EIMs and EICs pathologies are not fully understood (62).

A study using preclinical DSS-mediated colitis in different inbred mouse strains found worsened histological outcomes, including more severe lesions and hyperplasia, in male mice compared to their female counterparts (43). Similarly, another study reported that the C57BL/6 female mice with colitis had reduced disease activity index, lower macroscopic and histological scores, and lower tumor necrosis factor (TNF)- α concentration than C57BL/6 male mice with DSS-mediated colitis, demonstrating a biological impact of sex in this setting (63). However, the underlying mechanisms leading to sex-related differences in UC and colitis are unknown and will, therefore, require further investigation.

1.5. Sex hormones in UC

Sex hormones, including estrogens, progesterone, and androgens, exert pleiotropic functions in various tissues and organs, including the immune system and GI tract, and modulate the development and function of mammalian reproductive systems (64). These hormones are primarily synthesized in the gonadal organs (testis and ovaries), but also in lesser amounts by

tissues such as the placenta, skin, liver, heart, adipose tissue, adrenal glands, brain, and intestinal mucosa. Sex hormones fluctuate daily between sexes, across a lifespan, and during reproductive and non-reproductive periods, including puberty, menstruation cycle, pregnancy, post-partum, and menopausal transition (65,66).

The most predominant female sex hormones include progesterone (P4) and four main types of endogenous estrogens, including estrone (E1), 17 β -estradiol (E2), estriol (E3), and estetrol (E4) (65,67). E2 is the most potent and dominant female estrogen during the reproductive years (67). E1 is present in more significant amounts post-menopause, whereas E3 and E4 are primarily secreted during pregnancy. All three are weaker isoforms of estrogens (67). P4 and estrogens are also present in males but in lower concentrations. In males, P4 is mainly synthesized in the gonadal tissues and adrenal glands, while 80% of circulating estrogens arise from the extragonadal conversion of androgens into E1 and E2 within tissues (68,69).

Androgens are the predominant sex hormones in adult males while being produced in smaller amounts in females, and comprise testosterone, its more potent metabolite, dihydrotestosterone (DHT), as well as pro-androgens dehydroepiandrosterone (DHEA) dehydroepiandrosterone sulfate (DHEAS), and androstenedione (A4) (64). In males, testosterone, mainly produced in the testes, accounts for 90-95% of total circulating androgens (70). Circulating testosterone can be irreversibly converted to DHT, which has 5-10-fold greater affinity to androgen receptors (ARs), and in E2 by the 5 α -reductase and aromatase enzymes, respectively (70–72). The androgens with lower affinity to ARs, DHEA, DHEAS, and A4, produced by the adrenal cortex, can be metabolized through multiple pathways to produce additional testosterone or DHT in peripheral tissues (72,73). The most abundant circulating androgen hormones during the

reproductive period in females are, in order of concentration, the pro-androgens DHEAS, DHEA, and A4, and at significantly lower concentrations, testosterone and DHT (74). In females, pro-androgens are secreted primarily by the ovaries and adrenal cortex, while 50-75% of testosterone and DHT arise from extragonadal conversion in the peripheral tissues and circulation (74,75).

Sex hormone fluctuation throughout an individual's life span may translate into changes in the GI tract and contribute to the disparity between sexes in UC. Thus, in prepubertal, healthy pediatric individuals, studies showed that females had higher concentrations of E1, E2, and androgen metabolites (Androsterone, etiocholanolone, and DHT) than males, but testosterone concentrations were comparable in both sexes (76–78). As individuals progress through puberty (ages 11 to 16) and transition into adulthood, sex hormone levels increase, shift between sexes with elevated androgens in boys and higher estrogens in girls, and peak between 20-29 years (78,79). However, as individuals age, the concentration of sex hormones gradually decreases (79). Menopausal women experience a drastic decrease in E2 and P4 concentrations, similar to or lower than that observed in men (79–81). Similarly, the serum concentration of testosterone, E2, E1, and P4 also decreased with age in men, although not to the same extent as in women (79–81).

While still under investigation, current studies suggest a pivotal regulatory role for sex hormones in gut microbiota, intestinal epithelial barrier function, and immune response. These may result in sex-based differences in gastrointestinal functions, UC pathogenesis, and pathophysiology (**See Review (51)**).

Thus, fluctuation of sex hormones associated with the menstrual cycle can modulate GI tract function, leading to GI symptoms such as constipation, nausea, vomiting, abdominal pain,

flatulence, fatigue, irritability, and anxiety, among others, and likely worsen UC symptoms in women (82–84). Research suggests that uterine prostaglandin E₂, which increases during menstruation, promotes inflammation and consequently alters GI function in IBD by reducing microbiota-dependent intestinal Tregs. Clinical studies investigating the effect of oral contraceptive pills (OCPs) and hormone replacement therapies (HRT) on the risk of developing UC reported that women exposed to OCPs and HRT were more likely to develop UC compared to non-exposed women (85–89). Moreover, research showed that women with endometriosis, an estrogen-dependent condition, were more likely to develop UC than women in the general population. These studies suggest a potential role for elevated estrogen levels in UC etiopathology.

In the preclinical animal model of colitis, the effects of estrogen supplementation differed between the experimental models of colitis utilized (63,90,91). Studies reported that the C57BL/6 female mice with colitis had reduced disease activity index, macroscopic and histological scores, and lower TNF- α concentration than C57BL/6 male mice with DSS-mediated colitis (63). In the same study, E₂ supplementation in the ovariectomized female mice further improved their resistance to DSS-induced colitis but not in ovariectomized male mice (63). Consistent with these findings, E₂ treatment diminished the histological score and colitis severity in 2,4,6-trinitrobenzene sulfonic acid (TNBS), *Helicobacter hepaticus*, and acetic acid-induced mice colitis, as well as HLA-B27 transgenic rats (63,91). In contrast, another study found that treatment with a supraphysiological concentration of E₂, akin to levels observed during pregnancy, increased disease severity and histological scores in C57BL/6 female mice compared to untreated mice in DSS-induced colitis (90). While outcomes in males are consistently worse compared to females, E₂ treatment in ICR male mice with colitis-associated colon carcinogenesis promoted the

expression of genes associated with the mucosal barrier and tight junctions along with reduced cytokines gene expression (92). In addition to being model-dependent, the discrepancies resulting from E2 supplementation might also stem from variations in E2 doses administered and likely point toward the need to investigate the effect and underlying mechanism implicated in physiologically relevant estrogen doses in UC etiology and pathophysiology.

In contrast to the extensive research investigating the relationship between estrogens and UC, few studies explored the impact of progesterone and androgens on UC etiopathology. Moreover, less is known about their role in men. However, chronic states of low-grade inflammation are known to decrease testosterone levels (93). A cohort study conducted on men with prostate cancer who underwent androgen deprivation therapy (ADT) reported a lower risk for UC (94). Conversely, another clinical study found that higher testosterone levels did not correlate with women's risk of developing UC (95). A preclinical study reported a lack of effect for testosterone treatment in orchiectomized male mice with DSS-induced colitis (63). This finding indicates a potential role for androgen in UC pathogenesis and warrants further investigation.

1.6. Sex hormone receptors in UC

In addition to reproductive organs, sex hormones regulate many tissues, including the hematopoietic system and GI tract. Sex hormones mediate various physiological cellular activities by binding to their corresponding receptors, including estrogen receptor (ER) α , ER β , and membrane G protein-coupled estrogen receptors (GPER/GRP30), progesterone receptor (PR)-A, PR-B, and PR-C, and androgen receptors (AR) (66,96) (**Figure 2**). Beyond classical sex-hormone

receptor isoforms, several alternatively spliced isoforms of sex hormone receptors with incomplete functional domains have also been identified in different tissues, including the GI tract, yet the functional role of these variants in the context of IBD is still poorly understood (97,98). As part of the steroid hormone nuclear receptors family, sex hormone receptors located in the cytoplasm, cellular and nuclear membranes, are transcription factors that exert their intracellular effect through the classical genomic and non-genomic signalling pathways (96). Typically, sex hormones binding to their corresponding receptors cause conformation changes that lead to the dissociation of chaperone molecule heat shock proteins and activation of sex hormone receptors (99). Sex hormone receptors activated in the cytoplasm, translocate to the nucleus, where they dimerize and can either directly bind to corresponding and specific hormone-response elements (HREs) DNA sequences or interact indirectly with DNA by complexing with various coregulatory molecules and transcription factors (99). On the other hand, membrane-localized sex hormone receptors can also mediate physiological changes independent of their genomics action through interaction with kinase pathways to propagate a more rapid response and promote cellular processes such as autophagy, proliferation, apoptosis, survival, and differentiation, which all influence pathophysiology in UC (99). Of note, sex hormone receptors' genomic and non-genomic signalling pathways have reciprocal influence.

In recent years, a growing body of literature has proposed an essential role for sex hormone receptors, particularly ERs, in UC pathophysiology (100–104). Although all three estrogen receptors are co-expressed in the colon, ER- β is the most prevalent isoform expressed in a healthy colon (90,103,105–109). ER- β are detected in endothelial, enteric neuron, smooth muscle, immune and terminally differentiated epithelial cells, and potentially intestinal epithelial stem cells, ER α is

mainly expressed in the lamina propria by macrophages and enteric neuron. At the same time, the presence of GPER has been shown in a plethora of cells in the colon, including mast, enteric neurons, mesothelial, endothelial, smooth muscle and epithelial cells (103,107–111).

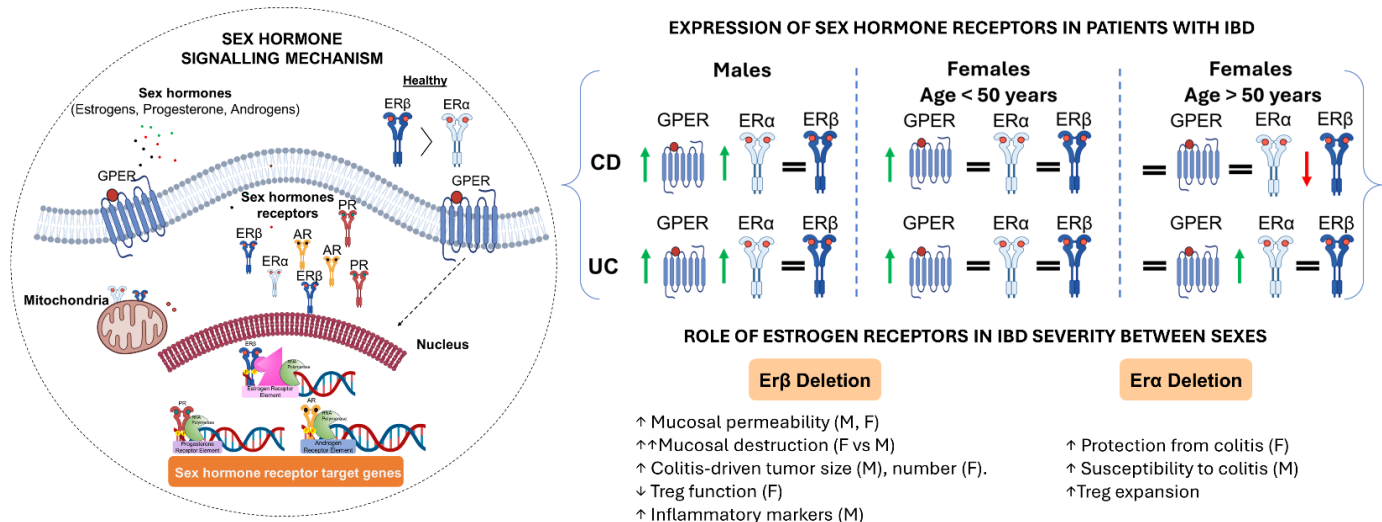


Figure 2: Role of estrogen receptors in the discrepancy of inflammatory bowel disease (IBD) severity between sexes. Sex hormones impact the gastrointestinal tract through the interaction with the nuclear receptor family, estrogen receptor (ER)α, ERβ, G protein-coupled estrogen receptors (GPER), progesterone receptors (PRs), and androgen receptors (ARs). Sex hormone receptors can function as transcription factors or form multiprotein complexes to initiate or suppress gene expression and promote epigenetic modification. Erβ is the most abundantly expressed estrogen receptor in healthy individual intestinal tissue. However, IBD alters the expression of estrogen receptors differentially between sexes in an age-dependent manner for females. “=” sign indicates a lack of significant difference in the expression of estrogen receptors in intestinal tissues from patients with IBD compared to healthy individuals.

Although clinical studies reported altered expression of ERs in patients with UC, these changes differ between studies and in a sex and age-dependent manner. Thus, several studies using biopsies from patients with UC reported a reduction in ERβ concomitant with elevated ERα in intestinal tissues from UC patients (111,112). By contrast, a study by Jacenik and colleagues showed

increased ER α and GPER expressions with no significant changes in ER β levels in intestinal tissues from UC, regardless of age and sex (113). However, when segregated by sex, men with UC had increased ER α and GPER protein levels compared to healthy individuals, while ER expression was age-dependent in women with UC (113). In women under 50 years of age, the expression of GPER was elevated in UC, whereas, ER α levels did not differ compared to healthy individuals (113). Conversely, over 50 years old, ER α was elevated in women with UC, while GPER remains unaltered (113).

Preclinical studies suggest fundamental differences between males and females in ER- α and ER- β signalling pathways. A finding by Goodman and colleagues described sex-dependent disparities in the role of ER α and ER β in DSS-mediated colitis (114). This study showed that the deletion of ER α protected female mice against DSS-induced colitis but promoted male mice's susceptibility to colitis. The discrepancy in disease severity between sexes in the absence of ER α was associated with sex-mediated differential expressions of estrogen-responsive genes suppressor of cytokine signaling (*Socs*)3, *Fos*, and cathepsin D (*Ctsd*) (114). Thus, loss of ER α led to reduced *Ctsd* gene expression in females, increased *Fos* in males but not females, and elevated *Socs3* gene expression in males and females with DSS-mediated colitis (114). By contrast, the same study reported that ER β loss-of-function exacerbated colitis severity in females but protected male mice. This finding contrasted in part with a study that revealed that activation of ER β promoted the NOD-like receptor pyrin family domain containing 6 (NLRP6)-mediated autophagy, which promoted the elimination of inflammasome, cytokines, and damaged mitochondria and, as a result, inhibited DSS-mediated colitis in male mice (115). Moreover, findings by Goodman and colleagues differ from a study that reported that in the absence of ER β , expression of inflammatory markers increased in both

sexes but most notably in male mice and correlated with an elevated number of colitis-driven tumors in males and tumor sizes in females (116).

In contrast to the extensive number of research investigating the role of ERs in UC etiology and pathophysiology, very few studies explored the role ARs and PRs in the GI tract and study investigating ARs and PRs signaling pathways in UC are lacking. However, studies have described a weak PR expression detected in rare mesenchymal cells located in the lamina propria and epithelial cells in the crypt (100,117). Similarly, ARs have been found expressed in smooth muscle cells, enteric neurons, and mesenchymal cells but not in the intestinal epithelial cells in males and along females colon (101,118). Moreover, a study reported that the activation of ARs in stromal cells resulted in the indirect stimulation of intestinal epithelial proliferation, which could influence the colon barrier function hence highlighting the need to investigate ARs and PRs functions in UC (118). (See **Annex II**)

1.7. Genetics in UC

Over the past decade, genome-wide association studies (GWAS) identified over 300 loci related to IBDs, of which 38 susceptibility loci uniquely correlated with an increased predisposition for UC (119–121). Many gene variants, altered in active UC, are closely related to genes encoding for immune process regulations or loci associated with intestinal mucosal barrier integrity and functions, including components of the mucus layer, tight junction, desmosomes, extracellular matrix, and regulating proteins (122). However, not all genetically susceptible individuals develop UC, meaning genetic factors account for only 7.5-8.2% of total disease variation (122,123).

Therefore, although genetic studies provide insight into disease mechanisms, environmental factors, including diet, drugs, pollution, stress, gut microbiota, and the microbiome interplay with the intestinal mucosal barrier, may be prominent factors in UC pathogenesis (124,125).

Research reports sex-specific genetic associations in autosomal loci encoding for cytokines interleukin (IL)10 and IL23R, autophagy proteins autophagy related 16 Like 1 (ATG16L1), Osteosarcoma Amplified 9 (OS9), and ER degradation-enhancing alpha-mannosidase-like 2 (EDEM2), involved in the endoplasmic reticulum (ER)-associated protein degradation during ER stress and unfolded protein (UPR) response, which is activated in UC and likely contribute to chronic inflammation in UC (126). However, while monogenic-linked defect is a central component in very early UC onset in infants, the relatively low genetic contribution to UC in adulthood reported between 7% to 17% raised the importance of other biological and environmental influences in UC pathogenesis (127,128).

1.8. Immune response in UC

The GI tract, which houses the innate and adaptive immune cells that maintain gut mucosal homeostasis, is considered the largest lymphoid organ of the body (129). Immune cells comprise multiple cells, many of which reside in the lamina propria and epithelium of the GI tract, where they orchestrate the immune response through cell-cell, cytokine, and chemokine-mediated communications (130). Thus, lymphocytes B and T cells, dendritic cells (DCs), macrophages, eosino-phils, and mast cells are found in the lamina propria, whereas the intestinal epithelium mainly contains mast cells and T cells (131). Although most key immune cell types are common

to all distinct regions of the GI tract, some display cell types and subtypes-specific spatial preferences (22). In homeostasis, the immune cells' spatial preference for the large intestine features higher frequencies of macrophages, dendritic cells subtypes, B cells, T cell subtypes classical forkhead box P3 (Foxp3⁺), and RAR-related orphan receptor gamma (RORγt) regulatory (Treg) T cells along with lower frequencies of intraepithelial lymphocytes (IELs) and granulocytes compared to the small intestine. However, in contrast to mice, in human lymphocytes T helper (Th) subtypes, Th17 cells are more abundant along the colon, while Th1 cells are more frequent in the distal colon (132).

Several studies have reported a regulatory role for myeloid cells, including intestinal DC and macrophages, in maintaining the large intestine homeostasis (133). DC populations in the GI tract comprise four subtypes, including plasmacytoid DCs, conventional DCs, CD103⁺CD11b⁺DCs, and the most prevalent subtype of DC in the colon, CD103⁺CD11b⁻DCs. Owing to their professional antigen-presenting cells (APCs) and cytokine-producing capacity, DCs play a critical role in initiating and regulating immune responses to pathogens (134). CD103⁺CD11b⁺DCs, despite their scarcity in the large intestine, have been shown to promote the T helper (Th)2 and Th17 responses in the mucosa, whereas CD103⁺CD11b⁻DCs serve as a support for CD4⁺T cells-dependent CD8⁺T cells responses owing to their high antigen-presenting capacity and promote tolerance to food and commensal bacteria antigens (133,134). Furthermore, retinoic acid-producing DCs can, in part, promote the development of Tregs.

Another key component of innate immunity, intestinal macrophages, broadly categorized into monocyte-derived mature macrophages, monocyte-derived inflammatory macrophages, and self-maintaining macrophages, are highly heterogeneous and display several functions that differ from

macrophages of other tissues and contribute to intestinal mucosa tolerance (135). Thus, intestinal macrophages do not respond to pathogen recognition receptors (PRRs) sensing toll-like receptors (TLRs) stimuli by promoting the production of proinflammatory cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO), nor do they upregulate expression of co-stimulatory molecules such as CD80, CD86 and CD40 (136). Studies reported that intestinal macrophages downregulate the expression of lipopolysaccharide (LPS) receptor, CD14, in the presence of Gram-negative bacteria to dampen their inflammatory response. They secrete the anti-inflammatory cytokines transforming growth factor (TGF)- β and IL-10, which inhibit proinflammatory responses and promote FoxP3⁺Treg survival, proliferation, and suppressive function in response to commensal bacteria-mediated TLR activation (131,135). Furthermore, resident macrophages display highly phagocytic and bactericidal activities while also promoting epithelial cell self-renewal and regulating epithelial integrity (133,136). In response to commensal bacteria, they also secrete IL-1 β , which supports group 3 innate lymphoid cells (ILC3) production of Granulocyte-macrophage colony-stimulating factor (GM-CSF), which in turn maintains Treg homeostasis by regulating DC and macrophages (134). Moreover, a subset of macrophages, macrophages reg cells, initiated effector T cells anergy state and suppressed T cells proliferation in a cell-cell contact-dependent manner. Overall, intestinal DCs and macrophages are critical mediators of intestinal tolerogenic response to commensal bacteria and food antigens (133).

Besides the myeloid population, several lymphoid cells are with the mucosa in the large intestine. As part of the adaptive immune response, the lymphoid B and T lymphocytes are critical players in the GI tract immune response. In homeostasis, the population of B cells, which are highly enriched in the proximal and distal compartment of the GI tract, are mainly comprised of

immunoglobulin (Ig)A (90% of total B cells population) and IgM-producing plasma cells (131). In addition to producing antibodies such as IgA and IgM, which prevent the infiltration of pathogens and regulate microbiota colonization in the intestinal mucosa, B cells can differentiate as regulatory B cells (Bregs) with immunosuppressive activities during inflammation (133,137).

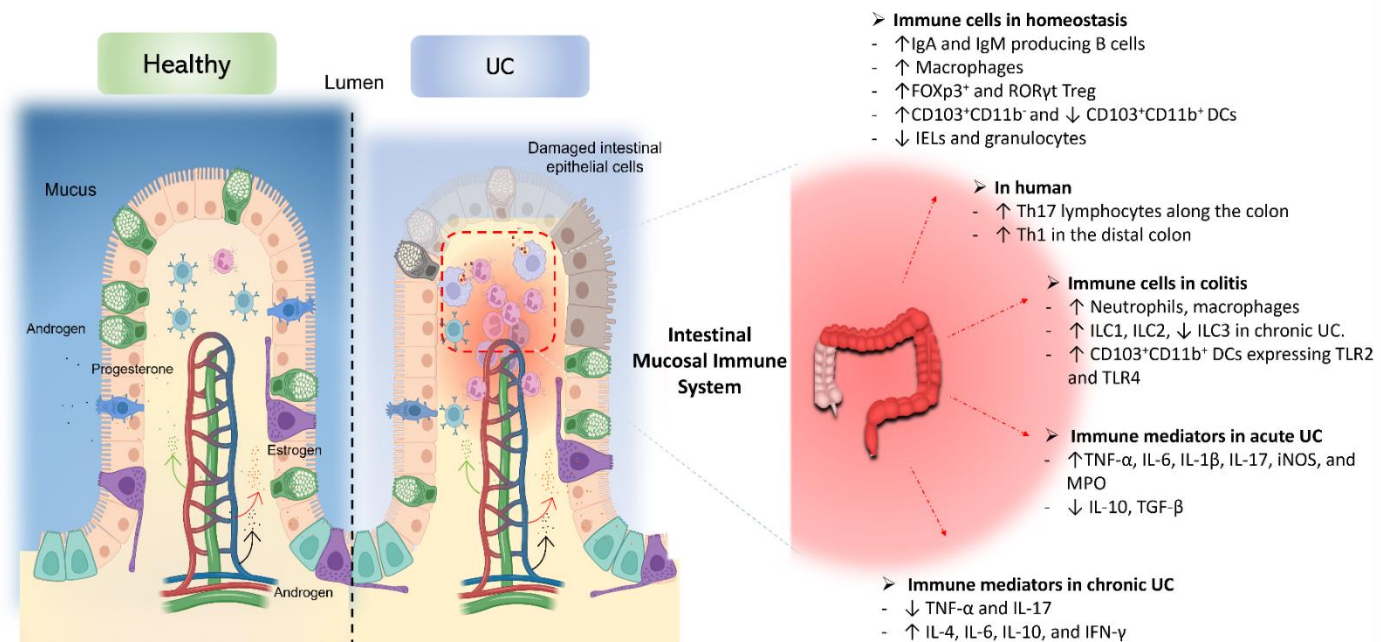


Figure 3. **Colonic immune response.** Schematic describing the colonic immune system at steady state and during ulcerative colitis.

Another key player of the adaptive immune response, T lymphocytes, can differentiate into four subtypes, Th1, Th2, Th17, and Treg, found throughout the GI tract with a density and composition that differs markedly. Th1 cells eliminate intracellular pathogens, Th2 cells regulate the humoral and allergic response and protect the intestinal tissues against parasite infections, Th17 cells protect against extracellular bacterial and fungal infections, and Treg cells are immunosuppressive and promote tissue repair (138). In the large intestine, the T-cell compartment comprises CD4⁺T and CD8⁺T cells, most of which are effector memory cells. Studies reported a higher proportion

of CD4⁺T subtypes FoxP3⁺ Tregs, IL-10, and IL-17-producing CD4⁺T in the colon, whereas Th17, more prevalent in the cecum than the distal colon, were inversely correlated with Treg numbers, both cells frequencies regulated by the microbiota (131). The intestinal mucosa's immune heterogeneous landscape is critical to protect the large intestine against pathogenic insults while maintaining strict control over inflammatory responses to commensal bacteria and promoting tolerance to food antigens. Therefore, any dysregulation of the immune compartment in the gut can alter individual susceptibility to disease and lead to pathology such as UC (139,140). Research suggests that compromised intestinal barrier function led to increased exposure of the intestinal mucosa to food and commensal microorganisms' antigens, resulting in a breakdown in immunological tolerance, dysregulated activation of innate and subsequently adaptive immunity, and the development of chronic inflammation in UC. The hallmark of dysregulated immune responses in the intestinal mucosa during UC encompasses increased recruitment of neutrophils, accumulation of inflammatory macrophages, infiltration of ILC1 and ILC2 in chronic UC, and a shift of DCs toward a proinflammatory profile associated with elevated TLR2 and TLR4 expression (141–143) (**Figure 3**).

Among the initial immune cells recruited to the inflamed tissue in UC by IL-1 α , IL-17, IL-22, GM-CSF, and granulocyte colony-stimulating factor (G-CSF), neutrophils represent the primary component of the infiltrated cells (133,141,143). In patients with UC, neutrophils become activated in response to several stimuli, including the bacterial endotoxin lipopolysaccharide (LPS), phagocytes, endothelial cells-produced NO and IL8, tumor necrosis factor (TNF)- α , and protein arginine deiminase 4 (PAD4), which is highly expressed in intestinal epithelial cells (141). Upon activation, neutrophils degranulate and release primary secretory granules containing

proinflammatory and antimicrobial proteins such as IL-8, TNF- α , IL-1 β , neutrophils elastase, myeloperoxidase (MPO), cathepsin G, defensins, and bacterial permeability-increasing protein (BPI) (141,144). Moreover, activated neutrophils release neutrophil extracellular traps (NETs), a mesh-like structure composed of DNA and histone embedded with secretory granules components such as MPO, neutrophils elastase, cathepsin G, and protease 3, which will encapsulate pathogens, activate the complement factors, and promote bacteria lysis. Despite the critical role of neutrophils in eliminating pathogens, their excessive accumulation and activation can damage the intestinal epithelium and impair the mucosa barrier function, exacerbating UC pathology and patients' symptoms (133).

In UC, the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) due to mucosal injury initiate the intestinal inflammation and recruitment of circulating lymphocyte antigen 6 complex (Ly6C)^{high}, CX3C chemokine receptor 1 (CX3CR1)^{low} monocytes in C-C motif chemokine receptor 2 (CCR2)-dependent manner and in response to IL8 and TGF- β constitutively secreted by intestinal epithelial cells (142). The recruited Ly6C^{high} CX3CR1^{low} macrophages express high levels of PRRs TLR2 and nucleotide-binding oligomerization domain containing (NOD)2, which enhance their sensitivity to bacteria and promote their differentiation into proinflammatory effectors cells that can produce proinflammatory molecules, including TNF- α , IL-6, IL-1 β , and inducible nitric oxide synthase (iNOS) (142). Interestingly, the resident Ly6C^{low} CX3CR1^{high} macrophages maintain their anti-inflammatory profile during intestinal inflammation (145).

In the context of colitis, studies have shown an increase in the numbers of ILC1 and ILC2 and reduced ILC3 in the inflamed intestinal mucosa of patients in both the early and chronic stages of

UC (146). The primary role of ILC1 is to eliminate viruses and bacteria by secreting interferon (IFN)- γ and TNF- α in response to DCs and stimulation with cytokines such as IL-12, IL-15, and IL-18 (141,146). ILC2, when stimulated by IL-25 and IL-33, secrete Th2-associated cytokines, IL-5 and IL-13, which contribute to neutrophil recruitment on the site of inflamed mucosa and impaired intestinal barrier function (141). In contrast, ILC3 becomes activated by cytokines including IL-1 β , IL-2, IL-6, IL-7, and IL-23 and, as a result, produces TNF- α and IL-17 in addition to being the primary sources of IL-22 (146).

In DSS-induced colitis, the acute phase of inflammation is mediated predominantly through a Th1/Th17 response associated with increased inflammatory mediators TNF- α , IL-6, IL-1 β , IL-17, and MPO activities along with decreased levels of IL-10, TGF- β , and mucin (17,147). On the other hand, chronic DSS elicits a predominantly Th2-mediated response associated with lower levels of TNF- α and IL-17, as well as elevated IL-4, IL-6, IL-10, and IFN- γ (147). Both models closely mimic human UC characterized by Th1/Th17 phenotypes associated with higher production of TNF- α , IL-1 β , and IL-6 at disease onset and a Th2/Th17 cytokine profile accompanied by increased IL-4, IL-5 mRNA expression levels in late disease (146).

Most immune cells express multiple sex hormone receptors, hinting at a potential modulatory role for sex hormones in differences in the immune response between males and females in UC (148). IBD is associated with a dysregulated innate immune response that leads to increased PRRs TLR2, TLR4, CD40, and chemokine receptors CCR7, which help promote the production of inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-18 and recruitment of neutrophils (149). Androgen and estrogen response elements have been identified in the promoters of innate immune genes, several of which are part of the PRRs, IFN, Janus kinase (JAK)2, and signal

transducer and activator of transcription (STAT)3 signaling pathways, which are known to play an important role in IBD pathogenesis (150–152). Moreover, studies reported a sex-mediated bias in innate immunity. Thus, studies have shown that males have higher levels of TLR4 expression and more robust inflammatory responses to LPS in macrophages and neutrophils than females, yet testosterone-sensitized macrophages display reduced TLR4 and TNF- α (14,153,154). Together, these studies suggest a role for ARs and ERs in regulating the innate immune response and, consequently, UC pathophysiology.

Sex-mediated differences also alter adaptive immunity in IBD. Thus, studies reported increased ER α and decreased ER β levels in T cells from patients with IBD compared to healthy individuals (111). Despite demonstrating no significant difference in the frequency of T cells, patients with active UC had markedly lower T cells and Treg expressing ER β than healthy controls and patients in remission, likely caused by the presence of IL-6 (111,155). Moreover, circulating T cells and CD4⁺ T cells expressing ER β proportions inversely correlated with disease activity and plasma concentration of inflammatory markers such as IL-6 and c-reactive protein (CRP) in patients with UC (111,155). Interestingly, patients with elevated T cell ER β expression were more responsive to anti-TNF treatment than unresponsive patients (111). In contrast, ER α expression did not correlate with anti-TNF therapy and proinflammatory cytokines (111).

Preclinical studies demonstrated that while naive T cells respond to estrogen through ER α and ER β , mature Treg mainly responds to estrogen through ER β (156). In Treg, activation of ER β can promote Foxp3 expression and production of IL-10 and TGF- β (156). Using a chronic model of DSS, studies found that the exclusive activation of ER β , could restore the population of CD4⁺ T expressing ER β and Treg cells while also inhibiting the infiltration of effector CD4⁺ and CD8⁺

T cells in the colon mucosa (155). Interestingly, the deletion of $E\alpha$ from the effector T cell population promoted the expansion of Treg cells by enhancing the expression of Foxp3 and Treg-associated genes while suppressing the expression of Foxp3 suppressor genes such as Transcription Factor 7 (Tcf7) and Special AT-rich sequence binding protein 1 (Satb1) (157). Consequently, Treg expansion limited effector T cell activation and expansion, reduced $\text{INF-}\gamma$ and IL-17A production, and ultimately alleviated colitis (157). These studies highlight the significance of maintaining a balance between ERs in UC to preserve intestinal mucosa tolerance in the gut.

1.9. Colonic mucosal structural organization and changes during ulcerative colitis

The colonic mucosa, comprising epithelium, mucus layers, and antimicrobial peptides, form a tight and highly regulated protective barrier that segregates the intestinal tract luminal content comprised of exogenous microorganisms, food antigens, and microbiota from the immune cells of the host located in the lamina propria (**Figure 3**) (158). The colonic epithelial barrier includes a monolayer of specialized epithelial cells tightly connected by tight junctions (TJ). These differentiated epithelial cells include the absorptive, colonocytes, microfold (M), secretory enteroendocrine, tuft, and goblet cells, which emerge from multipotent stem cells located at the crypt base of the colon (159). Owing to the short life span of colonic epithelial cells (CEC), 4 to 5 days, gut epithelium undergoes rapid self-renewal that preserves the colonic mucosal integrity and barrier function under homeostatic conditions. Thus, epithelium homeostasis is maintained through intestinal epithelial cell shedding in the lumen, restitution, proliferation, and

differentiation/maturation to replenish the constant loss of cells (25). Failure to elicit the colonic epithelial regeneration can lead to impaired barrier function, increased permeability to luminal antigens, dysregulated immune response, and, consequently, colonic inflammation, as seen in patients with UC (25).

1.9.1. Cell-to-cell junctional proteins complex and ulcerative colitis

As presented above, intestinal epithelia play a crucial role in regulating gut mucosa permeability by coordinating cell-to-cell junctional interactions, allowing for ions, nutrients, and water uptake while preventing pathogen infiltration (5). The junctional complexes formed of tight and adherens junctions (AJ), which interact with cytoskeletal linker scaffolding proteins such as zonula occludens (ZO), cingulin, afadin, Multi-PDZ domain protein 1 (MUPP1), and calcium/calmodulin-dependent serine protein kinase (CASK), located in the lateral face of polarized epithelial cells (160–164). TJ pathways can include bicellular and tricellular junctions. The bicellular junction occurs between two adjacent epithelial cells, while the tricellular junction emerges at the converging point between three epithelial cells (160). TJ, AJ, and desmosome expressions in the gut varied according to the intestinal region (small or large intestine) along the crypt axis and epithelial cell membrane (162). Moreover, their functions differ depending on the type of proteins involved. Thus, TJ, AJ, and desmosomes can control epithelial cells' cytoskeleton dynamics and leukocyte transmigration, along with colonocyte proliferation, differentiation, apoptosis and function, and communication between adjacent epithelial cells, all critical elements for the intestinal mucosal healing process (review in (162,165)).

TJs are the apical-most junctional transmembrane proteins, and they consist of claudins, junctional adhesion molecules (JAMs), angulin, the TJ-associated MARVEL proteins (TAMs) occludin, tricellulin, and marvelD3. TJ molecules regulate the selective paracellular transport of ions and solutes by sealing the space between contiguous epithelial cells through connecting plasma membranes (160–162).

Claudin, the first principal component of the TJ barrier, comprises a family of 27 proteins with varying properties and showcases differential expression across tissues and between species. Thus, claudin expressions differ along the large intestine of humans with claudins 1- 4, 7-8, 12, and 15 (166,167) and mice with claudins 1-5, 7-12, 14-15, and 17 (168). Claudins are subdivided into cation-permeable pore-forming and barrier-forming claudins (see review (165,169)). Studies have identified claudin-2, 7, 10, 12, and 15 as contributors to the formation of cation-permeable paracellular pores in contrast to other barrier-forming claudins (167,170). Claudins 2-4, 7, 8, 12, 14, and 15 expressions are higher in the large intestine. Early studies have shown that inflammation has a contrasting effect on claudins 2, 3, and 4 expression, as epithelial cells treated with TNF- α and IFN- γ *in vitro* increase claudin-2 levels but reduce claudins 3 and 4 expression, contrasting with the positive regulatory role of pro-inflammatory cytokine IL-13 (171). A recent study by Shashikanth and colleagues demonstrated that claudin-4 was dispensable for TJ barrier function but disrupted claudin-2, 7, 15, and 19 structures and pore-forming, thus reducing the epithelium paracellular permeability (172).

The adherens junctions underneath TJ consist of transmembrane cadherin such as E-cadherin that binds to cytoplasmic γ -catenin (also named plakoglobin) or catenin p120, β -catenin, and α -catenin to form the cadherin-catenin complex (173,174). The catenins bind directly to epithelial cells actins

(β , and γ -actin) and actin-associated proteins (formin-1, vinculin, α -actinin) and indirectly through the nectin/afadin complex or ZO molecules (173,175,176). Through its complex interaction, E-cadherin links the TJ to the cytoskeleton network of actin-myosin to promote cell-cell adhesion and vesicle transport critical in assembling adherens junctions protein desmosomes and cell polarity machinery (176).

Impaired intestinal epithelial junctional barrier function and loss of barrier integrity are the hallmarks of UC. Although it remains uncertain to what extent intestinal epithelial cells' junctional proteins contribute to UC pathology, several studies have linked UC with the downregulation of TJ, adherens junctions, desmosomes, and TJ scaffold proteins (5,177). Thus, the expression of claudins differs markedly in the colonic epithelium of UC patients. Although the signature of claudin expression is species and strain-specific, claudin expression in the murine model of UC colitis does not always reflect claudin expression in humans. Many studies showed an increased expression of pore-forming claudin-2 in the colon epithelium in biopsies from patients with UC (167,171,178). However, the TJ-forming claudins 1, 3, 4, 7, and 8 expressions have been either reduced or unaltered in colon tissue from UC patients (160,171,178). Occludin, ZO-1, Dsg2, Dsc2, and ZO expression were reduced in patients with active quiescent UC and associated with UC disease severity (177,179,180). Conversely, in DSS-induced murine colitis, reduced claudin-2, 3, and 8 contrasted with elevated claudin-4, whereas, lower Dsg2 upregulated claudin-2 through phosphoinositide-3-kinase (PI-3-kinase)/AKT signaling (160,166,171,178,181). Similarly, the Dsc2 expression was lower in inflamed tissue in DSS-induced murine colitis. Furthermore, its loss correlated with reduced cell adhesion and migration and impaired colonic mucosal healing, resulting in increased susceptibility to colitis (182).

1.9.2. *Intestinal restitution*

The intestinal epithelium repair strategies differ in response to the degree of injury severity of the mucosa. Thus, superficial mucosa lesions are rapidly repaired within minutes to hours by migrating adjacent intestinal cells that underwent dedifferentiation from epithelial-mesenchymal transition (EMT) and migrated in the injury site through epithelial restitution, which occurs without proliferation (183). Epithelial restitution is either mediated through a TGF- β -dependent pathway activated by a spectrum of molecules, including endothelial growth factor (EGF), VEGF, fibroblast growth factors (FGF), human growth factor (HGF), Glucagon-like peptide (GLP)-2, IL-1, IL-2, IFN- γ , and non-canonical Wnt-related MMTV integration site (WNT)5a or in a TGF- β -independent pathway likely mediated by regulating the E-cadherin/catenin complex and driven by proteins from the trefoil factors family (184,185). Activation of the TGF- β signaling downstream of the TGF- β type 1 receptor enhances the phosphorylation and translocation of Smad 3 in the nucleus, secretion of matrix metalloproteinase-1 (MMP-1) and MMP-10 to degrade the extracellular matrix proteins (ECMs), expression of fetal-like genes, changes calcium signaling which cause the reorganization of the cytoskeleton of intestinal epithelial cells surrounding the lesion and a robust contraction of the epithelium (186). Altogether, during the intestinal restitution, the epithelial cells surrounding the site of injury dedifferentiate, promote the shedding of damaged epithelial cells in the lumen, migrate within the lesion, and reorganize their cytoskeleton to expand to overlay the wound and reestablish intestinal epithelium. Interestingly, activation of cell migration mediated by TGF- β -signalling likely coordinated with suppression of intestinal cell

proliferation by promoting expression to potentially prevent the proliferation of cells with damaged DNA (185). Additionally, damage to the epithelium is associated with increased activation of the Wnt signaling pathway, which can inhibit the intestinal stem cell expansion and proliferation following the interaction with the mechanosensory Hippo YAP/TAZ, key regulatory effectors of cell proliferation, differentiation, and survival in tissue regeneration and wound healing process (187).

In the context of intestinal inflammation, the activation of the receptor suppression of tumorigenicity 2 protein (ST2) by its ligand IL-33, an endogenous alarmin released by stressed, damaged, or necrotic intestinal epithelial, endothelial, myofibroblast, and smooth muscle cells, promotes the epithelium repair and restitution through the production of microRNA (MIR)320A during DSS-mediated colitis recovery phase (188). However, studies reported a reduction in MIR320A, whose expression is downregulated by the PRR nucleotide-binding oligomerization domain-containing protein 2 (NOD2), during the acute phase of inflammation in patients with active UC. Similarly, adhesion desmosomal cadherins molecules, desmoglein (Dsg), and desmocollin (Dsc), which downregulated in UC patients and DSS-induced mice model, have also been described as contributors to cell adhesion, migration, and intestinal restitution during colitis recovery phase indicating a potential impairment in epithelial repair capacity in the inflamed colon (182). On the other hand, a study by Choi and colleagues demonstrated that in response to ulcerative mucosal injury, colonocytes increased the production of nonsteroidal anti-inflammatory drugs (NSAID)-activated gene (NAG)-1, a contributor of P53 apoptotic activity during acute genotoxic stress, thus promoting the expression of cytoskeleton modulator Ras-homolog family member guanosine triphosphate ((RhoA) GTPase)/ (TGF- β)-activated kinase 1 (TAK1)-linked

pathway, mediating early intestinal restitution (189). In the acute phase of inflammation, recruited neutrophils signal through the intestinal epithelial cells' adhesion proteins intercellular adhesion molecule 1 (ICAM-1) to activate AKT. AKT, in turn, phosphorylates β -catenin and enhances epithelial restitution (190).

1.9.3. *Intestinal proliferation*

As a result of their short lifespan, colonic epithelial cells must undergo constant self-renewal to prevent epithelial barrier dysfunction and promote mucosa healing (25). In homeostatic conditions, epithelial self-renewal is driven primarily by fast-cycling crypt base columnar (CBCs) leucine-rich repeat-containing G protein-coupled receptor (Lgr) 5-expressing stem cells, which are progenitors for terminally differentiated epithelial cells involved in the intestinal mucosa daily homeostatic regeneration (191). However, following the formation of severe lesions involving crypt loss, as seen in UC pathophysiology, CBCs Lgr5⁺ stem cells are depleted. As a result, colonic epithelial cell lineages are restored by a group of heterogenous slow-cycling damage-associated regenerative stem cells (DARSCs), expressing various markers such as telomerase reverse transcriptase (Tert), polycomb complex protein Bmi-1 (Bmi1), and homeodomain-only protein homeobox (Hox) (191,192). Moreover, over the past decade, compelling findings have proposed a model of intestinal epithelial repair based on plasticity and differentiation. Thus, lineage tracing studies reported that progenitors from secretory and absorptive lineages, differentiated epithelial cells, could restore the intestinal stem cell pool during epithelial regeneration through paligenosis (193–195). Whereby intestinal epithelial cells, through activation of the transcription effector Yap and Hippo signaling in response to mechanical forces, prostaglandin E2, pro-inflammatory cytokines, and TGF- β , acquire a fetal-like stem cells signature associated with expression of embryonic genes

such as clusterin (Clu), Anxa1, IL-33, Wfdc2 and lymphocyte antigen 6 A Ly6a (Ly6a/Sca1) (193–196).

In UC, colonic crypts undergo repeated damage that impairs the mucosal healing process, thus preventing long-term remission for UC patients. Multiple studies reported an increased susceptibility for CBCs Lgr5⁺ stem cells to mucosal injuries (197). Conditions in which the epithelium is directly and acutely injured, such as in UC and DSS-induced colitis, lead to the loss of CBCs Lgr5⁺ and DARSCs Hopx⁺ stem cells in involved and non-involved areas during the injury phase (196,197). However, damage to colonic crypts and loss of intestinal stem cells coordinated with activating plasticity mechanisms contribute to restoring intestinal epithelial stem cells after injury. In the DSS mice model of colitis, the colonic mucosal ulceration correlated with increased levels of Ly6a expressing epithelial cells, which expression was induced by inflammatory cytokines IFN- γ and IL-22 and, to a lesser extent, by TNF- α (197,198). However, loss of TNFR2 expression in the intestinal epithelial compartment led to higher retention of Ly6a⁺ stem cells and delay in secretory progenitor lineages and goblet cell restoration in the injured epithelium (199). Moreover, higher levels of Ly6a expression led to increased production of chemokines ligands such as CCL7 and CXCL5, which are linked to the recruitment of many innate immune cells and are associated with disease onset and progression in colitis (198,200).

The self-renewal and differentiation of colonic epithelial cells are primarily regulated by the Wnt, bone morphogenic proteins (BMPs), neurogenic locus notch homolog protein (Notch), and EGF signaling pathways (201). In the colon, Wnt ligands which include R-spondin (RSPO)1-4, Wnt2b, Wnt4, and Wnt5, produced by crypt mesenchymal cells such as myofibroblasts, engaged frizzled (FZD) and Lgr4-Lgr6 receptors along with the co-receptors low-density lipoprotein receptor-

related protein (LRPs) 5/6 expressed on intestinal epithelial stem cells to activate the non-canonical and canonical Wnt signaling pathway (25,202,203). Thus, the non-canonical Wnt signaling pathways, categorized into Wnt/planar cell polarization (PCPs) and Wnt/calcium signaling, control cytoskeleton remodeling, adhesion, and motility of differentiated cells in the upper crypt. Conversely, tissue repair in the colon highly depends on the canonical Wnt/ β -catenin signaling pathway, which plays a central role in colonic epithelial stem cells' self-renewal (190). Activation of the Wnt receptor rapidly inhibits the β -catenin destruction complex formed by adenomatous polyposis coli (APC), Casein Kinase 1 (CK1), axis inhibition protein (AXIN), and glycogen synthase kinase 3 (GSK3), which mediate phosphorylation and constitutive proteasomal degradation of β -catenin (190). As a result, dephosphorylated β -catenin accumulates in the cytoplasm and subsequently translocate in the nucleus where, as a cofactor, they interact with various transcription factors such as T-cell specific factor/lymphoid enhancer factor (TCF4/LEF) and promote expression of genes associated with intestinal epithelial cells stemness, cell cycle progression, and proliferation (204–206).

The intestinal epithelial proliferation rate, stable in homeostasis, is temporarily increased in the crypt adjacent to the lesion site before returning to a quiescent state following the healing of the mucosa (190). This observation is particularly pronounced in pathology that exhibits repeat cycles of mucosa ulceration and repair, such as UC. Therefore, studies reported that patients with IBD have significantly higher epithelial proliferation at the peak of inflammation, which decreases with a resolution of the inflammation (207,208). Similarly, DSS-induced acute colitis was associated with enhanced proliferation in the surrounding wound despite the tissue's overall reduction in epithelial proliferation correlating with inhibition of β -catenin/Wnt signaling (190,209). In colitis,

the proliferation process involves the mesenchymal cells and macrophages located within the ulceration, which produce prostaglandin known to inhibit GSK3 and stabilize β -catenin, in addition to enhanced production of Wnt ligand RSPO1. Consequently, this causes the proliferation of epithelial progenitor cells adjacent to the wound through the Wnt canonical pathway (190,201). Interestingly, immune cells recruited in intestinal mucosa in UC, including macrophages, DCs, and T cells, produce Wnt agonists. Thus, macrophages express the canonical Wnt ligands WNT1, WNT2B, WNT3A, and WNT10A, which promote CBC stem cell proliferation and intestinal mucosal repair through Wnt signaling. Although DCs and T cells expressed Wnt ligands that control inflammation, their effect on intestinal epithelial proliferation remains to be investigated (190).

In addition to Wnt ligands, inflammatory cytokines can regulate intestinal proliferation through the Wnt signaling pathway. Thus, studies showed that $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ can promote epithelial proliferation by activating β -catenin through AKT signaling during colitis. However, *in vitro* studies described a biphasic effect for $\text{IFN-}\gamma$. Thus, exposure to $\text{IFN-}\gamma$ first elicits epithelial cell proliferation by activating the Wnt signaling before promoting the expression of Wnt antagonist dickkopf (DKK) 1 after prolonged exposure (72h) to $\text{IFN-}\gamma$, which reduces Wnt signaling (190,209). Conversely, $\text{TNF-}\alpha$ does not induce DKK1 expression and can directly activate β -catenin-dependent transcription in the inflamed colon. However, $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ proliferative effects might be concentration-dependent as higher concentrations overall inhibit proliferation and promote intestinal apoptosis (210). Other cytokines that can regulate intestinal epithelial proliferation include IL-6, IL-22, and $\text{TGF-}\beta$. Thus, IL-6 and IL-22 can promote CBC stem cell proliferation and survival by activating the JAK/STAT pathway rather than β -catenin/Wnt

signaling pathways (190). Conversely, TGF- β can indirectly suppress intestinal epithelial cell proliferation by upregulating DKK levels (204). Together, these findings describe a complex interplay between the mesenchymal, intestinal epithelial, and immune cells, which all regulate the delicate balance between the promotion and suppression of the proliferation process indispensable for mucosal repair in UC.

1.9.4. *Intestinal differentiation*

The colonic stem cells' continuous renewal gives rise to progenitor cells, also called transit amplifying cells, which must undergo distinct differentiation and maturation processes to produce the colonic functional terminally differentiated epithelial cells.

The differentiation of intestinal progenitors to specific colonic epithelial cells' fate is tightly regulated and involves the BMP and Notch signaling pathway (205,206).

As part of the TGF- β superfamily of signaling molecules, BMPs encompass more than 15 members regulating biological development, stem cell homeostasis, and intestinal disorders (211). In the colon, BMP ligands are expressed in a polarized gradient with the highest signals at the peak of the crypt inversely from the Wnt and BMP extracellular antagonists, Gremlin (Grem1), sclerostin (SOST), uterine sensitization-associated gene 1 (USAG-1), Dante (Dte), protein related to DAN or Cerberus (PRDC/Grem2) and Coco, Noggin (Nog), Chordin (Chrd), follistatin (FST) and twisted gastrulation (Twsg), gradients (212). BMP4 homodimers or heterodimers can form a heterotetrameric complex that signals through canonical and non-canonical pathways using the type I and type II serine-threonine kinase receptors, which comprise type 1A BMP receptor (BMPR-1A), BMPR-1B, type 1A activin receptor (ActR-1A), type 2 BMP receptor, (BMPR-2),

type 2A activin receptor (ActR-2A), and (ActR-2B) (211). Thus, in the canonical signaling pathway, BMP agonists such as BMP2 and BMP4 bind preferentially to type I receptors and recruit the constitutively activated type II receptor to form a complex that will serve to phosphorylate and activate the type I receptor (211). The activated type I receptor can, in turn, recruit and phosphorylate the downstream molecules receptor-regulated suppressor of mothers against decapentaplegic (R-SMADs), comprised of SMAD1, SMAD5, and SMAD8. Subsequently, phosphorylated R-SMADs form a complex with the common SMAD, SMAD4, which translocates to the nucleus, where, as transcription factors, they regulate several target genes, including inhibitors of DNA binding (ID1-3) and Smad6 (212). Various co-activators, co-suppressors, and inhibitory SMAD6 and SMAD7, which can directly inactivate the R-SMAD-SMD4 complex, BMP receptors degradation, or repression of R-SMAD-SMD4-mediated transcription, can regulate BMP downstream signaling (213).

In the non-canonical pathway, BMP binding to BMPR-1A prompts the formation of a complex with X-linked inhibitor of apoptosis (XIAP) and TAK1-TAB1 to phosphorylate and activate the mitogen-activated protein kinases (MAPKs) pathway, comprising p38, c-Jun amino-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK1/2), which in turn, activate downstream co-factors ATF2, c-JUN, and c-FOS to regulate the transcription of targets genes (212,213).

Among all BMPs, BMP2 and BMP4 account for the most abundant BMP agonist in human intestines. BMP2, produced by terminally differentiated colonocytes, and BMP4, mainly expressed by mesenchymal cells in the colon, can regulate intestinal epithelial cell differentiation

and proliferation (212). Thus, *in vitro* studies reported that BMP2 promotes the differentiation and apoptosis of colonic epithelial cell lines while inhibiting their proliferation and β -catenin expression in an autocrine manner (212,214). Conversely, *in vivo* inhibition of BMP signalling with noggin revealed an inverse effect as the treatment promoted apoptosis and reduced the proliferation of epithelial cells, likely due to a compensatory mechanism (214).

Similarly, BMP4 specifically influences the epithelium, promoting intestinal mucosa homeostasis while limiting inflammation in the colon. Thus, a recent study demonstrated that BMP4 inhibition in gonadectomized male and female mice using the inhibitory effect of androgens in the intestinal stroma cell compartment promoted epithelial proliferation indirectly and, subsequently, mucosal homeostasis by downregulating several Wnt antagonists (118). Conversely, a study performed using female mice and a human fibroblast cell line revealed reduced BMP4 expression associated with DSS-induced colitis and proinflammatory cytokines, TNF- α and IL-1 β , which contrasted with IFN- γ enhanced fibroblast-mediated BMP4 expression (215). Furthermore, BMP expression differed in a time-dependent manner in DSS-induced colitis. Thus, at an early stage of colitis, enhanced BMP4 and SMAD4 expression was associated with increased colonic epithelial proliferation and preserved Lgr5⁺ stem cell population. However, at the late stage of the disease, BMP4 and SMAD4 were substantially diminished (216). Interestingly, overexpression of BMP4 in the intestinal crypt during DSS-induced colitis inhibited proliferation, enhanced differentiation, and impaired intestinal regeneration. However, mice treated with exogenous recombinant BMP4 proteins exhibited enhanced epithelial proliferation and preserved Lgr5⁺ stem cells in colitis by targeting the ID3 gene (216). Together, these findings highlight the complexity of BMP4 signaling and its antiproliferative and pro-proliferative effects influenced by multiple factors such as

concentration, intestinal mucosal microenvironment, availability of receptors on target tissue, and time course of disease.

The balance between self-renewal and differentiation is not only regulated by the Wnt/ β -catenin and BMP pathways but also by the Notch1 signaling pathway, which plays a central role in determining progenitor epithelial cells' commitment toward absorptive and secretory lineages. Notch receptors and ligands mRNA are present in epithelial and mesenchymal cells in intestinal tissues. Lineage tracing studies demonstrated that the Notch pathway in the colon is mediated through Notch 1 and Notch 2 receptors, signaling in the crypt-based stem cell population in response to Notch ligands delta-like (DLL)1 and DLL4 (217). A study by Shimizu and colleagues revealed that DLL1 and DLL2 are expressed in distinct locations along the crypt, with DLL1⁺ cells residing primarily in the lower crypt, whereas DLL2⁺ cells resided along the crypt (11). Furthermore, the intestinal epithelial stem and subset of goblet cells are likely one of the sources for both DLL1 and DLL2 in the colon (218).

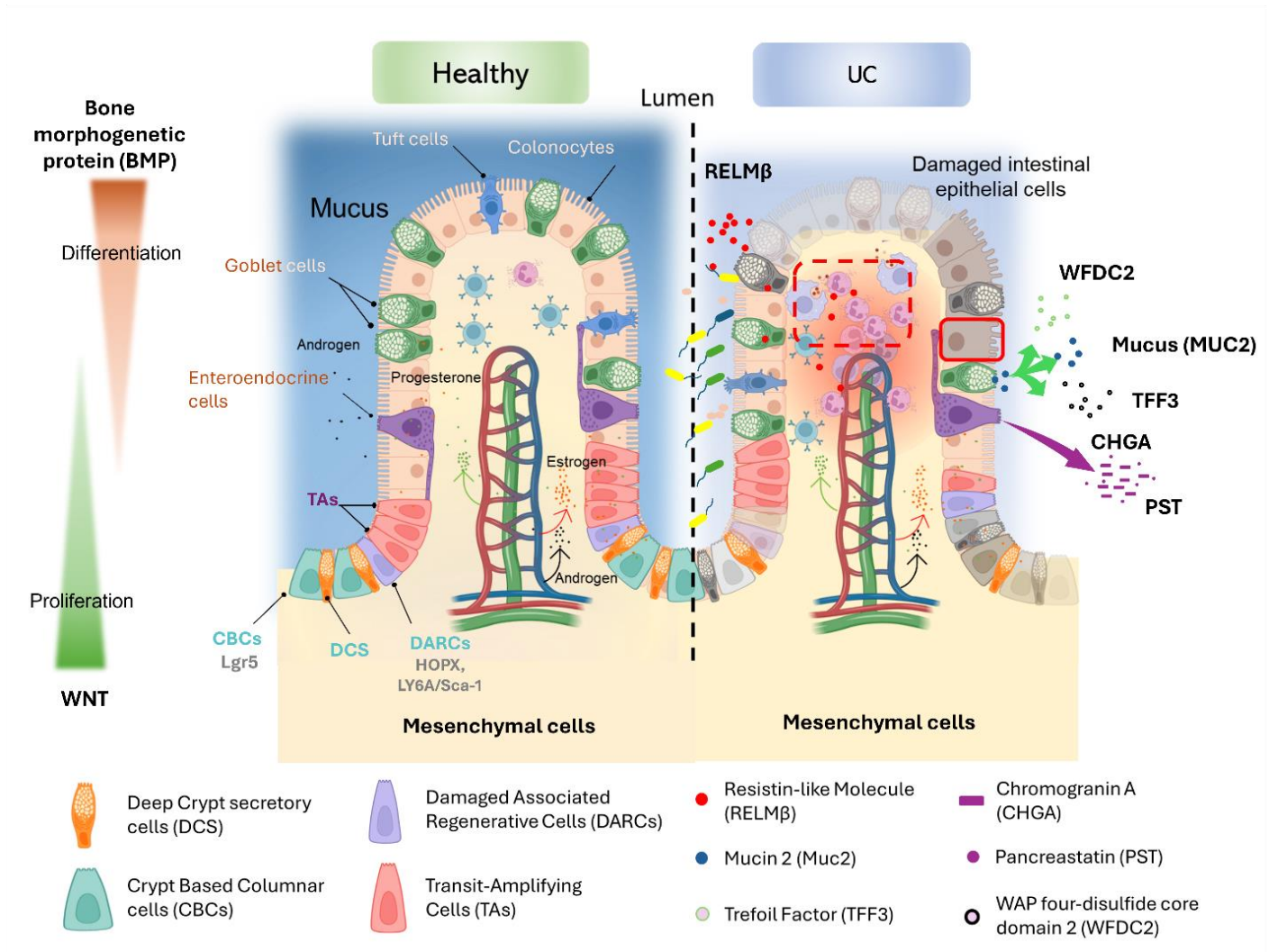
Activation of Notch signaling initiates the gene hairy/enhancer of split (Hes)1, a transcription factor that represses the transcription of atonal homolog 1 (Atoh1, also called Math1 or Hath1) and inhibits the progenitor cells' differentiation into a secretory lineage (219). Thus, activation of Notch signaling and Hes1 production drives colonic progenitor differentiation toward an absorptive colonocyte phenotype (206,220). However, below a threshold of Notch1 signaling, Atoh1 drives progenitor cells' fate toward a secretory lineage. Downstream Atoh1 signaling pathways, several transcription factors drive the progenitor cells toward different secretory cell lineages. Thus, growth factor-independent (GFI)1, Kruppel-like factor (Klf)4, and SAM pointed domain containing ETS transcription factor (Spdef) are critical in goblet cell differentiation,

whereas neurogenin (neurog)3 is essential for enteroendocrine cell differentiation (206,220). Interestingly, Atoh1 is dispensable for progenitor differentiation into tuft cells. However, transcription factors, POU domain, class 2, transcription factor 3 (POU2F3), and SRY-related HMG-box 4 (SOX4) are critical for tuft cell differentiation (219). Finally, the transcription factor SPI1-related protein B (Spib) activated by receptor activator of nuclear factor kappa B ligand (RANKL) secreted by intestinal stromal cells regulates M cell differentiation (219).

In UC and colitis, Notch signaling was abnormally elevated, which correlates with increased expression of Notch 1 mRNA. This upregulation is associated with a higher number of DLL4 and Hes1-expressing epithelial cells, leading to a reduction in the differentiation of secretory lineages, including goblet cells, highlighting the importance of regulating Notch signaling in inflammatory conditions like UC (218,221,222) .

1.10. Terminally differentiated colonic epithelial cells

The colonic epithelium is composed of a monolayer of several specialized terminally differentiated epithelial cells, including colonocytes, goblet, tuft, M, and enteroendocrine cells tightly connected by TJ, which play a critical role in the maintenance of the epithelium integrity and mucosal barrier



function (Figure 4).

Figure 4. **Colonic epithelium.** Schematic describing the cells and markers associated with colonic epithelium, in healthy and ulcerative colitis conditions.

1.10.1. Colonocytes

Colonocytes, also named enterocytes in the small intestine, are the most abundant cell type of the epithelium. As simple columnar cells, the functions of colonocytes include participating in the digestion process to ensure the uptake of ions, water, nutrients, vitamins, and unconjugated bile salts and regulating the immune response in the gut. Colonocytes shape the microbiota and promote the establishment and prosperity of obligate anaerobic bacteria by maintaining an anaerobiosis state in the intestinal lumen. These obligate anaerobic bacteria convert fiber into fermentation products, contributing to host epithelium nutrition, immune development, and protection against enteric pathogens (223). In a healthy gut, colonocytes retrieve energy from the degradation of the butyrate, a microbiota-derived short-chain fatty acid, using the catabolic beta-oxidation pathway (223,224). Beta oxidation of butyrate consumes a substantial amount of oxygen, which results in limited diffusion of oxygen from the gut mucosa to the lumen, promoting a hypoxic environment with an oxygen level lower than 1%, suitable for the successful colonization of the gut with obligate anaerobic bacteria (223,225). However, without butyrate, following crypt hyperplasia resulting from an increase in undifferentiated epithelial cells or during pseudomembrane formation, the colonocytes shift their energy production to converting glucose into lactate during anaerobic glycolysis (225). The anaerobic glycolysis process occurs in oxygen-deprived conditions, during which colonocytes consume a limited amount of oxygen, accumulating oxygen molecules in the colon lumen. Thus, the higher levels of oxygen availability in the lumen support the proliferation of facultative anaerobic bacteria such as the phylum *proteobacteria*. However, unlike obligate anaerobic bacteria, facultative anaerobic bacteria cannot digest fibers and produce short-chain fatty acids crucial for the host mucosa's

nutrition (223,225). Consequently, modification in colonocytes' metabolism causes dysbiosis and exacerbates colonic inflammation. Moreover, colonocytes produce several antimicrobial peptides, including Ly6/Plaur domain-containing 8 (Lypd8) and cathelin-related antimicrobial peptides (CRAMP) that also shape the microbiota.

Lypd8 is a glycosylphosphatidylinositol (GPI) anchored protein highly expressed on the apical surface of colonocytes. Shed constitutively into the lumen, Lypd8 binds to flagellated commensal bacteria and inhibits their motility and ability to invade the colonic mucosa (158). Recently, Lypd8 was shown to suppress enteric bacterial pathogens' ability to attach to colonic epithelial cells, preventing the formation of attaching and effacing lesions known to cause severe colitis accompanied by TH17 cells and neutrophil infiltration in the lamina propria (226). Moreover, decreased Lypd8 expression was reported in UC patients (158,227).

CRAMP is an antimicrobial peptide constitutively expressed by colonocyte progenitors in a homeostatic condition. However, in colitis, differentiated colonocytes, macrophages, and subepithelial myofibroblasts are the primary sources of CRAMP in the colon (228,229). Studies have shown that CRAMP expression is enhanced by SCFAs and increased colonocyte differentiation (228). Moreover, CRAMP regulates the microbiota homeostasis, preserves the mucus barrier, reduces the production of proinflammatory cytokines, suppresses epithelial apoptosis, and alleviates DSS-induced colitis (230). Furthermore, CRAMP promotes re-epithelialization and angiogenesis as an agonist to formyl peptide receptors (FPR2). CRAMP derived from colonocytes and macrophages contributes to colon homeostasis in distinctive manners (229). Thus, CRAMP derived from colonic epithelial cells supports the normal development of crypt, mucus production, colon epithelial proliferation, and mucosa repair. By

contrast, macrophage-derived CRAMP had a protective effect against colonic microbial invasion. (229).

1.10.2. Goblet cells

Goblets are highly polarized secretory cells found throughout the gut at a proportion that increases gradually from 4% in the duodenum to up to 50% in the distal colon, correlating with an increase in the number of microorganisms as we progress in the GI tract (159,231,232). As single-cell glands, goblets generate the first line of defense of the colonic mucosa through the production and secretion of the intestinal mucus layers (233). Interestingly, mucus structurally and functionally differs throughout the GI tract. A single-layered mucus system in the small intestine contrasts with the two-layered mucus structure found in the colon (234). Primarily composed of highly glycosylated gel-forming secretory mucins, with mucin 2 (MUC2) as the main component, the intestinal mucus layers form a mesh-like protective barrier critical to segregating the intestinal microbiota from the colonic epithelial cells (235). The colonic two-layered mucus structure encompasses the compact inner mucus layer, a net-like polymer sheet with small pore sizes and reduced bacterial penetrability, creating the physical barrier preventing contact between bacteria and the colonic epithelium (233,234). Far from the epithelia, on the luminal side, the inner mucus layer transformed into the outer loose mucus layer by proteolysis forms a soluble and less organized structure with larger pore sizes and multiple microbial attachment sites that permit bacterial infiltration (231). The outer loose mucus layer serves as an energy source for commensal bacteria and provides an ideal environment for microbiota growth (236). The fecal contents and intestinal microbiota composition within the mucus regulates the colonic mucus layers' organization and thickness (237,238). Thus, altered mucin2 gene expression and defective mucus

layer structure are associated with progressive inflammation, increased susceptibility to colitis, and exacerbated disease (238). Along with muc2, goblet cells produce several other mucosal defense factors, including trefoil factor 3 (TFF3), Resistin-like Molecule β (RELM β), and the antimicrobial peptide WAP four-disulfide core domain protein 2 (WFDC2) (230,231,239).

TFF3 is the second most abundant peptide produced by goblet cells and secreted with MUC2 in the gut. It participates in epithelial restitution and regeneration and promotes mucosal protection (231,240). Moreover, TFF3, found in monomeric and dimeric forms, can enhance intestinal epithelial barrier function by regulating tight junctions. In a clinical UC study, the increased TFF3 levels in the serum of patients with active UC correlated with disease activity. Similarly, ulceration sites in UC exhibited higher levels of TFF3 expressions (240). Moreover, TFF3 has been proposed as a biomarker of mucosal healing, with high levels positively correlating with a limited mucosal healing score (241).

RELM β , also named adipocyte-secreted factor or found in inflammatory zone 2 (FIZZ2), is exclusively produced in the gut and secreted apically in the lumen. Initiated by commensal microbiota, RELM β expression is upregulated in several models of gut inflammation and could be a biomarker of mucosal bacterial infiltration (232). RELM β deficiency can reduce mice susceptibility to DSS-mediated colitis, contrasting with a murine model of nematode infection mediating a TH2 response, RELM β possessed an antiparasitic activity mucosal response led to increased susceptibility to colitis (49,242). Interestingly, in DSS-induced colitis, studies demonstrated that RELM β could penetrate the colonic lamina propria through the disrupted epithelium and cause the infiltration of macrophages and granulocytes and the production of TNF- α (49). Furthermore, following bacterial infection, RELM β can mediate the recruitment of CD4⁺

T cells in the colon, where they promote intestinal epithelial cell proliferation through the production of IL-22 (235). Moreover, studies have shown that RELM β can upregulate the expression of the antimicrobial peptide RegIII β , which kills Gram-positive bacteria, thus causing microbiota dysbiosis and worsening colitis in Muc2^{-/-} mice model (242,243).

WFDC2 is a cross-class protease inhibitor typically highly expressed basally and apically by crypt-base goblet cells. As a protease inhibitor, WFDC2 inhibits serine and cysteine protease and can prevent the premature conversion of the mucus inner to the outer layer. Further, WFDC2 can block the proteolytic activity of metalloprotease MMP12 and 13, which are involved in tissue destruction in colitis. WFDC2's selective antimicrobial activity prevents mucosa invasion and epithelial barrier damage. WFDC2 demonstrated a particular antimicrobial activity by controlling bacteria such as *Staphylococcus aureus* and *Escherichia coli* at physiological levels (239). Moreover, in colitic conditions such as UC, WFDC2 expression is downregulated by proinflammatory factors such as IFN- γ (239).

1.10.3. Tuft cells

Tuft cells comprise 0.4% of all intestinal epithelial cells (244). Several newly identified biomarkers specific to Tuft cells include the chemosensing protein, Transient Receptor Potential Cation Channel Subfamily M Member (TRPM)5, tPG endoperoxide synthase 1 (Ptgs1), Pou2f3, Gfi1b, and Sialic acid-binding Ig-like lectin F (Siglecf), and the structural proteins doublecortin-like Kinase 1 (DCLK) and acetylated α -tubulin (206,245). Recent studies described tuft cells as the primary source of IL-25 in the gut. Hence, Tuft-constitutively expresses IL-25 work with IL-33 and Thymic stromal lymphopoietin (TSLP) to activate innate lymphoid cell 2 (ILC2) in helminth

infection. In response to IL-25, the activated ILC2 secretes IL-13, which regulates the progenitor cells' fate decision and promotes Tuft and goblet cells differentiation and expansion (246). Tuft cells can promote intestinal epithelial proliferation, enhance mucosal barrier integrity, and act as a reserve stem cell population (247). Thus, tuft cell deletion in the intestinal compartment caused impaired epithelial repair capacity after radiation-mediated mucosal injuries (248). In inflammatory conditions, DCLK⁺ deletion impaired epithelial proliferation and repair and exacerbated bacterial-induced spontaneous colitis (249). In DSS-induced colitis, the lack of DCLK1 correlated with dysregulated Wnt/B-catenin pathway, loss of intestinal epithelial stem cell compartment, and increased mucosal permeability, leading to worsened colitis (250).

1.10.4. Microfold (M) cells

M cells are primarily found in the epithelium site overlaying lymphoid tissues, where they capture and transport luminal antigens and particles by transcytosis through the GP1 from the apical face to the basolateral side of the cell to the immune cells, such as B and dendritic cells, to elicit either a mucosal immune response or mucosal tolerance (251). In inflammatory conditions, M gene expression specific to M cells increased due to proinflammatory cytokines in vitro Caco-2 cell lines. Additionally, there was a correlation between DSS-induced colitis and increased M cell numbers mediated by TNF- α (252).

1.10.5. Enteroendocrine cells

Scattered along the GI tract, enteroendocrine cells represent approximately 1% of the total gut epithelium, forming the most comprehensive endocrine system in the body (253). Enteroendocrine cells are secretory storage granules that co-express several hormones, including CHGA, serotonin,

somatostatin, ghrelin, and gastrin, exploited as biomarkers for enteroendocrine cell identification and categorized into 12 broad subtypes based on their major secretory hormones (253,254) As the principal producer of serotonin in the body, enteroendocrine cells generate several additional hormones, including chromogranin A (255). By releasing the contents of their secretory storage granule, enteroendocrine cells regulate nutrient absorption, intestinal immunity, epithelial barrier response, and colonic motility, which are crucial in gut hemostasis. Enteroendocrine cells express several receptors, including G protein-coupled receptors (GPCRs), toll-like receptors (TLRs), taste receptors (TR), and specialized sensory microvilli that serve to sense the microbiota, microbiota-derived components, and intestinal luminal nutrients (253). Moreover, morphological studies proposed that enteroendocrine cells modulate neighboring intestinal neurons and epithelial cells by releasing hormones or direct action through a paracrine effect. Enteroendocrine cells display several long pseudopod-like elements and neuropods extending from the enteroendocrine cells' basal surface and interacting with neighboring epithelial cells and neurons (255).

It is becoming increasingly evident that profound interactions occur between the intestinal immune system and enteroendocrine cells (17,253,256). Thus, Previous studies reported increased enteroendocrine cell numbers in UC patients (257). Transcriptomic analysis of biopsies from UC patients demonstrated that enteroendocrine cells acquire an increased capacity for protein synthesis, antigen procession, and presenting functions (255). Moreover, altered enteroendocrine cell functionalities associated with increased secretion of serotonin and CHGA have been reported in UC patients (253,258–260). However, the exact role of enteroendocrine cells in UC pathophysiology is still unknown.

1.11. UC therapy and mucosal healing

In recent years, extensive research has focused on developing treatments to control disease symptoms and maintain UC patients in symptomatic remission (9,10). Expansion of IBD therapeutic options include anti-inflammatory medications (5-aminosalicylates and corticosteroids), immune system suppressors such as T lymphocyte cells inhibitors (thiopurines), DNA synthesis inhibitors (methotrexate), calcineurin inhibitors that inhibit T cells effects, promotes macrophages apoptosis, and biologics such as anti-tumor necrosis factors (TNF)- α and anti-interleukin (IL)-12/23, and oral small molecules include Jak and integrin inhibitors (12). However, despite the advances in UC therapeutics, an estimated 38% of UC patients are refractory or lose response to therapy, and 80-85% fail to achieve complete mucosal healing (9–11). Growing evidence describes epithelial cells as critical in gut mucosa homeostasis (5,6). Complete regeneration of the colonic mucosa in UC patients predicts long-term remission, minimizes hospitalizations, lowers the risk for surgical treatment, and improves patients' quality of life (6,7). These factors accentuate the need to discover effective therapeutic strategies that offer complete mucosal healing and long-term remission with favorable adverse event profiles to advance UC patients' quality of life.

1.12. Chromogranin and its cleavage products

Mainly secreted by the enteroendocrine cells, CHGA is an acidic glycoprotein ubiquitous to the nervous, endocrine, and immune systems. Located on chromosome 14q32.12 of the human genome, the *CHGA* gene, formed of eight exons and seven introns, is transcribed and translated

into a prohormone of 439 amino acids with a molecular mass estimated between 70-85 kDa (261–263). As a prohormone, CHGA can undergo post-translational modifications to give rise to numerous biologically active peptides, including Pancreastatin (PST) (hCHGA₂₅₀₋₃₀₁), catestatin (CST), chromofungin, and vasostatin (261,264). The post-translational modifications of CHGA, regulated by enzymes such as prohormone convertases, aminopeptidase, carboxypeptidases, kallikrein, plasmin, and thrombin, can occur in the intragranular matrix or following secretion in the extracellular medium (263,264).

Several studies reported that CHGA and its proteolytic fragments exert a broad spectrum of regulatory functions affecting several organs, including the gastrointestinal and immune systems (262). Thus, CHGA peptides catestatin suppress inflammation, inhibit obesity, and promote insulin sensitivity and the paracellular barrier function, whereas pancreastatin (PST) has the opposite effect as it promotes obesity, insulin intolerance, and mucosal permeability (265).

In inflammatory conditions, CHGA and CHGA-derived peptide PST are elevated in plasma, feces, and intestinal tissue of patients with UC and experimental animal models of colitis, leading to the proposal of using CHGA as a biomarker for UC (16–18,256,258,259).

In the clinical setting, elevated expression of CHGA and PST correlated with enhanced intestinal inflammation and mucosa apoptosis, which was associated with increased disease activity and worsened severity in UC patients (17,256,258,259). These observations were validated experimentally using a murine model of colitis (17,256). However, their influence on colon mucosal repair remains unknown.

CHAPTER II

GLOBAL HYPOTHESIS AND OBJECTIVES

2.1. Gap in knowledge

A lack of understanding prevails about the effect of CHGA and PST on colonic epithelial barrier function and gut mucosal integrity and how their effect differs between sexes.

2.2. Hypothesis

I hypothesize that CHGA and PST compromise the integrity and barrier function of the colon mucosa, thereby hindering intestinal mucosal healing in UC in a sex-dependent manner.

2.3. Objectives

To study CHGA and PST as potential novel molecular targets in UC treatment. To evaluate the therapeutic potential of pancreastatin inhibitor 8 (PSTi8) in targeting PST and mitigating PST's biological effects in the context of sex-based differences in the colon mucosal barrier integrity and function. To attain this objective, I developed three specific aims.

2.4. Specific aims

Aim 1: To investigate the influence of sex-based differences on colonic epithelial integrity, mucosal barrier function, mucosal healing capacity, and immune system compartmentalization along the large intestine. These will be evaluated in homeostatic and colitic conditions using male

and female C57BL/6 mice treated with DSS to induce chemically acute colitis, a well-accepted animal model of UC.

Aim 2: To phenotype the impact of the lack of CHGA on colonic mucosa using male and female C57BL/6 CHGA knockout mice treated with DSS to induce acute colitis.

Aim 3: To evaluate PSTi8 as a therapeutic strategy to promote mucosa healing in acute colitis using male and female C57BL/6 mice treated intrarectally with PSTi8, a competitive inhibitor of CHGA-derived peptide PST, and DSS to induce colitis.

CHAPTER III

GLOBAL MATERIALS AND METHODS

3.1. Peptides

PSTi8 (PEGKGGEQEHSSQQKEEEEEEMAV-amide) purified by reverse-phase high-performance chromatography to a purity level of >98% will be used in this study (Pepmic Co., Suzhou, China).

3.2. Mice

This study was approved by the University of Manitoba Animal Ethics Committee (Protocol number: 23-023). All experiments followed the Canadian Council on Animal Care (CCAC) guidelines for animal research. For this study, 12-20 weeks old male and female C57BL/6 CHGA-global knockout (CHGA^{-/-}) (266) and wild-type control (CHGA^{+/+}) mice came from the colonies at the University of Manitoba, Winnipeg, Manitoba. Animals were housed in a 12h dark/light cycle and fed *ad libitum*. Thirty-two male and Thirty-two female mice, randomly assigned into four experimental groups of 8 mice, included untreated male and female CHGA^{+/+} (WT control), CHGA^{-/-} (CHGA^{-/-} control), and DSS-treated CHGA^{+/+} (WT DSS) and CHGA^{-/-} (CHGA^{-/-} DSS) mice.

3.3. Mice treatment with Pancreastatin inhibitor 8 (PSTi8)

Female and male C57BL/6 wild-type (WT) mice randomly assigned into experimental groups received daily administration of 2.5 mg/kg/day PSTi8 or 1% phosphate buffer saline (PBS) intrarectal (*i.r.*) route under anesthesia starting one day before DSS-induction (18). Four groups of

female and male C57BL/6 WT (n=8) mice received daily administration of 2.5 mg/kg/day PSTi8 (*i.r.*) for 6 days. Two groups of PSTi8-treated mice were given 5% DSS in their drinking water to induce colitis for 5 days. The second two groups received pasteurized water as a PSTi8-control group. The control experimental groups received daily treatment of 1% PBS (*i.r.*) and either pasteurized water or DSS.

3.4. Induction and assessment of experimental colitis

DSS-induced colitis was performed in mice following a well-established protocol. In brief, mice were given 5% DSS (molecular weight: 40-50kDa, ThermoFisher Scientific Chemicals Inc, Catalog #: J14489-22) in their drinking water for 5 days to induce colitis (18,256,267). The control group received pasteurized water. Features of disease activity, including weight loss, stool consistency, and bleeding, were monitored daily and served to calculate the disease activity index (DAI) as previously described (268). All mice euthanized by cervical dislocation were assessed for colon length and macroscopic score, including rectal bleeding, rectal prolapse, diarrhea, and colon bleeding, on day five after colitis induction (267). The colon was divided into proximal, medial, and distal sections and utilized as outlined next. The proximal segment was used to evaluate the mucosa paracellular permeability. The medial segment was used for histological analysis, immunofluorescence staining, and enzyme-linked immunoassay (ELISA). The distal segment used for real-time reverse transcription-PCR (RT-qPCR) and for the Ussing chamber measurements.

3.5. RNA extraction, purification, and quantification

Segments of the distal colon placed in TRIzol reagent (Invitrogen, ThermoFisher Scientific, MA, USA), were homogenized, sonicated, mixed with phenol/chloroform, and centrifugated for 20 minutes at 12000 rpm at 4°C. RNA collected in the aqueous phase was diluted in 70% ethanol and purified using the PureLink RNA mini kit (Invitrogen, ThermoFisher Scientific, MA, USA) according to the manufacturer's instructions. The yield and purity of RNA eluted in RNase-free water were measured using the NanoDrop spectrophotometer (ThermoFisher Scientific, MA, USA). The purified RNA preparation was reverse transcribed into cDNA using the SuperScript VILO cDNA Master mixed (Invitrogen, ThermoFisher Scientific, MA, USA) and stored at -80°C until used.

3.6 Real-time qPCR

The mRNA expression of the gene of interest was quantified by RT-qPCR of cDNA samples using the LightCycler 96 instrument (Roche Life Science, Basel, CH) using Power SYBR green master Mix (Applied Biosystems™, Life Technologies, Burlington, ON. CA). For each sample, the cycle threshold value (C_q) expressed over 2 (2^{C_q}) was divided to that of the sample's corresponding housekeeping gene, TATA-binding protein (TBP) (269), 2^{C_q} and expressed as the relative ratio of the targeted gene expression. Markers of colonic epithelial cells' structure and functions, including antimicrobial activities, bacterially induced peptides, and colonic stem cells, were evaluated. (Annex I Table 1).

3.7. Cell purification

Colon tissues open longitudinally were incubated twice in the epithelial cells isolation buffer composed of phosphate buffer saline (PBS), 2mM Ethylenediaminetetraacetic acid (EDTA), 43.4mM sucrose, and 0.5 mM dithiothreitol (DTT) (Sigma Aldrich, Carlsbad, CA, USA) for 15 minutes at 4°C. Tissues rinsed in cold PBS and minced were digested for 40 minutes at 37°C in RPMI supplemented with 10% fetal bovine serum (FBS), 50 mg/mL penicillin/streptomycin (all ThermoFisher Scientific, San Diego, CA, USA), 1.5 mg/mL collagenase (Sigma Aldrich, Carlsbad, CA, USA), and 50 µg/mL DNase I (Roche, Pleasanton, CA, USA). The digested tissues resuspended in complete RPMI, filtered through a 70 µm cell strainer, and resuspended in flow buffer containing PBS supplemented with 1% FBS and 1% sodium Azide were stained with various antibodies as described next.

3.8. Flow cytometry

Single-cell suspension resuspended in flow bluffer were incubated for 10 minutes with Fc Receptor Binding Inhibitor Polyclonal Antibody (ThermoFisher Scientific, San Diego, CA, USA) (270), stained with surface antibodies (**Annex I Table 2**) for 30 minutes, then fixed with 2% neutral buffered formalin (ThermoFisher Scientific, San Diego, CA, USA) overnight. For intracellular staining, cells stained with surface antibodies were permeabilized for 15 minutes with PBS buffer containing 0.5 % saponin, incubated with intracellular antibodies for 30 minutes, and resuspended in a flow buffer. For immunoglobulin (Ig)M, IgA, and IgG staining in B cells, single-cell suspension was washed using ice-cold acetate at pH4 to eliminate surface immunoglobulins, as

previously described then stain for surface and intracellular markers as described above (271). The sample was acquired using the CytoFlex system (Beckman Coulter, Inc., Indianapolis, IN, USA).

3.9. The Ussing Chambers, forskolin and FITC-Dextran

The proximal and distal colon samples were cut longitudinally and gently rinsed in Krebs buffer supplemented with 10 mM glucose, mounted in the tissue holder was inserted between chambers of the voltage-clamp apparatus (VCC MC8; Physiologic Instruments, San Diego, CA). Krebs/glucose solution was added in each chamber and oxygenated with carbogen (95% O₂ and 5% CO₂) through data acquisition. The transepithelial electrical resistance (TEER) was calculated using the Acquire and Analyze software (Physiologic Instruments, San Diego, CA). Forskolin (Cat#: F3917, Sigma-Aldrich, ST-Louis, MO, USA) was added in the apical chamber for a final concentration of 10 μ M during data acquisition. FITC-dextran 4kDa (Cat#: FD4, Sigma-Aldrich, ST-Louis, MO, USA) was added in the apical chamber for a final concentration of 100 μ g/ml and collected every 30 minutes for 90 minutes from the basolateral chamber to evaluate the mucosal permeability (272).

3.10. Protein extraction and Enzyme-linked immunosorbent assay (ELISA)

A segment of the colon placed in PBS supplemented with protease inhibitors (Roche, Pleasanton, CA, USA), was homogenized, sonicated, and centrifuged for 20 minutes at 4°C. The collected supernatant protein concentrations were measured using the Quick Start™ Bradford Protein Assay Kit (Cat#: 5000201, Bio-Rad Laboratories Inc., Hercules, CA, USA) and stored at -20 °C for later analysis. Commercial mouse conventional ELISAs for IL-6 (Cat#: DY206-05, R&D Systems), TNF- α (Cat#: DY410-05, R&D Systems), TGF- β (DY240-05, R&D Systems), IL-22 (Cat#:

436304, BioLegend), IL-25 (Cat#: 447104, BioLegend) and multiplex ELISA kits for interferon-gamma (IFN- γ), tumor necrosis factor (TNF)- α , interleukins (IL)-1 β , IL-6, IL-17A, IL-17C, IL-17F, IL-21, IL-22, IL-25 (U-PLEX TH17 Combo 2, Meso Scale Diagnostics, Rockville, MD, USA) used according to manufacturer instructions.

3.11. Tissue processing for histology and immunofluorescence

To perform the alcian blue, hematoxylin, and eosin (AB/H&E) staining of immunofluorescence staining, the OCT-embedded frozen tissue section of 6 or 10 μ m was fixed with either cold Carnoy's solution (60% absolute ethanol, 30% chloroform and 10% acetic acid) for 5 minutes or 4% Formalin buffer for 15 minutes. The frozen sections fixed with Carnoy's solution were rehydrated in subsequent passage through increasingly diluted sequences of ethanol-water solutions and rinsed with deionized water for 1 minute.

To perform the alcian blue, hematoxylin, and eosin (AB/H&E) staining, the frozen section of 6 μ m fixed in Carnoy's solution and rehydrated were stained for 15 minutes with alcian blue solution (pH 3) (Molecular probe, Sigma-Aldrich, ST-Louis, MO, USA), washed and stained with Mayer's hematoxylin solution (Molecular probe, Sigma-Aldrich, ST-Louis, MO, USA) for 3 minutes, washed and incubated in Scott's solution for bluing. The washed tissues incubated in 95% ethanol solutions for 1 minute and eosin Y 1% alcohol solution for 30 seconds (Sigma-Aldrich, ST-Louis, MO, USA) were dehydrated in a series of ethanol solutions with increased concentration, cleared with xylene, and mounted. The images were acquired using an Olympus DP camera.

3.12. Immunofluorescence

Frozen tissue sections of 10 µm thickness fixed in 4% PFA incubated in a permeabilized solution (0.25% gelatin and 0.2% triton-100X diluted in PBS) for 10 min. All samples were blocked with a blocking solution containing 5% bovine serum albumin (BSA) diluted in permeabilization buffer or PBS for 2-4 h at room temperature and were incubated with the appropriate primary antibodies in the blocking buffer overnight at 4 °C (**Annex I Table 3**). Tissue labeled for MUC2 were fixed in the Carnoy's buffer and along with tissues labeled for LGR5, and DCLK1 were not permeabilized before staining. The slide sections were washed in PBS with 0.1% Triton-100x for 10 minutes and incubated with the appropriate secondary antibody labeled with Alexa fluor (Molecular probe, Sigma-Aldrich, ST-Louis, MO, USA) for 1 hour followed with one µg/ml of the 4, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, ST-Louis, MO, USA) diluted in PBS for 10 minutes at room temperature in the dark. The tissues section rinsed in washing buffer were incubated in an anti-autofluorescence buffer (10 mM CuSO₄ and 50 mM NH₄Cl) and Milli-Q water for 10 minutes, air dried, and mounted using the ProLong Gold Antifade Mountant (ThermoFisher Scientific, MB, Canada). The images acquired using the EVOS Cell Imaging Systems (ThermoFisher Scientific, Winnipeg, MB, Canada) were processed using FIJI 2.16.0 ImageJ software.

3.13. Statistical analysis

Statistical analyses were performed on all data using GraphPad Prism Version 9.0.0 software (GraphPad Software, San Diego, CA, USA). Flow cytometry data were analyzed using FlowJo software (BD, Franklin Lake, NJ, USA). The flow data were gated on a nucleated, singlet, live,

CD45⁺, CD45⁻ followed by the population of interest (**Please see Chapter V, Figure 1 Supp and Annex I Table 2**). The values from colitic samples were divided by the average of control values for normalization. Continuous variables expressed as the mean $\pm$ standard deviation (S.D) were tested for statistical significance defined as $P < 0.05$ between groups using one-way or two-way ANOVA followed by Tukey-post hoc analysis. All data were evaluated for outlier values using robust regression and outlier analysis. Data were square root-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten (273).

LIST OF ARTICLES PUBLISHED AND UNDER PREPARATION

1. Tshikudi, D.M.; F. Olayinka-Adefemi, C. Zhang, A. Marshall: JE. Ghia. **Regional and sex-dependent immune profiling across the colon in a mouse model of ulcerative colitis.** *Biol. Sex Diff.* (Under review).
2. Tshikudi MD, Bernstein NC, Mishra S, Ghia JE, Armstrong KH. **Influence of biological sex in inflammatory bowel diseases.** *Nat Rev Gastroenterol Hepatol.* 2025. (Published).
3. Tshikudi, D.M.; Hutchison. H.: Ghia, JE. **Pancreastatin Inhibition Alters the Colonic Epithelial Cells Profile in a Sex-Dependent Manner.** *Int. J. Mol. Sci.* 2024, 25, 12757. (Published).

Manuscript under preparation

4. Tshikudi, D.M.; JE. Ghia. **Sex difference influences susceptibility to colitis through altered colon mucosal integrity function.**
5. Tshikudi, D.M.; O. Johnson, F. Hesampour, N. Eissa, JE. Ghia. **Loss of chromogranin A protects males but exacerbates colitis severity in females.**

BRIDGE TO CHAPTER IV

There is increasing evidence of sex-related differences in UC pathophysiology. Moreover, these differences likely influence the capacity of intestinal epithelial cells to repair in acute inflammation. Understanding how intestinal repair processes differ between sexes could offer new insights to improve therapeutic strategies for UC. Accordingly, **Chapter IV** details our efforts to characterize colonic epithelial integrity and mucosal function between sexes in a widely used mouse model of colitis.

CHAPTER IV

Sex difference influences susceptibility to colitis through altered colon mucosal integrity function

Diane M. Tshikudi, and Jean-Eric Ghia

Authors' contributions: JE.G. and D.M.T. participated in the study conceptualization, and D.M.T. carried out the RT-qPCR, ELISA and Ussing experiment, data collection and formal analysis. D.M.T prepared the figures, performed the statistical analysis. JE.G. provided the resources. JE.G. administrated the project and provided funding. (**Manuscript under preparation for publication in the Cell Death & Differentiation Journal**).

4.1. Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by acute inflammation of the large intestine limited to the mucosa. Clinical studies report that men are more likely to develop UC than women. In an experimental murine model of colitis, the elevated susceptibility of male mice to colitis correlated with increased dysregulated mucosal barrier function. Yet the underlying mechanism that regulates sex-related differences in UC is not well understood. Therefore, this study characterized the possible sex differences in the intestinal mucosal barrier function and epithelial integrity in acute colitis. Male and female C57BL/6 wild type (WT), administrated with 5% dextran sodium sulfate in their drinking water to induce colitis or water for the control group for 5 days, were monitored for disease activity score daily. Inflammatory cytokines, epithelial-associated mediators, and markers specific to differentiation, proliferation, differentiated epithelial cells, and stem cells were characterized using ELISA, qRT-PCR, and immunofluorescence techniques. Here, we show that females are less susceptible to DSS-induced colitis than males. Females expressed higher levels of stem cell markers at homeostatic and colitic conditions than males. During colitis, females enhanced the mRNA expression of goblet and tuft cells, all known to play a critical role in maintaining the colon mucosal barrier function. Conversely, males displayed an increased number of differentiating cells compared to females in colitic conditions. This increased differentiation correlates with higher mRNA expression of colonocytes, M cells, and stem fetal-like cell markers. This finding indicates that sex-related differences in UC pathogenesis are likely associated with a differential healing process.

4.2. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) associated with fluctuating periods of relapse characterized by acute inflammation of the large intestine limited to the mucosa, diarrhea, rectal bleeding, abdominal pain, and weight loss, followed by periods of remission (13,29). Epidemiological clinical studies report that men are more likely to develop UC than women (50,274). Moreover, women exposed to hormone therapies such as oral contraceptive pills, whose formulations include progesterone-only or oestrogen-progesterone, are more likely to develop UC (51). Similarly, men undergoing androgen deprivation therapy have a lower risk of developing UC (51). These studies suggest a potential role for sex-related differences in the onset of UC. Preclinical murine models of colitis reported that male mice displayed a higher susceptibility to colitis associated with shorter colon length, worsened stool score, and histological injury, all hallmarks of impaired mucosal barrier function, than female mice (63). Yet the underlying mechanism associated with sex-related differences in UC is not well understood.

Although the mechanism that mediates UC etiology remains unknown, current research suggests that impaired colonic epithelial integrity, likely associated with disrupted epithelial repair and compromised mucosal barrier function, contributes to the pathogenesis and pathophysiology in UC (6). Growing evidence suggests that the epithelium, which overlays the colon mucosa, is crucial for gut barrier homeostasis (5,6). Moreover, longitudinal clinical studies associate the gut mucosal healing, defined as the endoscopic and histologic resolution of the colon mucosal ulcer in UC patients, as a predictor of reduced risk for surgery, hospitalization, long-term remission, and improved patients' quality of life (6,7).

The colonic mucosa, consisting of epithelium, mucus layers, and mucosal defense factors, forms a tight and highly regulated protective barrier that segregates the intestinal tract luminal content comprised of exogenous microorganisms, food antigens, and microbiota from the immune cells of the host (158). The colonic epithelial barrier is a monolayer of specialized epithelial cells tightly connected by tight junctions (275). These differentiated epithelial cells include the absorptive, colonocytes, microfold (M), secretory enteroendocrine, tuft, and goblet cells, which emerge from multipotent stem cells located at the crypt base of the colon (159). Owing to the short life span of colonic epithelial cells (CEC), 4 to 5 days, gut epithelium undergoes rapid self-renewal that preserves the colonic mucosal integrity and barrier function under homeostatic conditions. Thus, epithelium homeostasis is maintained through intestinal epithelial cell shedding in the lumen, restitution, proliferation, and differentiation to replenish the constant loss of cells (25). Failure to elicit the colonic epithelial regeneration can lead to impaired barrier function, increased permeability to luminal antigens, dysregulated immune response, and, consequently, colonic inflammation, as seen in patients with UC (25).

Epithelial self-renewal is driven primarily by fast-cycling crypt base columnar (CBCs) leucine-rich repeat-containing G protein-coupled receptor (Lgr) 5-expressing stem cells, which are progenitors for terminally differentiated epithelial cells involved in the intestinal mucosa daily homeostatic regeneration (191). However, following the formation of severe lesions involving crypt loss, as seen in UC pathophysiology, CBCs Lgr5⁺ stem cells are depleted. As a result, the depleted colonic epithelial cells are restored by a group of heterogenous slow-cycling damage-associated regenerative stem cells (DARSCs), expressing various markers, including homeodomain-only protein homeobox (Hox) (191,192). Moreover, progenitors from secretory

and absorptive lineages and differentiated epithelial cells could restore the intestinal stem cell pool during epithelial regeneration through paligenosis (193–195). Whereby intestinal epithelial cells acquire a fetal-like stem cell signature associated with embryonic genes such as IL-33, Wfdc2, and lymphocyte antigen 6 A Ly6a (Ly6a/Sca1) expression (193–196).

Although extensive studies focused on characterizing the epithelium repair process under homeostatic conditions, studies investigating the mechanism of mucosal healing in acute inflammatory conditions such as those seen in colitis are limited. Moreover, the role of sex-related differences in the mucosal healing process in colitis has yet to be fully understood.

Therefore, this study aimed to characterize the possible sex differences in the intestinal mucosal barrier function and epithelial integrity in the murine model of colitis. We investigated the influence of sex-related differences on the mucosal healing process in a murine model of UC induced by dextran sulfate sodium. Our study focused on profiling features related to mucosal barrier function and epithelial integrity in both female and male mice at steady-state conditions and during colitis. By characterizing the mucosal healing process under colitic conditions for both sexes, we aim to provide a more comprehensive understanding of the influence of biological sex on mucosal healing in the colon, using a mouse model that mimics the pathogenesis UC.

4.3. Results

4.3.1 Males displayed worsened disease severity in DSS-induced colitis, contrasting with the resistance observed in females

The mounting evidence suggests a dichotomy in colitis severity between males and females. To investigate whether the difference in disease severity between sexes results from variations in mucosal barrier function, I examined the impact of DSS-induced acute colitis in C57BL/6 female and male mice. In colitic conditions, both groups showed increased weight loss, DAI, macro-score, and reduced colon length compared to the corresponding control groups (**Figures 1A-D**). However, male mice displayed significant weight loss 24 hours earlier ($P=0.05$), which persisted and worsened compared to females ($P<0.0001$) (**Figure 1A**). Similarly, males showed a higher increase in DAI and macro-score than female mice as DSS treatment persisted ($P<0.04$; $P=0.02$), despite females exhibiting a marked early increase in DAI, 24 hours after colitis induction, compared to male mice ($P=0.001$) (**Figure 1B-C**). Colon length was not significantly different between male and female mice in colitic conditions (**Figure 1D**). The mRNA level of Tff3, a marker with intestinal mucosal inflammation, was substantially increased in colon tissues from male mice but not female mice in colitis compared to control conditions ($P=0.0002$). Consequently, this caused higher Tff3 mRNA levels in males than female mice in colitis ($P=0.02$) (**Figure 1E**). The level of proinflammatory cytokine IL-6 increases, whereas TNF- α and Th2-derived cytokines IL-25 did not differ significantly in colitis compared to control conditions in both groups in colitis. However, females showed a trend toward higher levels of all three cytokines than male mice in both control and colitic conditions (**Figures 1F-H**). TNF- α levels were

significantly higher in colitic females than colitic male mice, whereas IL-25 was markedly elevated in female mice in control and colitic conditions compared to male mice (**Figures 1G-H**).

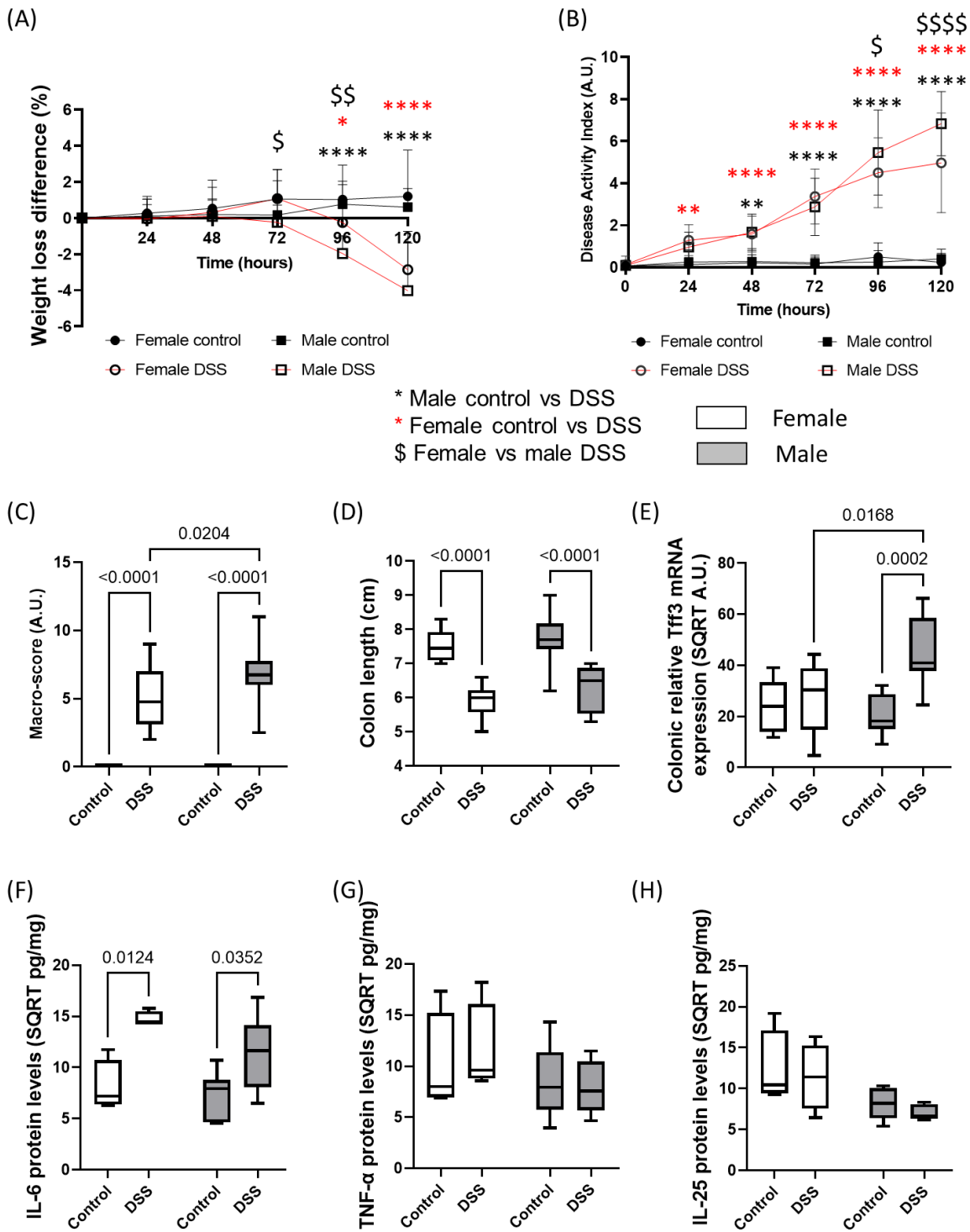


Figure 1. **Males, unlike female mice, are more susceptible to DSS-induced colitis.** Male and female C57BL/6 mice received 5% dextran sulfate sodium (DSS) daily for 5 days (n=7-31). Weight loss (A) and disease activity index (DAI) (B) were calculated from daily scoring of weight loss,

bleeding, and loose stool. Macro score (C) was calculated from rectal bleeding, rectal prolapse, colon bleeding, stool consistency, and colon length (D) after euthanizing the mice. Colon tissues collected were tested for trefoil factor 3 (TFF3) (E) mRNA expression by RT-PCR and level of cytokines (F-H) interleukin (IL)-6, tumor necrosis factor (TNF- α) and IL-25 by ELISA. Data were square root-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten. All data was extracted from 2-3 independent experiments with four mice per group for n=8-12. (A-D) n=12, (E) n=8-12, (F-G) n=8. Statistical significance determined using ordinary two-way ANOVA and Tukey's multiple comparisons test corresponds to $P < 0.05$. All figures exclusively display values that meet a $P < 0.05$. *, \$ $p < 0.05$, **, \$\$ $p < 0.01$, ****, \$\$\$\$ $p < 0.0001$.

Next, we evaluated the mucosal barrier function (**Figure 2**). Our preliminary data showed that, unlike female mice, male mice displayed increased permeability to FITC-dextran in control and colitic conditions (**Figure 2A**). Mucosal resistance and response to CFTR transporter agonist forskolin did not differ significantly during colitis compared to control conditions in male and female mice. In contrast, female mice showed increased mRNA levels of cotransporter Na-K-Cl1 (Nkcc1) and tight junction regulator claudin 4 (Cld4) in colitic compared to control conditions. However, NKCC1 and Cld4 mRNA expression showed no significant differences between both conditions in male mice (**Figures 2D-E**). While the bacterial-induced proteins and antimicrobial peptides Relm β and Cramp mRNA levels did not differ significantly between colitic and control conditions in female mice, the mRNA expression of these molecules was substantially higher in colitic male mice (**Figures 2E-F**). Lypd8 mRNA levels did not show marked differences between colitis and control conditions in male and female mice (**Figure 2G**). Together, these results suggest that males develop exacerbated impaired mucosal barrier function in response to DSS-induced colitis compared to females.

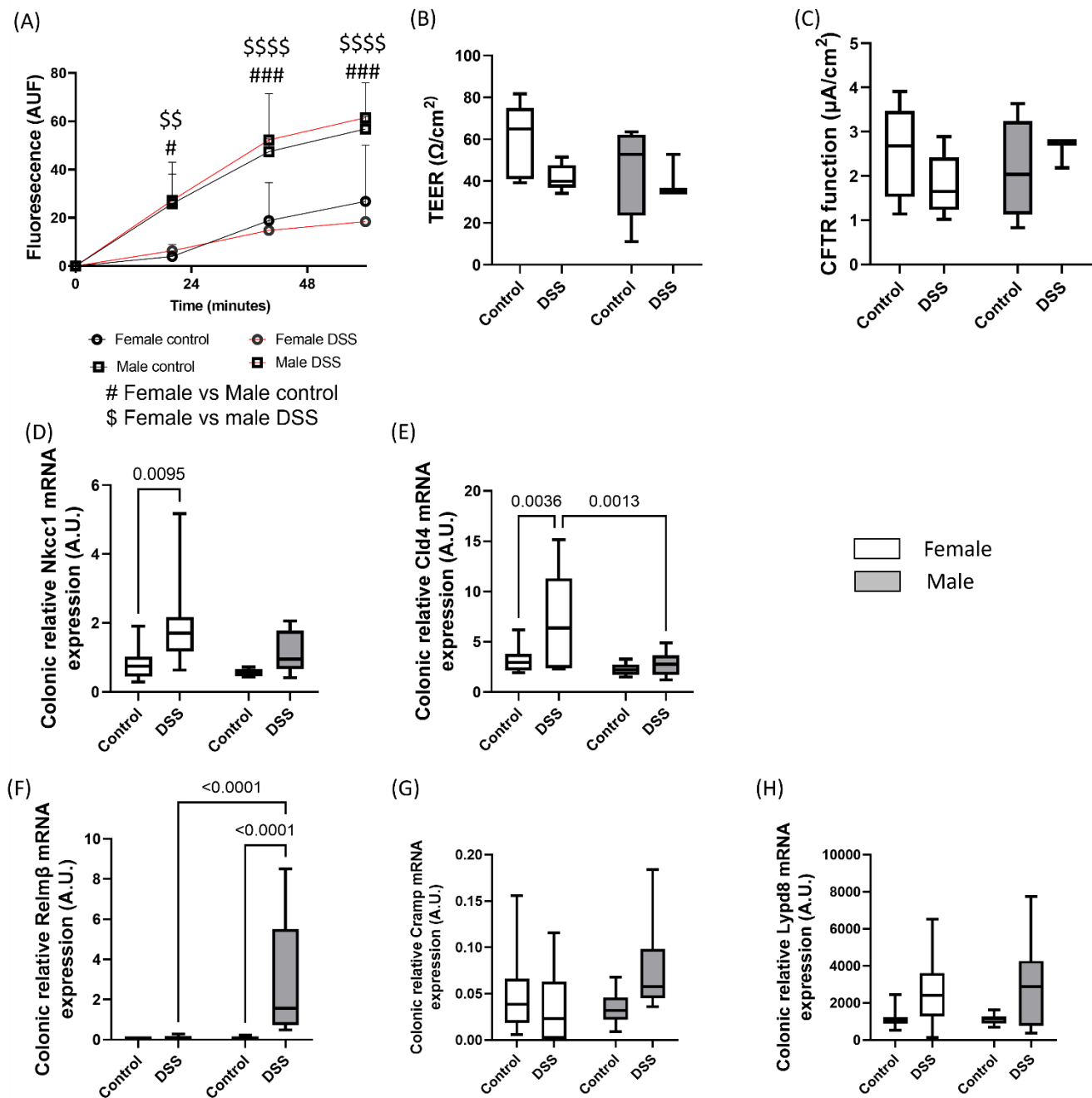


Figure 2. Males display exacerbated loss of mucosal barrier function during colitic conditions compared with female mice. Male and female C57BL/6 mice received 5% dextran sulfate sodium (DSS) for 5 days (n=8-12). Transepithelial electrical resistance (TEER) (A), response to forskolin, and (B) mucosal permeability measured using FITC-dextran 4kDa (C) were evaluated in colon tissues using the Ussing chamber. Gene expression of Na-K-Cl (Nkcc)1 cotransporter (D), tight

junction claudin (Cld)4 (E), bacterially modulated protein and antimicrobial peptides Relm β (F), Cramp (G) and Lypd8 (H) were characterized from colon samples by RT-qPCR. Data expressed as the mean $\pm$ standard deviation. Data were extracted from 1 (A-C) to 2-3 (D-H) independent experiments with 3-4 mice per group for n=3-12. (A) n=3-8, (B) n=1-2, (C) n=2-6. Statistical significance determined using ordinary two-way ANOVA and Tukey's multiple comparisons test corresponds to $P < 0.05$. All figures exclusively display values that meet a $P < 0.05$. Outliers were determined using the robust regression and outlier method. # $p < 0.05$, \$\$ $p < 0.01$, ****, ### $p < 0.001$, #####, \$\$\$\$ $p < 0.0001$.

4.3.2. The expression of intestinal epithelial cell markers is elevated in female colon tissues in control and colitis but not in males.

The epithelium homeostasis is maintained through cell shedding in the lumen and colonic stem cell proliferation and differentiation to replenish the constant loss of cells and preserve the mucosal barrier. Given the discrepancy observed in intestinal mucosal barrier function between both sexes in colitis, we first characterized the differentiation and proliferation of the epithelium and then evaluated the expressions of markers specific to terminally differentiated intestinal epithelial cells in colon tissues from male and female mice in control and colitic conditions.

In control conditions, the intensity of Ki67 immunostaining, a marker for proliferation, was markedly elevated in female compared to male mice (**Figures 3AI, BI**). The intensity of cytokeratin 20 (CKRT20), a marker of intestinal epithelial cell differentiation, did not differ substantially among all groups in control conditions. Proliferation was markedly lower in colitic than control conditions in male and female mice. However, the reduced proliferation was substantially more in colitic male than female mice. The differentiation of epithelial cells was markedly higher in male than female mice in colitic conditions (**Figures 3AII, BII**).

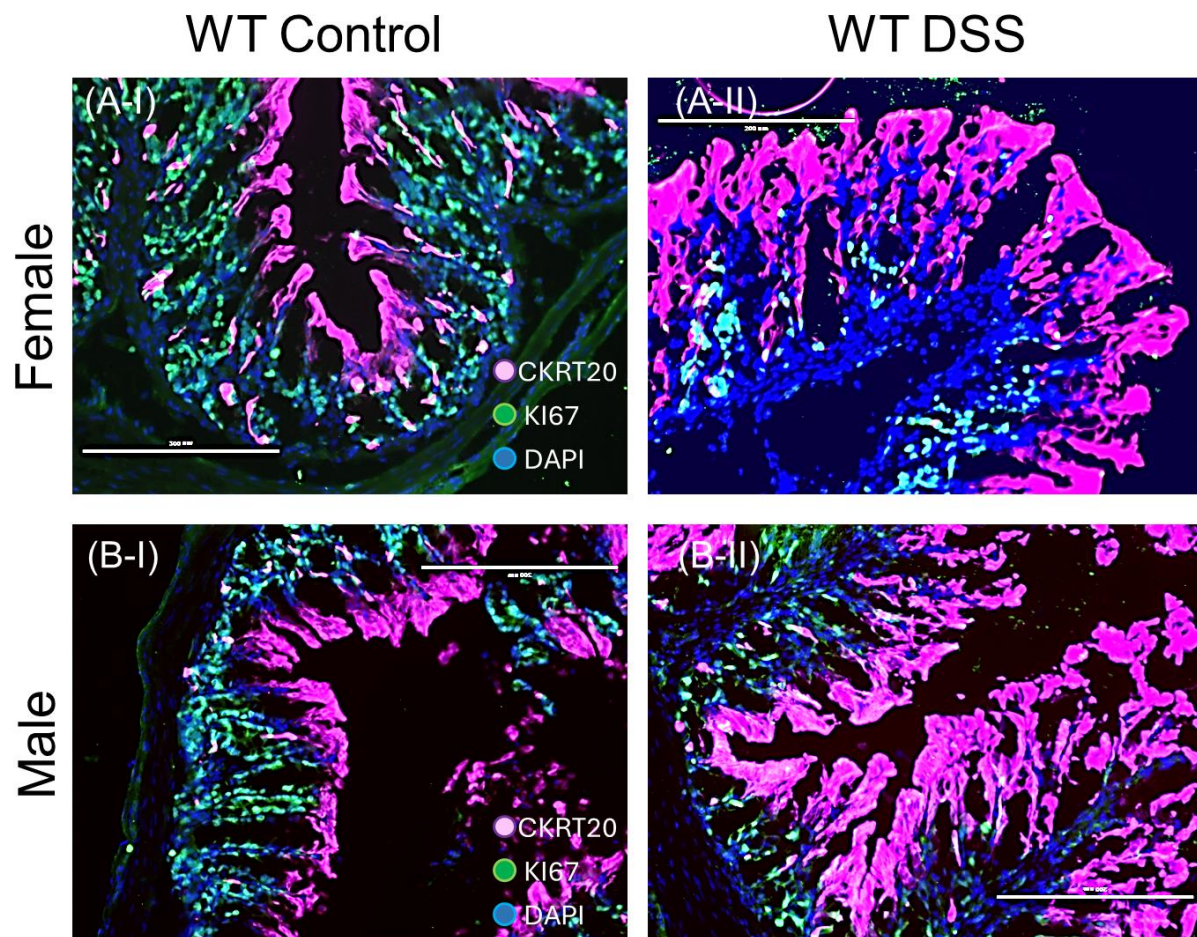


Figure 3. The **female mice exhibit enhanced proliferation at a steady state whereas the male mice present increased differentiation under colitic conditions**. Male and female C57BL/6 mice received 5% dextran sulfate sodium (DSS) for 5 days. Protein levels of proliferation (KI67) and differentiation (cytokeratin 20, CKRT20) were assessed by immunofluorescence (IF) (AI–AII) in females and (BI–BII) male mice. Scale bar: 200 μm. Data extracted from one independent experiment with three mice for a total of n=3.

The mRNA expression levels for goblets, tufts, colonocytes, enteroendocrine, and M cells markers, which included mucin 2 (Muc2), doublecortin-like kinase 1 (Dclk1), carbonic anhydrase 2 (Ca2), CHGA, and transcription factor Spib (Spib), respectively were evaluated (**Figure 4**). The level of all differentiated epithelial cell markers did differ significantly in control conditions. However, in colitis, Muc2 and Dclk1 mRNA levels were increased in colon tissues from female mice compared to control conditions ($P=0.0008$; 0.0002), whereas colonocyte marker Ca2 mRNA expression did not change significantly between both conditions (**Figures 4A-C**). In male mice, Ca2, Muc2, and Dclk1 mRNA levels did not differ markedly in colitic conditions compared with control conditions. As a result, Muc2 and Dclk1 mRNA expression were significantly higher in female colon tissues than in male mice in colitis ($P=0.01$). CHGA mRNA levels increased at similar levels in both male and female colon tissues in colitis compared with control conditions ($P<0.001$) (**Figure 4D**). Although Spib mRNA levels did not differ significantly in colitis compared to control conditions in both sexes, colon tissue from colitic male mice exhibited higher Spib mRNA expression than in female mice ($P=0.006$) (**Figure 4E**).

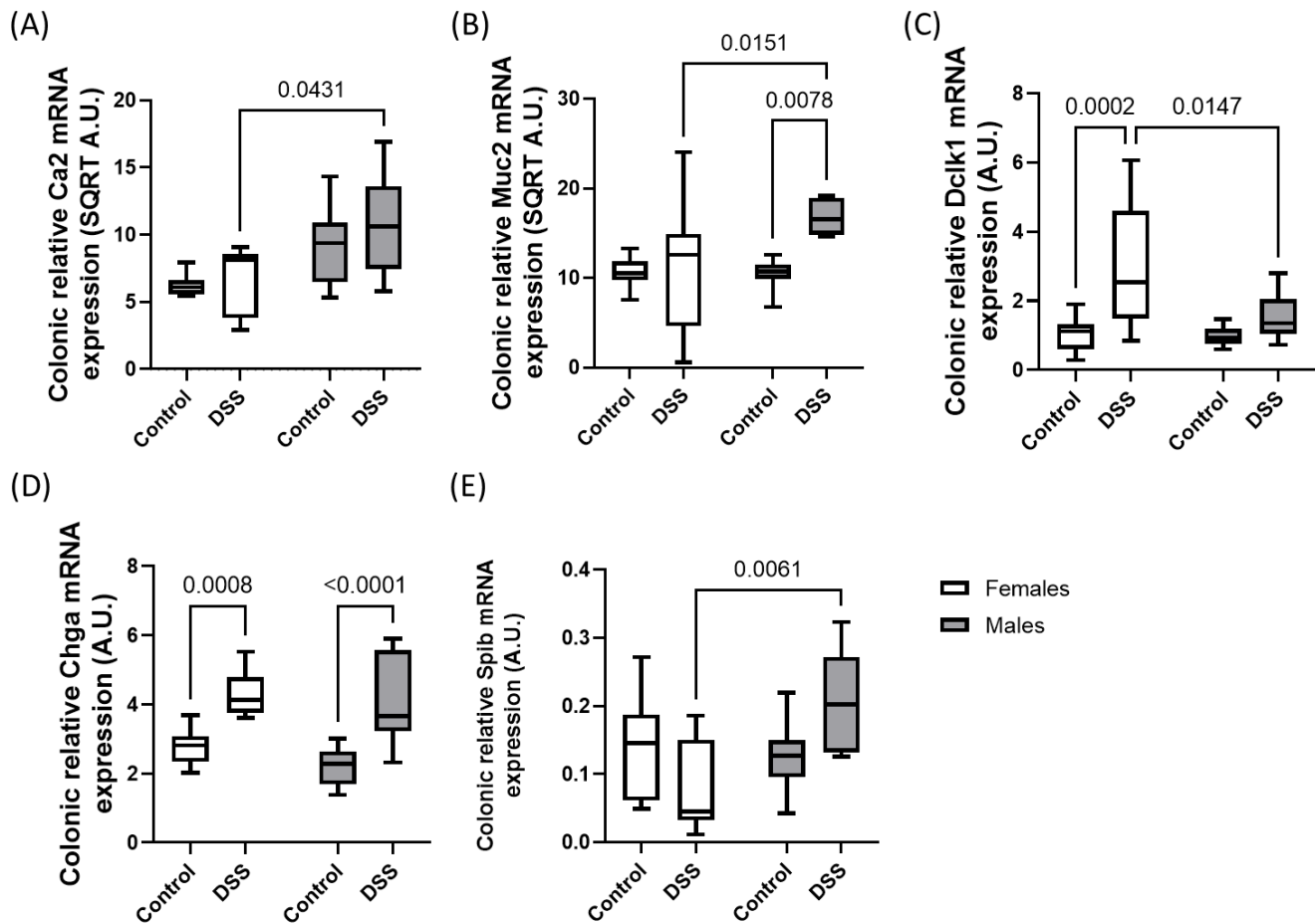


Figure 4. Females display increased expression of differentiated cell markers in colitis. Male and female C57BL/6 mice received 5% dextran sulfate sodium (DSS) for 5 days (n=6-12). Biomarkers specific to (A) mature goblets mucin (Muc) 2, (B) colonocytes (carbonic anhydrase (CA) 2) (C), tuft cells doublecortin-like kinase 1 (Dclk1), and (D) enteroendocrine cells chromogranin A (CHGA) were assessed in colon tissues by real-time qPCR. Data from 2-4 independent experiments with 3-4 mice per group for n=8-12. (A) n=7-8, (B) n=8-12, (C) n=6-8, (D) n=7-12, (E) n=7-10, expressed as the mean $\pm$ standard deviation and square root-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten. Statistical significance determined using ordinary two-way ANOVA and Tukey's multiple comparisons test corresponds to $P < 0.05$. All figures exclusively display values that meet a $P < 0.05$. Outliers were determined using robust regression and outlier methods.

To examine whether the change in differentiated epithelial cells profile was a result of the altered profile in the stem cell compartment, we assess the mRNA expression of markers Lgr5, Hopx, and Ly6a specific to CBC, damage-associated regenerative (DARCs) and fetal-like stem cells in both control and colitic female and male mice (**Figure 5**). In control conditions, female mice exhibited higher Lgr5 and Hopx mRNA levels than male mice, but Ly6a mRNA expression did not differ significantly between sexes (**Figure 5A-B**). Following colitis induction, Lgr5 and Hopx mRNA expression were downregulated compared to control conditions in both female and male mice, although the reduction in their expression was markedly exacerbated in males than in females (Lgr5: $P=0.009$; Hopx: $P=0.01$). By contrast, increased Ly6a mRNA levels were observed in both sexes, although more significantly in male than in female mice ($P<0.0001$ vs $P=0.09$) (**Figure 5C**). Thus, the resistance to colitis observed in female mice likely stems from their ability to preserve intestinal epithelial proliferation and differentiation capacity.

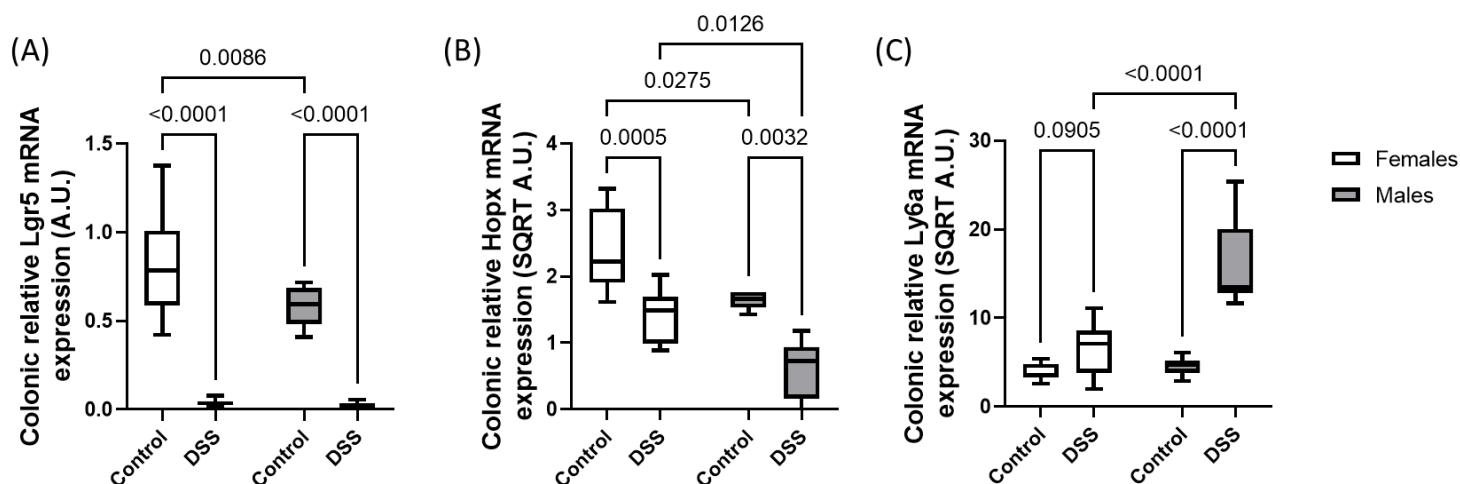


Figure 5. The female mice display elevated stem cell markers at steady state and during colitis compared to male mice. Male and female C57BL/6 mice received 5% dextran sulfate sodium (DSS) for 5 days (n=6-12). Biomarkers specific to (A) crypt base columnar (Lgr5), (B) damage-associated regenerative (Hopx), and (C) fetal-like (Ly6a) stem cells were assessed from colon samples by real-time qPCR. Data from 2-4 independent experiments with 3-4 mice per group for n=8-12. (A) n=7-8, (B) n=8-12, (C) n=6-8, (D) n=7-12, (E) n=7-10, expressed as the mean $\pm$ standard deviation and square root-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten. Statistical significance determined using ordinary two-way ANOVA and Tukey's multiple comparisons test corresponds to $P < 0.05$. All figures exclusively display values that meet a $P < 0.05$. Outliers were determined using robust regression and outlier methods.

4.2. Discussion

Evidence of sex-related differences in UC and colitis disease courses reported in human population-based clinical and preclinical animal studies showed a higher risk for developing UC than women (50,63,274). Many preclinical murine models of colitis reported that male mice displayed a higher susceptibility to colitis associated with shorter colon length, worsened stool score, and histological injury, which are all hallmarks of impaired mucosal barrier function, than female mice (63). Yet the underlying mechanism associated with sex-related differences in UC is not well understood. Therefore, this study aimed to characterize the possible sex differences in the intestinal mucosal barrier function and epithelial integrity in the murine model of colitis.

This study demonstrated that, unlike males, female mice resist DSS-induced colitis. This is likely due to a higher repair potential mediated by enhanced proliferation and differentiation, which causes increased mucosal barrier function. These studies indicate that sex-related differences in UC pathogenesis are likely associated with a differential healing process.

Consistently, our findings showed that males, in response to experimental colitis, exhibit severe weight loss along with elevated DAI and macro-score index, which are features of exacerbated disease severity when compared to females. A cross-sectional clinical study in patients with UC proposed TFF3, a mucin-associated peptide, as a biomarker of mucosal healing (241). Thus, the study found elevated TFF3 levels in the serum of UC patients with active mucosal injury, which was reduced, in part, after mucosal healing as defined by endoscopy remission (241). In line with the previous study, we find that males exacerbated disease severity correlated with a substantial increase in TFF3 levels, which contrasted with the lack of change in TFF3 expression observed in

females, Suggesting that males undergo more severe DSS-induced mucosa destruction than females. Shortening of the colon and proinflammatory cytokines such as IL-6 and TNF- α are well-known characteristics of inflammation during colitis (276). As both males and females had reduced colon length and increased IL-6 levels in colitis, these changes did not differ between sexes, likely suggesting either a lack of sensitivity to potential alteration mediated by sexes or that sex-mediated differences do not influence these factors. As part of the IL-17 family, studies have shown that IL-25, a Th2-mediator secreted mainly by tuft cells and subepithelial macrophages, promotes the production of Th2 cytokines IL-4, IL-5, and IL-13, enhances epithelial cell hyperplasia, in addition to facilitating the recruitment of inflammatory cells (246). IL-25 also reduced the production of proinflammatory cytokines from monocytes isolated in inflamed tissue of UC patients (246,277). Furthermore, IL-25 expression is reduced in the colon of UC patients with active disease (277). Clinical studies have reported lower IL-25 expression in the colon of UC patients with active disease (278). Conversely, a study by Reynolds and colleagues revealed that deficiency in IL-25 in male mice of a C57BL/6 background conferred protection from inflammatory destruction, mucosal remodeling, and leukocyte infiltration (246,277,279). Here, the level of IL-25 did not change substantially between colitis compared to homeostatic conditions in both male and female mice. The discrepancy between reports can be explained by the influence of regional differences in the expression of IL-25 along the large intestine, as we recently demonstrated that IL-25 levels differ along the large intestine (51).

Defective mucosal barrier function may increase intestinal epithelial permeability to luminal antigens, initiating the immune response and promoting inflammation (280). Component of the intestinal mucosa barrier encompassed the adhesion molecules which regulate the paracellular

transport, mucosa electrogenic transports, antimicrobial peptides, and mucus layers (280,281). During inflammation, altered mucosal barrier function correlates with increased paracellular permeability caused by changes in adhesion molecules such as tight junction claudin 4 (Cld4), dysregulated electrogenic transports mediated by cystic fibrosis transmembrane conductance regulator (CFTR) protein and Na-K-Cl 1 (NKCC1) co-transporter, along with changes in the antimicrobial peptide expressions (160,280,281). Our findings showed that males exhibit a trend toward increased permeability to 4kDa fluorescein isothiocyanate-dextran (FD4), reduced resistance and lower CFTR function in colitis. These findings align with previous studies that reported increased mucosal permeability to FD4 and a tendency toward reduced resistance and lower CFTR expression in UC patients with active disease and mice with DSS-induced colitis (48,160,282). However, compared to control conditions, females did not display marked changes in mucosal resistance, FD4 permeability, and CFTR activity in colitis.

NKCC1, is a co-transporter that tightly regulates the transport of ions Na^+ , K^+ , and Cl^- across plasma membrane of many cells, including intestinal epithelial cells. In recent years, studies have suggested that the loss of NKCC1 in the colon may reduce colon hydration, dysregulate the release of mucus from goblet cells, impair the formation of the mucus barrier, and consequently promote intestinal inflammation (283). Moreover, NKCC1 have been shown to downregulate CLD2, a pore-forming TJ, associated with increased intestinal permeability, suggesting a critical role for NKCC1 in preserving intestinal mucosa barrier function (284). Similarly, studies demonstrated that Cld4 may disrupt the polymerization of pore-forming TJ CLD2, 7, 15 and 19, thus indirectly prompting a decrease in mucosa permeability and increase function (172). Cld4 expressions differ among studies, some reported reduced Cld4, whereas others reported that its expression remained

unchanged in UC (160,167,171,178). Here, we showed that females upregulate *Nkcc1* and *Cld4* mRNA expression, contrasting with the lack of changes observed in males in colitis. These results probably indicate that females preserve their ability to maintain their intestinal mucosa barrier function in colitis partly through the increase in NKCC1 and CLD4 in colitic conditions. Females' ability to preserve their mucosal barrier function in colitis likely causes the absence of marked changes in *Relmβ* and *Cramp* mRNA expression, two proteins whose expression is highly upregulated by the microbiota compared to control conditions (49,242). On the other hand, the marked increase of both markers in males is likely a consequence of worsened loss of mucosal barrier functions.

The colonic mucosa comprises multilayer tissues, among which the intestinal epithelium, a single layer of epithelial cells, serves as the first line of defense against food, microbiota, and pathogen antigens (158). In homeostasis, the maintenance of intestinal epithelium integrity occurs through a process of continuous regeneration involving the self-renewal of rapidly proliferating stem cells along with differentiation of progenitor cells into terminally differentiated epithelial cells, including goblet, colonocytes, tuft, enteroendocrine, and microfold cells (159). In UC or colitis, the lesions are confined mainly in the mucosa and caused by epithelial damage, dysregulated mucosal barrier, and initiate the epithelial wound repair or healing to re-establish the colonic mucosa homeostasis (25). Similarly, coordinated by many mediators of the mucosal regeneration process, the epithelial wound healing process requires activation of the epithelial cells' migration, proliferation, and differentiation to restore intestinal mucosa homeostasis (25). Here, we showed that females exhibit an elevated number of proliferating cells at steady state, which correlated with substantially more mRNA expression of crypt-based columnar (CBCs) *Lgr5*⁺ and reserve stem

(RSCs) Hpx⁺ stem cells compared to males. Although, differentiation did not differ markedly between both sexes. These results hint toward a higher mucosal healing potential for females than males. This finding, validated by our subsequent study, showed that despite the expected reduction in CBCs and RSCs associated with inflammation in colitis, females conserved part of Lgr5 and Hpx mRNA expression compared to males. Moreover, females increased the mRNA expression of most differentiated epithelial cells, including goblet and tuft cell makers, then males in colitis. The role of these differentiated epithelial cells is critical to preserve the intestinal mucosal barrier as goblet cells produce the mucus layer, which serves as a physical barrier that limits the interaction between the luminal contents and epithelium, hence preventing inflammation and mucosal injury (25). Similarly, studies reported that tuft cells could promote intestinal epithelial cell proliferation and maintain mucosal integrity through the upregulation of tight junctions, along with serving as a reserve stem cell population that can reconstitute intestinal crypt through paligenosis (247). Overall, the maintenance of stem cells and increased goblet and tuft cell expression in the intestinal mucosa of females in colitis might offer a better capacity for mucosal healing and increased resistance to DSS-mediated colitis.

On the other hand, males exhibit an increased number of differentiating cells compared to females in colitic conditions, which correlated with elevated mRNA levels of colonocytes, M cells, and fetal-like markers. These observations are consistent with previous studies that documented an increase in immature and less differentiated atypical colonocytes, a higher number of M cells, and elevated levels of Ly6a⁺ fetal-like stem cells in UC and DSS-induced colitis in response to inflammation and severe epithelial damage (197,198,252,285). These atypical colonocytes, highly susceptible to apoptosis, displayed enhanced antigen transport and epithelial permeability and

correlated with mucosal inflammation severity in UC (285,286). M cells act as an antigen entry site for mucosal immune surveillance. Thus, at a steady state, M cells sample and transfer the luminal particles through the epithelial barrier to the underlying antigen-presenting immune cells by transcytosis (251). However, the role of M cells in inflammatory conditions such as UC is still poorly understood. Due to the importance of M cells functions in homeostatic conditions, we can hypothesize that an excessive number of M cells in the intestinal epithelium could be detrimental to colitis. Thus, M cells could serve as a portal for pathogens to invade the intestinal mucosa resulting in mucosal damage (251). Moreover, M cells might potentially exacerbate inflammation and disease severity by promoting dysregulated immune cell activation. Similar to previous findings, colitis was associated with elevated CHGA mRNA expression in both sexes, suggesting an absence of influence for sex-related differences in CHGA expression.

4.3. Conclusion

In summary, males demonstrate enhanced susceptibility to colitis, which correlates with elevated levels of inflammation, bacterial infiltration in the mucosa, a marked reduction in stem cell populations, and an excessive differentiation towards absorptive cell lineages. In contrast, females show resistance to DSS-induced colitis, characterized by preserved stem cell pools, elevated secretory differentiated cells, and enhanced expression of mediators that promote barrier-forming tight junctions, all key indicators of maintained epithelium integrity and effective mucosal repair.

BRIDGE TO CHAPTER V

UC can arise from various factors, including over-activation of the immune response due to impaired mucosal barrier function and increased infiltration of microbiota in the colonic mucosa. Overactivation of the immune response can lead to uncontrolled inflammation and damage to the mucosa along the large intestine. As outlined by **Chapter I**, males and females display different immune responses that may influence the severity of UC. Moreover, in **Chapter IV**, we demonstrated that males displayed increased susceptibility to colitis, which correlated with marked loss of epithelium integrity and mucosal barrier function, as well as elevated levels of colonic mucosal inflammatory mediators. Several studies report significant heterogeneity in the immune system along the gastrointestinal tract, likely resulting from regional differences in intestinal anatomical structure, function, and microenvironment. Therefore, determining how immune cells and responses differ across segments of the large intestine under healthy and diseased conditions between sexes is essential for understanding UC pathophysiology. In **Chapter V**, we characterized the immune cell and cytokine profiles along regions of the large intestine in male and female mice under colitic and control conditions. Our findings reveal distinct immune cells and cytokines spatial patterns across segments of the LI that differ between sexes during colitis, suggesting a link between biological sex, the compartmentalization of immune cells, and responses along the LI, and likely explain the susceptibility of males to DSS-induced colitis. Furthermore, this study thoroughly explains how the immune system is compartmentalized along the large intestine and highlights the differences between sexes in relation to colitis.

CHAPTER V

Regional and sex-dependent immune profiling across the colon in a mouse model of ulcerative colitis

Diane M. Tshikudi, Fola Olayinka-Adefemi, Christine Zhang, Aaron Marshall, Jean-Eric Ghia

Authors' contributions: JE.G., D.M.T., and A.M. participated in the study conceptualization, and D.M.T. carried out the panels one to three flow cytometry and the cytokines study data collection and formal analysis. F.O.A carried out the B cells subset data collection and analysis. C.Z. provided support in all flow cytometry data collection and analysis. D.M.T. prepared the figures, performed the statistical analysis, and drafted the manuscript. JE.G. and A.M. provided the resources. JE.G. administrated the project and provided funding. All authors have read and agreed to the published version of the manuscript.

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5.1. Abstract

Background: Ulcerative colitis (UC), an incurable inflammatory bowel disorder, is characterized by a dysregulated immune system leading to mucosal damage along the large intestine. Sex-mediated differences in the immune responses might influence UC pathogenesis and disease course. Given the distinctive immune profile across the gastrointestinal tract, determining how the immune system changes along the large intestine in healthy and disease conditions between sexes is critical for understanding UC pathophysiology. **Aim:** Here, we characterized the spatial distribution of immune cells and cytokines along the large intestine between sexes using a UC mouse model. **Methods:** Segments of the large intestine, ceca, proximal, medial, and distal colons collected from male and female C57BL/6 mice treated with 5% dextran sodium sulfate to induce colitis or water (control) were analyzed to assess the immune cells, anti-inflammatory, proinflammatory, and T helper 17 (TH17) cytokines using flow cytometry and Multiplex ELISA. **Results:** At steady-state conditions, males had higher CD8⁺ in the proximal colon and a gradual increase in germinal center B cells, cytokines interleukin (IL)-6, IL-17C, IL-17F, IL-25, IL-10, and tumor growth factor (TGF)- β along the large intestine. Females exhibited enriched innate cells, T regulatory (Treg), T follicular cytotoxic (Tfc), and higher interferon (IFN)- γ levels along the large intestine. However, dendritic cells (DCs) were higher in the cecum and proximal colon than the distal colon, contrasting with the gradual increase of monocyte-macrophages and granulocytes from the cecum to the distal colon. In colitis, granulocytes and monocyte-macrophages increased and correlated with increased levels of proinflammatory cytokines across most regions of the large intestine in both sexes. In males, tolerogenic T lymphocytes increased in the distal colon. M2 macrophages were reduced and accompanied by elevated TH17 cytokines levels in the colitic

cecum. Females had elevated CD11B⁻CD103⁺DCs and lower B cells along the tissue associated with increased TGF- β levels at the distal colon in colitis. **Conclusion:** The immune profile differs along the large intestine between males and females at steady-state and in colitic conditions, highlighting the importance of considering the regional difference between sexes during diagnosis while assessing new therapies' efficacy using the UC mouse models.

5.2. Background

Ulcerative colitis (UC), a subset of inflammatory bowel disease (IBD), is a chronic inflammatory disorder restricted to the large intestine (LI) of unknown etiology, thought to result from a dysregulated immune response to commensal microorganisms in genetically susceptible individuals (140). As one of the largest tissues in the gastrointestinal (GI) tract, subdivided into the cecum, colon, and rectum in humans, the LI's primary function is to reabsorb water and serve as a reservoir for commensal microorganisms (20,287). The LI forms a continuous tube lined by a mucosa consisting of a single layer of epithelium overlaying the lamina propria and the submucosa (20). Along with other tissues of the GI tract, the LI encompasses 3-4% of the body's total immune cells, located within organized secondary lymphoid structures known as gut-associated lymphoid tissue (GALT) or scattered throughout the mucosa and submucosa (288,289). Several studies report significant heterogeneity in the immune system along the GI tract, likely resulting from regional differences in intestinal anatomical structure, function, and microenvironment (131,287,290).

During inflammation, innate immune cells are recruited and activated in the intestinal mucosa. This activation is associated with the expression of numerous costimulatory molecules and microbial receptors, and overexpressed proinflammatory cytokines (138,291). The resulting inflammatory environment likely leads to a loss of immune tolerance to commensal microorganisms and abnormal activation, expansion, and maturation of T and B cells, promoting UC pathogenesis and disease progression. RNA sequencing studies using large datasets have revealed regional differences in immune cell profiling across intestinal tissues associated with enriched innate and adaptive immune cells in the colon and rectum in UC patients (292). Mounting evidence showed that sex bias can influence the immune system independently of the gut microbiota in lymphoid organs such as the spleen, thymus, mesenteric lymph nodes, and Peyer's Patches, highlighting the link between the gut, sex difference, and immune system (293) . However, the exact profiling of immune cells' landscape along the LI and whether sex can influence their profile remains poorly understood in an inflammatory condition such as UC, as presented in our recent review (51).

The dextran sulfate sodium (DSS) model of colitis is widely used to study UC pathogenesis and pathophysiology due to its cost-effectiveness and ease of manipulation. However, reconciling data between studies can be challenging, as samples are often collected from different locations within the LI, making direct comparisons difficult. In this study, we investigated the influence of sex-related differences in the immune system along the LI in DSS-induced colitis by profiling the distribution of innate and adaptive immune cells in the cecum and across segments of the LI in female and male mice, both at steady state and during colitis. Given that cytokines can shape the profile of immune cells and promote colitis, we also characterized cytokine levels along the LI.

By standardizing sample collection locations and profiling immune cell distribution and cytokine levels, our study aims to provide a more comprehensive understanding of sex differences in a mouse model mimicking UC pathogenesis.

5.3. Materials and Methods

5.3.1. Mice

Male and female C57BL/6 mice, 12-20 weeks old, were obtained from colonies housed and bred under conventional conditions at the animal house of the University of Manitoba. Animals were kept in filter-top cages, fed a standard diet, received water *ad libitum*, and housed in a 12-hour dark/light cycle under controlled humidity and temperature. All animal experiments performed during the light cycle were conducted according to the Canadian Council on Animal Care (CCAC) guidelines for animal research, which follow the Animal Research Reporting of In Vivo Experiments (ARRIVE) guideline. This study was approved by the University of Manitoba Animal Ethics Committee (**Protocol #: 23-023**).

5.3.2. Induction and assessment of DSS colitis

Male and female mice were assigned randomly into treated and control groups, each containing 4-8 mice. The treated groups received 5% DSS (molecular weight: 40-50kDa, ThermoFisher Scientific, Catalog #: J14489-22) in their drinking water for five days to induce colitis. The control groups received water. Animals' disease activity index (DAI) was monitored daily for weight loss, stool consistency, and bleeding, as previously published (268). On day five following DSS

induction, animals were euthanized by cervical dislocation. Macroscopic score was determined (267). The large intestine was collected, and colon length was assessed. The tissues cleared of feces were divided into four samples (Figure 1); the colon was equally subdivided into the proximal, medial, and distal colon and the cecum for flow cytometry and enzyme-linked immunosorbent assays (ELISA) analysis.

5.3.3. Immune cells isolation and antibody staining

Washed colon tissue segments open longitudinally were incubated twice in the epithelial cells isolation buffer composed of phosphate buffer saline (PBS), 2mM Ethylenediaminetetraacetic acid (EDTA), 43.4mM sucrose, and 0.5 mM dithiothreitol (DTT) (Sigma Aldrich, Carlsbad, CA, USA) for 15 minutes at 4°C. Samples rinsed in cold PBS were minced and digested for 40 minutes at 37°C in RPMI supplemented with 10% fetal bovine serum (FBS), 50 mg/mL penicillin/streptomycin (all ThermoFisher Scientific, San Diego, CA, USA), 1.5 mg/mL collagenase (Sigma Aldrich, Carlsbad, CA, USA), and 50 µg/mL DNase I (Roche, Pleasanton, CA, USA). The digested samples, resuspended in cold complete RPMI buffer, were filtered through a 70 µm cell strainer. Cells resuspended in flow buffer containing PBS supplemented with 1% FBS and 1% sodium Azide were stained with antibodies as previously described. In Brief, cells resuspended in flow bluffer were incubated with Fc Receptor Binding Inhibitor Polyclonal Antibody (ThermoFisher Scientific, San Diego, CA, USA) for 10 minutes (270), stained with surface antibodies (BioLegend and ThermoFisher Scientific, San Diego, CA, USA) (antibodies complete list is available on Additional file Supp. Table 1) for 30 minutes, then fixed with 2% neutral buffered formalin (ThermoFisher Scientific, San Diego, CA, USA) overnight. For

intracellular staining, cells were permeabilized with PBS buffer containing 0.5 % saponin for 15 minutes, incubated with intracellular and extracellular antibodies for 30 minutes, and resuspended in a flow buffer. Flow cytometry for surface and intracellular staining of immunoglobulin (Ig)M, IgA and IgG in B cells was performed as described above, except for the inclusion of an acid wash step before FC blocking using ice-cold acetate at pH4 to eliminate surface immunoglobulins, as previously described (271). The CytoFlex system was used for sample acquisition (Beckman Coulter, Inc., Indianapolis, IN, USA).

5.3.4. Protein isolation and cytokines measurement

Minced colon samples resuspended in PBS supplemented with protease inhibitors (Roche, Pleasanton, CA, USA) were homogenized mechanically and centrifuged for 20 minutes. Supernatants were collected and stored at -20 °C for later analysis. Commercial mouse conventional ELISAs for IL-10 and TGF- β (R&D Systems, Inc., Minneapolis, MN, USA) and multiplex ELISA kits for interferon-gamma (IFN- γ), tumor necrosis factor (TNF)- α , interleukins (IL)-1 β , IL-6, IL-17A, IL-17C, IL-17F, IL-21, IL-22, IL-25 (U-PLEX TH17 Combo 2, Meso Scale Diagnostics, Rockville, MD, USA) were used according to manufacturer instructions.

5.3.3. Data analysis

Simple size, estimated at $n=7$, was calculated using an effect size of 0.497, estimated standard deviation of 0.286, type I and II error rate of 0.05 (two tails) and 0.2, respectively. The effect size was calculated from the difference in neutrophils abundance in the medial colon between control and DSS conditions in female mice. Flow cytometry data were analyzed using FlowJo software

(BD, Franklin Lake, NJ, USA). Data were gated on a nucleated, singlet, live, CD45⁺, CD45⁻ followed by the population of interest (Supp. Figure 1) (A complete antibodies list is available in Additional file in Supp. Table 1). Four panels were established to characterize immune cells and subsets. Flow cytometry and ELISA data from colitic samples were normalized to the data measured in control conditions. The normalization was performed by dividing data measured from the colitic samples to the average of control data. Statistical analysis robust regression and outlier, Z score, one and two-way ANOVAs followed by a post-hoc Tukey test were performed when appropriate using Microsoft Excel and GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). Results are expressed as the mean $\pm$ standard deviation of the mean. Data were significant at $P < 0.05$. Data were square-root-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten (273).

5.4. Results

5.4.1. Distribution of major immune cells subsets along male and female mice's large intestine in homeostatic conditions.

We profiled the major immune cell lineages along male and female mice LI in homeostatic conditions. The relative fraction of dendritic cells (DCs), monocytes/macrophages, granulocytes, natural killer (NK), NKT, B, CD4⁺, and CD8⁺T cells within the CD45⁺ cells compartment differed significantly along the LI between sexes (Figure 1B, Supp. Figures 4A1-H2). DCs, monocytes/macrophages, and granulocytes were more enriched in female than male LI mice. Conversely, NK and CD8⁺T cells were more abundant in males than in female mice distal and

proximal colon, respectively (Figure 1C, Supp. Figures 4A1-C2; D1-D2). Along the LI, DC abundances were higher in the cecum, peaked in the proximal colon ($P=0.0032$), and abruptly decreased in the medial and distal colon in female mice ($P<0.0001-0.05$). Conversely, macrophage frequencies were significantly higher in the medial and distal colon, whereas neutrophils peaked in the distal colon compared to the cecum and proximal colon in female mice ($P=0.004$; $P<0.0001$). The relative proportion of NK cells remains unaltered along the LI in both sexes except for the peak in the male distal colon ($P=0.03$). Conversely, NKT peaked markedly in the proximal colon in male mice ($P=0.01$). B cell abundances peaked in the cecum and drastically reduced in the proximal colon in both sexes ($P<0.0001$). $CD4^+$ and $CD8^+$ T cell frequencies did not differ significantly along the LI in both sexes, whereas DCs and macrophages were distributed similarly along male mice LI.

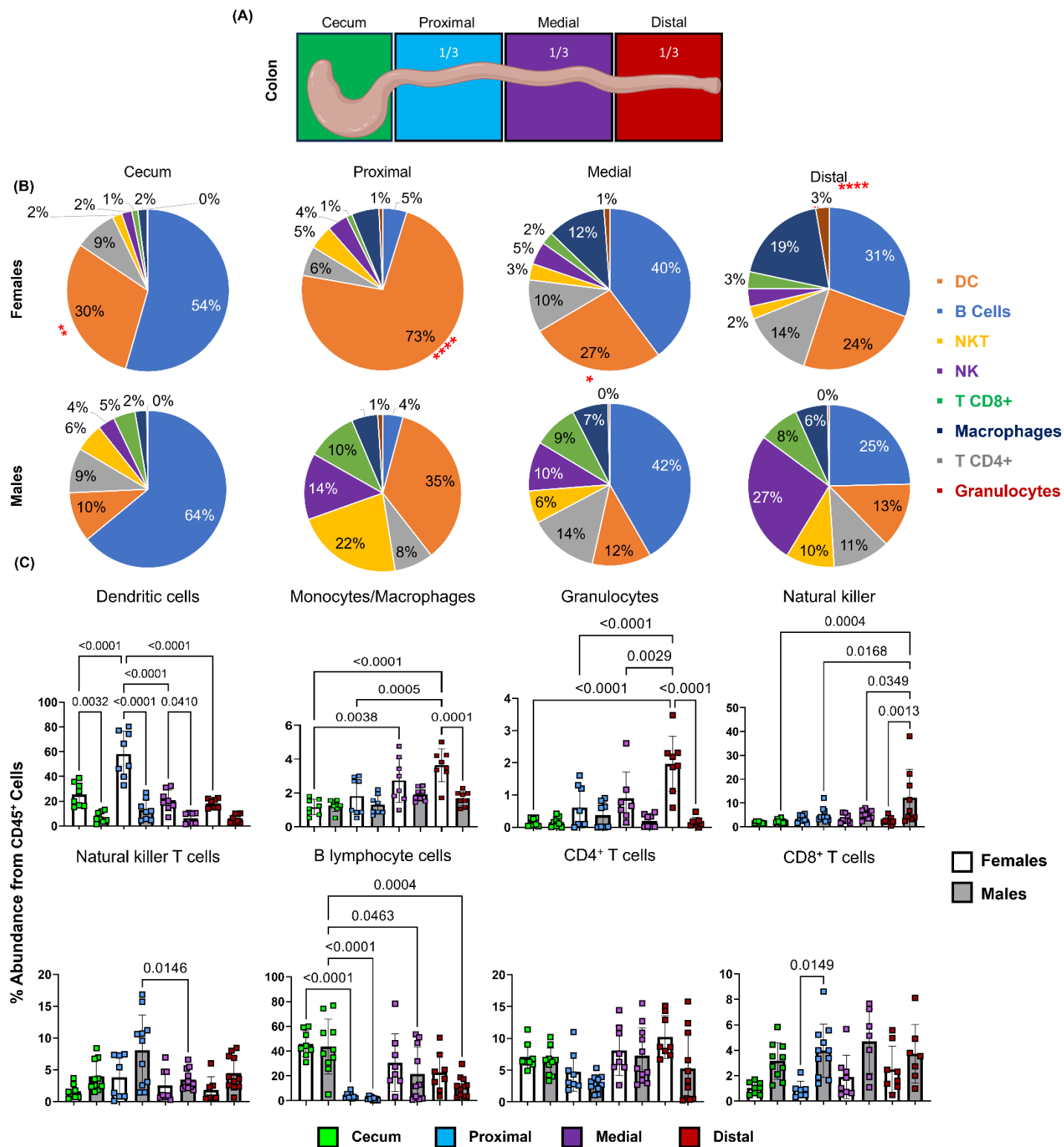


Figure 1: Major immune cell profiles differ across the large intestine in a sex-dependent manner in homeostatic conditions. (A) Diagram describing the segmentation of the large intestine for sample collection. The profiles of male and female mice's innate and adaptive immune cells within the large intestine, including the cecum, proximal, medial, and distal colon. (B) Relative fractions of immune cells across all segments of the large intestine in male and female mice. (C) Comparison of the abundance of major immune cell lineages within the CD45⁺ cells compartment along the large intestine between sexes. Data from two (females) and 3 (males) independent experiments with 3-4 mice per group for n=8-11 expressed as the mean $\pm$ standard deviation. Outliers were determined using robust regression and outlier methods. Statistical significance is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Red stars on the pie chart indicate the significantly enriched cell population between male and female mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

5.4.2. Distribution of major immune cells subsets along large intestine in male and female mice in homeostatic conditions

When profiling subsets within the macrophage and lymphocyte lineages at homeostasis, we observed striking differences in the abundance of CD11B⁻CD103⁺DCs, CD11B⁺CD103⁺DCs, F4/80⁺CD11B⁺CD86⁺, F4/80⁺CD11B⁺CD209⁺, Treg, Tfh, Tfc and PD1⁺CD4⁺, and PD1⁺CD8⁺ lymphocytes T cells along the LI between sexes (Figure 2; Supp Figures 5A1-I2). Overall, DC and macrophage subsets were more abundant in the LI in females compared to male mice ($P < 0.05$) (Figure 2A-B). Thus, CD11B⁻CD103⁺DCs were enriched along the LI ($P < 0.0001$), whereas CD11B⁺CD103⁺DC subsets were more abundant across the colon in female than male mice ($P < 0.05$) (Figure 2A). Moreover, CD11B⁻CD103⁺DCs distribution peaked in the proximal colon compared to the cecum, medial, and distal colon in females ($P < 0.0001$). Similarly, F4/80⁺CD11B⁺CD86⁺ macrophages were more enriched in the distal colon ($P = 0.0006$), whereas F4/80⁺CD11B⁺CD209⁺ macrophages were more abundant along the LI in females than male mice ($P = 0.02-0.00001$) (Figure 2B). F4/80⁺CD11B⁺CD86⁺ macrophages showed a trend toward increased frequency in female mice distal colon. However, this trend contrasted in male mice with

higher abundances of F4/80⁺CD11B⁺CD86⁺ macrophage in the cecum and proximal compared to the medial and distal colon ($P<0.01$). CD11B⁺CD103⁺DC and F4/80⁺CD11B⁺CD209⁺ macrophage abundances did not differ along females LI. Similarly, CD11B⁻CD103⁺DCs, CD11B⁺CD103⁺DC, and F4/80⁺CD11B⁺CD209⁺ macrophage frequencies did not change significantly along males LI.

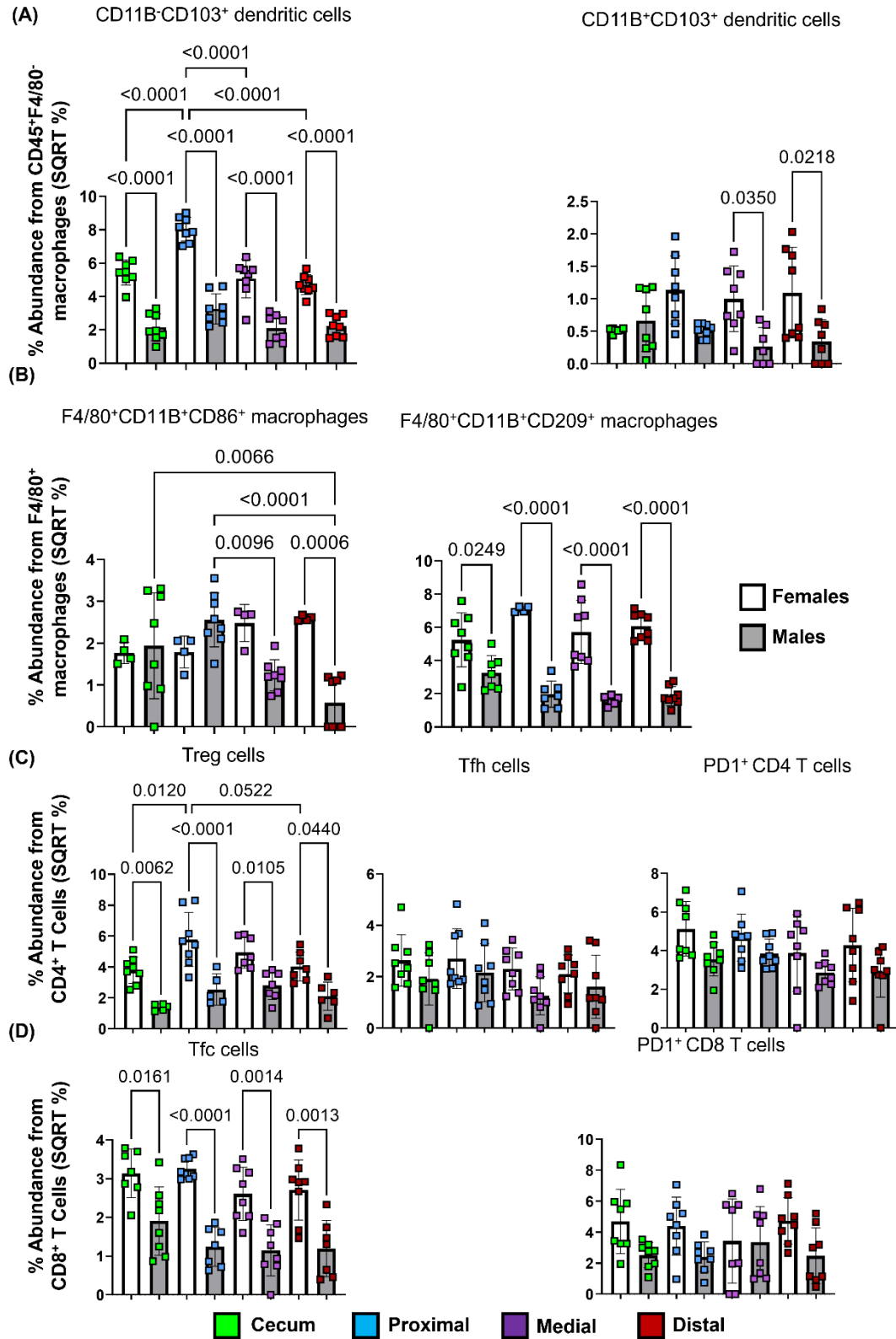


Figure 2: Females have greater innate and adaptive immune cell subsets along the large intestine than males in homeostatic conditions. Comparison of the abundance of subsets of (A) dendritic cells, (B) macrophages, (C) CD4⁺, and (D) CD8⁺ T lymphocytes populations calculated within respectively the F4/80⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ cell compartments across the cecum, proximal, medial, and distal colon between male and female mice. Data from two independent experiments with four mice per group for n=8, except for data from female groups in Figure B (F4/80⁺CD11B⁺CD86⁺), which was from one experiment. All data were expressed as the mean $\pm$ standard deviation and square root-transformed (SQRT) when the standard deviation was at least three times different between groups with sample sizes smaller than ten. Outliers were determined using robust regression and outlier methods. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures.

Subsets of lymphocyte T Treg and Tfc were more enriched along the LI in females compared to male mice, whereas Tfh, PD1⁺CD4⁺, and PD1⁺CD8⁺T cells abundance did not differ markedly along the LI between sexes (Figure 2C-D). Treg frequencies peaked in the proximal colon compared to the cecum in females ($P=0.01$). Tfh, Tfc, PD1⁺CD4⁺, and PD1⁺CD8⁺T cell distributions did not change significantly along the LI in both sexes. Within the B cells subsets (Figures 3A-C, Supp. Figures 5J1-O2), germinal center B cells were more abundant in the medial and distal colon in female than male mice ($P=0.009$; 0.02 respectively) (Figure 3A). Although the abundance of other B cell subsets showed no significant differences between sexes, we observed changes in their distribution along the LI. Thus, in both male and female mice, effector B cells were more abundant in the cecum, while IgA-secreting plasma B cells were significantly higher in the colon. In contrast, memory B cells, antibody-secreting plasma cells, and IgG-secreting B cell distributions did not change significantly across male and female mice LI.

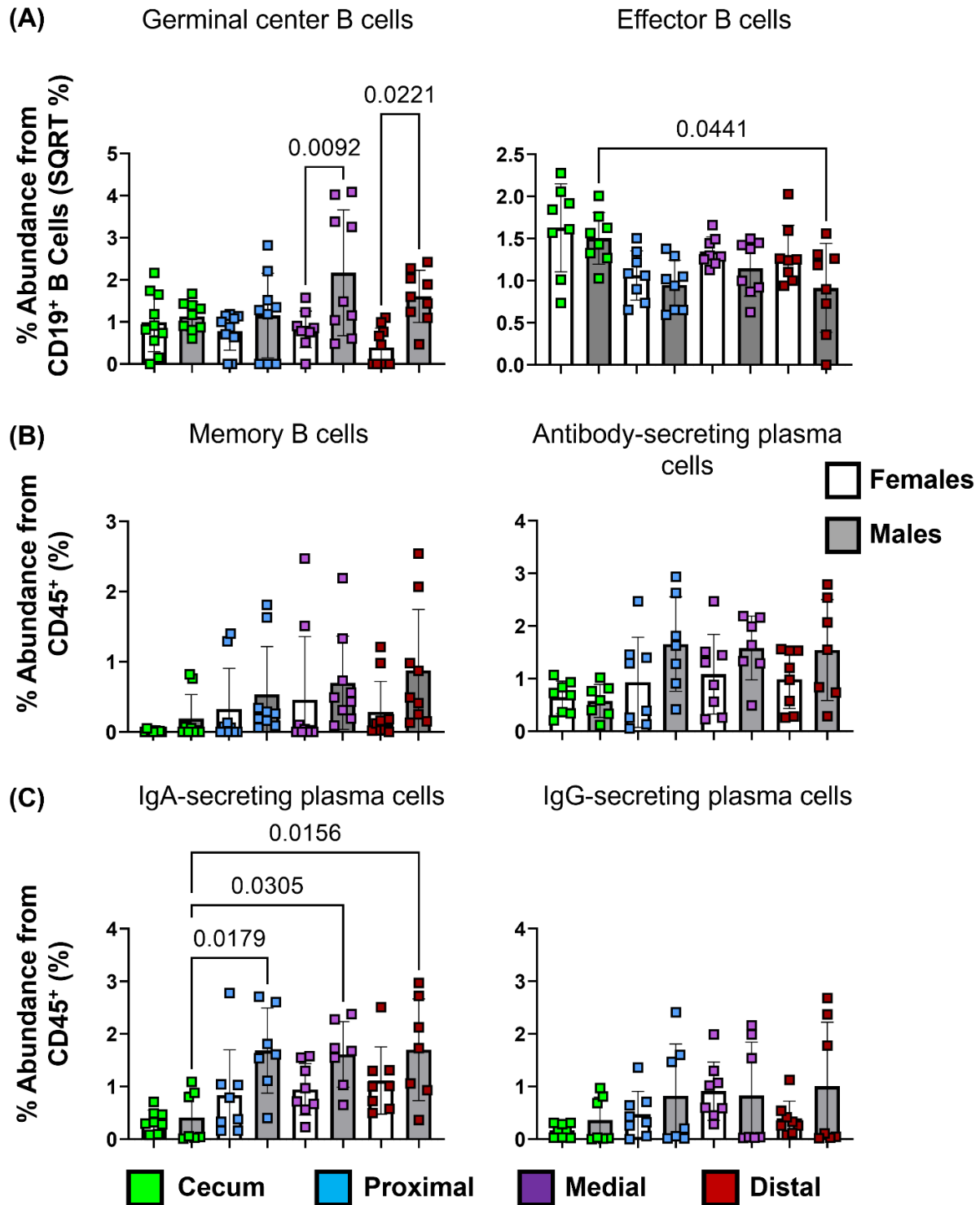


Figure 3: **In homeostatic conditions, germinal center B cells are more abundant in male mice distal colons.** Comparison of the abundance of B cells subsets calculated within the CD19⁺ (A) and CD45⁺ cells (B-C) compartments across the cecum, proximal, medial, and distal colon

between male and female mice. Data from two independent experiments with four mice per group for $n=8$, expressed as the mean $\pm$ standard deviation. Data measuring the abundance of germinal center and effector B cells were square root transformed (SQRT) when the standard deviation was at least three times different between groups with sample sizes smaller than ten. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are displayed in the figures.

5.4.3. Cytokines profile along large intestine under homeostatic conditions

Given the intrinsic regional and sex-dependent difference in immune cell landscape observed along the LI, we investigated whether these differences altered the local profile of anti-inflammatory, TH1, and TH17 cytokines along the LI in male and female mice in homeostatic conditions. Z score analysis showed that anti-inflammatory, TH1, and TH17 cytokine levels change substantially between sexes along the LI (Figure 4A). Thus, TNF- α , IFN- γ , and IL-17A levels were, overall, significantly elevated in female than male mice LI ($P < 0.05$ -0.0001) and contrasted with the higher level of TGF- β , IL-10, IL-6, IL-17C, IL-17F, and IL-25 in male compared to female mice LI ($p < 0.03$ -0.0001) (Figures 4A-D, Supp. Figure 6). When investigating the profile of cytokines along the LI, IL-1 β was elevated in the cecum and reduced in the colon in both sexes ($P < 0.04$ -0.0001). TNF- α and IL-17A levels peaked in the cecum and reduced in the colon in female mice ($P < 0.04$ -0.0001). Conversely, IL-17C, IL-17F, and IL-25 levels gradually increased from the proximal to the distal colon, while the concentration of IL-6 peaked in the medial colon in male mice ($P < 0.05$ -0.0001). IFN- γ level was higher in the cecum and distal colon compared to the proximal and medial colon in females, while IL-10 levels gradually increased along the colon in both sexes. TNF- α , IFN- γ , and IL-17A levels did not differ significantly along the LI in male mice, IL-6 in females, and TGF- β in both sexes. IL-22 level was below the detection limits in both sexes.

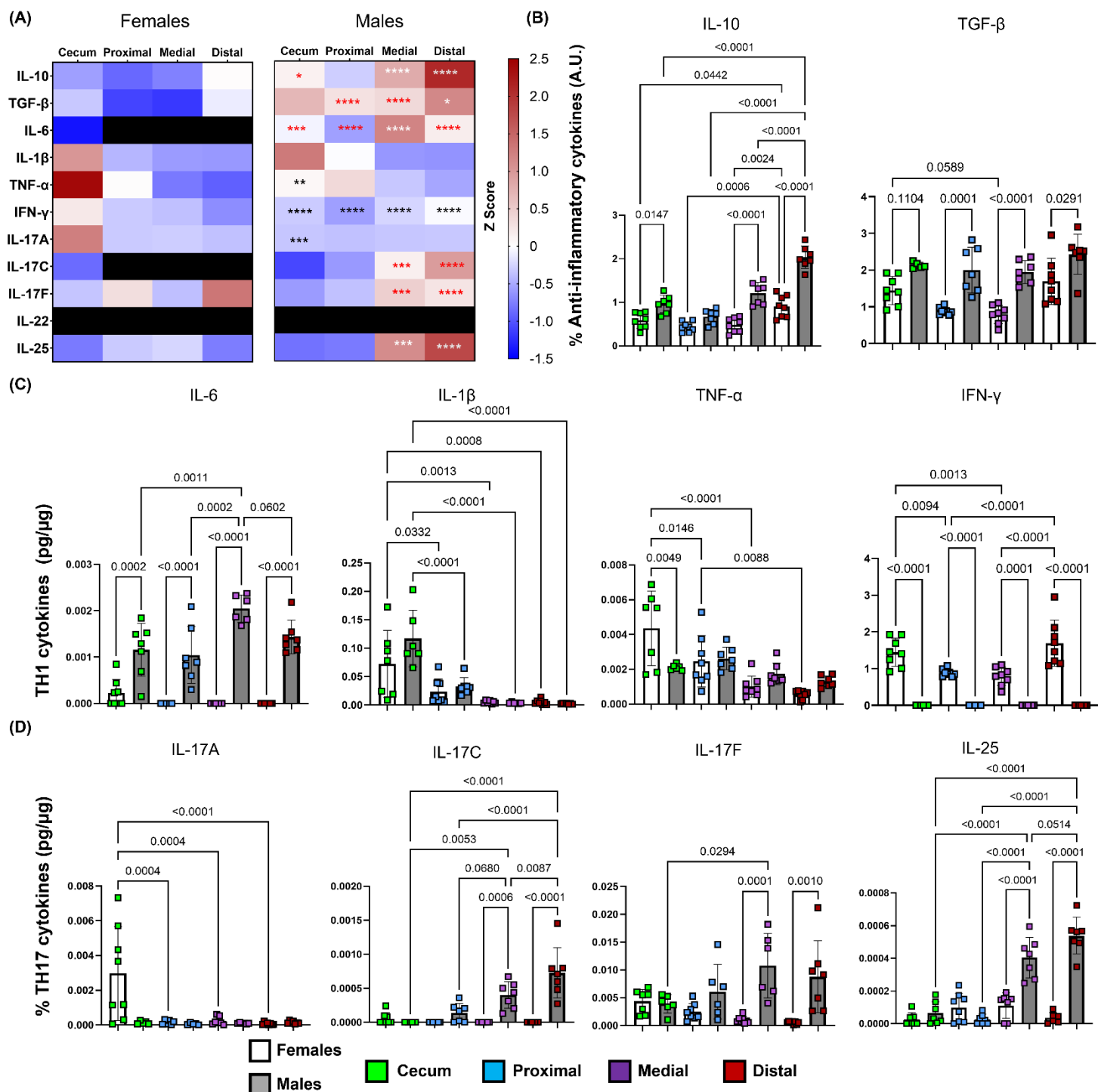


Figure 4: The concentration of colonic mucosal cytokines elevated along the large intestine of male mice in homeostatic conditions. (A) Heat map visualizing the Z score concentration of anti-inflammatory, TH1, and TH17 cytokines within the cecum, proximal, medial, and distal colon from male and female mice. Comparison between concentration of (B) TGF- β , IL-10, (C) IL-6,

IL-1 β , TNF- α , IFN- γ (**D**) IL-17A-C, IL-22 and IL-25 in male and female mice large intestine segments. Data from two independent experiments with 3-4 mice per group for n=7-8 expressed as the mean $\pm$ standard deviation. Outliers were determined using robust regression and outlier methods. In the heat map, red significance markers indicate increased cytokine concentration, while black markers indicate reduced cytokine levels between sexes. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.4.5. Cytokines profile throughout the large intestine in homeostatic conditions

Mounting evidence suggests altered innate and adaptive immune cell distributions along the GI tract in colitis. Here, we find that colitic males displayed a trend toward higher DAI and significantly elevated macro-score compared to colitic female mice (Figures 5A-B). Colon length did not differ between colitic male and female mice (Figure 5C). These results suggested a higher susceptibility to DSS-induced colitis for male compared to female mice.

As our results show sex-mediated differences in hematopoietic and myeloid cell proportions along regions of the LI at steady state, we first evaluated whether these differences were altered and associated with changes in major innate and adaptive immune cell profiles in colitis. Results normalized to control conditions showed that NKT, CD8⁺T, and NK cells proportions decreased, respectively, in the cecum, medial, and distal colon in males than in female mice in colitis ($P < 0.01$ for all) (Figure 5D). Conversely, granulocyte abundances peaked in the medial and distal colon ($P < 0.0001$; $P < 0.01$), while B cells increased in males compared to females ' proximal colon ($P = 0.006$). DCs, monocytes/macrophages, and CD4⁺T cell fold changes did not change between sexes in colitis. This data showed a sex-dependent regional alteration in granulocytes, NK, NKT, B, and CD8⁺T cells abundance during colitis.

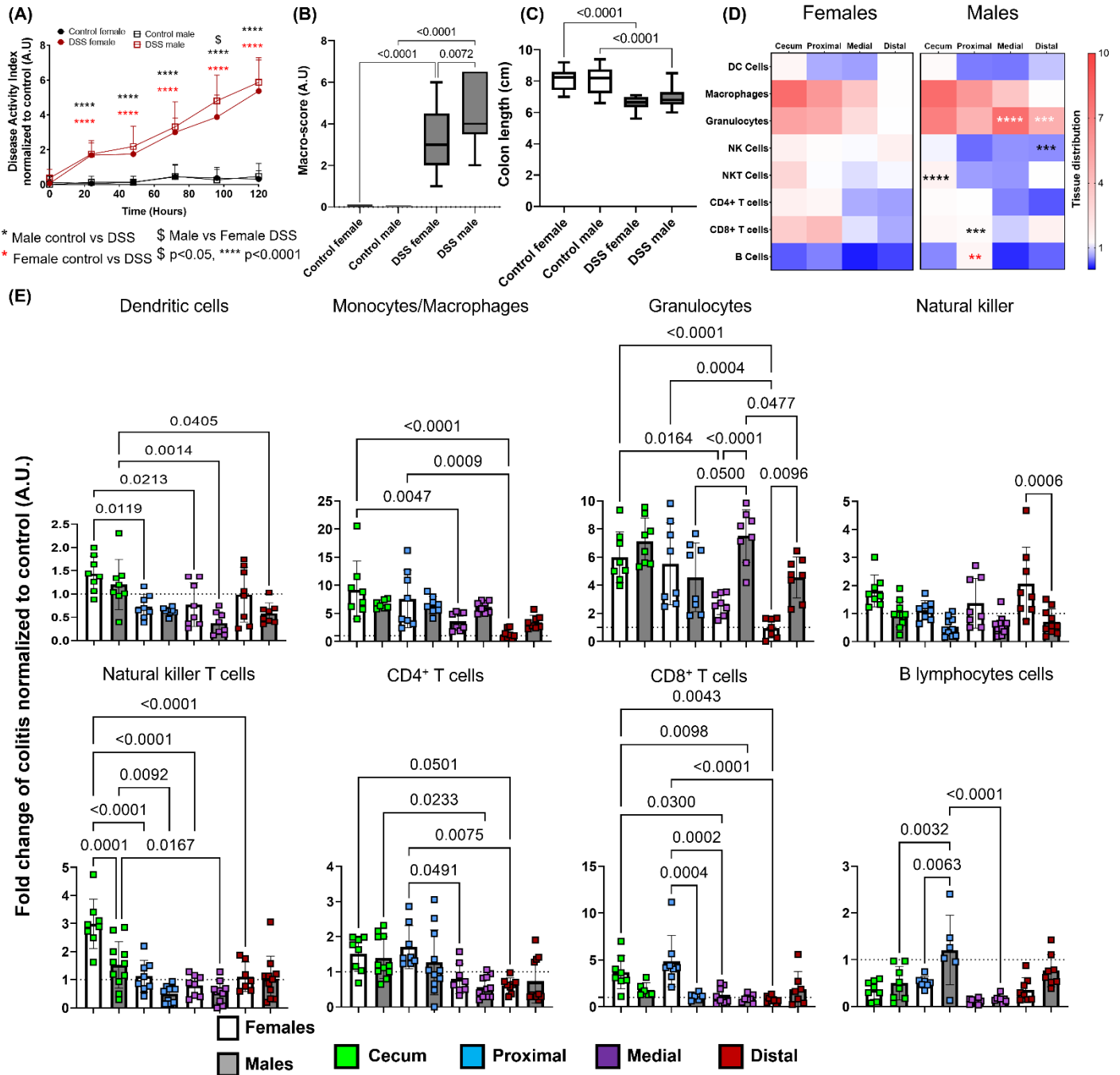


Figure 5: Male mice display more severe colitis associated with altered immune cell profiles that differ along the large intestine in a sex-dependent manner. The clinical assessment of male and female mice during DSS-induced colitis. (A) The disease activity index was calculated daily from weight loss, stool consistency, and bleeding scores. On the day of the experiment, (B) the macro score index, calculated from rectal bleeding, rectal prolapse, diarrhea, and (C) colon bleeding scores, and (C) colon length, was evaluated for each mouse. Profiling of colitic male and

female mice innate and adaptive immune cells within the large intestine cecum, proximal, medial, and distal colon. **(D)** Heat map visualizing the fold change of major immune cells normalized to control across segments of males and females' large intestines. **(E)** The relative abundance of major immune cell lineages within the CD45⁺ cells compartment along the large intestine in colitic conditions divided by control conditions. In the heat maps, red significance indicates increased cell abundance, whereas black indicates reduced cell abundance between sexes. Fold change values from neutrophils are transformed into square roots in both the heatmap and histogram. Data from two (females) and 3 (males) independent experiments with 3-4 mice per group for n=8-11 expressed as the mean $\pm$ standard deviation. Outliers were determined using robust regression and outlier methods. Statistical significance was determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures. ^s $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$, ^{****} $p < 0.0001$.

We next evaluated whether colitis was associated with changes in innate and adaptive immune cell abundances along the LI in male and female mice. First, we characterized the immune cell profiles in colitis compared to control conditions (Supp. Figures 3A1-H2). Second, we evaluated the immune cell population distribution along the LI in colitic conditions normalized to control conditions between sexes.

Within the innate immune compartment, DC frequencies did not change along male and female mice LI in colitic compared to control conditions (Supp. Figures 5A1-A2). Macrophage abundances increased in female proximal and medial colon ($P=0.02$; $P=0.003$) and along males LI in colitis compared to control conditions ($P<0.03$) (Supp. Figures 5B1-B2). Similarly, granulocyte frequencies expanded across all segments of the LI in both sexes, except for females' distal colon, in colitis compared to control conditions (Supp. Figures 5C1-2). When normalized to control conditions, DC fold changes along the colitic LI in both sexes peaked in the cecum and decreased in the colon ($P<0.05$). Similarly, macrophage proportions increased in females' ceca and proximal colons and diminished in the medial and distal colons while not changing significantly

along LI during colitis in male mice. However, males had significantly more granulocytes in the medial and distal colon compared to the gradual decrease in this population in female mice (Figure 5E). While NK abundance did differ significantly along females LI between colitic and control conditions, NK cells were less abundant in males' distal colon in colitis than in control conditions, resulting in more NK proportion in female than male mice distal colon in colitis ($P=0.0006$) (Supp. Figures 3D1-D2).

Within the adaptive immune compartment, NKT and CD4⁺T cell abundances did not differ significantly between colitic and control conditions along the LI in female and male mice (Supp. Figures 5E1-F2). CD8⁺T cells increased in female and male mice ceca and female proximal colons in colitic compared to control conditions (Supp. Figures 5G1-G2). Conversely, B cells were less abundant across all segments of female mice LI, except for the proximal colon, while not changing significantly along males LI in colitic compared to control conditions (Supp. Figures 5H1-H2). When normalized to controls (Figure 5E), NKT cell fold changes peaked in the cecum, while CD4⁺T cell ratios increased in the cecum and proximal colon in males and female mice with colitis. CD8⁺T cell fold changes increased in the cecum and proximal colon and drastically decreased in the medial and distal colon in female mice, contrasting with the lack of significant changes in CD8⁺T cell ratios along males LI in colitis. Conversely, B cell ratios peaked in males' proximal colon but did not change along female LI in colitis. Colitis is associated with changes in innate and adaptive immune cell distributions along the LI, which differs between sexes.

5.4.6. Immune cell subsets distributions along the large intestine in colitic conditions

Given that colitis altered major immune cell distributions along the LI in a sex-dependent manner, we profiled the immune cell subsets along the LI between sexes in colitis (Figure 5A). Results normalized to control conditions showed that CD11B⁻CD103⁺DCs proportions were lower along males than females LI, except for the proximal colon, in colitis. While F4/80⁺CD11B⁺CD86⁺ and F4/80⁺CD11B⁺CD209⁺ macrophage fold changes increased in the distal colon in both sexes ($P=0.0002$; 0.0004), F4/80⁺CD11B⁺CD209⁺ macrophage ratios were also lower in males compared to female mice ceca ($P=0.005$). T cell subsets were increased differently along the LI in males compared to female mice in colitis. Briefly, Treg cell proportions were higher in the proximal colon ($P=0.02$), PD1⁺CD4⁺T ratio increased across all segments of the LI, save for the proximal colon ($P<0.02$), PD1⁺CD8⁺T ratio peaked in the distal colon ($P=0.01$) in male compared to female mice. Tfh and Tfc cell fold change along the LI did not differ.

Next, we investigated the immune cell subsets profile along the LI in colitic compared to control conditions (Supp Figures 5A1-O2). CD11B⁺CD103⁺DC abundances increased in male mice ceca ($P=0.04$) but were not altered along females' LI in colitic compared to control conditions (Supp Figure 3B1, B2). In colitis, F4/80⁺CD11B⁺CD86⁺ macrophages were significantly reduced along the colon in females ($P<0.02$) and the proximal colon in male mice ($P=0.0009$). Conversely, F4/80⁺CD11B⁺CD209⁺ macrophage abundances decreased in males' ceca but did not change significantly along females' LI in colitis compared to control conditions (Supp. Figures 5C1-D2). The frequency of CD11B⁻CD103⁺DCs did not differ along the LI in female and male mice during colitis compared to control conditions (Supp. Figures 3A1-A2). When normalized to control

conditions, CD11B⁻CD103⁺DCs ratios peaked in females' cecum but did not change along males' LI in colitis. CD11B⁺CD103⁺DCs fold changes did not differ along male and female mice LI in colitis (Figure 6B). F4/80⁺CD11B⁺CD86⁺ macrophage ratios did not change significantly across segments of males and females, LI safe for the marked increase in males' distal colons ($P=0.005-0.0002$). Conversely, F4/80⁺CD11B⁺CD209⁺ macrophage fold changes were lower in the cecum and increased along males' colons but did not differ along females' LI in colitis ($P=0.006-<0.0001$) (Figure 6C). Compared with myeloid cells, CD4⁺T cell subsets were mainly regulated along males than female mice LI in colitis. In female mice, Treg, Tfh, Tfc, PD1⁺CD4⁺T, and PD1⁺CD8⁺T cells did not change significantly along the LI in colitic compared to control conditions (Supp Figures 5E1-I1). Conversely, Tfh and CD4⁺PD1⁺T cell abundances increased in the medial colon and along the LI, respectively, and Treg, Tfc, and PD1⁺CD8⁺T cell frequencies did not change significantly along the LI in male mice in colitic compared to control conditions (Supp. Figures 5E2, 6F2-I2). Analysis of T lymphocyte subsets distribution along the LI in colitic conditions showed reduced Treg cell fold changes in males' distal colons, contrasting with the increased CD4⁺PD1⁺T proportions in male mice medial and distal colons and no significant changes in T lymphocyte subsets along the LI in female mice (Figure 6D-E).

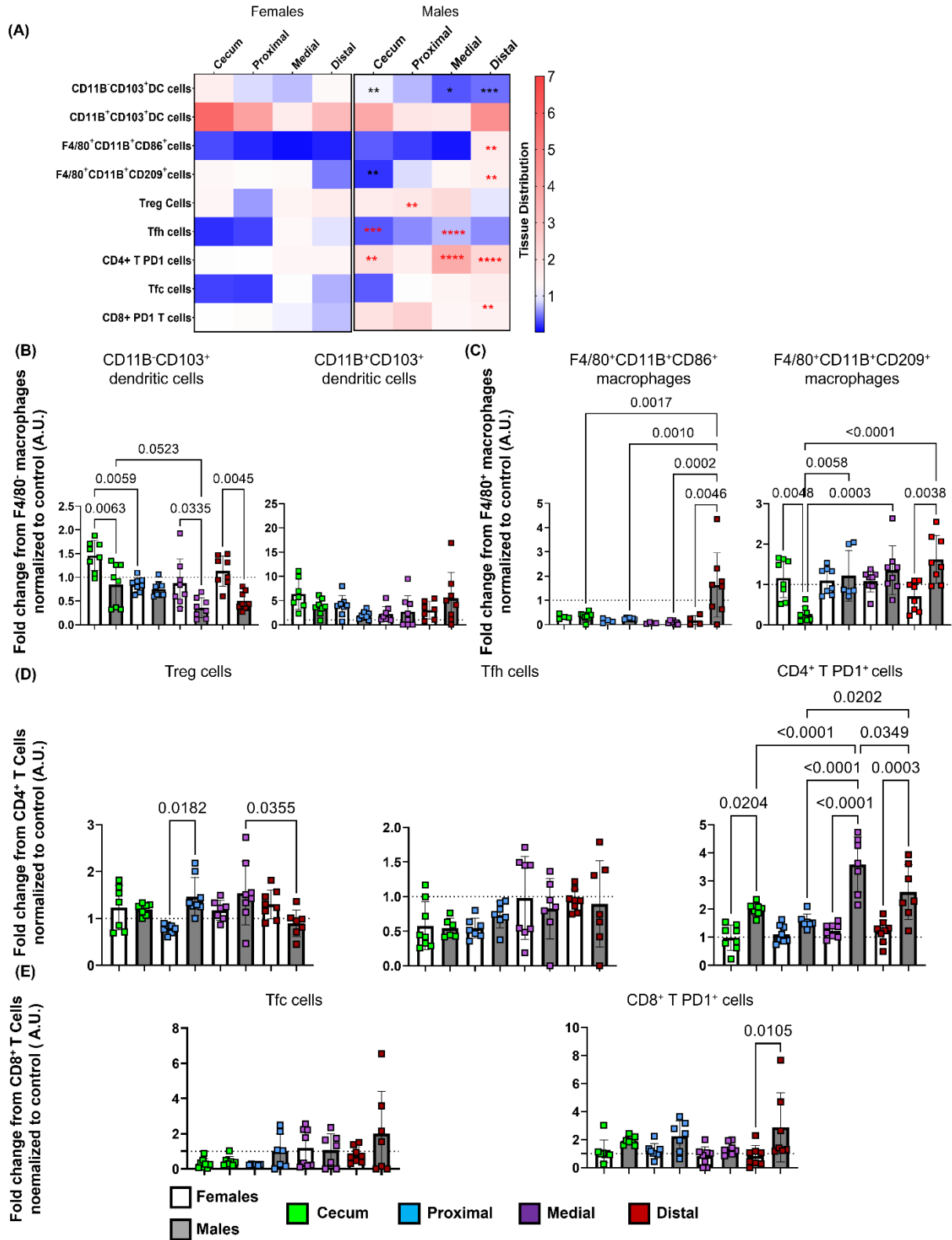


Figure 6: Colitis led to increased follicular helper and exhausted T cells in males and enriched

dendritic cells in female mice along the large intestine. (A) Heat map visualizing the relative abundance of macrophages and T cell subsets normalized to control across segments of colitic males and females' large intestines. Comparison of the abundance of subsets of (A) macrophage, (B) CD4⁺, and (C) CD8⁺ T lymphocytes population calculated within respectively the F4/80⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ cell compartments across segments of colitic male and female mice' large intestine. Data from two independent experiments with 3-4 mice per group for n=7-8 expressed as the mean $\pm$ standard deviation, except for data from female groups in figure C (F4/80⁺CD11B⁺CD86⁺), which was from one experiment. Outliers were determined using robust regression and outlier methods. Fold change values from T regulatory (Treg) cells are square root transformed and expressed as the mean $\pm$ standard deviation. In the heat map, red significance indicates increased cell abundance, whereas black indicates reduced cell abundance between sexes. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In contrast to T lymphocytes, B cell subset frequencies were mainly altered along females' LI in colitis. Thus, B cell subset abundances along males' LI remains unaltered in colitis (Supp. Figures 5J2-O2), contrasting with the increased cecal effector B cells along with higher memory B cells, IgA⁺, and IgG⁺ plasma cells in the medial colon in colitic compared to control female mice (Supp. Figures 5K1-O1). GC B cell frequencies did not differ along females' LI in colitic compared to control conditions (Supp. Figure 5J1). Analysis of B cell subset distributions along the LI in colitis (Figures 7A-D) showed increased memory B cell fold changes in the cecum and medial colon, along with elevated IgA⁺ plasma cell ratios in the medial colon in female mice and higher effector B cell fold changes in the cecum in male and female mice, although not significant in females.

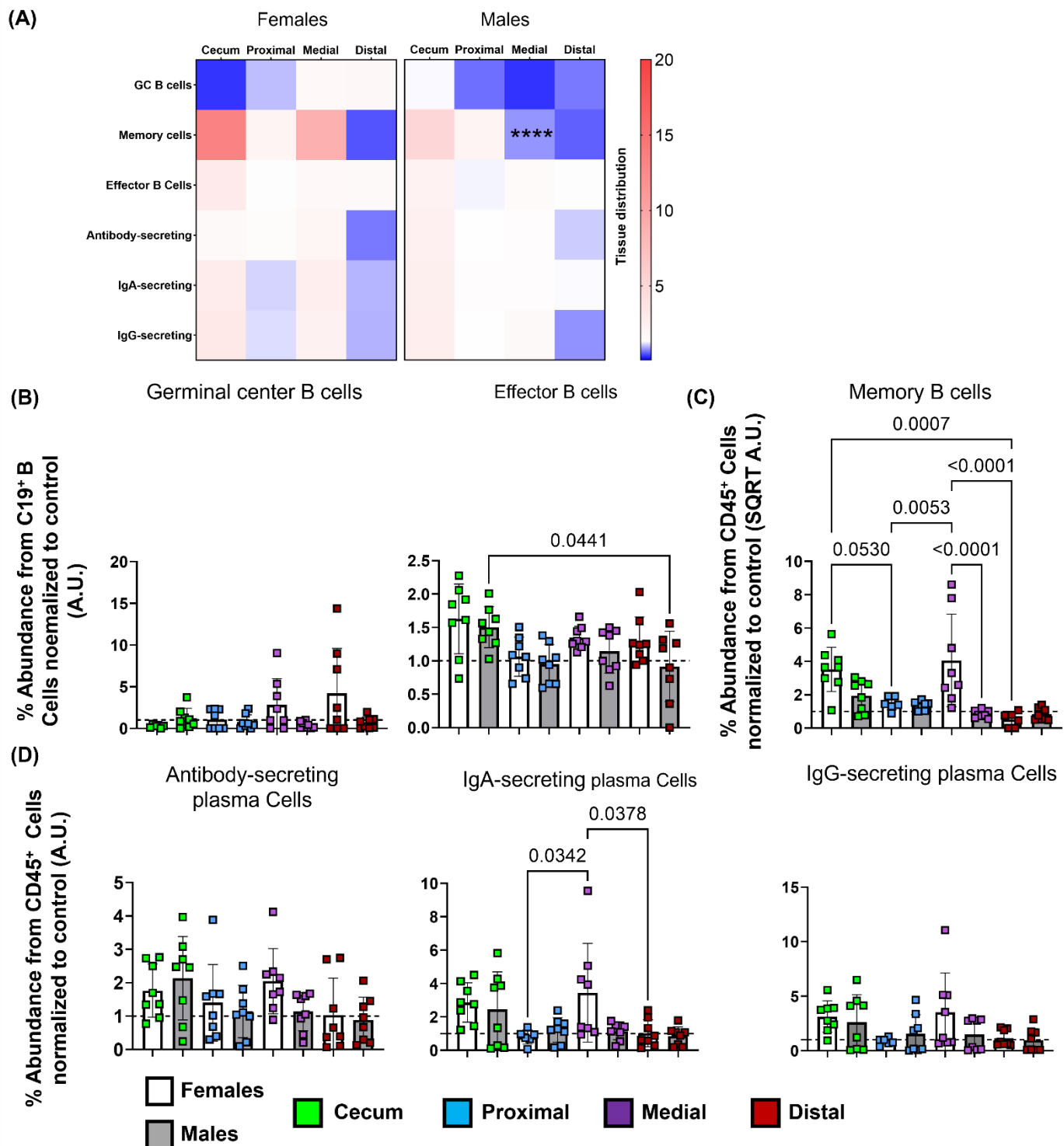


Figure 7: **Memory B cells enriched in the medial colon of colitic female mice.** (A) Heat map visualizing the relative abundance of B cells subset normalized to control across segments of colonic males and females' large intestines. Comparison of B cell subsets abundance calculated

within the CD19⁺ (A) and CD45⁺ cells (B-C) compartments across segments of colitic male and female mice's large intestines. Data from two independent experiments with 3-4 mice per group for n=7-8 expressed as the mean $\pm$ standard deviation. Outliers were determined using robust regression and outlier methods. In the heat map, the black significance marker indicates reduced cell abundance between sexes. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures. **** $p < 0.0001$.

5.4.7. Cytokines profile along large intestine in male and female mice's in colitic conditions.

Colitis is known to alter cytokine profiles in the GI tract. Thus, we investigated the anti-inflammatory, proinflammatory, and TH17 cytokine profiles along the LI in colitic conditions between sexes. In colitis, female mice had elevated TGF- β and IL-1 β levels in the distal and medial colon, respectively, compared to male mice ($P=0.04$; 0.001) (Figure 8A). Conversely, colitic male mice had higher IL-17C in the cecum ($P < 0.0001$) and IL-25 levels in the cecum and proximal colon ($P=0.001$; 0.04) than colitic female mice. IL-10, IL-6, IL-22, TNF- α , and IL-17A Z scores did not differ significantly between sexes.

Profiling of cytokines levels along the LI in colitic compared to control conditions showed increased IL-10 and TGF- β levels in females distal colon, higher IL-6, IL-1 β , and TNF- α levels across all segments of males and females LI, safe for the distal colon, and elevated IFN- γ levels in the cecum in both sexes (Supp. Figures 6A-E). TH17 cytokines levels were modulated in a sex-dependent manner along the LI during colitis. Thus, IL-22 was the only TH17 cytokines to increase in females' ceca and medial colons in colitis compared to control conditions ($P=0.04$; 0.001) (Supp. Figure 6I). Conversely, in male mice, IL-17A and IL-22 levels increased in the cecum ($P=0.001$; <0.0001), while IL-17C and IL-25 levels rose in the cecum and proximal colon in colitis

compared to control conditions ($P < 0.001$ for all) (Supp. Figures 4G-I; 5L). IL-17F level did not differ significantly between colitic and control conditions in both sexes (Supp. Figure 4J).

Analysis of cytokine distributions along the LI in colitis showed a gradual increase in TGF- β Z scores from the cecum to the distal colon in both sexes (Figure 8B). In colitic female mice, IL-1 β and IL-6 Z scores peaked at the medial colon, while TNF- α and IFN- γ Z scores increased in the cecum and drastically diminished in the colon (Figure 8C). In colitic male mice, IL-1 β and IL-6 Z scores peaked in the proximal colon, while TNF- α , IL-17C, and IL-25 Z scores were higher in the cecum and decreased in the colon (Figure 8D). IL-17C and IL-25 cytokine distributions did not differ significantly along female mice' LI in colitis, while IL-10, IL-17A, and IL-22 Z scores remained unaltered along the LI in both sexes. These data suggest a modulatory role for sex-related differences in anti-inflammatory and TH17 cytokine regional distributions along the LI in colitis.

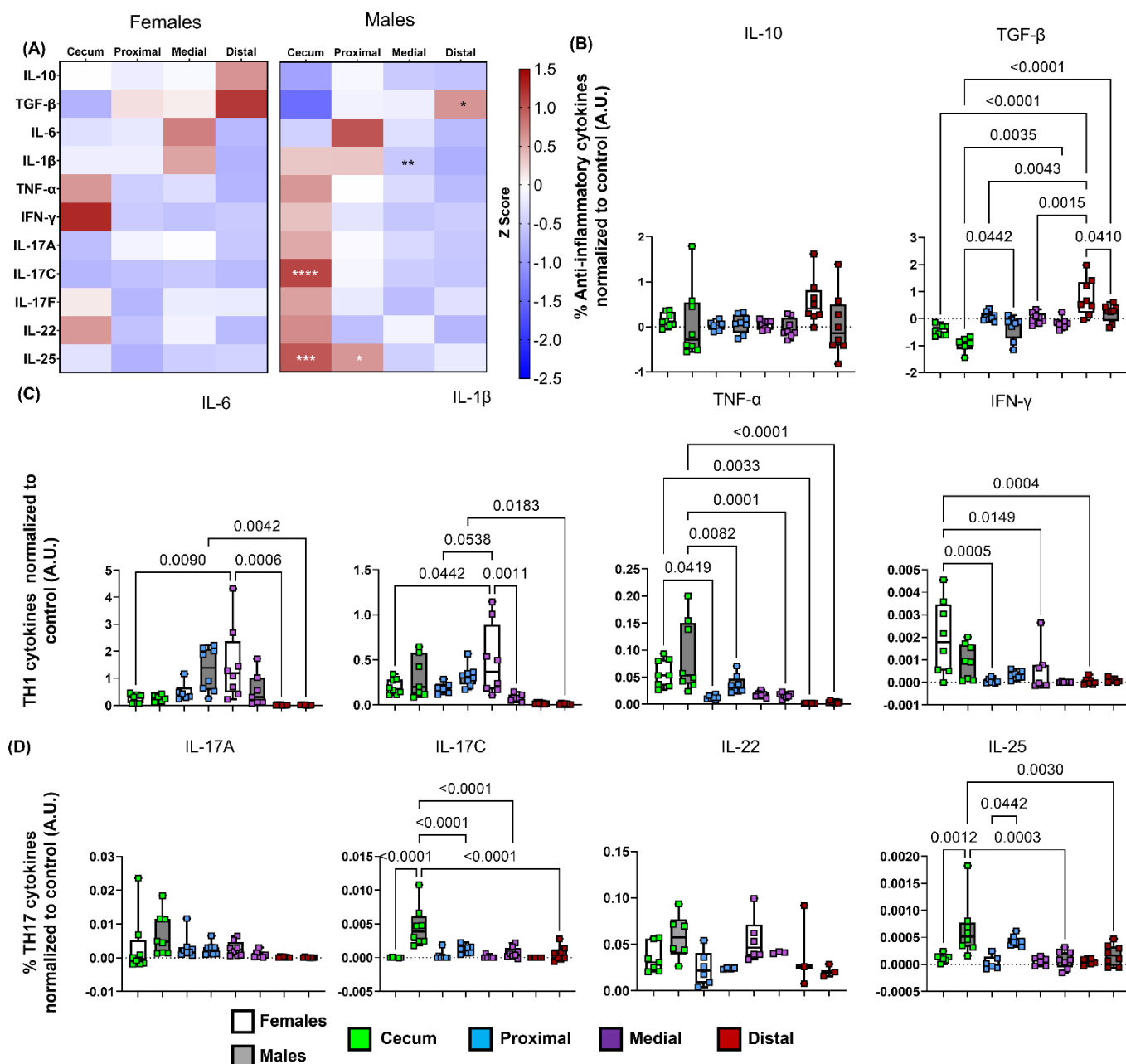


Figure 8: Colitic male mice have increased concentrations of TH17 cytokines in the proximal large intestine. (A) Heat map representing Z scores of anti-inflammatories, TH1, and TH17 cytokine concentrations normalized to control, measured from colitic male and female mice ceca, proximal, medial, and distal colons. Concentration of (B) TGF-β, IL-10, (C) IL-6, IL-1β, TNF-α, IFN-γ (D) IL-17A-C, IL-22 and IL-25 compared across segments of male and female mice' large intestine. Data from two independent experiments with 3-4 mice per group for n=7-8 expressed as the mean ± standard deviation. Outliers were determined using robust regression and outlier methods. IL-22 values transformed into square roots. In the heat map, red significance markers

indicate increased cytokine concentration, while black markers indicate reduced cytokine concentration between sexes. Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

5.5. Discussion

The current paradigm suggests that abnormal immune response in the LI might drive UC pathogenesis (138). Inherent sex bias exists in the immune system in non-pathological and pathological conditions across various tissues (14,294). This study profiled immune cells and cytokines compositions within the cecum and across female and male colons in mice under homeostatic and DSS-mediated colitic conditions. Our findings reveal distinct immune cells and cytokines spatial patterns across segments of the LI that differ between sexes during experimental colitis, suggesting a link between biological sex, the compartmentalization of immune cells and responses along the LI, and the potential susceptibility of males to colitis (63).

The compartmentalization of immune cells along the LI here concurs with recent studies demonstrating inherent regional differences in the immune cell profile across segments of the LI (290,292,295). The disparity in immune cell distribution likely stems from various factors such as regional differences in the draining lymph nodes (LNs), GALTs structure and distribution, colonic specialized epithelial cells abundance, and microbiota diversity and density (290).

The draining LNs play a significant role in regulating the immune response in the gut. The LNs draining regions along the LI differ anatomically and mediate distinct regional immune responses resulting from differential exposure to distinctive environmental elements such as food and microbiota antigens (287,296). Lymph from the cecum, ascending (proximal) colon, and ileum drains to the distal segments of the mesenteric LNs (MLNs), whereas the transverse colon and

duodenum drain to the duodenopancreatic LNs (DPLNs). The lymph from the descending (distal) colon and rectum drains to the caudal LN (131). Proper trafficking of immune cells such as DCs, neutrophils, macrophages, and T and B lymphocytes within the LNs is crucial for pathogen clearance and activation of the adaptive immune system (296). Previous studies have demonstrated that the anatomical segregation of lymphatic drainages in the gut dictates the trafficking of DCs from either the small intestine or colon to distinct LNs (287). Differences in the LNs microenvironment influence the frequency, immune response, and metabolic activities of the population of DC subsets between tissues, promoting more the anti-inflammatory CD11B⁻CD103⁺DCs population in the colon, compartmentalizing T cells expansion and altering Treg subsets profile across the gut (287,297). Therefore, the regional segregation in immune distribution observed here may reflect the differences in the microenvironment across the LI, highlighting the benefit of investigating the immune response in distinct segments of the LI drained by different LNs separately.

Although all current studies show differences in immune cell landscape along the LI at steady state, the profile of immune cells characterized between studies differs significantly (290,295). In our study, the characterization of immune cells and cytokines landscape along segments of the LI revealed differences in the immune profiling between the proximal and distal colon, exacerbated in a sex-dependent manner. Moreover, our study highlights the similarity in the immune cell profile shared between the cecum and regions of the colon. We found that the proximal colon was enriched with DCs, CD11B⁻CD103⁺DCs, and NKT and had few B cells in males and females. Our findings align with a study by Sirvinskas *and colleagues*, performed with female mice, that suggested a different immunological state for the proximal colon associated with lower B cells

(295). While DCs were enriched only in the proximal colon in females, no significant differences in NKT and B cell populations existed between the sexes, suggesting that the role of the sex-mediated difference may only influence DCs profiling in the proximal colon as opposed to other LI compartments.

Characterization of myeloid, CD4⁺T lymphocytes, and NK lineages along the LI revealed higher frequencies of these populations in the medial and distal colon, although mediated in a sex-dependent manner. Thus, the numbers of monocytes/macrophages and granulocytes were only higher along the medial and distal colon in females, contrasting with the NK cells enriched in the distal LI in males only. Although not significant, CD4⁺T lymphocytes were more abundant in the medial and distal colon in both sexes. Interestingly, the distribution of CD8⁺T lymphocytes did not differ along the large intestine in both sexes, despite males having markedly higher numbers of CD8⁺T lymphocytes than females. Moreover, the distribution of IgA-secreting plasma cells showed a similar sex-mediated bias, as males had significantly fewer IgA-secreting plasma cells in the cecum compared to the colon but did not differ along females' LI. Our findings differ from previous studies, which, in turn, diverge among them. A transcriptomic study performed in young C57BL/6 female mice reported increased CD4⁺T cells but reduced naïve CD4⁺T cells in the proximal colon with no difference in the abundance of myeloid lineage along the LI, contrasting in part with our current results that report a trend toward lower CD4⁺T in the proximal colon but altered number of myeloid cells along the LI (295). Conversely, another study performed using human biopsies reported, in line with our finding, a higher proportion of CD4⁺T cells located in distal LI, but, in contrast to our data, higher accumulation of monocytes/macrophages/DCs, granulocytes, B lymphocytes, and CD8⁺T cells in the proximal colon (290). Foxp3⁺ cells and the

proliferating and activated CD4⁺T and CD8⁺T cells were higher in females' GALT and jejunum than males at steady state (298). This study supports our finding, which shows that Treg and T_{fc} were all markedly more abundant in females than in males LI at steady state. Conversely, in line with our current finding, a mice study showed reduced CD8⁺T lymphocytes and NK cells in females than males in Peyer's patches (299). Moreover, sex did not affect the frequency of DCs and macrophages found within Peyer's patches, in contradiction with our current finding, which found that females' LI had more DCs, macrophages, CD11B⁺CD103⁺DC, CD11B⁻CD103⁺DC, F4/80⁺CD11B⁺CD86⁺, and F4/80⁺CD11B⁺CD209⁺ macrophages (299). Our analysis points to a significant influence of sex-mediated differences in the abundance of DCs, NK, and T lymphocyte lineages along the large LI. Overall, the regional compartmentalization of hematopoietic cell lineages likely differs in a species, sexes, tissue, and environmental-dependent manner and might explain the discrepancies observed among all studies.

Cytokines, secreted in response to various stimuli by intestinal epithelial cells and innate and adaptive immune cells, can support intestinal homeostasis or promote intestinal inflammation in pathological conditions (300). As we profiled the cytokines levels along the LI, we found sex-mediated regional differences in the level of several cytokines, including IL-1 β , IL-6, IL-10, TNF- α , IFN- γ , IL-17A, IL-17C, IL-17F, and IL-25. Despite regional differences and sex-mediate disparity in their levels, IL-10, TGF- β , and IL-1 β were among the only cytokines with similar trends in expression between sexes.

We demonstrate a gradual increase in IL-10 level from the cecum to the distal colon, a trend more pronounced in males, that correlated with our finding showing sex and regional-mediated bias in macrophages/monocytes distribution and abundance, in agreement with previous findings

proposing macrophages as a major source of IL-10 (291). By contrast, TGF- β , a pluripotent cytokine produced by many immune and non-immune cells, from Treg, microbiota-activated DCs, short-chain fatty acids (SCFAs)-stimulated intestinal epithelial cells to activated fibroblasts, were evenly distributed along the LI although, in higher levels in male than females (301). These results contrast with previous studies, which reported either no difference or higher levels of TGF- β in females compared to males (298,302,303). The discordance between our data and other studies may suggest that alteration in the level of TGF- β between sexes may be not only sex-mediated but also influenced by factors including species, strain, and microbiota-specific (299). Altogether, data suggest a role for sex-related difference in regulating anti-inflammatory cytokines secretion along the LI.

Owing to the diverse cells producing inflammatory cytokines, the profile of IL-1 β , IL-6, TNF- α , and IFN- γ levels contrasted significantly in a regional and sex-dependent manner. Produced by cells such as monocytes/macrophages and intestinal epithelial cells in response to commensal bacteria and damage-associated molecular patterns, IL-1 β plays a crucial role in gut homeostasis and pathogenesis. Studies have shown that IL-1 β , constitutively expressed by resident macrophages, is critical for intestinal TH17 cell differentiation at a steady state (304). Additionally, females GI tract is described as having a robust immune response associated with increased levels of proinflammatory cytokines IL-1 β , IL-17A, TNF- α , and IFN- γ compared to males, indicating an inherent sex-based difference predisposing females to inflammatory associated diseases (298). In that context, our results described enriched levels of IL-1 β in the cecum in both sexes that correlated with higher cecal TH17 cytokines, IL-17A, and TNF- α in female mice ceca. Notably, the increased inflammatory immune profile in female mice was also accompanied by

higher IFN- γ levels likely driven by T cells as it correlated with the higher percentage of T lymphocyte subsets along female mice LI.

The cecum, which is significantly larger in mice than the smaller vestigial cecum in humans, is a site of extensive microbiota-mediated fermentation of dietary materials and production of SCFAs in mice. In humans, fermentation processes are restricted to the colon (21). Fermentation processes occur in separated regions along the LI in response to carbohydrate and protein availability. Thus, carbohydrate fermentation and proteolytic fermentation, performed by distinct anaerobic commensal bacteria along the LI, occur in the proximal and distal colon, respectively (20,132). Metabolic by-products of carbohydrate fermentation, SCFAs gradually decreased from the cecum to the distal colon, owing to their absorption along the tissue by colonocytes, and have been shown to differ between sexes (20,132). Moreover, SCFAs can exert both anti and pro-inflammatory effects in the gut by limiting the production of pro-inflammatory cytokines, inducing differentiation of Tregs and production of IL-10 and TGF- β while simultaneously promoting neutrophils recruitment, inflammasome activation, TH17 cells response and released of IL-1 β and IL-10 (299,305). By contrast, ammonia produced during the proteolytic fermentation process reduced gut mucosal barrier integrity and function and increased the expression of inflammatory cytokines (21). Here, we show that TH17 cytokines levels differed significantly along the LI in a sex-dependent manner. While IL-17A was enriched only in the cecum of female mice, other TH17 cytokines, IL-17C, IL-17F, and IL-25, gradually increased along the LI from the cecum to the distal colon in males but remained unaltered along females LI. Studies showed the production of the IL-17 family of cytokines in response to the gut microbiota, SCFAs, IL-6, and IL-21 by intestinal epithelial cells, $\alpha\beta$ CD4⁺Th17 cells, and $\gamma\delta$ T cells (306). While gut microbiota is critical

for the maintenance and functions of conventional Th17 cells across the intestinal compartments, the response of $\gamma\delta$ T cells from various intestinal compartments to the microbiota differs (304,307). A previous study demonstrated a suppressive effect in $\gamma\delta$ T cell production of IL-17A and IL-22 by SCFAs in the cecum and colon (307). Thus, the gradual decrease of SCFA levels from the cecum to the distal colon may explain the consistent increase in IL-17C, IL-17F, and IL-25 levels observed along the LI in males (308).

Sex-related differences in TH17 cytokines production may also result from differences in the composition of the fecal microbial community, as both males and females responded differently to dietary prebiotics, exemplified by studies that reported that oligofructose supplementation in female rats increased the abundance of Bacteroidetes, which used ammonia metabolism as a nitrogen source for growth (303). Conversely, oligofructose supplementation in males enhanced the proteolytic fermentation, consequently increasing intestinal epithelial permeability and gut microbiota mucosal infiltration that might lead to heightened proinflammatory responses in males, in line with our finding of increased levels of TH17 and IL-6 cytokines in males distal colon (303).

The inflammatory environment resulting from the activation of innate immune cells can lead to a loss of immune tolerance to commensal microorganisms and abnormal activation, expansion, and maturation of T cells and B cells, promoting pathogenesis and progression of UC (309). Moreover, with the development of RNA sequencing technology, evidence of distinct regional-specific immune cell profiles across the GI tract in UC has been reported (292).

In colitis, our results revealed a spatial distribution of immune cells and cytokines that differ significantly across segments in the LI between sexes and compared to steady-state conditions.

The myeloid cell compartment in male and female mice showed increased recruitment of

monocyte/macrophages and granulocytes along the LI, in line with studies that reported a role for neutrophils and macrophage infiltrates in the inflammatory response during colitis (310,311). However, the distribution of monocytes/macrophages and granulocytes along the LI differs in a sex-dependent manner. Thus, in females, both myeloid subsets were higher in the cecum and gradually decreased along the colon, inversely to the gradual reduction of only monocyte/macrophages in males, indicating a sex-dependent role in granulocyte infiltration within the colon. Macrophages are broadly classified as an F4/80⁺CD11b⁺CD86⁺ M1-like phenotype, associated with enhanced inflammatory response and increased antigen-presenting ability, or F4/80⁺CD11b⁺CD209⁺ M2-like phenotype with strong phagocytic and tissue repair ability (312). Interestingly, colitis was associated with a considerable reduction in F4/80⁺CD11b⁺CD86⁺ and no change in F4/80⁺CD11b⁺CD209⁺ macrophages along the LI in females. By contrast, in males, colitis led to a drastic decrease in F4/80⁺CD11b⁺CD86⁺ macrophages in the cecum and proximal and an increase in the distal colon. F4/80⁺CD11b⁺CD209⁺ macrophages, drastically reduced in the cecum, were restored along the colon. The discrepancy between the increased infiltration of macrophages and the reduced number of F4/80⁺CD11b⁺CD86⁺ macrophages might stem from the fact that gut macrophages do not fit readily into the classical M1/M2 paradigm as they share features of both F4/80⁺CD11b⁺CD86⁺ and F4/80⁺CD11b⁺CD209⁺ macrophages (313). Therefore, anti-TNF- α treatments in patients with IBD can induce the highly immunosuppressive regulatory macrophages (Mregs), which expressed both F4/80⁺CD11b⁺CD86⁺ and F4/80⁺CD11b⁺CD209⁺ markers such as F4/80, CD11b, CD86, and CD209, indicating that inflammation dampened this macrophage population. Thus, the reduction of F4/80⁺CD11b⁺CD86⁺ might result from diminished Mreg subsets. Our finding suggests a role for sex-mediate differences associated with

more changes in the profile of granulocytes and macrophage subsets distribution along the LI in males compared to females.

Conventional DCs (cDCs), found throughout the LI within the lamina propria, are subdivided into two major subsets, CD11b⁺CD103⁺ and CD11b⁻CD103⁺, whose expression differ along the intestinal tract (314). Thus, CD11b⁺CD103⁺ accounts for the major DCs subset within the small intestine, whereas CD11b⁻CD103⁺ is more predominant within the LI (314). CD11b⁻CD103⁺DCs maintain T lymphocytes within the intestinal compartment besides promoting CD4⁺, CD8⁺, and intraepithelial CD8⁺T cells production of IFN- γ , which, in turn, repress inflammation by modulating the intestinal epithelial cells production of the anti-inflammatory IFN- γ -inducible proteins, indoleamine 2,3 dioxygenase enzyme and the decoy protein interleukin-18-binding protein (315). Moreover, CD11b⁻CD103⁺DCs can induce a Th2 immune response in the colon (316). By contrast, CD11b⁺CD103⁺DCs drive IL-6-dependent Th17 cells differentiation and induce Th17 and Th2 responses in the small intestine. Moreover, CD11b⁺CD103⁺DCs promote T-dependent IgG and IgA response in MLNs (316–318). A study using a Muc2 knockout mice model of spontaneous colitis reported a marked reduction in CD11b⁻CD103⁺ population along the colon with no significant changes in CD11b⁺CD103⁺DCs during colitis. However, extensive colitis was associated with increased CD11b⁺CD103⁺ numbers in the distal colon. Moreover, CD11b⁺CD103⁺DCs isolated from colitic tissue promoted the differentiation of Th17 cells only, whereas CD11b⁻CD103⁺DCs induced the differentiation of both Th1 and Th17 cells (319). Consistent with this study, our analysis revealed a trend toward reduced CD11b⁻CD103⁺DCs along the colon and a marked increase in cecal CD11b⁺CD103⁺DCs in males during colitis. Similarly, colitis was associated with a trend toward higher cecal CD11b⁺CD103⁺DCs, yet CD11b⁻

CD103⁺DCs did not change significantly in females, suggesting more extensive colitis in males than females.

Characterization of lymphocytes in colitic conditions revealed significant differences along the LI compartment between sexes. A previous study described the direct trafficking of adoptive transferred NK cells to the inflamed colon, where they dampened neutrophil recruitments, reduced proinflammatory activities, and played a protective role in DSS-mediated colitis (320). However, this data contrasts with our finding that revealed an absence of changes in NK cell abundance in colitis along most regions of male and female colons, save for the reduced NK cells in males' distal colon that correlates with increased neutrophils, hinting toward more severe inflammation in male's distal colon. Several studies reported that NKT can provide resistance or increased susceptibility to colitis depending on the disease model. Studies found that NKT cell deficiency could either increase or not susceptibility to DSS-induced colitis. Moreover, NKT activation could confer a resistance to DSS-induced colitis. However, NKT effects are likely influenced by the microbiota as NKT-deficiency dysregulated and promoted a proinflammatory microbiota associated with increased susceptibility to colitis (321). In our study, colitis correlates with increased NKT cell abundance in the cecum in both sexes, although more abundant in females than males. These results indicate that, besides the microbiota, sex-mediated differences can also influence NKT recruitment in colitis.

Altered lymphocyte profiles In IBD might modulate the disease severity (292). Thus, several studies described increased CD8⁺ cells in the rectum with no significant changes in the colon of UC patients and during DSS-mediated colitis (292,310). Moreover, a transcriptomic study reported increased effector CD8⁺T cells, reduced tissue-resident memory CD8⁺T cells, and elevated CD8⁺-

activated PD1 pathway in UC patients (322). Similarly, our results showed that CD8⁺T cells are elevated in the cecum and proximal colon in females, yet not in males in colitis. Sex-mediated differences influenced CD8⁺-expressing PD1 cell frequencies. Thus, as previously described studies, colitis was associated with higher CD8⁺PD1⁺T cell infiltration in the lower region of the LI in males but not in females, resulting in a higher abundance of CD8⁺PD1⁺T cells along the LI in males than females, likely in response to dysregulated immune response and increased inflammation in males. Conversely, Tfc cell abundances, despite a trend toward reduced numbers in females' ceca and proximal colon, did not differ significantly along the LI during colitis, in agreement with a precedent study (323). Our findings suggest sex-mediated differences in CD8⁺ lymphocyte recruitment along the LI during colitis. In line with previous studies, the percentage of CD4⁺T cells and their subsets were unchanged along the LI in both sexes in colitis (310).

B cells are the primary source of IgA and IgG, which neutralize pathogens in the gut. Accumulating findings report a role for B cells in IBD. Thus, studies reported increased B cells and IgM⁺B-1-like cells in inflamed tissue in UC patients (324,325). Transcriptomic studies showed enriched rectal B cells owing to increased naïve, plasma, and memory B cells, but no changes in this population in the colon of UC patients (292). A preclinical study demonstrated that B cell profiles in colitis differ in response to DSS concentration, as a high dose of DSS (5%), associated with acute colitis, and correlated with reduced Ig-containing plasma cells, IgA, and IgG-producing cells. Conversely, a low dose of DSS (2.5%) correlated with increased Ig-containing plasma and IgA-producing cells, reduced IgM-producing cells, and no change in IgG-producing cells (310). Consistent with this study, we found lower B cells along most segments of the LI in females and medial colon in males in colitis. However, colitis was also associated with increased cecal effector

B cells, higher memory B cells, IgA, and IgG-expressing plasma cells in the medial colon in females, and no significant changes along males LI. Studies have shown that DSS can directly promote some B cell subsets' proliferation, differentiation, and activation, besides enhancing their sensitization to LPS (310). Therefore, the direct effect of DSS on B cells likely explains the discrepancy existing between the reduced B cell frequency and the regional increase in B cell subsets observed here.

Dysregulated cytokine levels are associated with colitis onset and progression in animal models of IBD. Acute colitis led to increased inflammatory immune response and cytolytic activities. Our results extended this knowledge as we demonstrated the influence of sex bias and LI segments in cytokines levels. Thus, IL-6 and TNF- α levels were elevated in the cecum and along the colon, except for the distal colon, in both sexes. IL-1 β was only enriched in the proximal and medial colon in female mice than in male mice, with higher IL-1 β levels in the cecum. IFN- γ was mainly secreted in the cecum in both sexes, while Th17 cytokines were markedly elevated in male ceca but not in females in colitis. Moreover, our findings demonstrate that the distal colon in females is markedly enriched with IL-10 and TGF- β but did not differ in males. These results hint toward a more severe inflammatory response in males in comparison to females in DSS-mediated colitis.

This study has limitations that will require further investigation in the future. Here, the profile of sex hormones, including estrogens, progesterone, and androgens, was not characterized. However, studies have shown that sex hormones can influence both innate and adaptive immune responses, which in turn can modulate the disease severity in IBD (14,153,155,157). Moreover, sex hormones can also regulate the microbiota composition, diversity, and abundance, which might influence the intestinal immune response. As aforementioned, gut microbiota and microbial metabolites are

crucial regulators of colon mucosal homeostasis and the pathophysiology of ulcerative colitis (326). The gut microbiota and microbial metabolites can regulate the GI tract immune cell distribution profile and response directly or by altering the intestinal epithelial integrity. Therefore, further studies will be necessary to investigate the interaction between sex hormones, microbiota, and immune cell distribution and response along the LI.

5.6. Perspectives and Significance

DSS-mediated colitis in mice is a preclinical model frequently utilized to investigate the underlying mechanism in IBD etiology and pathophysiology. Moreover, the preclinical colitis mice model can also serve in drug development and evaluating drug efficacy. Histological findings and the characterization of the immune response are among the most widely used characteristic evaluation indices and are crucial to assessing treatment outcomes in preclinical studies (327). The current study highlights the significance of considering sex and region-specific disparity in the distribution of immune cells and the profile of cytokines along the LI while assessing the histological findings and therapeutic responses to established and new therapies in DSS-related studies.

5.7. Conclusion

Immune cell distribution and cytokines levels differ regionally along the LI at steady state and in DSS-mediated colitis in a sex-dependent manner. These differences highlight the significance of considering sex and region-specific biases in immune cells and cytokine profiles in the LI while

investigating DSS-induced pathogenesis and immune responses to established and new therapies in DSS-induced colitis.

5.8. Supplementary file

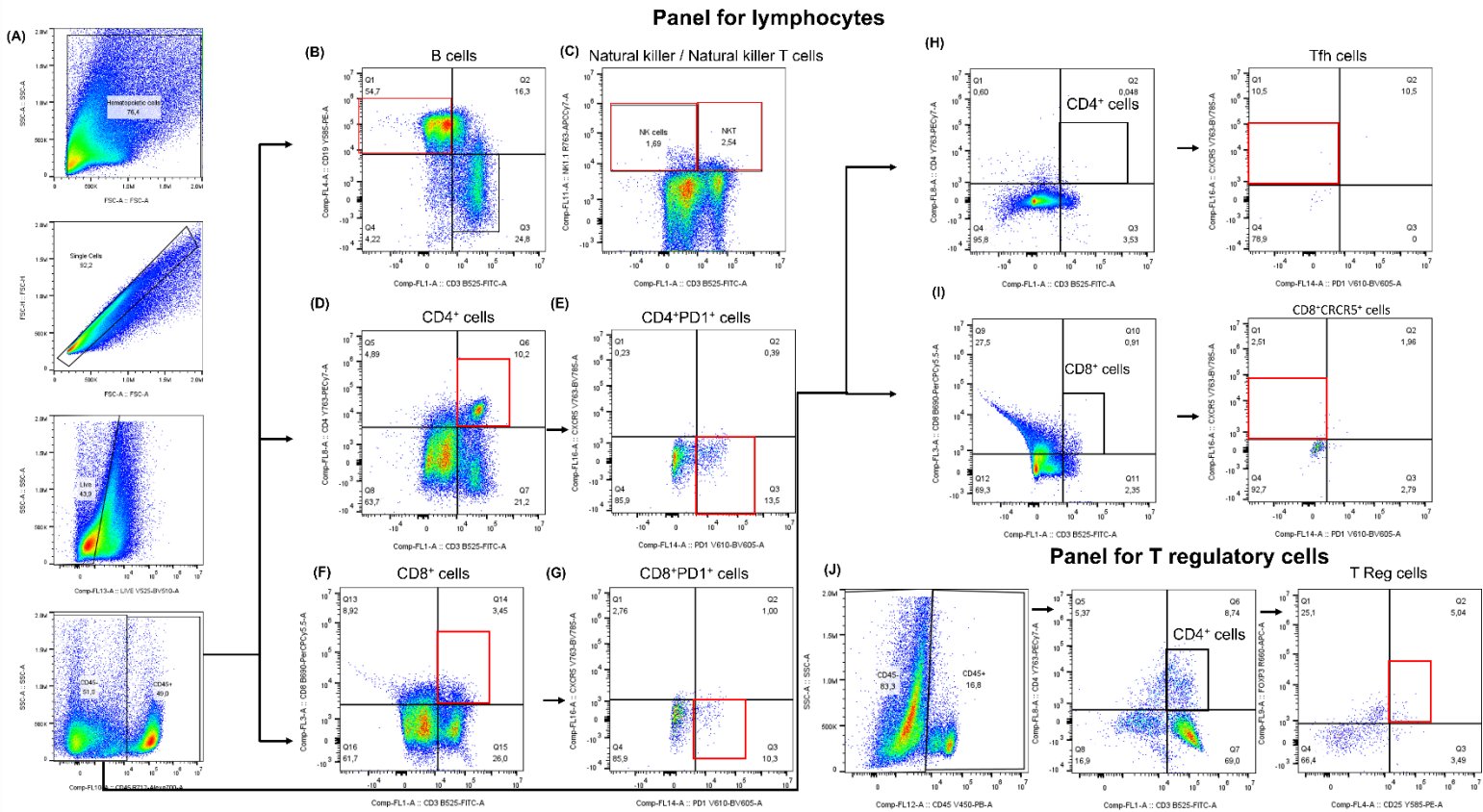


Figure 1 Supp: Gating strategy to characterize the lymphocytes immune cell subsets along the large intestine. (A) Data gated based on their size using the forward side scatter plot, followed by singlet, live-cells, and CD45⁺/CD45⁻ cells. (B) B, (C) natural killer (NK) and NKT selected by gating the CD19 and NK1.1 to the CD3⁻ and positive cells from the CD45 positive compartment. (D-I) Lymphocytes CD3 positive cells gated for CD4⁺ and CD8⁺ cells from CD45 positive and negative cells, then PD1, CXCR5 assessed within CD4 and CD8⁺ cells. (J) CD25⁺FOXP3⁺ assessed within CD4⁺ population.

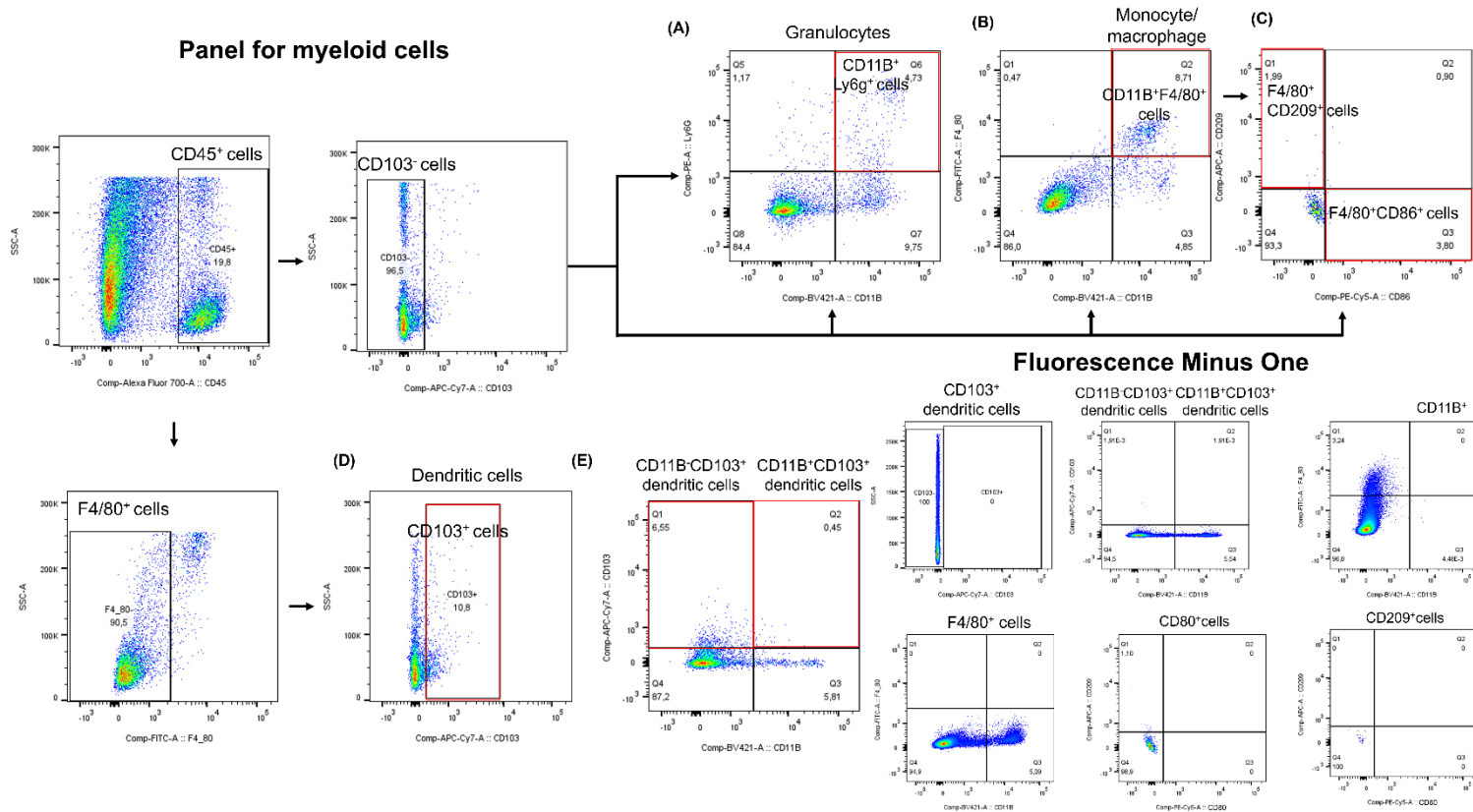


Figure 2 Supp: Gating strategy to characterize the myeloid immune cell subsets along the large intestine. (A-C) Macrophage and granulocytes selected from the CD103⁻, CD11b⁺, and F4/80⁺ cells, and then the expression of CD209⁺ or CD86⁺ cells gated from F4/80⁺ cells. (D-E) Dendritic cells (DCs) gated from F4/80⁻, then CD103⁺ cells within CD45⁺ cells, and CD11B⁻ and CD11B⁺CD103⁺ cells assessed within CD45⁺ F4/80⁻ population.

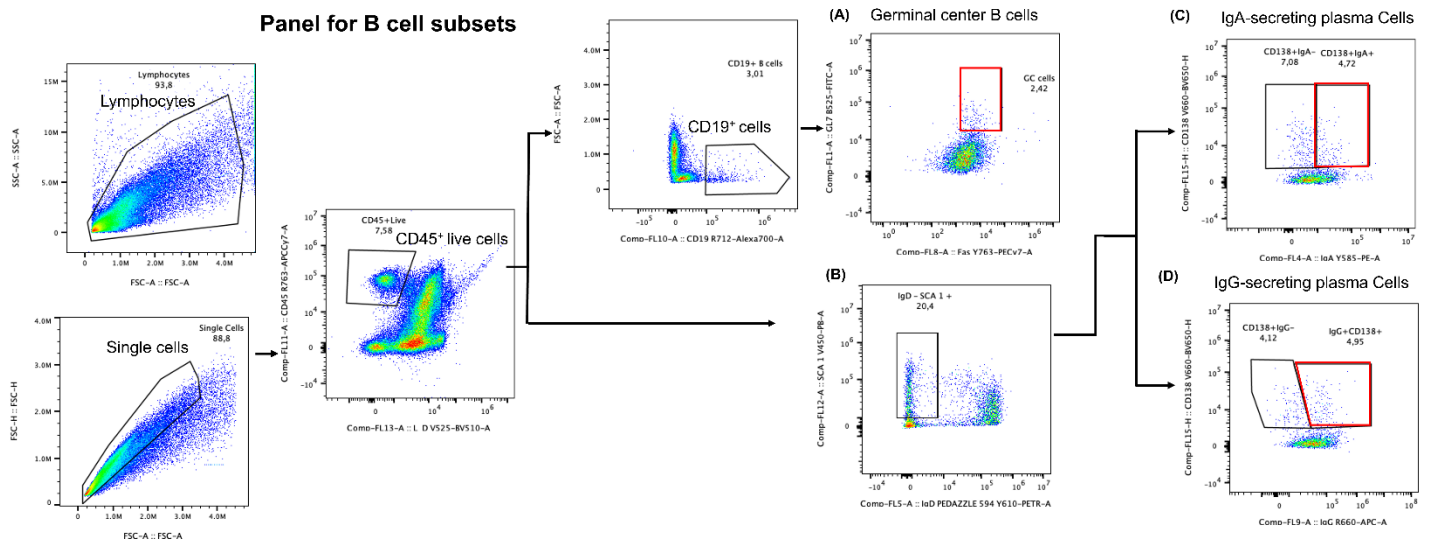


Figure 3 Supp: Gating strategy to characterize the B immune cell subsets along the large intestine. (A) Germinal center B cells selected from the CD19⁺, (B-D) IgA and IgG-secreting plasma selected from IgG⁺SCA1⁺ cells followed by CD138⁺IgA⁺ and CD138⁺IgG⁺.

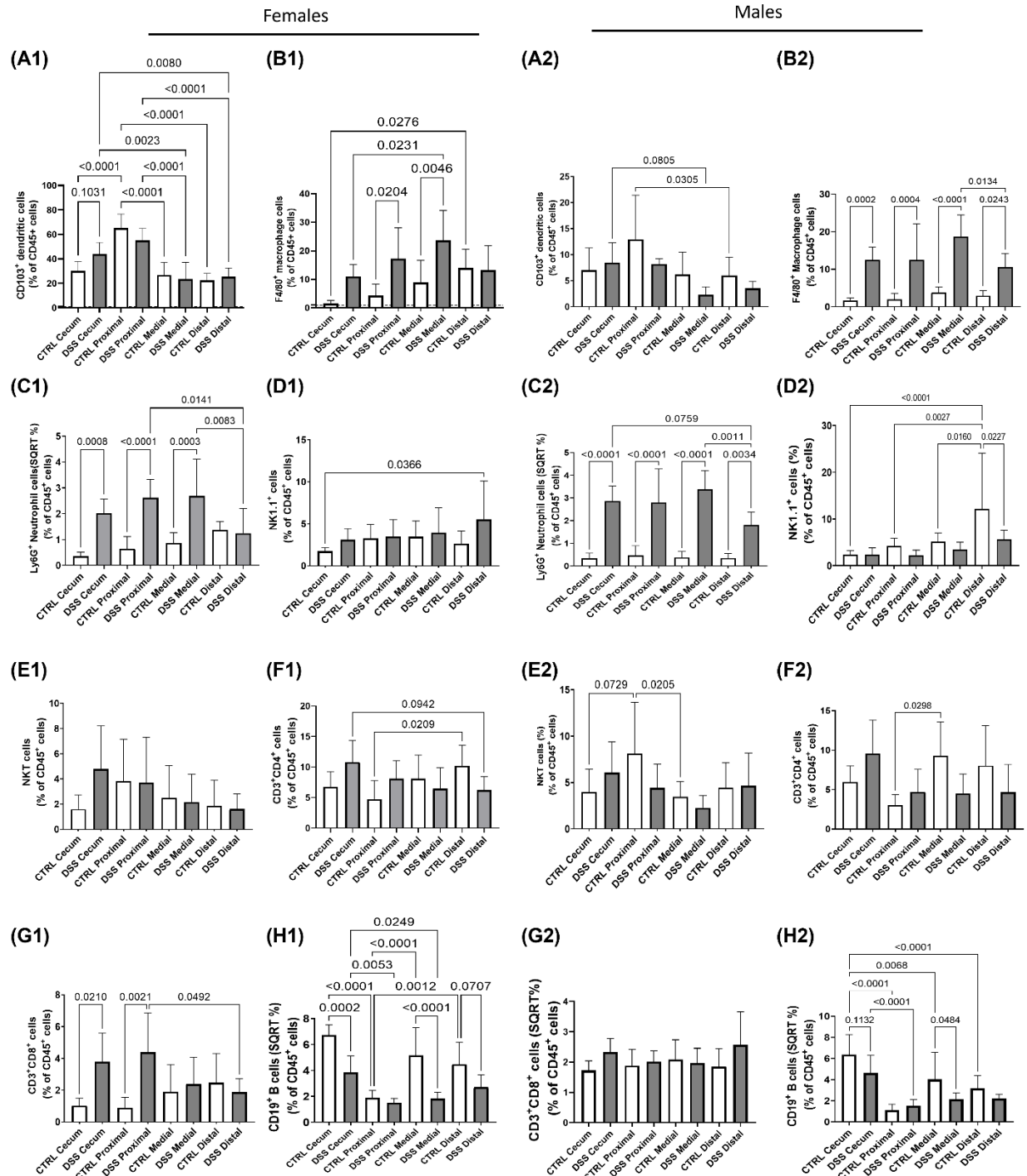


Figure 4 Supp: Characterization of key immune cell types across the large intestine during steady state and colitis in male and female mice. Mice were treated with water (control group) (CTRL) or dextran sulfate sodium (DSS) to induce colitis. Comparison of the abundance of subsets of (A1-2) Dendritic cells (DC), (B1-2) macrophages, (C1-2) neutrophils, (D1-2) Natural killer

(NK), **(E1-2)** Natural killer T (NKT), **(F1-2)** CD4⁺ T cells **(G1-2)** CD8⁺ T cells and CD19⁺ B cells population calculated within CD45⁺ cells compartments across the cecum, proximal, medial, and distal colon between male (1) and female (2) mice. When indicated, data were transformed into root transformed. Data expressed as the mean $\pm$ standard deviation (n = 6-11). Statistical significance, defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test.

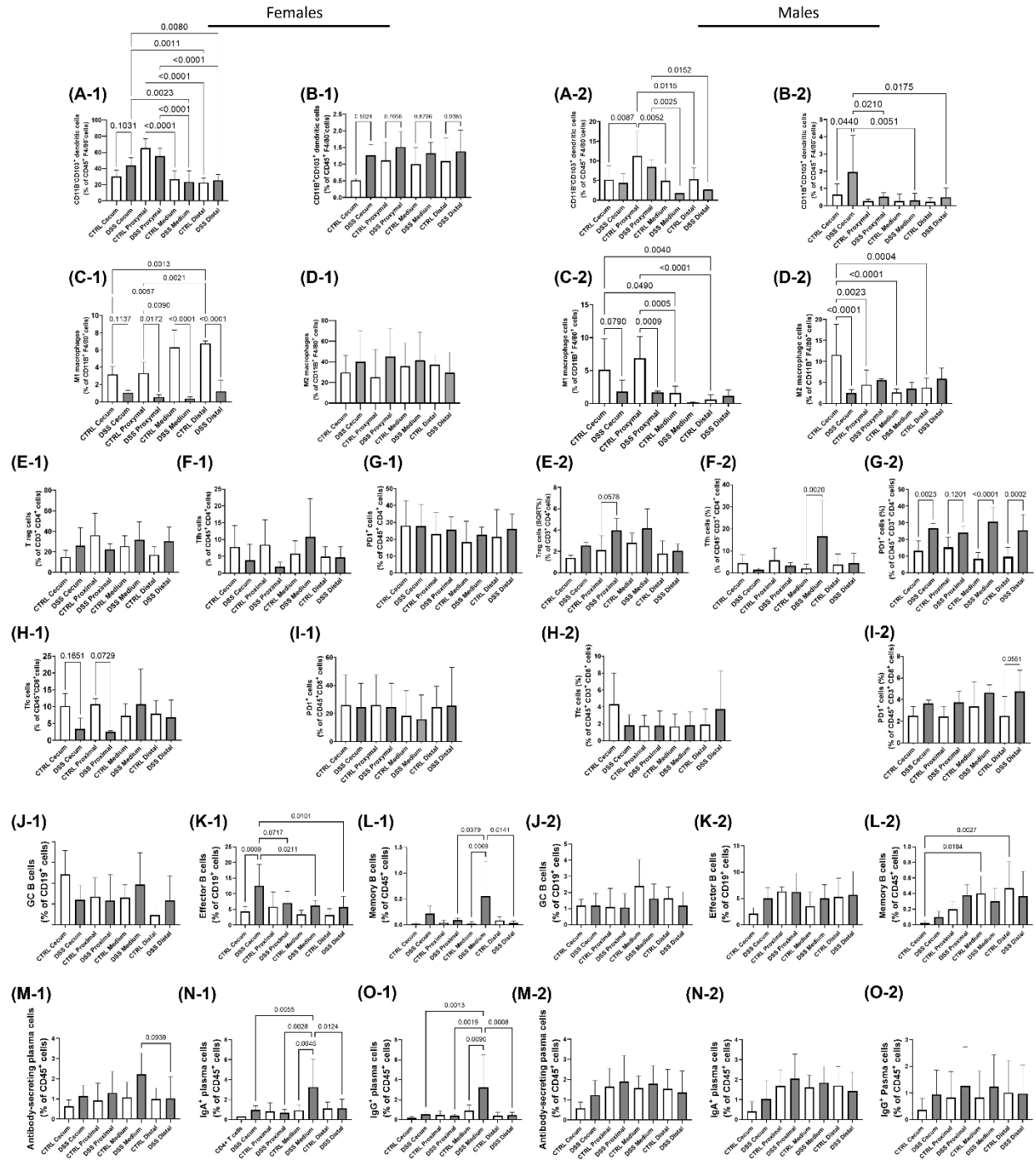


Figure 5 Supp: Profiling of immune cell subtypes across segments of the large intestine in male and female mice evaluated at steady-state and during colitis. Mice were treated with water (control group (CTRL)) or dextran sulfate sodium (DSS) to induce colitis. (A1-B2) Percentage of CD11B⁺CD103⁺ and CD11B⁺CD103⁺ dendritic cells, (C1-D2) M1 and M2

macrophage from F4/80⁺ cells, **(E1-G2)** Foxp3⁺ Regulatory T cells (Treg), CXCR5⁺, and PD1⁺ from CD4⁺ T cells, **(H1-I2)** CXCR5⁺ and PD1⁺ from CD8⁺ T cells, **(G1-K2)** germinal center and effector B cells from CD19⁺ T cells **(L1-O2)** memory, antibody-secreting plasma cells, IgA⁺ and IgG⁺ plasma cells from CD45⁺ cells were calculated across the cecum, proximal, medial, and distal colon between male and female mice. When indicated, data were transformed into root transformed. Data expressed as the mean $\pm$ standard deviation (n = 4-11). Statistical significance, defined as p<0.05, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test.

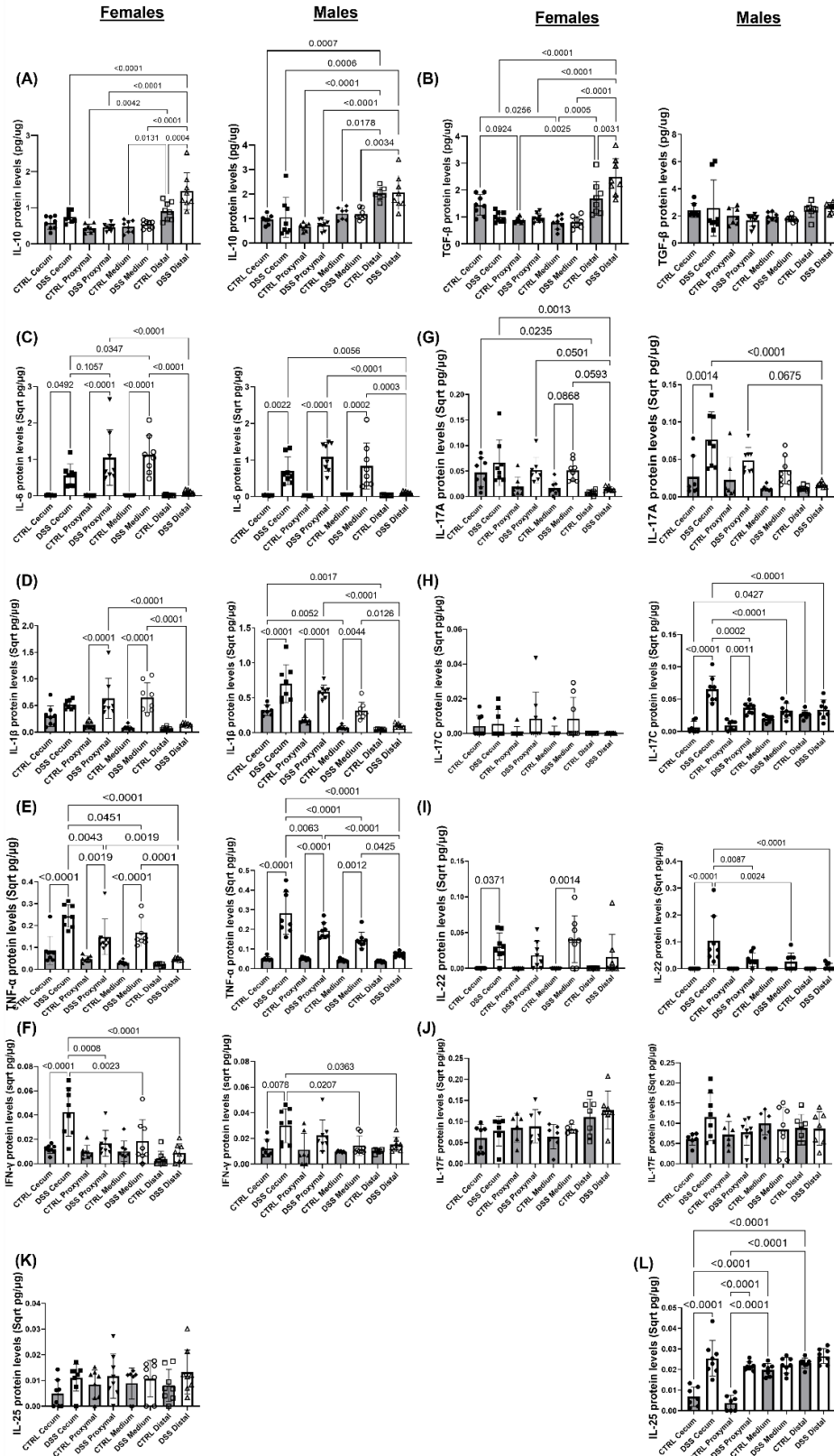


Figure 6 Supp: Anti-inflammatory, TH1 and TH17 cytokine levels along the large intestine of female and male mice in homeostatic and colitic conditions. Concentration of (A-B) TGF- β , IL-10, (C-F) IL-6, IL-1 β , TNF- α , IFN- γ (G-L) IL-17A-C, IL-22 and IL-25 within the cecum, proximal, medial, and distal colon from male and female mice. Data expressed as the mean $\pm$ standard deviation (n = 8). Statistical significance defined as $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test.

BRIDGE TO CHAPTER VI

As demonstrated in Chapters **IV** and **V**, males display increased susceptibility to DSS-induced colitis, which likely results from impaired mucosal barrier function and exacerbated inflammation correlating with increased granulocyte infiltration and a trend toward reduced tolerogenic dendritic cells along males' large intestine compared to females. Furthermore, our findings demonstrate that the distal colon in females was more enriched with IL-10 and TGF- β than those from males, suggesting more extensive colitis in males than females.

CHGA is a pro-hormone secreted by enteroendocrine cells scattered through the colonic epithelial layer. We and others have reported increased levels of CHGA in colon tissue from patients with active UC and in a preclinical murine model of UC. Elevated CHGA levels correlated with worsened colitis severity, increased mucosa-associated cytokines, and downregulation of tight junctions. Furthermore, the absence of CHGA ameliorated colitis and preserved epithelial barrier function by maintaining tight junctions in male mice. However, the role of CHGA in the healing process of the intestinal mucosa in both males and females remains unknown. Therefore, **Chapter VI** explored the influence of CHGA deletion on the colonic mucosa repair process during acute injury in both sexes using a CHGA knockout murine model of colitis. Our finding in this Chapter revealed that CHGA deletion influences the intestinal epithelial integrity, mucosal barrier function, and immune cell type profile in acute colitis in a sex-dependent manner.

CHAPTER VI

Loss of chromogranin A protects male but exacerbates colitis severity in female mice.

Diane M. Tshikudi, Olivia Johnson, Fatemeh Hesampour, Nour Eissa and Jean-Eric Ghia

Authors' contributions: JE.G., D.M.T., and N.E. participated in the study conceptualization, and D.M.T. carried out the RT-qPCR study data collection and formal analysis. O.J., F. A., and D.M.T performed the histology and immunofluorescence experiment, data collection, and analysis. D.M.T. performed the ELISA, flow cytometry, Ussing experiment, data collection, and analysis. D.M.T. prepared the figures and performed the statistical analysis. JE.G. provided the resources. JE.G. administrated the project and provided funding. (**Manuscript under preparation for submission to Acta Pharmacologica Sinica**).

6.1. Abstract

Ulcerative colitis (UC) is a gut inflammatory disorder associated with loss of gut barrier integrity and reduced epithelial repair. Epidemiological studies have shown that UC affects more men than women. Chromogranin A (CHGA), a pro-hormone highly expressed in the colon of UC patients, positively correlates with inflammatory mediators, known to disrupt gut barrier integrity and function in UC. However, the effect of CHGA on colonic mucosal barrier integrity and function at steady state and in acute colitis between sexes is poorly understood. Here, we characterize the impact of CHGA deletion on colonic epithelial integrity and mucosal function in CHGA knockout (*CHGA*^{-/-}) mice using an experimental model of colitis. Male and female C57BL/6 wild-type (WT) and *CHGA*^{-/-} mice (13-17 weeks old) were treated for 5 days with 5% dextran sodium sulfate to induce colitis or water for the control group. Monitoring and recording of disease activity scores were daily. Epithelial-associated mediators and markers specific to differentiation, proliferation, differentiated epithelial cells, and stem cells were assessed using ELISA, immunofluorescence, and qRT-PCR techniques. The profile of colonic immune cells was characterized using flow cytometry. Here, we showed that CHGA deletion ameliorates colitis in males as a consequence of increased production of anti-inflammatory cytokines interleukin-25 and Tumor growth factor (TGF)- β , lower mucosal infiltration of granulocytes, improved epithelial integrity and mucosal barrier function by preserving the tight junction regulators claudin 4, along with diminished expression of biomarkers specific to intestinal mucosal healing (trefoil factor 3) and bacterially-induced bactericide, resistin-like molecule (RELM) β . By contrast, in females, CHGA deletion worsened colitis owing to loss of epithelial barrier integrity, which correlated with lower claudin

4, mucosal resistance, and increased RELM β . This study shows that CHGA deletion alters the colonic epithelial integrity and mucosal barrier function in a sex-dependent manner in acute colitis.

6.2. Introduction

Ulcerative colitis (UC), one of the two main types of inflammatory bowel disease (IBD), is a chronic and incurable disorder of the gastrointestinal (GI) tract, characterized by severe inflammation of the mucosa of the large intestine and associated with cyclical periods of remission and relapse (13). Moreover, population and clinical-based studies reported a higher incidence rate for UC in males than women (51). Although the mechanism that mediates UC etiology is still unknown, mounting evidence suggests that impaired intestinal mucosal barrier function and dysregulated immune response contribute to UC pathophysiology (13,25).

The GI tract contains the largest endocrine organ in mammals, formed of many specialized enteroendocrine cells scattered amongst the epithelial cells that overlay the intestinal mucosa throughout the large intestine (328). These enteroendocrine cells secrete a plethora of prohormones and biologically active peptides in response to neurotransmitters and luminal stimuli such as ingested nutrients, microbial metabolites, and hormones (329). The GI neuroendocrine mediators modulate various intestinal physiological functions, including appetite, GI motility, visceral sensitivity, intestinal mucosal secretion, absorption and proliferation, and local immune response (330).

One such peptide is chromogranin A (CHGA), an acidic and soluble secretory glycoprotein of 431 residues and a mass molar of 49-52 kDa, stored and released from not only secretory granules from enteroendocrine cells but also by the pituitary gland, neurons, fibroblast, neutrophils, and macrophages (17,331). In the pituitary gland, CHGA levels were strongly reduced by circulating estrogen, highlighting sex-related differences in CHGA expression (332). CHGA has autocrine, paracrine, and endocrine activities in addition to giving rise to various bioactive peptides as a result of post-translational proteolytic processing (330). Although the exact function of CHGA is yet to be fully understood, mounting evidence suggests that CHGA plays a critical role in secretory granule formations, which serve to store hormones such as catecholamine, serotonin, and histamine in endocrine cells (333). Thus, studies reported that CHGA contributes to the increases in hypertension and promotes Ca^{2+} storage within the secretory granules in endocrine cells (264,334). Moreover, the depletion of CHGA correlated with reduced intestinal mucosal paracellular permeability, lower expression of tight junctions and desmosome, and elevated production of anti-inflammatory cytokines such as TGF- β in the colon tissues in homeostatic conditions (265).

Many reports described pathologic increases in CHGA levels in neurological, cardiovascular, gestational, and inflammatory disorders such as IBD (334–336). Thus, high levels of CHGA reported in plasma, feces, and intestinal tissues from patients with UC and animal models of colitis positively correlated with active disease along with elevated leukocyte mucosal infiltration, proinflammatory cytokines secretion, and epithelial apoptosis (17,259,336). Furthermore, in the chemically induced male rodent model of acute colitis, the lack of CHGA was associated with increased macrophage differentiation in the M2 profile and elevated productions of anti-

inflammatory mediators such as IL-10 (17). However, the mechanism that governs CHGA action on colon mucosal integrity and function during acute colitis has yet to be fully understood. Moreover, the role of sex-mediate differences in the CHGA effect in colitis remains unclear. Therefore, we sought to characterize the impact of CHGA deletion on colonic mucosal barrier integrity and function in both male and female mice at steady state and in acute colitis.

6.3. Results

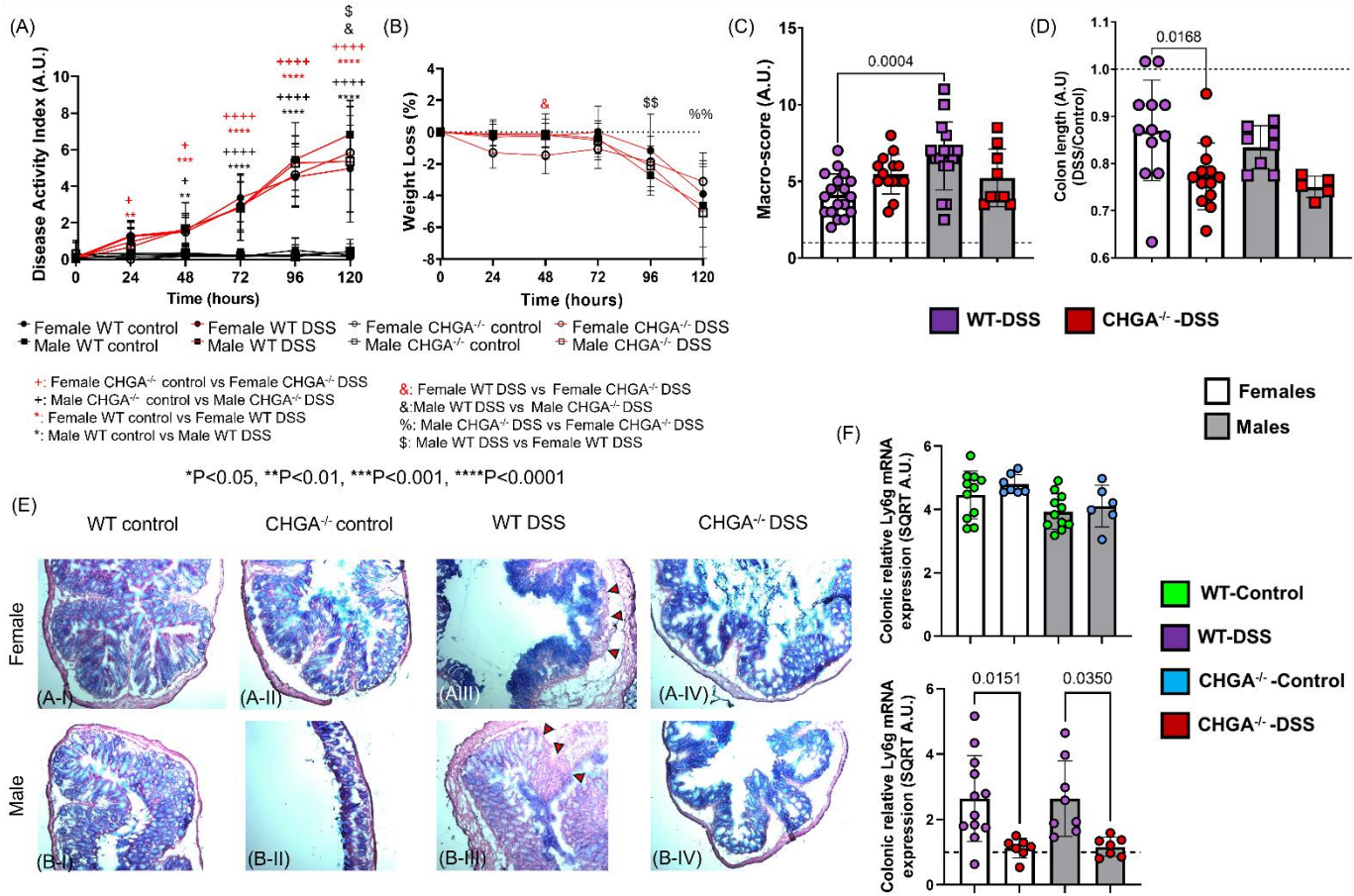
6.3.1 CHGA deficiency is associated with ameliorated colitis in males but not in female mice

Mounting evidence reports a regulatory effect for CHGA in colitis severity. Here, we assessed the impact of CHGA ablation on DSS-induced acute colitis in CHGA^{-/-} male and female mice. As expected, DSS-treated mice showed significant increases in DAI, weight loss, and macro-score, as well as reduced colon length compared to controls in both sexes (**Figures 1A-D**). However, CHGA deficiency correlated with notable sex-dependent differences in the disease severity. We find reduced DAI in CHGA^{-/-} male compared to WT male mice, 120 hours following colitis induction (day 5), whereas DAI did not differ significantly between CHGA^{-/-} and WT female mice throughout DSS treatment ($P<0.05$) (**Figure 1A**). Compared to males, CHGA^{-/-} and WT female mice showed increased DAI 24 hours after the induction of colitis ($P<0.05$). CHGA^{-/-} female mice had a faster weight loss induced by colitis than WT female mice in the first 48 hours ($P=0.05$), which slowed and was significantly less marked at day-5 compared to CHGA^{-/-} colitic male mice ($P=0.004$) (**Figure 1B**). CHGA^{-/-} female mice exhibited a trend toward increased macro-score

and significantly smaller colon length than WT female mice in colitis ($P=0.02$) (Figures 1C-D).

In contrast, the macro-score and colon length did not differ between CHGA^{-/-} and WT colitic males.

These data suggest that CHGA deficiency protects male mice from colitis but aggravates colitis



severity in female mice.

Figure 1: The lack of CHGA is associated with improved colitis in males, whereas females displayed a trend toward enhanced susceptibility to colitis. Disease severity was characterized in C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice with DSS-induced colitis and compared to control groups. (A) The disease activity index was calculated daily using a composite of (B) weight loss, stool consistency, and bleeding scores (n=12-31). (C) the macro-score index, calculated for rectal bleeding, rectal prolapse, diarrhea, colon bleeding scores, and (n=9-24) (D) colon length, was assessed for each mouse (n=5-12). (E) Mucus production and leukocyte mucosal infiltration were examined using H&E and alcian blue histological staining. (F)

Expression of granulocyte marker Ly6g was assessed by QRT-PCR. The relative mRNA expression of Ly6g in colitis was normalized to control conditions and transformed into square roots. The dashed line described a ratio of 1, representing a lack of difference between colitis and control conditions. Data for DAI, weight loss, and macro-score were obtained from four (WT) and five (CHGA^{-/-}) independent experiments. Ly6G mRNA expression was calculated from data from two independent experiments (n=6-13). Data expressed as the mean $\pm$ standard deviation. Outliers were determined using robust regression and outlier methods. Statistical significance determined using ordinary one-way ANOVA and Tukey's multiple comparisons test is $P < 0.05$. Only statistically significant results ($p < 0.05$) are presented in the figures. *, \$, & $p < 0.05$, **, \$\$, %% $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Colon tissues stained with alcian blue showed no significant changes in mucus between female groups (**Figures 1E; AI-IV**). Conversely, tissues from WT colitic males displayed substantially reduced mucus than WT control males (**Figures 1E; BI, BIII**). However, mucus was restored in tissues from CHGA^{-/-} colitic males, exhibiting no significant difference in staining intensity compared to CHGA^{-/-} control male mice (**Figures 1E; BII, BIV**). Moreover, colitis was associated with increased leukocyte infiltration in WT male and female mice compared to corresponding control groups. However, leukocytes infiltrated during colitis were markedly lower in CHGA^{-/-} male and female mice than in WT male and female mice. These results, confirmed by qRT-PCR analysis, showed that colitis resulted in a trend toward reduced fold change in granulocyte marker Ly6g mRNA levels in CHGA^{-/-} females and significantly lower Ly6g mRNA ratio in CHGA^{-/-} males compared to their WT male and female counterparts (**Figure 1F**). These results suggest a trend toward increased susceptibility to DSS-induced colitis in females associated with potentially reduced mucosal regeneration in the absence of CHGA, contrasting with the enhanced resistance of male mice to colitis.

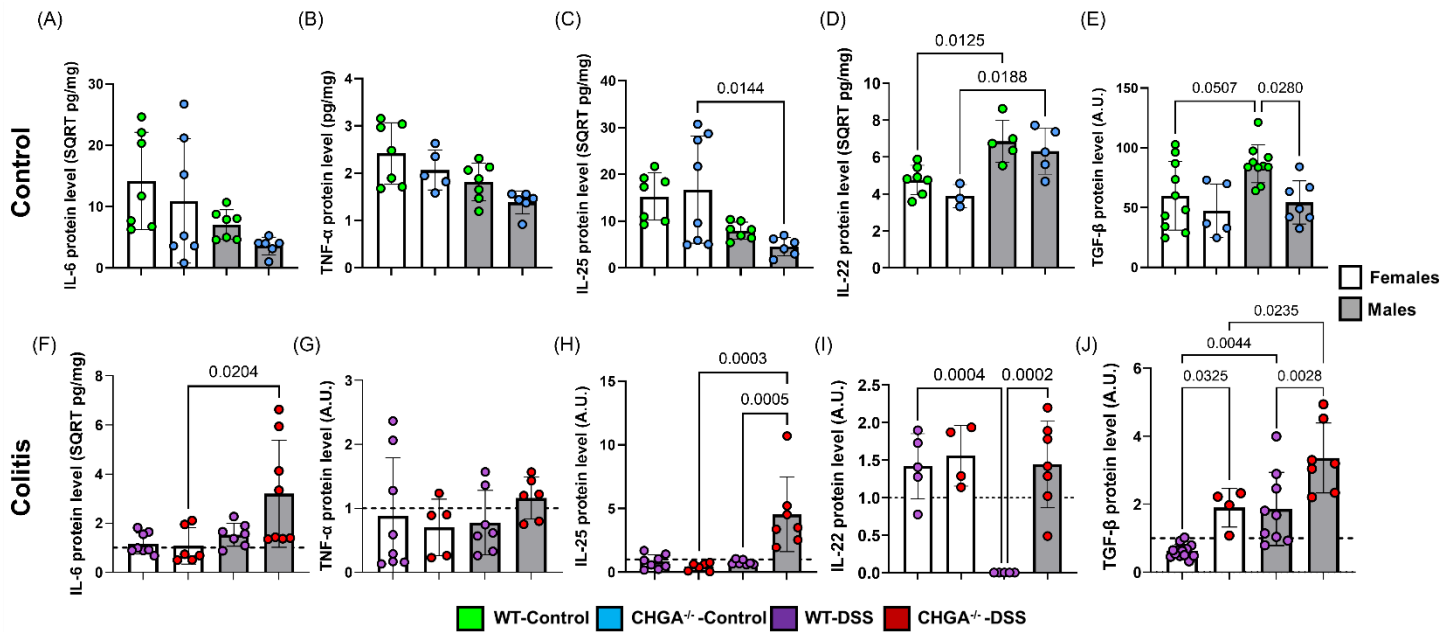


Figure 2: The lack of CHGA alters the level of cytokines regulating the intestinal mucosa integrity and functions in a sex-dependent manner in DSS-induced colitis. Cytokine levels were analyzed in homogenized colon tissues from C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice with DSS-induced colitis and compared to control groups. The levels of proinflammatory, Th2, and anti-inflammatory cytokines (A) IL-6, (B) TNF- α , (C) IL-25, (D) IL-22, and (E) TGF- β were assessed by ELISA. The levels of (F) IL-6, (G) TNF- α , (H) IL-25, (I) IL-22, and (J) TGF- β in colitis were normalized to control conditions. The dashed line described a ratio of 1, representing a lack of difference between colitis and control conditions. Data from 2-3 independent experiments with 3-4 mice per group for n=7-9 expressed as the mean $\pm$ standard deviation. IL-6, IL-25, and TGF- β data in the control condition are square root transformed. The level of IL-22 and TGF- β in several samples was below the instrument detection limit. Robust regression and outlier detection were calculated to identify outliers. Statistical significance, $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Only statistically significant results ($p < 0.05$) are presented in the figures.

Given the marked contrast in disease severity observed between males and females in the absence of CHGA, we investigated whether these differences alter the profile of proinflammatory and anti-inflammatory cytokines IL-6, TNF- α , IL-25, IL-22, and TGF- β in homeostatic and colitic conditions in colon tissues (**Figure 2**). In homeostatic conditions, the levels of all evaluated

cytokines did not differ significantly between WT and CHGA^{-/-} mice in both sexes, although the level of IL-25 was reduced in colon tissues from CHGA^{-/-} females compared to CHGA^{-/-} male mice ($P=0.004$, 0.009) (**Figures 2A-E**). In colitis, IL-6, TNF- α , IL-25, and IL-22 levels in WT and CHGA^{-/-} female mice did not change significantly compared to control conditions (**Figures 2F-I**). Moreover, IL-6, TNF- α , IL-25, and IL-22 ratios did not differ significantly between WT and CHGA^{-/-} female mice with DSS-induced colitis. By contrast, TGF- β levels in WT and CHGA^{-/-} female mice were not significantly different between colitic and control conditions, yet the level of TGF- β in colitis, normalized to those in controls, was substantially higher in CHGA^{-/-} females than in WT female mice ($P=0.0007$) (**Figure 2J**). In WT male mice, IL-6, TNF- α , and IL-25 did not change significantly in colitis compared to control conditions. On the other hand, CHGA^{-/-} male mice had more IL-25 in colitis than in control conditions. Moreover, CHGA^{-/-} male mice displayed a significantly higher IL-25 ratio than WT male mice in colitis. IL-22 level was depleted in WT male mice but substantially increased in CHGA^{-/-} male mice in colitis compared to control conditions ($P<0.003$ for all). Consequently, in males, the ratio of IL-22 was markedly higher in CHGA^{-/-} than in WT mice in colitis ($P<0.0001$). Moreover, CHGA^{-/-} males displayed a higher IL-22 ratio than female mice in colitis ($P=0.04$). WT male mice demonstrated a trend toward increased TGF- β levels, while TGF- β levels were significantly higher in CHGA^{-/-} male mice in colitis compared to control conditions ($P<0.0001$). Interestingly, unlike WT females, WT male mice presented with significantly elevated levels of TGF- β ($P=0.01$), which rose substantially in the colon of CHGA^{-/-} mice in colitis ($P=0.001$). These findings likely indicate that CHGA modulates the expression of cytokines, which regulates mucosal barrier integrity in a sex-dependent manner.

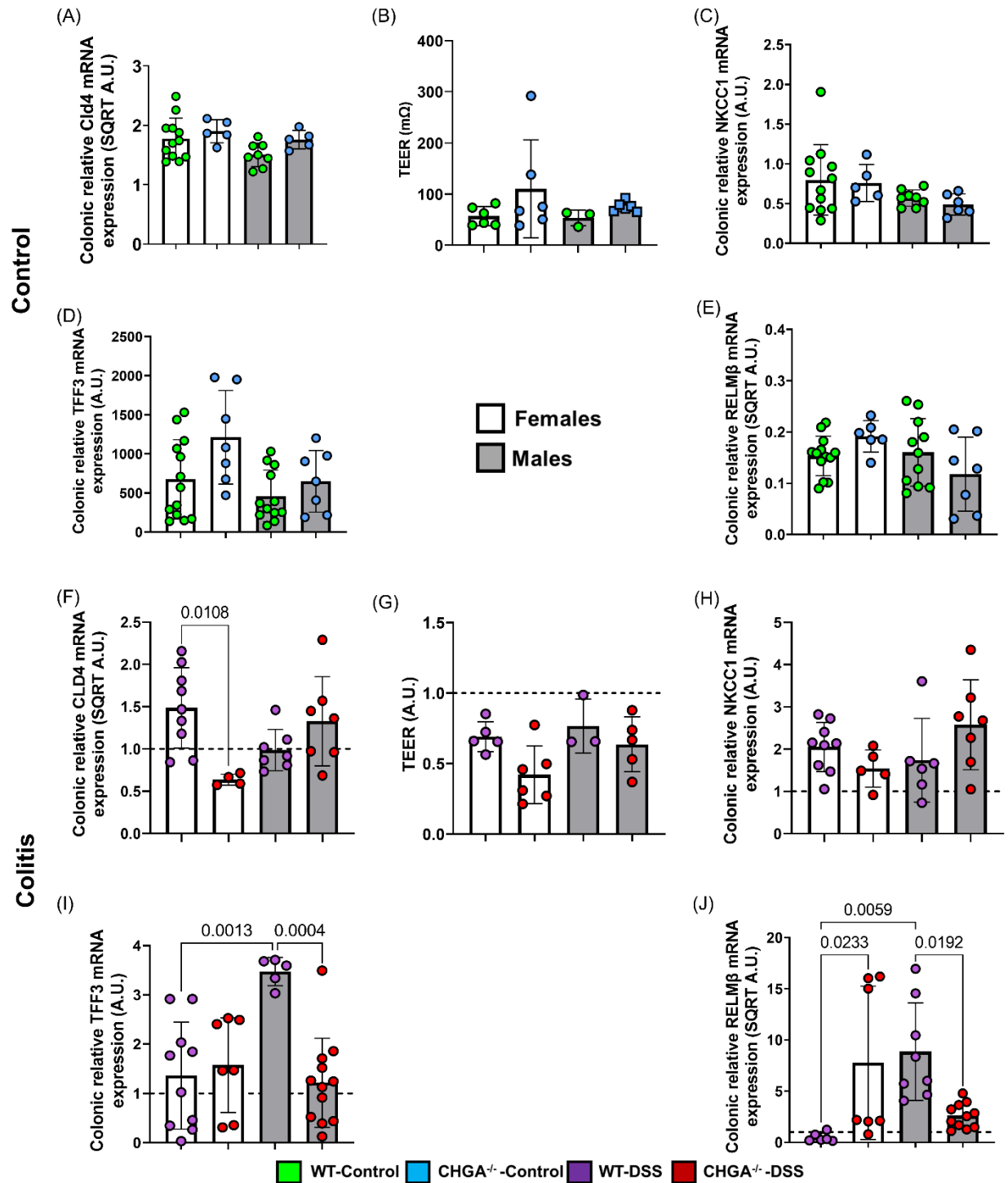


Figure 3. CHGA deletion correlates with exacerbated loss of mucosal barrier integrity in females but improved mucosal healing in males. C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice with DSS-induced colitis and compared to control groups. (A) mRNA

levels of tight junction regulator claudin 4 (Cld4) were assessed in colon tissue. (B) Transepithelial electrical resistance (TEER) was performed using the Ussing chamber. Gene expression of (C) Na-K-Cl (Nkcc)1 cotransporter (D), mucosal healing biomarker trefoil factor (Tff3), and (E), bacterially induced Relm β , were characterized from colon samples by RT-qPCR in control conditions. The values from F) Nkcc)1, (G) TEER, (H) Tff3, and (E) Relm β mRNA levels and Ussing measurement in colitis were normalized to control conditions. The dashed line described a ratio of 1, representing a lack of difference between colitis and control conditions. Data from 2-3 independent experiments for n=3-12 expressed as the mean $\pm$ standard deviation. Data expressed as the mean $\pm$ standard deviation. Statistical significance, $p < 0.05$, was assessed using ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Robust regression and outlier' detection were used to identify outliers. Only statistically significant results ($p < 0.05$) are displayed in the figures.

6.3.2. Ablation of CHGA correlates with preserving mucosal barrier function in males but enhances the colitis-mediated loss of mucosal barrier function in females

The unexpected alteration in TGF- β and IL-22, two critical promoters of mucosal barrier integrity, prompted the assessment of the epithelial barrier function in CHGA^{-/-} mice. We first characterized the mRNA expression of tight junction Cld4 and cotransporter Na-K-Cl1 (Nkcc1) as well as biomarkers of mucosal inflammation Tff3 and goblet-derived bacterially induced Relm β in CHGA^{-/-} mice in control and colitic conditions (**Figure 3**). Furthermore, we measure the epithelial cells' barrier integrity using transepithelial electrical resistance (TEER) measurement. In control conditions, Cld4, Nkcc1, Tff3, and Relm β mRNA levels did not differ significantly across all groups (**Figures 3A-E**). DSS-induced colitis resulted in elevated Cld4 and Nkcc1 mRNA levels ($P=0.02$; 0.003) and no significant changes in mucosa resistance, Tff3, and Relm β mRNA levels in colon tissues from WT female mice compared to control conditions. In contrast, CHGA^{-/-} female mice colon displayed a trend toward reduced tissue resistance and Cld4 mRNA expression, a marked increase in Realm ($P= 0.003$), and no significant changes in Nkcc1 and TFF3 mRNA

levels in colitis compared to control conditions. Results normalized to control conditions showed reduced *Cld4* ($P=0.01$) and higher *Relm β* ($P=0.02$) mRNA ratio, whereas epithelial resistance was lower in *CHGA*^{-/-} compared to WT female mice ($P=0.003$) (**Figures 3F-G, J**). *Nkcc1* and *TFF3* ratios did not differ between both groups in colitis (**Figures 3H, I**). In contrast, *CHGA*^{-/-} male mice did not display significant changes in *Cld4* and *Nkcc1* mRNA ratio as well as epithelial resistance but showed a marked decrease in *TFF3* and *Relm β* mRNA expression proportion compared to WT male mice in colitis ($P=0.0004$, $P=0.02$).

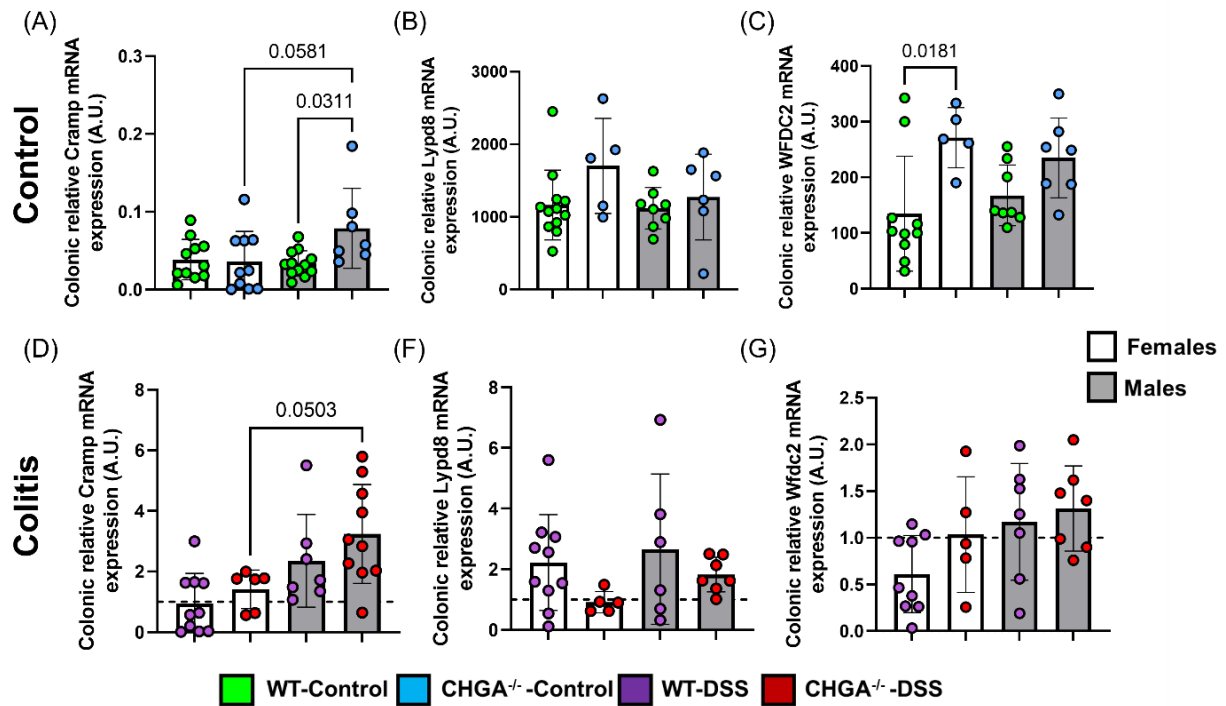


Figure 4. Homeostatic mucosal antimicrobial activities differ in a sex-dependent manner in the absence of CHGA. Biomarkers specific to antimicrobial peptides were quantified from colonic tissues from male and female C57BL/6 (WT) and CHGA knockout (CHGA^{-/-}) mice treated with DSS or water (control group). Goblet-secreted antimicrobial (A) Wfdc2, (B) Cramp, and (C) colonocytes-secreted antimicrobial Lypd8 mRNA expression was characterized from colon samples using RT-qPCR in control conditions. The values from (D) Wfdc2, (E) Cramp, and (F) Lypd8 mRNA levels in colitis were normalized to control conditions. The dashed line described a ratio of 1, representing a lack of difference between colitis and control conditions. Data from 2-3 independent experiments for n=8-12 were expressed as the mean $\pm$ standard deviation. The level of IL-22 and TGF- β in several samples was below the instrument detection limit. Statistical significance, $p < 0.05$, is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Statistical analysis using robust regression and outliers was performed to identify outliers. Only statistically significant results ($p < 0.05$) are displayed in the figures.

Next, we investigated whether changes in the intestinal epithelial barrier function alter the expression profile of antimicrobial proteins in healthy and colitic colons of CHGA^{-/-} mice (**Figure 4A-G**). In control conditions, increased Wfdc2 mRNA levels were found in CHGA^{-/-} compared to WT female mice ($P=0.02$), whereas Cramp and Lypd8 mRNA levels did not differ markedly between both groups (**Figure 4A-C**). Conversely, CHGA^{-/-} male mice displayed higher Cramp mRNA levels but no significant differences in Lypd8 and Wfdc2 mRNA expression compared to WT males ($P=0.03$). In colitic conditions, Cramp, Lypd8, and Wfdc2 mRNA fold changes did not change significantly between all groups despite the significant increase in Cramp mRNA levels observed in colitic compared to control conditions in CHGA^{-/-} male mice ($P=0.0007$) (**Figure 4D-G**). Together, these results indicate that CHGA likely modulates the intestinal epithelial barrier function and mucosa integrity in a sex-dependent manner in colitis.

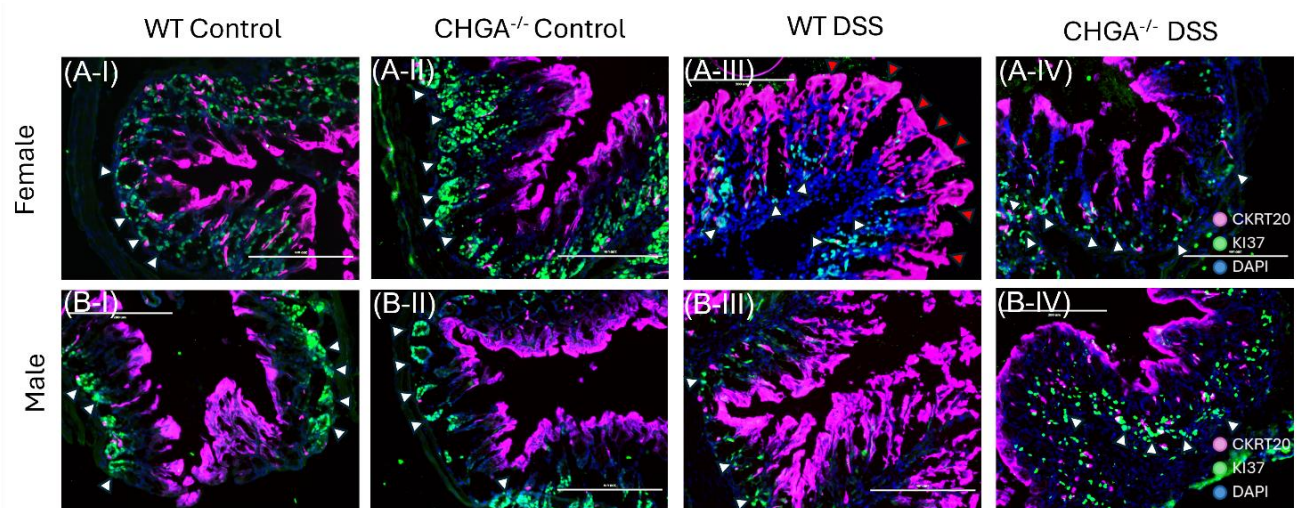


Figure 5. **CHGA deletion correlates with enhanced proliferation at a steady state in females and reduced differentiation in males and females in colitic conditions.** C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice received 5% dextran sulfate sodium (DSS) for 5 days. Protein levels of proliferation (KI67) and differentiation (cytokeratin 20, CKRT20) were assessed by immunofluorescence (IF) (A-I–A-IV) in females and (B-I–B-IV) male mice. Scale bar: 200 μ m.

6.3.3. CHGA-deficient male and female mice with colitis harbor distinct intestinal epithelial cell profile

Proliferation and differentiation of new intestinal epithelial cells are critical to sustain mucosal barrier function and promote tissue repair after injury. We investigated the impact of CHGA on colonic mucosal repair capacity (**Figure 5**). In control conditions, the intensity of Ki67 immunostaining, a marker for proliferation, was markedly elevated in CHGA^{-/-} than WT female mice but not significantly altered between CHGA^{-/-} and WT male mice (**Figures 5AI-II, BI-II**). The intensity of cytokeratin 20 (CKRT20), a marker of intestinal epithelial cell differentiation, did not change significantly among all groups in control conditions. Proliferation was markedly lower in colitis than control conditions in male and female WT and CHGA^{-/-} mice. However, the reduction in proliferation was substantially more in colitic compared to control conditions in CHGA^{-/-} female mice. Furthermore, proliferation was markedly higher in CHGA^{-/-} compared to WT male mice in colitic conditions.

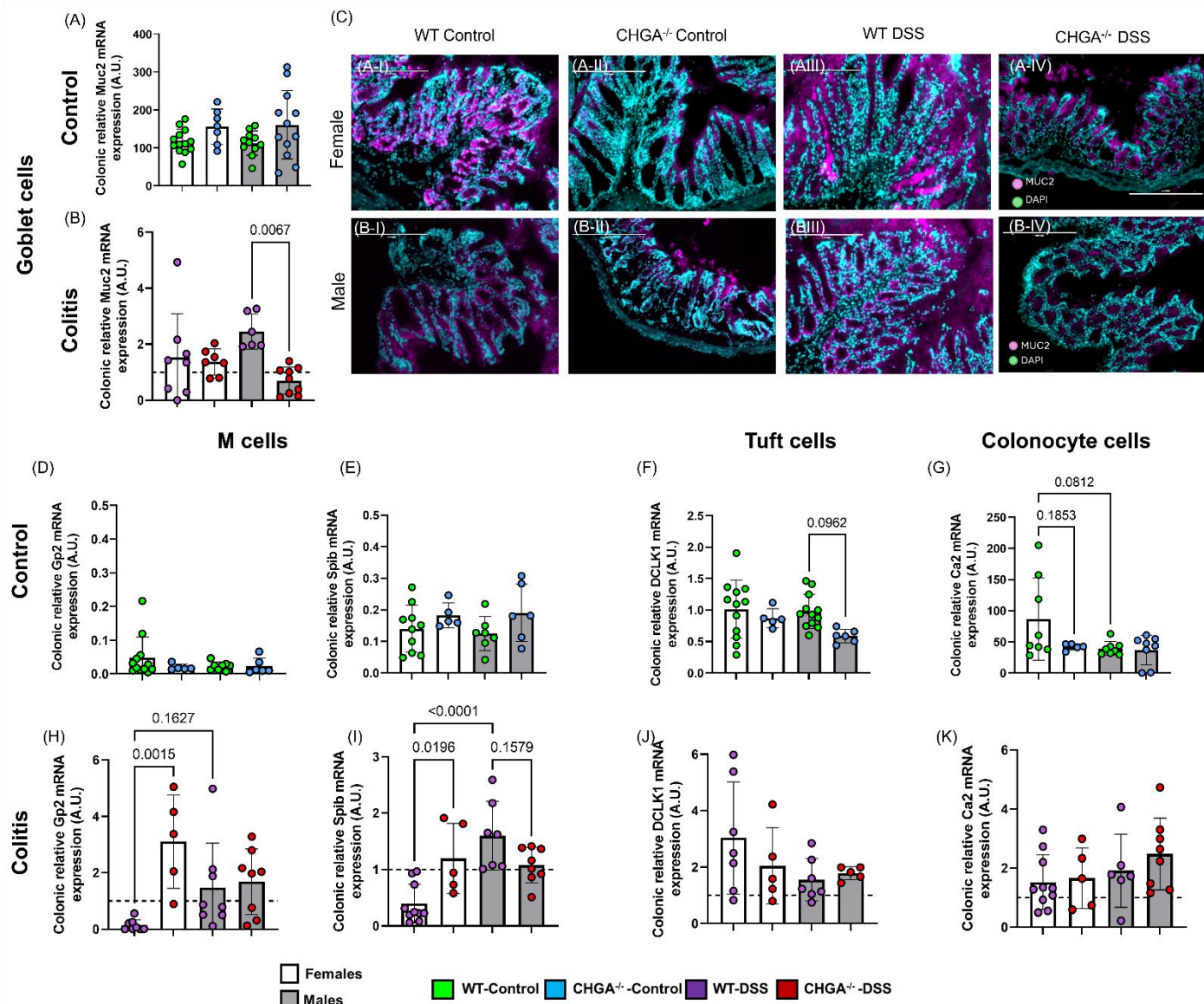


Figure 6. The lack of CHGA is associated with higher M cells in females and reduced goblet cells in males and females in colitic conditions. (A-K) Expression of markers specific to differentiated colonic epithelial cells was evaluated. Colonic tissues collected from C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice were treated with 5% dextran sulfate sodium (DSS) for 5 days to induce colitis and compared to control groups. mRNA and protein levels of markers specific to goblet cells (A-C), mucin (MUC2)2, (D, E) M cells Gp2 and Spib, (F) Tuft cells doublecortin-like kinase (DCLK) 1, and (G) colonocytes Ca2 were characterized by RT-qPCR and immunofluorescence (IF) (n = 3), scale bar: 200 μ m. The values from (H) Muc2, (I) Gp2, (J) Spib, and (K) Ca2 mRNA levels in DSS-induced colitis were normalized to data from control mice. The dashed line in each figure represents a ratio of 1, indicating no difference

between DSS-induced colitis and control conditions. The histogram represents the means $\pm$ standard deviation of data from 2-3 independent experiments for $n=8-12$. One-way ANOVA followed by Tukey's post hoc was performed between groups. Only statistically significant results ($p < 0.05$) are displayed in the figures.

Next, we examined the profile of terminally differentiated intestinal epithelial cells in colon tissues (**Figure 6A-K**). In control conditions, the mRNA level of marker specific to goblet cells mucin 2 (Muc2), tuft cells doublecortin like kinase 1 (Dclk1), colonocytes carbonic anhydrase 2 (Ca2), as well as M cells glycoprotein 2 (Gp2) and transcription factor Spib (Spib) did not show significant differences across all groups (**Figures 6A-B, D-K**). Similarly, the intensity of MUC2 immunostaining did not differ significantly between female and male CHGA^{-/-} and WT mice counterparts (**Figures 6C AI-II, BI-II**). However, Gp2 mRNA levels increased markedly in CHGA^{-/-} female mice in colitis compared to control conditions ($P=0.005$). Moreover, Spib and Gp2 mRNA fold changes were significantly elevated in colon tissues from CHGA^{-/-} females compared to WT female mice in colitis (Figure 5H-I) ($P<0.02$). On the other hand, CHGA^{-/-} male mice displayed a reduced Muc2 mRNA ratio compared to WT male mice, which was also seen at the protein levels in colitic conditions (Figures 5B-C). Of note, the proportion of Muc2 mRNA levels did not differ significantly between CHGA^{-/-} and WT female mice with colitis, yet MUC2 protein levels were lower in colon tissues from CHGA^{-/-} females compared to WT females with colitis (**Figures 6C AIII-IV**). The ratio of Ca2 and Dclk1 mRNA levels did not differ significantly between all groups in colitic conditions, suggesting that the effect of CHGA mainly modulates goblet mucus production and M cell expansion in a sex-dependent manner.

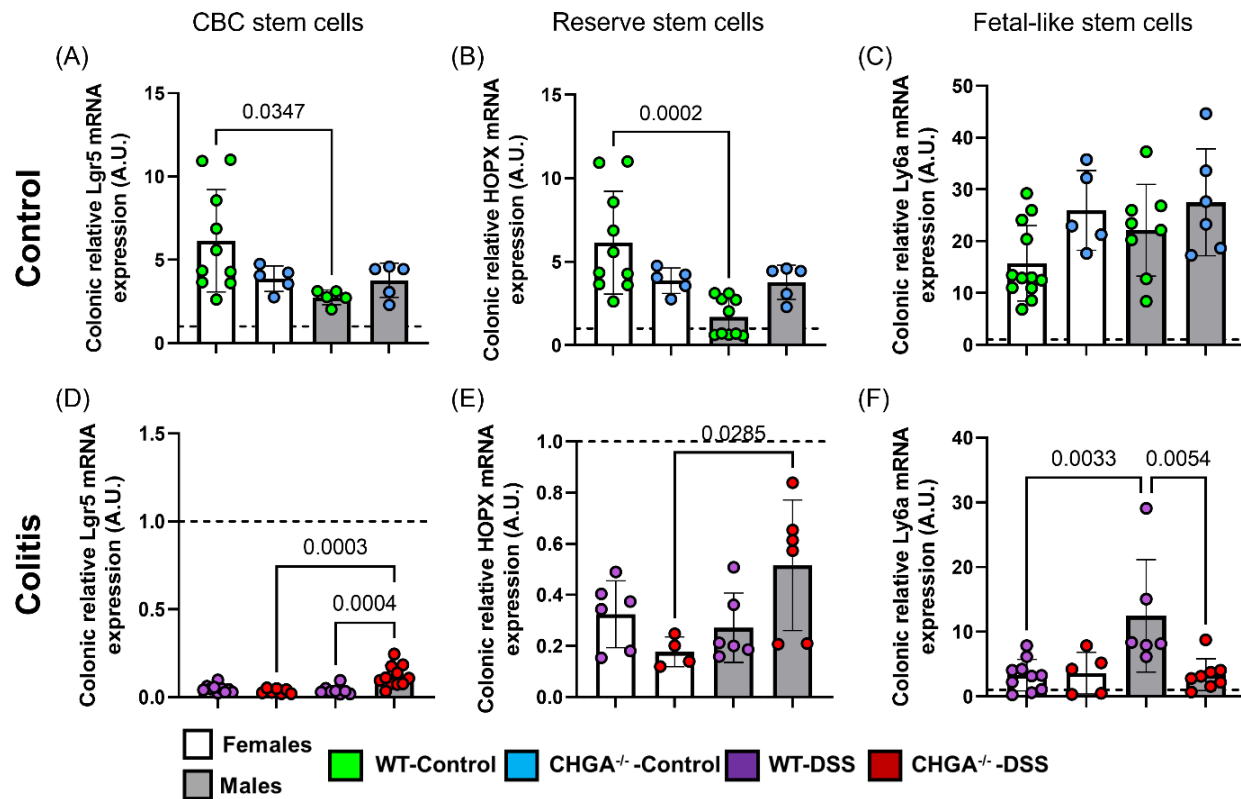


Figure 7. The lack of CHGA is associated with higher stem cells maintained in males than females in colitic conditions. Markers associated with colonic epithelial stem cells were evaluated. Colonic tissues collected from C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice were treated with 5% dextran sulfate sodium (DSS) for 5 days to induce colitis and compared to control groups. Colon tissue extracted on day five post-DSS treatment was analyzed using RT-qPCR. Biomarkers specific to (A) crypt base columnar (Lgr5), (B) damage-associated regenerative (Hopx), and (C) paligenosis (fetal-like) (Ly6a) colonic stem cells were evaluated. (D) Lgr5, (E) Hopx, and (F) Ly6a data from DSS-treated mice were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. The histogram represents the mean $\pm$ standard deviation from 2-3 independent experiments for n=8-12. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis using robust regression and outliers was performed to identify outliers. Only statistically significant results ($p < 0.05$) are displayed in the figures.

Next, we investigated whether perturbation in terminally differentiated intestinal epithelial cell profiles in the absence of CHGA was associated with changes in the stem cell compartment

(**Figure 7**). Thus, mRNA expression of markers specific to crypt-based columnar (CBCs), reserve and fetal-like stem cells, *Lgr5*, *Hopx*, and *Ly6a*, respectively, did not differ significantly between *CHGA*^{-/-} males and WT mice in control conditions independent of sexes (**Figures 7A-C**). *Lgr5* and *Hopx* mRNA expression were downregulated in WT and *CHGA*^{-/-} males male and female mice in colitic compared to control conditions, whereas *Ly6a* mRNA expression was primarily significantly enhanced in colitic male mice ($P < 0.01$ for all). However, when normalized to the control conditions, the mRNA levels of *Lgr5* and *Hopx* were higher in *CHGA*^{-/-} males compared to WT male mice in colitis, although not significantly for *Hopx* ($P = 0.0004$; $P = 0.09$) (**Figures 7D-E**). Conversely, the fold change in *Ly6a* mRNA expression was lower in *CHGA*^{-/-} males compared to WT male mice with colitis (**Figure 7F**). The ratio in *Lgr5*, *Hopx*, and *Ly6a* mRNA levels did not differ between WT and *CHGA*^{-/-} female mice with colitis. Altogether, these results suggest a role for CHGA in regulating the intestinal epithelial cell repair process during inflammation, yet its effects differ between sexes.

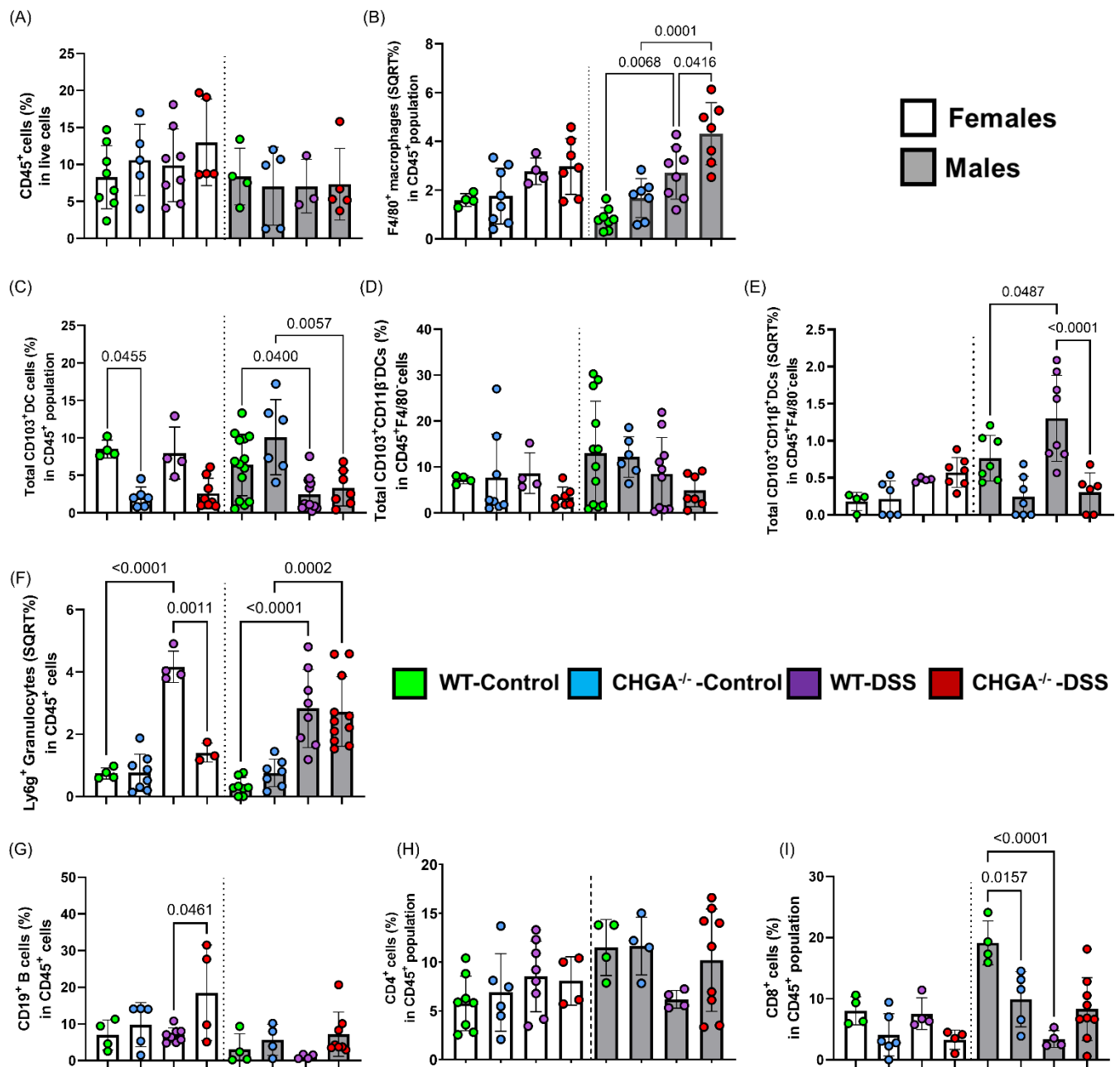


Figure 8: CHGA deletion leads to reduced myeloid compartments in males and females in colitic conditions. The profile of innate and adaptive immune cells measured in the medial colon from C57BL/6 (WT) and CHGA knockout CHGA^{-/-} male and female mice treated with 5% dextran sulfate sodium (DSS) for 5 days to induce colitis and compared to control groups. Abundance of hematopoietic cells (A) CD45⁺ and major immune cell types (B) F4/80⁺ macrophages, (C) CD103⁺ DCs, (D) CD103⁺CD11B⁺DCs, (E) CD103⁺CD11B⁺DCs subtypes, (F) LY6G⁺ granulocytes, (G)

CD19⁺ B cells, (H) CD4⁺ T cells, and (I) CD8⁺ T cells were evaluated by flow cytometry. Data expressed as the mean $\pm$ standard deviation, 2-3 independent experiments, n=3-9. Statistical significance is determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Statistical analysis using robust regression and outliers was performed to identify outliers. Only statistically significant results ($p < 0.05$) are displayed in the figures.

6.3.4. Loss of CHGA correlates with a substantial reduction in hematopoietic immune cells in female mice with colitis

Given the critical role played by the epithelium in coordinating the intestinal immune responses, we investigated whether the ablation of CHGA modulates the profile of immune cells in male and female mice in control and colitic conditions. In control conditions, preliminary data showed that CD45⁺ hematopoietic cells, F4/80⁺ macrophages, Ly6⁺ granulocytes, CD19⁺, and CD8⁺ lymphocytes did not differ significantly between WT and CHGA^{-/-} female mice (**Figures 8A-K**). However, lower CD4⁺ cells and dendritic cells (DCs), along with the reduced frequency of CD103⁺CD11b⁺DCs and DC103⁺CD11⁻DCs, two of the most common intestinal DC subtypes, were found in the medial colon of CHGA^{-/-} females compared to WT female mice (**Figures 8C-E, H**). In colitis, abundance of most innate immune cells, including macrophages, neutrophils, DCs, and DCs subtypes, were all drastically diminished in the colon tissues of CHGA^{-/-} females compared to WT female mice in colitis, in agreement with the reduced in CD45⁺ cells frequency CHGA^{-/-} females. In the adaptive compartment, the abundance of CD4⁺, CD19⁺, and CD8⁺ cells did not differ between WT and CHGA^{-/-} females in colitis. Similarly, leukocyte abundance was not significantly different between WT and CHGA^{-/-} males during colitis. Moreover, they did not differ compared to control conditions. Thus, CHGA might dampen innate immune cell recruitment in the colon mucosa of female mice during colitis.

6.4. Discussion

We recently demonstrated that CHGA regulates colonic inflammation by controlling the plasticity of macrophages and altering mucosa apoptosis. However, the effect of CHGA on colonic epithelial integrity, mucosal functions, and healing remains to be characterized in both females and males. Here, we showed that CHGA deletion ameliorates colitis in males due in part to reduced inflammation mediated by the increased levels of IL-25 and TGF- β , reduced neutrophil recruitment, improved epithelial barrier integrity, function, and mucosal healing associated with elevated IL-22 and Cramp, preserved Cld4, along with reduced Tff3 and Relm β . By contrast, in females, CHGA deletion worsened colitis owing to loss of epithelial barrier integrity, which correlates with lower Cld4, mucosal resistance, and increased Relm β .

Stored and secreted in abundance by predominantly enteroendocrine cells and, to a lesser extent, by immune and neuroendocrine cells, CHGA is a biomarker associated with the disease activity in UC patients (258,259). Previous studies have reported a higher level of CHGA and a rise in the number of enteroendocrine cells in UC patients (258–260). In the current study, CHGA expression was elevated in colon tissues of males and females following DSS-induced colitis, in agreement with precedent observations (17,258,259).

We and others previously demonstrated that increased CHGA levels correlated with worsened disease severity in UC patients and male mice during DSS-induced colitis (17,258,331,337). Moreover, CHGA deletion correlated with improved colitis and reduced inflammation, neutrophil infiltration, and proinflammatory mediators. Similarly, colitis improved, the colon length maintained, reduced neutrophils infiltrated, and interestingly improved mucus production in males

with deleted CHGA (17). Additionally, the mediators of intestinal integrity TGF- β , IL-25, and IL-22 were all increased in the colon of males in the absence of CHGA. TGF- β , an immune suppressive cytokine produced by many cells, including dendritic cells (DCs), intestinal epithelial cells, myofibroblast, and Treg, regulates multiple cellular functions as a suppressor of the immune response, promoter of intestinal epithelial integrity and wound healing (338). Thus, TGF- β promotes differentiation of Treg and Th17 cells, which, in turn, produces TGF- β and IL-22 cytokines (338). IL-22, a proinflammatory cytokine, promotes antimicrobial peptide secretion, improves goblet cell mucus production, and promotes tissue regeneration by downregulating apoptosis, all critical to intestinal epithelial integrity and mucosal barrier function (339). Moreover, TGF- β upregulates the intestinal epithelial cells' production of IL-25 Th2 cytokines, which also contribute to limiting inflammation in colitis (277). Therefore, the improved colitis observed in males could likely result from the substantial increase in TGF- β observed in the absence of CHGA. Moreover, high TGF- β levels could also explain the reduced mRNA expression of the mucosal healing biomarker Tff3 and bacterially induced protein Relm β , whose high expression correlates with active disease and severe inflammation for Tff3 or following mucosal infiltration by bacteria for Relm β (49,241). Moreover, IL-22 higher levels may also explicate the substance increase in the antimicrobial peptide Cramp observed in males deficient in CHGA in colitic conditions.

CHGA depletion promoted slightly contrasting outcomes in females compared to males. Thus, the lack of CHGA in females was associated with rapid weight loss in the early phase of colitis, substantially shorter colon, and a trend toward increased macro-score, all indicating a slight increase in susceptibility to colitis. Similarly to males, CHGA deletion in females correlated with enhanced TGF- β production in DSS-induced colitis, despite a trend toward lower levels compared

to males, and likely explains the reduced neutrophil infiltration with female intestinal mucosa. However, in contrast to males, in females, IL-25 and IL-22 expression did not differ in the absence or presence of CHGA in colitic conditions, suggesting a distinct effect for TGF- β in the colonic mucosa between sexes. This observation is further validated by finding that showed a lack of changes in Tff3 mRNA expression in females with CHGA deficiency in colitis, likely because inflammation might not play a primary role in the female's increased susceptibility to colitis in the absence of CHGA. On the other hand, the lack of CHGA in females correlated with substantial loss of mucosa integrity associated with reduced pore-forming TJ regulator Cld4 mRNA expression and mucosal resistance, along with higher Relm β mRNA expression in colitis, all features of impaired mucosal integrity. These findings indicate that CHGA might maintain the colonic mucosal integrity by regulating the TJ proteins in females but not males.

Studies demonstrated that TGF- β downregulates proliferation during the mucosal healing process to promote epithelium restitution in the early stage of the mucosal healing process, which also comprises epithelial cell proliferation and differentiation in UC (25,204). Therefore, we investigated whether colonic epithelial cells differed in the absence of CHGA in colitic conditions. In males, the lack of CHGA correlated with substantially lower differentiation and increased proliferation, which corresponded with reduced Muc2⁺ cells and higher preservation of CBC and reserve stem cell population, likely due to reduced inflammation and mucosal damage given that both stem cell populations are highly susceptible to depletion mediated by DSS-induced colitis (190,209). Thus, reduced mucosal damage was likely linked to fewer bacteria infiltration of the mucosa, leading to less Muc2 expression. Reduced mRNA expression of fetal-like stem cells further confirmed these findings, whose expression correlates with colitis severity (197,198).

Interestingly, immunofluorescence staining in colon tissues from males deficient with CHGA showed a reduced Muc2⁺ cell population, whereas histological staining with alcian blue demonstrated increased intensity in mucus staining. The divergence between both techniques results likely for the difference in specificity, as alcian blue, in addition to detected Muc2, can also stain for other acidic mucin proteins such as MUC1, MUC3, MUC10, and MU13, which are all regulated by IL-22 (340). In females, CHGA deletion correlated with increased proliferating cells in homeostatic conditions and a trend toward higher Ly5a. Additionally, females exhibited higher WFDC2 mRNA expression, demonstrated to promote estrogen drive proliferation in ovarian cancer (341). Similarly to males, CHGA deficiency was associated with reduced differentiating cells in females, which correlated with lower Muc2⁺ cells at the protein level. Moreover, in the absence of CHGA, females displayed increased M cell mRNA markers, which indicates increased mucosal damage, consistent with previous studies. Stem cell marker expression was not altered in females deficient in CHGA in colitic conditions. These findings suggest an indirect effect of CHGA intestinal epithelial cells, which could explain the differences between sexes.

As expected, colitis correlated with increased neutrophils and macrophages in males and females. However, the frequency of myeloid cells in the mucosa was substantially lower in CHGA-deficient males and females. Moreover, this effect was markedly more drastic in the DC population. These observations suggest a critical role for CHGA in regulating the myeloid compartment, which effect is likely independent of sexes.

6.5. Conclusion

This study indicates that CHGA deletion affects the intestinal epithelial integrity and mucosal barrier function in acute colitis in a sex-dependent manner. Thus, in the absence of CHGA, males displayed reduced colitis severity associated with diminished myeloid cell recruitment in the mucosa and reduced inflammation, likely due to high TGF- β production, leading to less epithelial damage and consequently improved mucosal integrity and functions. In contrast, in females, the lack of CHGA exacerbates colitis through dysregulated TJ and impaired mucosal integrity. This study highlights the critical role played by CHGA in regulating the integrity and function of the colon mucosa during colitis. Additionally, it described the sex-related differences in the effect of CHGA on colon mucosa. This research underscores the significance of considering biological sex in UC research.

BRIDGE TO CHAPTER VII

The impaired mucosal barrier is a hallmark of UC pathophysiology, revealing a sex bias in epidemiology and pathophysiology. In **Chapter VI**, we reported that CHGA deletion influences intestinal epithelial integrity and mucosal barrier function in a sex-dependent manner. Thus, the lack of CHGA correlated with high TGF- β production, reduced colitis severity, lower myeloid cell recruitment and inflammation, and consequently improved mucosal integrity and functions in males. Conversely, in females, the lack of CHGA exacerbates colitis through dysregulated TJ and impaired mucosal integrity. This study highlights the critical role of enteroendocrine mediators such as CHGA in regulating mucosal integrity and function in colitis. In precedent publications, we reported that patients with UC overexpress the enteroendocrine CHGA-derived mediator pancreastatin (PST), which correlates with increased disease severity, altered tight junctions, and delayed epithelial closure after injury in males. To investigate whether inhibiting PST could contribute to reducing colitis and improve the mucosa integrity and barrier function during colitis similar to CHGA, we performed a preliminary study, which indicated that male mice treated daily intrarectally with pancreastatin inhibitor (PSTi)8, a competitive inhibitor of PST, displayed markedly reduced colitis. Therefore, with these promising findings in mind, in **Chapter VII**, we sought to investigate the impact of PSTi8 treatment on colon mucosal barrier function and epithelium integrity in both sexes under homeostatic and colitic conditions using murine models of colitis. Our data revealed that PST inhibition with PSTi8 influences the intestinal epithelial integrity and mucosal barrier function in homeostatic and colitic conditions in a sex-dependent manner, similar to findings in Chapter VI.

CHAPTER VII

Pancreastatin inhibition alters the colonic epithelial cells profile in a sex-dependent manner

Diane M. Tshikudi, Hannah Hutchison, and Jean-Eric Ghia

Authors' contributions: J-E.G. and D.M.T. conceptualized the study, and D.M.T. carried out the animal experiment, the cytokines and RT-qPCR experiment, data collection, and formal analysis. H. U. and D.M.T. carried out the immunofluorescence experiment, data collection, and formal analysis. D.M.T. prepared the figures, performed the statistical analysis, and drafted the manuscript. J-E.G. provided the resources. J-E.G. administrated the project and provided funding. All authors have read and agreed to the published version of the manuscript.

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7.1. Abstract

The impaired mucosal barrier is a hallmark of ulcerative colitis (UC), an inflammatory colonic disorder with epidemiological and pathophysiology sex bias. UC patients overexpress the colonic epithelial cells (CECs)-derived peptide pancreastatin (PST). Pancreastatin inhibitor 8 (PSTi8), an inhibitor of PST, has shown promising anti-inflammatory effects on UC. However, no data exists in the context of CEC barrier function and integrity. We investigated the impact of PSTi8 treatment on CECs in homeostatic and colitic conditions. PSTi8 (2.5 mg/mL/kg, i.r.) or PBS treatment started one day before colitis induction (5% dextran sodium sulfate for five days) in male and female C57BL/6 mice. The disease activity score was assessed daily. Epithelial-associated cytokines, markers specific to differentiation, proliferation, differentiated CECs, stem cells, CECs regulators, and the PSTi8/G-protein coupled receptor 78 (GPR78) signaling pathway, were evaluated using ELISA, immunofluorescence and qRT-PCR. PSTi8 treatment reduced the epithelial-associated cytokines and differentiated CECs while promoting CEC proliferation and self-renewal in females at a steady state through the GRP78 signaling pathway. PSTi8 treatment exacerbated colitis severity and increased CEC differentiation while reducing proliferation in colitic females. Conversely, PSTi8 treatment reduced males' susceptibility to colitis by preserving stem cells and differentiated CECs. PST regulated colonic mucosal maintenance in a sex- and disease-dependent manner.

7.2. Introduction

The gastrointestinal (GI) mucosa, formed of an epithelium overlaid by a mucus layer, serves as a selectively permeable barrier whose functions are primordial to restrict mucosal infiltration by luminal microorganisms, dietary antigens, and other cellular insults (192). The GI epithelium regulates the absorption of electrolytes, nutrients, water, and other beneficial molecules while creating a physical, immunological, and chemical barrier between the luminal contents and host immune cells in the lamina propria. The colon epithelium is organized along the crypt axis, with stem cell niches that reside at the base of the crypts and terminally differentiated colonic cells positioned apically from the crypt base (206). With a relatively short lifespan of 3–5 days, the colonic epithelial cells undergo constant self-renewal mediated by the Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5⁺) crypt base columnar stem cells (CBCs) to preserve the epithelial barrier homeostasis (342). In colonic inflammatory pathologies resulting in severe epithelium injury that lead to depletion of Lgr5⁺ cells, damage-associated regenerative cells (DARCs) such as ulcerative colitis (UC), homeodomain-only protein homeobox (HOPX⁺), and fetal-like lymphocyte antigen (LY6A⁺) stem cells can rescue CBCs loss, promote mucosa repair and prevent epithelial barrier dysfunction (25,192). Epithelial stem cells can give rise to terminally differentiated cells subdivided into absorptive colonocytes and the secretory mucus-secreting goblets, chemosensory Tufts, and hormones-producing enteroendocrine cells (159,342,343). These terminally differentiated cells maintain the colonic mucosal barrier integrity and function, hence promoting colonic mucosa homeostasis (342). Interestingly, recent studies reveal that sex hormones such as estrogen, progesterone, and testosterone modulate intestinal barrier function (108,344–346). Therefore, impaired colonic mucosal barrier function can promote the development of GI pathologies such as inflammatory bowel diseases, which include UC (25).

UC is a chronic inflammatory disorder with a higher incidence rate in males than in females (50,274). Characterized by severe mucosal ulceration along the colon and rectum, UC is associated with periods of remission and relapse symptoms such as bloody diarrhea, abdominal pain, and fecal urgency (347). Although still under investigation, many studies suggest a central role for compromised gut mucosal barrier function in UC etiopathology (6,348). Thus, the impaired colonic mucosal barrier in UC is associated with an unresolved endoplasmic reticulum (ER) stress and an unfolded protein response (UPR), a lower number of mature goblets cells, defective mucus production, along with an enhanced epithelial permeability linked to increased expression of pore-forming tight junction (TJ) Claudin 2 and lower expression of regulators of pore-forming TJ Claudin 4 (CLD4) (25,171,172,349). Moreover, several studies associate UC pathogenesis with altered enteroendocrine cell functions (239,255). Recent studies revealed that intestinal enteroendocrine cells from UC patients acquire antigen-presenting function through major histocompatibility complex (MHC) class I and MHC II pathways, in addition to undergoing a functional shift that increases their capacity to synthesize proteins and secrete hormones such as serotonin and chromogranin A (CHGA) (255,258). Ubiquitously distributed in enteroendocrine cells, CHGA can undergo post-translational proteolytic processing and give rise to numerous biological effector molecules, which are known to influence the colonic mucosa integrity and function in homeostatic and pathological conditions (6,333). One such bioactive peptide, pancreastatin (PST), has been shown to govern glucose homeostasis in the pancreas, skeletal muscle, liver, adipose tissues, and heart, likely through the insulin receptor substrate (IRS)1/2-PI3-kinase-Akt signaling pathway and the (UPR) chaperon molecule glucose-regulated protein of 78 kDa (GRP78) (333,350–353). In the GI tract, patients with active UC had significantly higher PST

levels than healthy control, which positively correlated with epithelial cell-associated inflammatory cytokines (18). Moreover, we and others demonstrated that PST supplementation in the CHGA-knockout mice model increases the intestinal mucosal permeability at a steady state, reduces TJ mRNA expression and worsens disease severity in the dextran sodium sulfate (DSS) experimental model of colitis (18,265,354). However, the influence of PST on colonic epithelial cell functions remains to be investigated. PST inhibitors could help study the effect of PST on the colonic mucosa integrity and functions. So far, pancreastatin variant 1 (PSTv1) and pancreastatin inhibitor 8 (PSTi8), two truncated forms of PST, have been shown as promising competitive inhibitors of PST peripheral metabolic action (352,355). PSTv1 and PSTi8, N-terminally and C-terminally truncated peptides, could be potential anti-inflammatory mediators in inflammatory-mediated pathologies such as UC (354). Although several studies report the effect of PST inhibition with PSTv1 and PSTi8 in the context of diet-induced obesity, insulin resistance, and diabetes, studies investigating their impact on inflammatory disorders are limited (354). While PSTv1-mediated PST inhibition has been found to suppress adipose tissues' macrophage-mediated inflammation, the mechanism of action by which PSTv1 mediates its inhibitory action is still poorly understood (355). By contrast, PSTi8, a recently developed anti-diabetic molecule, has been found to compete with PST for binding to the GRP78 receptor active site, attenuates insulin resistance and improves glucose homeostasis while reducing pro-inflammatory cytokine levels (352). Moreover, studies suggest that PSTi8 treatment alters the IRS1-2-phosphatidylinositol-3-kinase-AKT-Glycogen synthase kinase-3 beta (GSK3 β) signaling pathway, known to control cell growth, proliferation, and apoptosis, all of which are known to play a significant role in UC and colitis-associated cancer pathophysiology (356).

Growing evidence suggests that gut mucosal healing in UC patients correlates with an increased risk of hospitalization and surgery (28). In UC patients, amelioration of gut mucosa healing could improve clinical outcomes and long-term remission for UC patients (6,8). Therefore, it is crucial to understand factors that influence the mucosal integrity and colonic epithelial barrier function to provide novel molecular targets for UC therapy. In the present study, we explore the potential effects of PST inhibition with PSTi8 on colonic epithelial cell integrity and mucosal barrier functions in male and female mice at a steady state and during DSS-mediated acute colitis.

7.3. Materials and Methods

7.3.1. Peptides

PSTi8 (PEGKGGEQEHSQQKEEEEEMAV-amide) purified by reverse-phase high-performance chromatography to a purity level of >98% was used in this study (Pepmic Co., Suzhou, China).

7.3.2. Mice

All experiments were conducted according to the Canadian Council on Animal Care (CCAC) guidelines for animal research. For this study, 12-week-old male and female C57BL/6 wild-type mice were obtained from the colonies at the University of Manitoba, Winnipeg, Manitoba. Animals were kept in a 12 h dark/light cycle and fed ad libitum. Forty-eight male and fifty-two female mice were randomly assigned into four groups. The groups included PBS- and PSTi8-treated control

male and female groups (PBS-control and PSTi8-control) and PBS- and PSTi8-Dextran sulfate sodium (DSS)-treated groups (PBS-DSS and PSTi8-DSS) mice.

7.3.3. Treatment of Mice with Pancreastatin Inhibitor 8 (PSTi8)

Female and male mice were randomly separated into four groups of 12–16 mice. All mice anesthetized with 3% isoflurane and 1L oxygen (352,357) received daily intra-rectal (i.r.) administration of 2.5 mg/kg/day (n = 28 females and n = 24 male mice). Daily administration of PSTi8 was shown to have a more potent inhibitory effect than a single acute administration of PSTi8 in a mice model of insulin resistance and diabetes (352,357), or phosphate buffer saline (PBS) (n = 24 females; n = 24 male mice) for six days (modified from previous publications) (18,352).

7.3.4. Induction and Assessment of Experimental Colitis

One day after starting i.r. administration of PSTi8 (n = 16 females; n = 12 males) or PBS (females and males: n = 12), following a well-established protocol, mice were given 5% DSS (molecular weight: 40–50 kDa; ThermoFisher Scientific, Winnipeg, MB, Canada, Catalog #: J14489-22) in their drinking water for 5 days to induce acute colitis (18,256,267). PSTi8- and PBS-treated control groups (females and males: n = 12) received pasteurized water. Weight loss, stool consistency, and bleeding were monitored daily as previously described and were used to calculate disease activity index (21). On day five, all anesthetized mice were euthanized by cervical dislocation. Colon macroscopic score was determined using rectal bleeding, rectal prolapse, diarrhea, and colon bleeding (22). The colon collected, clear of feces, segmented into proximal, medial, and distal colons were stored at –80°C

for subsequent assays. The proximal, medial, and distal colons were used for Western blotting, enzyme-linked immunoassay (ELISA), and real-time qPCR (RT-qPCR). Tissue samples were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA, USA) and stored at -80°C for histology and immunofluorescence analysis.

7.3.5. Serum Preparation

Blood samples, allowed to clot at room temperature for 30 minutes, were centrifuged at 20,000 RPM for 10 minutes at 4°C . Supernatants were collected and stored at -20°C until analyzed.

7.3.6. Procedure for RNA Extraction, Purification, and Quantification

Minced colon samples resuspended in 1mL TRIzol reagent (Invitrogen, ThermoFisher Scientific, Winnipeg, MB, Canada) were mechanically homogenized by sonication, mixed with phenol/chloroform and centrifuged at 12,000 rpm and 4°C for 20 minutes. RNA collected in the aqueous phase was diluted with 70% ethanol and purified using an RNA column-based purification kit (PureLink RNA Mini Kit, Invitrogen, ThermoFisher Scientific, Winnipeg, MB, Canada) according to the manufacturer's instructions. The yield and purity of the extracted RNA eluted in RNase-free water were assessed using the NanoDrop Spectrophotometer (ThermoFisher Scientific Winnipeg, MB, Canada). The purified RNA samples were reverse transcribed into cDNA using the SuperScriptTM IV VILOTM Master Mix (Invitrogen, ThermoFisher Scientific) and stored at -80°C for later analysis.

7.3.7. Epithelial Cells Associated Markers Gene Expression

cDNA samples were analyzed using RT-qPCR on LightCycler 96 instrument (Roche Life Science, Basel, BS, Switzerland) with the Power SYBR™ Green PCR Master Mix (Applied Biosystems™, Life Technologies, Burlington, ON, Canada). The cycle threshold value (Cq) for each gene of interest was normalized to that of the housekeeping gene, TATA-binding protein (TBP), and expressed as the relative ratio of expression. The list of primer sequences is provided in Table 1.

Table 1. Narratives and primer sequences of selected genes.

TATA box protein (<i>Tbp</i>)	Forward	ACCGTGAATCTTGGCTGTAAAC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	GCAGCAAATCGCTTGGGATTA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Differentiated epithelial cells			
Resistin-like molecule β (<i>RelmB</i>)	Forward	CCATTTCTGAGCTTTCTGG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	AGCACATCCAGTGACAACCA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Carbonic anhydrases (<i>Ca2</i>)	Forward	CAAGCACAAACGGACCAGA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	ATGAGCAGAGGCTGTAGG	95 °C, 30 s/52 °C, 30 s/72 °C, 45 s
Mucin 2 (<i>Muc2</i>)	Forward	GAT GGC ACC TAC CTC GTT GT	95 °C, 30 s/52 °C, 30 s/72 °C, 45 s
	Reverse	GTC CTG GCA CTT GTT GGA AT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Chromogranin A (<i>Chga</i>)	Forward	CAGGCTACAAAGCGATCCAG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	GCCTCTGTCTTTCCATCTCC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Doublecortin Like Kinase 1 (<i>Dcl1</i>)	Forward	CTGGGTAAATGATGATGGTCTCC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	ACAGAAACTCCTGCTGCAGT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Stem epithelial cells			
Leucine-rich repeat-containing G-protein coupled receptor 5 (<i>Lgr5</i>)	Forward	CTTCCGAATCGTCGATCTTC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	AACGATCGCTCTCAGGCTAA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
homeodomain-only protein homeobox (<i>Hopx</i>)	Forward	TCTCCATCCTTAGTCAGACGC	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
	Reverse	GGGTGCTTGTTGACCTTGTT	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
Lymphocyte antigen-6 A (<i>Ly6a/Sca1</i>)	Forward	AGGAGGCAGCAGTTATTGTGG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	CGTTGACCTTAGTACCCAGGA	95 °C, 30 s/52 °C, 30 s/72 °C, 45 s
Lineage commitments			
hairy and enhancer of split 1 (<i>Hes1</i>)	Forward	CTTATGAAAGTCAAGTAAAGGACG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	ATAGGCTTTGATGACTTTCTGTG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s

Atonal homolog 1 (<i>Atoh1</i>)	Forward	GACAAATATCCCTGCACCCT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	CAGAGGCAGAGATACGACAT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Neurogenic locus notch homolog protein 1 (<i>Notch 1</i>)	Forward	CTGAGAGCTCCTGCTTCAAT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	AGTACCATAGTGTCTTGGC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Bone morphogenetic protein 4 (<i>Bmp4</i>)	Forward	TTCCTGGTAACCGAATGCTGA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	CCTGAATCTCGGCGACTTTTT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Growth Factor Independent 1B (<i>Gfi1b</i>)	Forward	ATGCCACGGTCCTTTCTAGTG	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	GGAAGGCTCTGGTTCAGCAA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Krüppel-like factor 4 (<i>Klf4</i>)	Forward	CAGACCAGATGCAGTCACAA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	GTTTCTCGCCTGTGTGAGTT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
Neurogenin 3 (<i>Neurog3</i>)	Forward	TCTCGCCTCTTCTGGCTTTC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	AAGTCGGTGAAGAACGGACAA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
SRY-Box Transcription Factor 9 (<i>Sox9</i>)	Forward	CAAGCACAAACGGACCAGA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	CAGCGCCTTGAAGATAGCATT	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
GRP78 signaling pathway			
Activating transcription factor 6 (<i>Atf6</i>)	Forward	CTGGGCTCGGTAGTTTGTATC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	AGACCTGAATGGCTGCTTAC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
X-box binding protein 1 (<i>Xbp1</i>)	Forward	CCTTCAGTGACATGTCTTCTCC	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
	Reverse	CCCAGTGTTATGTGGCTCTTTA	95 °C, 30 s/55 °C, 30 s/72 °C, 45 s
C/EBP homologous protein (<i>Chop</i>)	Forward	GGAGGTCCTGTCCTCAGATGAA	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
	Reverse	GCTCCTCTGTCAGCCAAGCTAG	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
Glycogen synthase kinase-3 beta (<i>Gsk3b</i>)	Forward	GAGCCACTGATTACACGTCCAG	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
	Reverse	CCAACTGATCCACACCACTGTC	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
Mammalian target of rapamycin (<i>mTor</i>)	Forward	AGAAGGGTCTCCAAGGACGACT	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s
	Reverse	GCAGGACACAAAGGCAGCATTG	95 °C, 30 s/59 °C, 30 s/72 °C, 45 s

7.3.8. Proteins Extraction

Minced colon samples were homogenized in PBS buffer containing a protease inhibitor cocktail (cOmplete™, Mini Protease Inhibitor Cocktail, Roche, Basel, BS, Switzerland. The homogenate samples were centrifuged at 20,000 rpm for 10 minutes. Supernatants were collected, and the protein concentrations were measured using the Pierce BCA protein assay according to the

manufacturer's instructions (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were stored at -20°C for later analysis.

7.3.9. Cytokines Analysis

Concentrations of interleukin (IL)-6, IL-18 (Duoset, DY206-05 and DY1709, R&D Systems, Minneapolis MN, USA), IL-22 (436304, BioLegend, San Diego, CA, USA), and pancreastatin (MBS267929, MyBiosource, San Diego, CA, USA) were measured by ELISA on extracted proteins from the medial segments of colons according to manufacturers' instructions.

7.3.10. Immunofluorescence

OCT-embedded frozen tissue sections with a $10\text{ }\mu\text{m}$ thickness were fixed in 4% formalin for 15 minutes and incubated in a permeabilized solution (0.25% gelatin and 0.2% triton-100X diluted in PBS) for 10 minutes. The permeabilized tissues were blocked with a blocking solution containing 5% bovine serum albumin (BSA) diluted in permeabilization buffer for 2–4 h at room temperature. Tissues labeled for MUC22 were fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform, and 10% acetic acid) for 5 minutes. Moreover, tissues labeled for LGR5 and MUC2 were not permeabilized. All samples were incubated with a primary antibody diluted in the blocking buffer overnight at 4°C . Slide sections rinsed in PBS containing 0.1% Triton-100x for 10 minutes were incubated in appropriate labeled secondary antibodies diluted using the blocking buffer for 1 h and in 4',6-diamidino-2-phenylindole (DAPI) $1\text{ }\mu\text{g/mL}$ diluted in PBS for 10 minutes at room temperature in the dark. The stained sections rinsed in washing buffer were incubated in an anti-autofluorescence buffer (10 mM CuSO_4 and 50 mM NH_4Cl) and Milli-Q water for 10 minutes and

mounted using the ProLong Gold Antifade Mountant (ThermoFisher Scientific). The images acquired using the EVOS Cell Imaging Systems (ThermoFisher Scientific) were processed using FIJI 2.16.0 ImageJ software. The list of antibodies used in this study is shown in Table 2.

Table 2. List of antibodies.

Antibodies	Sources	Locations	Identifier Catalogue Numbers
Rat Ki-67 Monoclonal Antibody (SolA15)	Invitrogen	Winnipeg, MB, CA	14-5698-82
Rabbit Keratin 20 (D9Z1Z) XP [®] antibody	Cell Signaling Technology	Danvers, MA, USA	13063S
Rabbit Anti-MUC2 (EPR23479-47) antibody	Abcam, Inc	Waltham, MA, USA	ab272692
Rabbit Chromogranin A antibody	Invitrogen	Winnipeg, MB, CA	PA5-16685
Mouse Doublecortin (E-6) antibody	Santa Cruz	San Diego, CA, USA	sc-271390
Rabbit LGR5/GPR49 antibody	Bio-Techne	Minneapolis, MN, USA	NBP1-28904
Rat LY-6A/E (D7) antibody	Santa Cruz	San Diego, CA, USA	SC-52601
Mouse HOP (E-1) (HOPX) antibody	Santa Cruz	San Diego, CA, USA	SC-398703
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Winnipeg, MB, CA	A11006
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 647	Invitrogen	Winnipeg, MB, CA	A21247
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Winnipeg, MB, CA	A11034
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 647	Invitrogen	Winnipeg, MB, CA	A21244
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Winnipeg, MB, CA	A11001

7.3.11. Statistical Analysis

The statistical analysis was performed with GraphPad Prism Version 9.0.0 software (GraphPad Software, San Diego, CA, USA). Data was expressed as the mean $\pm$ standard deviation

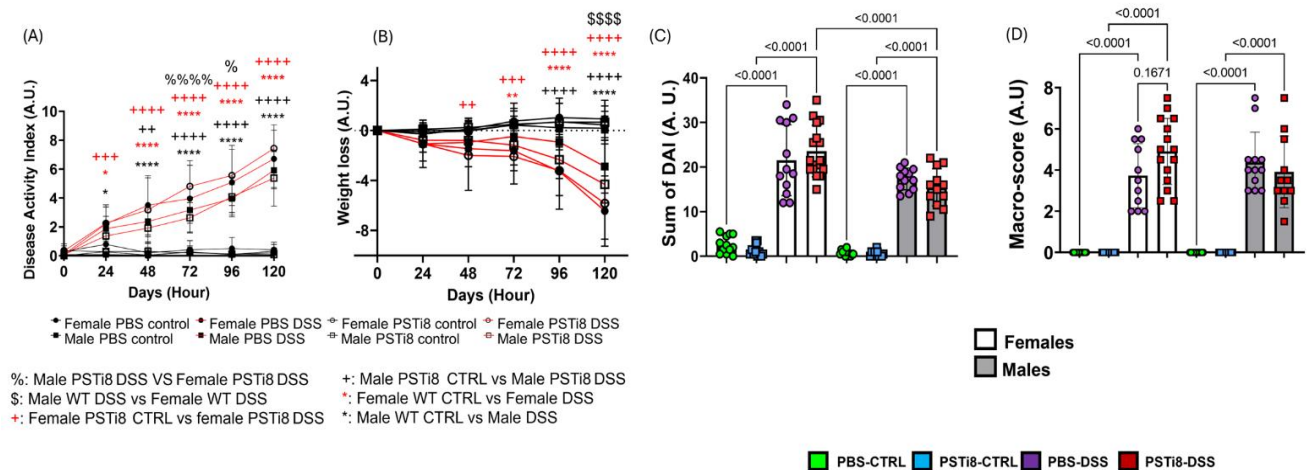
(S.D.). One-way ANOVA followed by Bonferroni or Tukey-post hoc analysis was performed to calculate the significance between groups. Statistical significance was defined as $p < 0.05$. All data were evaluated for outlier values using robust regression and outlier analysis. Data were log-transformed when the standard deviation was at least three times different between groups with sample sizes smaller than ten or square root-transformed with values equal to 0 (273).

7.4. Results

7.4.1. PST inhibition is associated with delayed colitis onset in male mice but moderately worsens disease severity in female mice

Male and female mice treated with DSS to induce colitis were administrated with PSTi8 to investigate the contribution of PST to colitis severity between sexes. All DSS-treated mice showed a progressive increase in the disease activity index (DAI) score and weight loss along with an elevated sum in DAI score and macro-score compared to non-colitic control mice ($p < 0.05$) (Figure 1A–D). However, colitis severity differed moderately between sexes following treatment with PSTi8. Prophylactic therapy with PSTi8 in males had a trend toward a delayed colitis onset with DAI that shifted from 24 to 48 h compared to PBS-male mice during colitis. By contrast, DSS-mediated colitis was associated with elevated DAI scores at 72 and 96 h ($p < 0.05$) along with early and more severe weight loss in PSTi8-treated females compared to in PSTi8-treated male mice and PBS-treated female and male groups. A non-significant difference was observed in the sum of DAI and macro-score in PSTi8-treated females compared to in their PBS-treated female counterparts and

PSTi8-treated male mice (Figure 1C,D). Interestingly, PSTi8-treated male mice had significantly higher weight loss occurring 24 h earlier than PBS-treated male mice in colitic conditions.



7.4.2. PST inhibition correlated with reduced levels of several colonic mucosal cytokines in female mice at a steady state

treatment on levels of PST, interleukin (IL)-6, interleukin (IL)-18, and interleukin (IL)-22 (Figures 2(A1–D2) and S1). Except for PST, these cytokines are known to modulate mucosal integrity and function in UC in male and female mice at a steady state and during colitis. The levels of IL-6 and IL-22 in colonic tissues from PSTi8-treated females were significantly lower than those measures in tissue from PBS-treated female mice at a steady state ($p < 0.01$). PSTi8 treatment did not alter IL-6 and IL-22 levels in male mice at a steady state. However, the levels of IL-6 and IL-22 were significantly lower and higher, respectively, in PSTi8-treated or PBS-treated males when compared to in PSTi8-treated or PBS-treated female mice at a steady state ($p < 0.05$) (Figure 2(B1,C1)). No significant differences in IL-18 and PST levels were observed between PSTi8-treated and PBS-treated female groups (Figure 2(A1,D1)). However, PSTi8-treated males had significantly higher levels of PST at a steady state than PBS-treated male groups (Figure 2(A1)). Interestingly, PBS-treated male mice had markedly lower IL-18 levels than PBS-treated female groups at a steady state (Figure 2(D1)). The levels of IL-6 and IL-18 did not differ markedly in colonic tissues between PSTi8-treated and PBS-treated male groups in homeostatic conditions.

Colitis in PBS-treated female mice was associated with no significant difference in IL-6 and significantly higher IL-18 levels, whereas PST and IL-22 expression were markedly reduced ($p < 0.05$) compared to in the non-colitic PBS-treated female groups (Figure S2A,B,E,F). However, no significant differences in PST, IL-6, IL-22, and IL-18 levels were observed between PSTi8-treated and PBS-treated female groups in colitic conditions (Figure 2(A2–D2)). In PBS-treated males, colitis resulted in markedly elevated IL-6 and no significant changes in PST, IL-22, and IL-18 levels compared to in the corresponding non-colitic PBS-treated mice (Figure S1E–H). Moreover, the levels of all four

cytokines were not altered significantly between PSTi8-treated and PBS-treated male mice in colitic conditions.

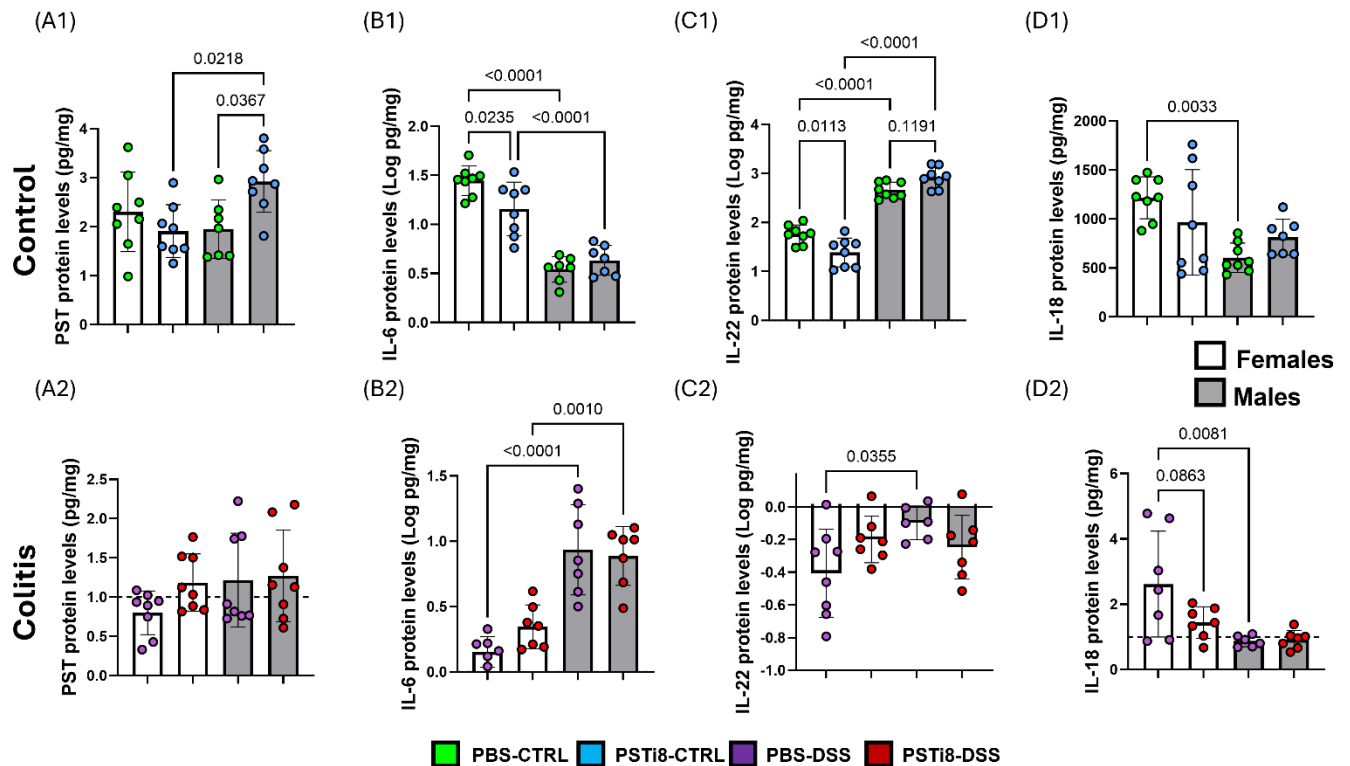


Figure 2. PSTi8 treatment altered the profile of colonic mucosal cytokines in a sex-dependent manner at a steady state and in colitic conditions. Male and female C57BL/6 (WT) mice (n = 6–8) were administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS for five days to induce colitis. Colon tissues were collected and homogenized to extract proteins. Protein levels of PST (A1, A2) and mucosal regulatory inflammatory cytokines, interleukin (IL)-6 (B1,B2), IL-18 (C1,C2), and IL-22 (D1,D2), were evaluated by ELISA. Cytokine concentrations in colitic conditions were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. Data were expressed as the mean $\pm$ standard deviation. Statistical significance was determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Statistical analysis to determine outliers was performed using robust regression and outlier methods.

7.4.3. PST inhibition is associated with changes in the expression of several colonic mucosal repair markers that differ at steady state and in colitic conditions between sexes

To investigate whether PSTi8 treatment altered the colonic mucosal barrier function, we characterized the mRNA expression of TJ protein claudin 4 (*Cld4*) as well as cytokine Resistin-like protein β (*Relm\beta*), used here as an indicator of colonic epithelial cells' response to colonic mucosa colonization by microbiota (232). PSTi8 treatment altered the expression of markers associated with mucosal integrity differentially in male and female mice at a steady state and colitic conditions (Figures 3 and S2). PSTi8-treated female groups had significantly lower *Relm\beta* levels ($p < 0.008$) and no significant changes in *Cld4* expression when compared to PBS-treated female groups at a steady state (Figure 3A,B). By contrast, PSTi8-treated male mice exhibited reduced *Cld4* mRNA expression ($p < 0.02$) and a non-significant difference in *Relm\beta* transcript level at a steady state. Although only a minor difference in *Relm\beta* transcript levels was observed in PBS-treated female mice between colitic and steady-state conditions, PSTi8-treated female mice presented with significantly elevated *Relm\beta* transcript levels in colitic as compared to under steady-state conditions ($p < 0.02$) (Figure S2A). However, no significant difference in *Relm\beta* and *Cld4* mRNA expression levels was observed between PSTi8- and PBS-treated female groups in colitic conditions (Figure 3C,D). By contrast, PBS-treated male mice had significantly elevated *Relm\beta* and lower *Cld4* mRNA expressions in colitis as compared to in steady-state conditions ($p < 0.04$) (Figure S2C,D). However, PSTi8 treatment altered *Relm\beta* and *Cld4* mRNA expressions. Hence, PSTi8-treated male mice had a trend toward reduced *Relm\beta* and a significant increase in *Cld4* mRNA expression ($p = 0.04$) in colitic compared to in steady-state conditions (Figure S2C,D). Interestingly, despite showing a trend toward reduced *Relm\beta* transcript levels, *Relm\beta* and *Cld4* mRNA expressions did

not differ significantly between PSTi8-treated and PBS-treated male mice in colitic conditions (Figure 3C,D).

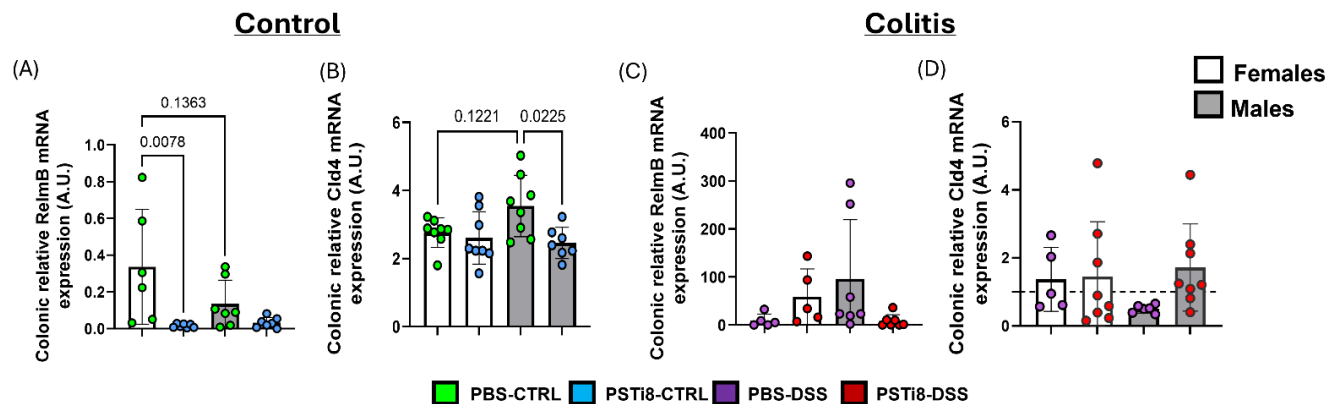


Figure 3. PST inhibition modulated the expression of markers associated with mucosal integrity at a steady state, but not in colitic conditions. (A–D) Colonic tissue collected from males and female C57BL/6 (WT) (n = 4–8) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used (control group, CTRL). (B–D) mRNA expression levels of markers linked to microbiota mucosal colonization, Resistin-like protein β (*relm β*), and tight junction, claudin 4 (*Cld4*) were measured by qRT-qPCR. Data obtained from DSS-treated mice were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. Results represented the means $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed to compare group averages. Statistical analysis using robust regression and outliers was performed to identify outliers.

7.4.4. PST inhibition is associated with no significant changes in the colonic mucosal antimicrobial activities in male and female mice at steady state and colitic conditions

As presented above, we demonstrated that PSTi8 treatment correlated with significant regulation of IL-22 levels and *Relm β* expression at a steady state and to a lesser extent in colitic conditions. Therefore, we investigated whether PSTi8 could regulate mucosal antimicrobial activities at a steady state and colitic conditions. We characterized antimicrobial peptides Ly6/Plaur domain containing 8 (*Lypd8*), WAP four-disulfide core domain protein (*Wfdc2*),

cathelin-related antimicrobial peptide (*Cramp*), regenerating islet-derived protein 3 β (*RegIII β*), and *RegIII γ* mRNA expressions. PSTi8 treatment was not associated with altered mRNA expression of all tested antimicrobial peptides in both male and female mice at a steady state (Figure S3). *Cramp* and *RegIII β* transcript levels were significantly increased in colonic tissues from PBS-treated female mice in colitic compared to in non-colitic conditions (Figure S3(C1,D1)). Levels of gene transcripts of *Cramp* and, to a lesser extent, *RegIII β* , were elevated in tissue from PSTi8-treated female mice in colitic compared to in non-colitic conditions. No significant differences in the transcript level of all tested antimicrobial peptides were observed between PSTi8-treated female mice and their PBS-treated female counterpart in colitic conditions (Figure S3(F2–J2)). By contrast, PBS-treated males had significantly higher level of *Lypd8* transcripts in colitic compared to in non-colitic conditions ($p < 0.0001$) (Figure S3 (F1)). However, although not significant, *Lypd8* and *Wfdc2* transcript levels were lower in PSTi8-treated male mice than in PBS-treated male mice in colitic conditions. Higher *Cramp* mRNA expression and a trend toward elevated *RegIII β* and *RegIII γ* were observed in colitic compared to in non-colitic PSTi8-treated male mice (Figure S3(H1–J1)). No significant difference in *Cramp*, *RegIII β* , and *RegIII γ* expression between colitic PSTi8- and PBS-treated male mice was observed (Figure S3(H2–J2)).

7.4.5. PST inhibition is associated with changes in the expression of colonic mucosal repair markers between sexes at steady state and during colitis

Immunofluorescence analysis to assess the colonic mucosal repair revealed a significantly lower level of cytokeratin 20 (CKRT20), a marker of differentiation in the colon (24), along with increased levels of the proliferative marker Ki67 in PSTi8-treated female mice in comparison to

in the PBS-treated female mice at a steady state ($p < 0.0001$ and $p = 0.0004$ respectively) (Figure 4A,B,D,E). Ki67 levels were also significantly higher in colon tissue from PSTi8-treated male mice than PBS-treated male mice at a steady state ($p = 0.0008$) (Figure 4D,E). DSS-mediated colitis was associated with a marked reduction in CKRT20 levels ($p < 0.03$ to <0.0001) and nonsignificant differences in Ki67 levels in colitic PBS-treated female and male mice in comparison to in their non-colitic PBS-treated counterparts (Figure 4A,C,D,F). Similarly, expression levels of CKRT20 and Ki67 were significantly lower in colitic PSTi8-treated compared to in the non-colitic PSTi8-treated females and male mice ($p < 0.0001$). Interestingly, Ki67 levels were more significantly reduced in colitic PSTi8-treated female and male mice compared to in colitic PBS-treated female and male mice ($p < 0.0001$) (Figure 4F).

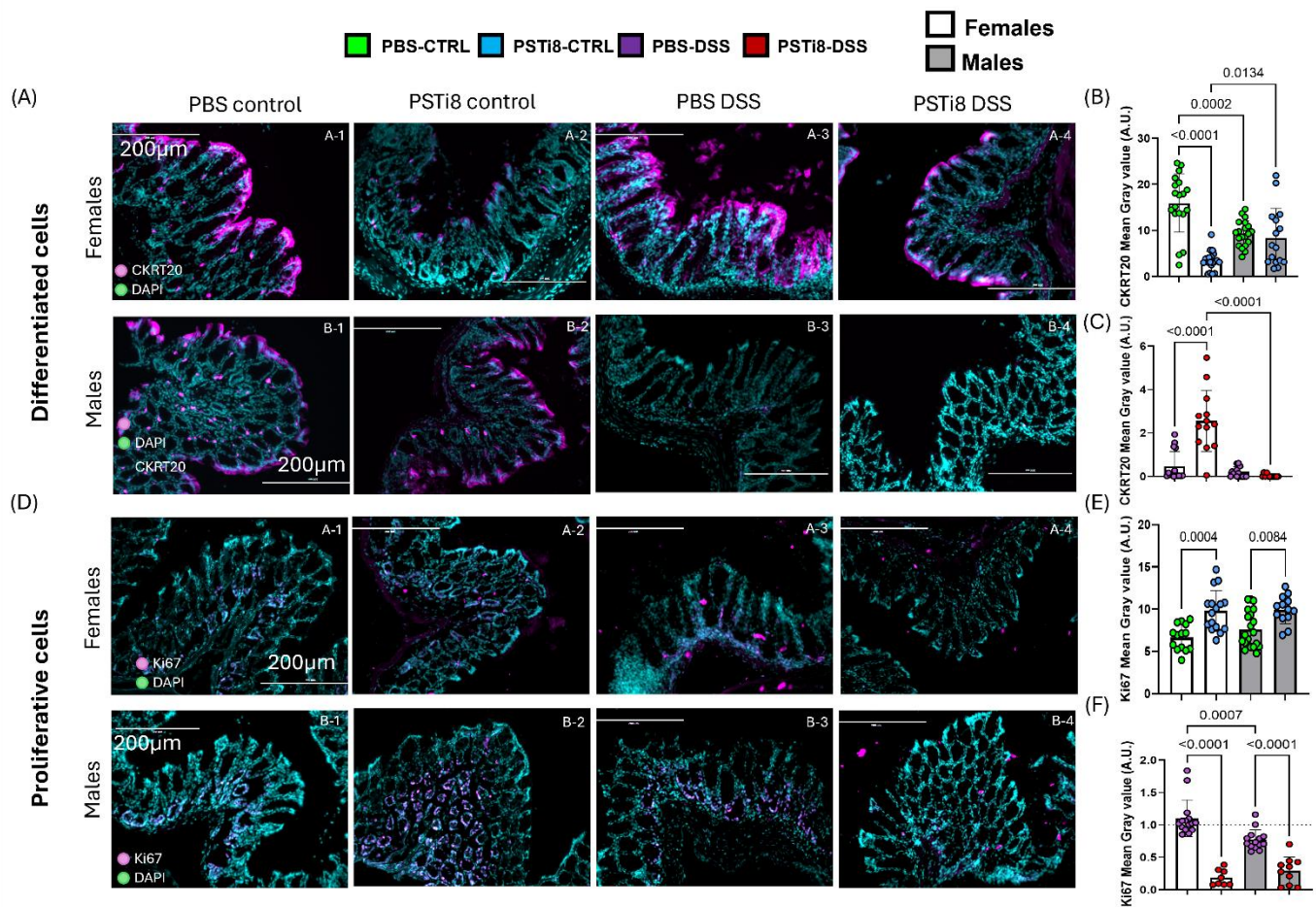


Figure 4. PST inhibition correlated with altered expression of colonic mucosal repair markers between sexes at a steady state and colitic conditions. (A–F) Colonic tissue collected from males and female C57BL/6 (WT) ($n = 3$) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used (control group, CTRL). Protein levels of proliferation (Ki67) (A–C) and differentiation (cytokeratin 20, CKRT20) markers (D–F) were assessed by immunofluorescence (IF). Scale bar: 200 μ m. Data from DSS-treated mice were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. Results represented the means $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis using robust regression and outliers was performed to identify outliers.

7.4.6. PST inhibition results in distinct colonic differentiated epithelial cells that vary between sexes under steady state and colitic conditions

Our finding suggests that PST inhibition may regulate the profile of terminally differentiated epithelial cells at a steady state and during colitis. We characterized the levels of mucin 2 (MUC2), CHGA, and double cortin-like kinase 1 (DCLK1) markers for goblet, enteroendocrine, and tuft cells, respectively, in colonic tissues from PSTi8-treated male and female mice in homeostatic and colitic conditions. qRT-PCR and immunofluorescence analysis revealed significantly lower *Muc2* RNA expression in PSTi8-treated females and reduced MUC2 protein levels in PSTi8-treated male mice compared to in their PBS-treated counterpart at steady state ($p < 0.05$; $p < 0.0001$) (Figure 5A,C,D). Despite showing a trend toward lower MUC2 protein levels, no significant differences in MUC2 expression were observed at protein levels in PSTi8-treated females and at mRNA levels in PSTi8-treated male mice. No marked differences in the transcript and protein expression of CHGA were observed in PSTi8-treated female mice compared to in PBS-treated female mice. By contrast, CHGA protein levels were significantly lower in PSTi8-treated male mice in comparison to in PBS-treated male mice at a steady state (Figure 5F,H,I). We also find that DCLK1 levels were

markedly lower in PSTi8-treated female and male mice than in their PBS-treated counterparts at the protein level in both sexes and at an mRNA level in males (Figure 5K,M,N).

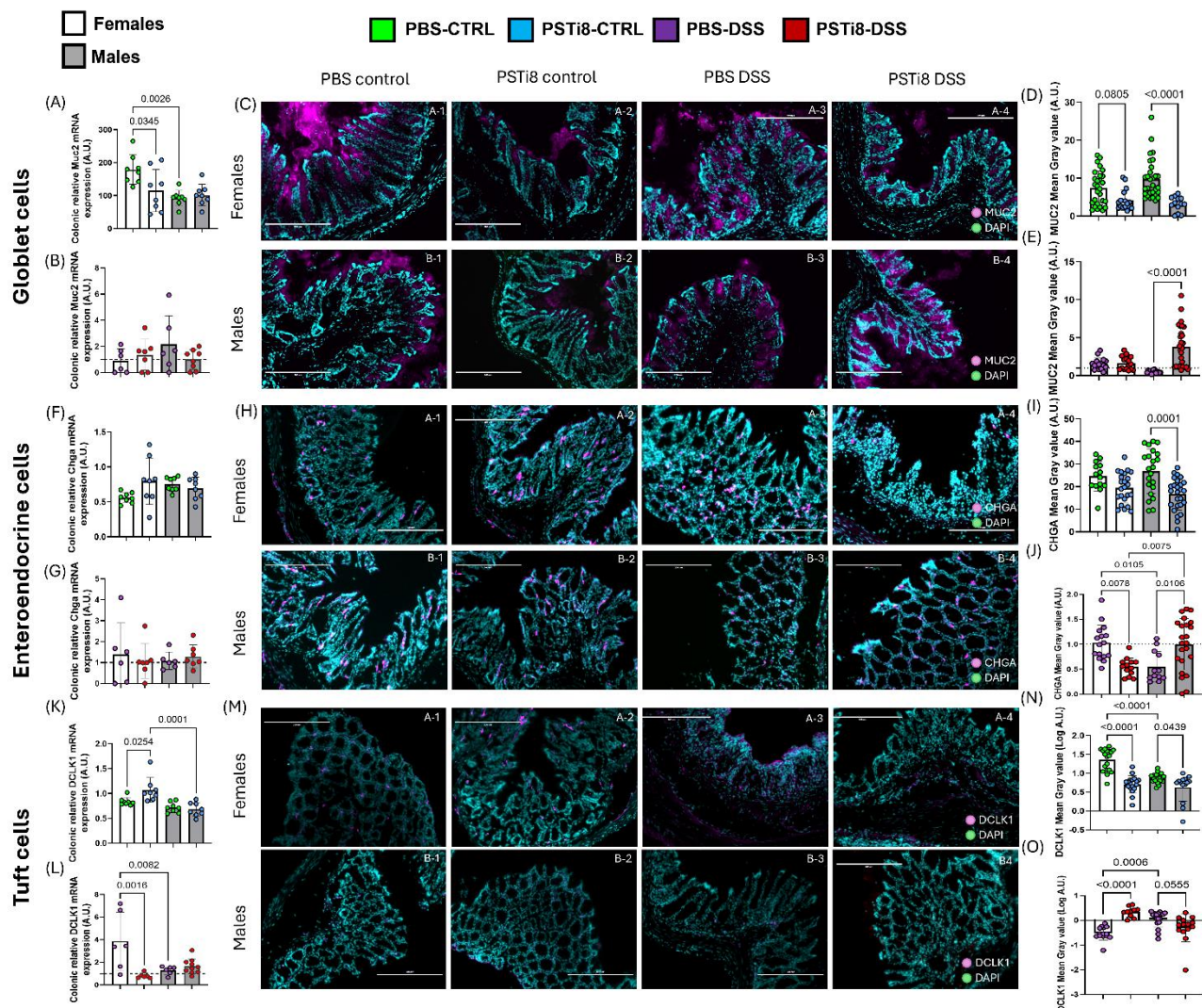


Figure 5. PSTi8 treatment was associated with changes in the expression of several terminally differentiated colonic epithelial cell markers between sexes at a steady state and colitic conditions. Expression of markers specific to differentiated colonic epithelial cells was evaluated. (A–O) Colonic tissues collected from males and female C57BL/6 (WT) (n = 5–8) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used (control group, CTRL). mRNA and protein levels of markers specific to goblet cells (A–E), mucin (MUC2)2, enteroendocrine cells (F–J), chromogranin A (CHGA), and Tuft cells (K–O), doublecortin-like kinase (DCLK) 1, were characterized by RT-qPCR and immunofluorescence (IF) (n = 3). Scale bar: 200 μ m. Data from DSS-treated mice were normalized to data from control mice. The dashed line in each figure represents a ratio of 1, indicating no

difference between DSS-induced colitis and control conditions. The histogram represents the means $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis using robust regression and outliers was performed to identify outliers.

Colitis was associated with no significant changes in *Muc2*, *Chga*, and the colonocyte marker, carbonic anhydrase (*CA*)2, mRNA levels, and increased *Dclk1* expression ($p = 0.02$) in PSTi8-treated female mice compared to in PSTi8-treated female mice in non-colitic conditions (Figure S4(A1–D1)). However, differentiated epithelial cell marker levels were substantially altered at protein levels in colitis (Figure S4(A2–C2)). Thus, results showed increased MUC2 along with decreased DCLK1 levels in PBS-treated females in colitic compared to in non-colitic conditions ($p < 0.05$) (Figure S4(A2,C2)). On the other hand, PSTi8-treated female mice displayed significantly reduced CHGA and elevated DCLK1 levels in colitis in comparison to in non-colitic conditions ($p < 0.01$) (Figure S4(B2,C2)). When normalized to their control conditions at a steady state, PSTi8-treated female mice presented with no significant difference in the gene expression of most differentiated epithelial cells except for the marked reduction in *Dclk1* mRNA expression compared to PBS-treated females and PSTi8-treated male mice (Figure 5B,G,L). However, protein levels of CHGA declined, and those of *Dclk1* increased in PSTi8-treated female mice compared to in PBS-treated female mice in colitis (Figure 5E,J,O). PBS-treated male mice had increased *Dclk1* and *Ca2* mRNA expression ($p < 0.05$) and no significant changes in *Muc2* and *Chga* gene expression in colitic compared to in non-colitic conditions (Figure S4 (E1,H1)). However, MUC2 and CHGA protein levels were significantly diminished in PBS-treated male mice in colitis compared to in steady-state conditions (Figure S4(E2,F2)) ($p = 0.04$; $p = 0.0009$). On the other hand, PSTi8-treated male mice did not display significant changes in the gene expression of all four markers compared to their PBS-

treated counterparts in colitic conditions. However, at the protein level, we found that PSTi8-treated male mice had significantly higher MUC2 levels in colitic compared to in non-colitic conditions ($p = 0.0001$) (Figure S4(E2)) along with increases in MUC2 and CHGA levels compared to colitic PBS-treated male mice ($p < 0.05$) (Figure 5E,J).

7.4.7. PST inhibition is associated with changes in several stem cell populations in a sex-dependent manner at steady state and during colitis

We explore whether the altered colonic mucosa proliferative activity observed at a steady state and in colitis, as demonstrated in Figure 4, resulted from modifications in the profile of the stem cell population. Thus, as shown in Figure 6, PSTi8-treated female mice had significantly elevated *Lgr5* and *Hopx* expression levels and lower *Ly6a* mRNA expression than the PBS-treated female groups at a steady state ($p < 0.01$) (Figure 6A,C,D,F,H,I,K,M,N). However, no significant changes in the levels of all three stem cell markers were observed at the protein level at a steady state. Conversely, although no significant changes in the expression levels of all three markers were observed at the transcript level, protein levels of LGR5 and LY6A were lower in PSTi8-treated male mice compared to in PBS-treated male mice ($p < 0.05$). By contrast, *Hopx* levels were higher in PSTi8-treated male mice compared to PBS-treated male mice at a steady state ($p < 0.05$). PBS-treated female mice displayed a significant downregulation of LGR5 expression ($p < 0.001$) and increased LY6A expression in colitic compared to in no-colitic conditions ($p < 0.001$) (Figure S5(A1–B2)). No changes in HOPX expression were observed at the mRNA and protein levels in colitic compared to in non-colitic conditions (Figure S5(C1,C2)). Similarly, LGR5 expression at the mRNA and protein levels diminished in colitic PSTi8-treated female mice compared to in their non-colitic PSTi8-treated

female counterparts ($p < 0.05$; Figure S5). However, no significant differences in the level of LY6A and HOPX were observed between colitic and non-colitic PSTi8-treated female mice. *Lgr5*, *Ly6a*, and *Hopx* gene expression did not differ between PSTi8-treated and PBS-treated female mice during colitis (Figure 6B,G,L). However, PSTi8-treated female mice had significantly reduced LY6A and HOPX protein levels compared to PBS-treated female mice during colitis ($p < 0.05$) (Figure 6H,M,J,O). During colitis, *Lgr5a* and *Hopx* mRNA expression levels were markedly reduced, while *Ly6a* expression remains unaltered in both PSTi8-treated and PBS-treated male mice in comparison to in their non-colitic counterparts ($p < 0.0001$) (Figure S5(D1,F1)). The mRNA results were, in part, validated at the protein level. Thus, LGR5 protein levels were reduced in colitic PBS-treated male mice, whereas HOPX levels declined in colitic PSTi8-treated male mice compared to in their PSTi8-treated non-colitic counterparts (Figure S5(D2–E2)). However, unlike mRNA results, LY6A protein levels were downregulated in PBS-treated male mice (<0.0001). In contrast, no significant differences in all three stem cell markers were observed in PSTi8-treated male mice between colitic and steady-state conditions (Figure S5(E2,F2)). The transcript levels of three stem cell markers did not differ between colitic PBS-treated and PSTi8-treated male mice. However, PSTi8-treated male mice preserved *Lgr5* protein levels compared to PBS-treated mice (Figure 6E).

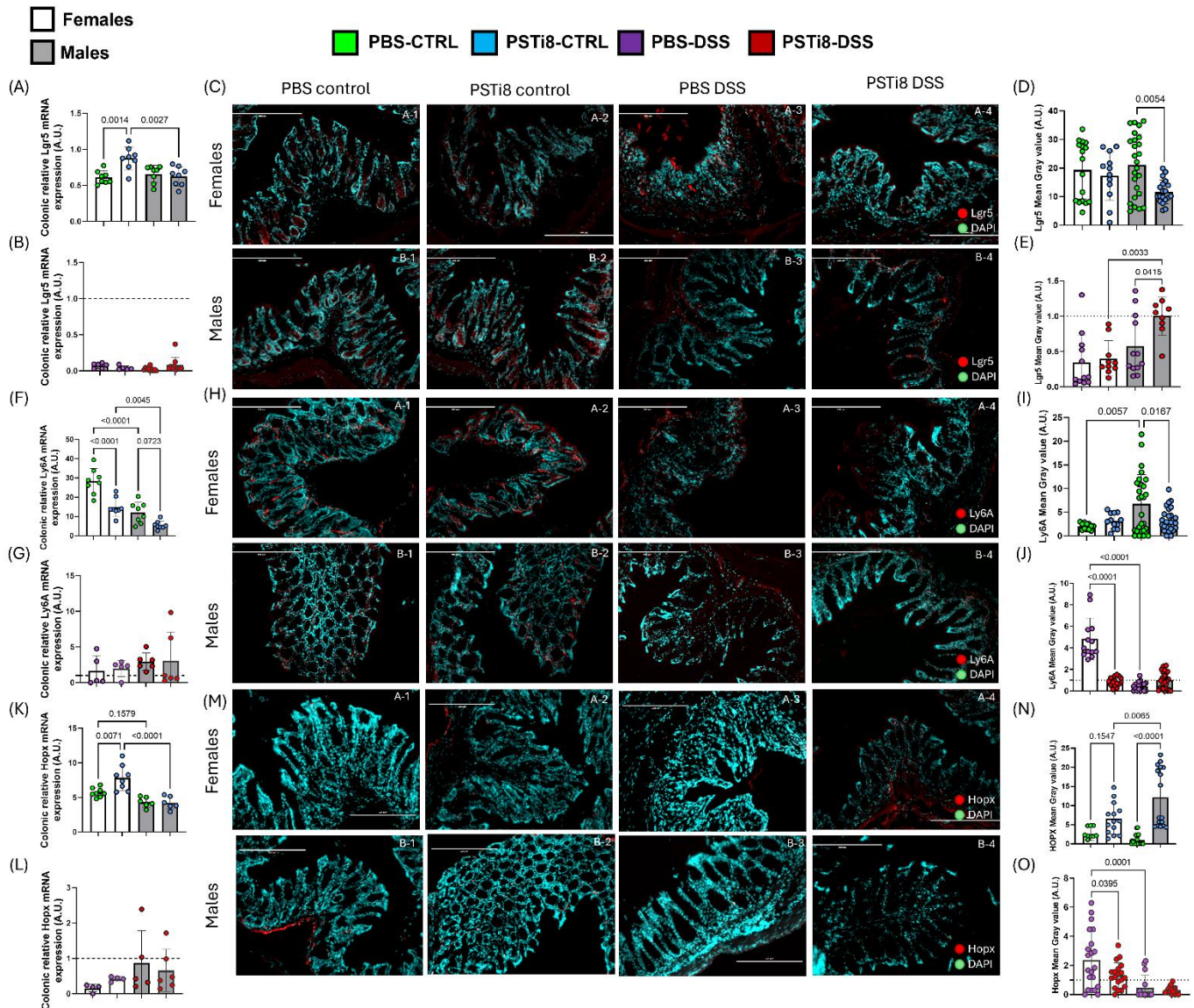


Figure 6. PST inhibition correlated with an altered profile of several colonic stem cells markers in a sex-dependent manner at a steady state and colitic conditions. The expression levels of markers associated with colonic epithelial stem cells were evaluated. Male and female C57BL/6 (WT) (n = 4–8 mice per group) were utilized for this study. Mice were treated with intrarectal injection with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days. One day after starting PSTi8 treatment, mice received, for five consecutive days, 5% dextran sulfate sodium (DSS) added in the drinking water to induce colitis or with water for the control group (CTRL). Colon tissue extracted on day five post-DSS treatment was analyzed using RT-qPCR and immunofluorescence (n = 3). Scale bar: 200 μ m. Biomarkers specific to crypt base columnar (LGR5) (A–E), paligenosis (fetal-like) (LY6A) (F–J), and damage-associated regenerative (HOPX) colonic stem cells (K–O) were evaluated. Data from DSS-treated mice were normalized to control conditions. The dashed

line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. The histogram represents the mean $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis using robust regression and outliers was performed to identify outliers.

7.4.8. PST inhibition correlates with altered expression of colonic mucosal self-renewal and differentiation markers between sexes at steady-state and colitic conditions

To investigate the mechanism underlying PSTi8 effect on colonic epithelial cells lineage commitments, transcription factors including NOTCH1, bone morphogenetic protein (BMP), hairy and enhancer of split-1 (*Hes1*), atonal homolog 1 (*Atoh1*), Sry-related HMG box 9 (*SOX9*), Krüppel-like factor 4 (*KLF4*), Neurogenin3 (*NEUROG3*), and Growth factor independence 1b (*GFI1b*) were characterized. The results showed that *Hes1* and *Gfi1b* mRNA expression levels were higher while *Atoh1* transcript expression was reduced in colon tissues of PSTi8-treated female mice compared to PBS-treated female mice at a steady state ($p = 0.02$, 0.0005 , and 0.005) (Figure 7(B1,E1,H1)). By contrast, in males, colonic tissues from PSTi8-treated mice had significantly lower *Bmp4* and *Atoh1* expression compared with those from PBS-treated mice ($p = 0.03$ and 0.0003) (Figure 7(D1,E1)). PBS- and PSTi8-treated male mice expressed markedly higher levels of *Sox9* and *Klf4* transcripts than their PBS- and PSTi8-treated female counterparts at a steady state (Figure 7(A1,F1)).

We further characterized the effects of PSTi8 on colonic epithelial cells lineage commitment in colitic conditions (Figures 7 and S6). *Hes1*, *Bmp4*, and *neurog3* mRNA expression levels were significantly downregulated in colonic tissues from both PSTi8- and PBS-treated female mice in colitis compared to in non-colitic conditions (Figure S6). *Atoh1* expression was elevated, whereas *Klf4* and *Gfi1b* mRNA expressions were lower in colonic tissues from PBS-treated female mice in

colitic compared to in non-colitic conditions. By contrast, significantly lower *Gfi1b* mRNA expression was observed in tissue from PSTi8-treated female mice in colitic compared to in non-colitic conditions (Figure S6(H1)). No significant differences were observed between PSTi8-treated and PBS-treated female mice in colitic conditions (Figure 7(A2–H2)). Similar to females, *Hes1*, *Atoh1*, and *Neurog3* transcript levels were significantly reduced in colon tissue from both PSTi8- and PBS-treated male mice in colitic compared to in non-colitic conditions (Figure S6(B2,E2,G2)). Moreover, no significant differences were observed in *Sox9*, *Notch1*, *Klf4*, and *Gfi1b* mRNA expression levels in tissues from colitic PBS-treated male mice; *Sox9* and *Klf4* transcript levels were significantly upregulated in colon tissues from PSTi8-treated male mice in colitic compared to in non-colitic conditions ($p \leq 0.04$ and 0.02) (Figure 7(A2–H2)). No marked differences in the expression of all transcription factors were observed between PSTi8- and PBS-treated male mice in colitis, whereas *Klf4* and *Gfi1b* expression levels were significantly higher in PSTi8-treated male than in their female counterparts in colitis (Figure 7(F2, H2)).

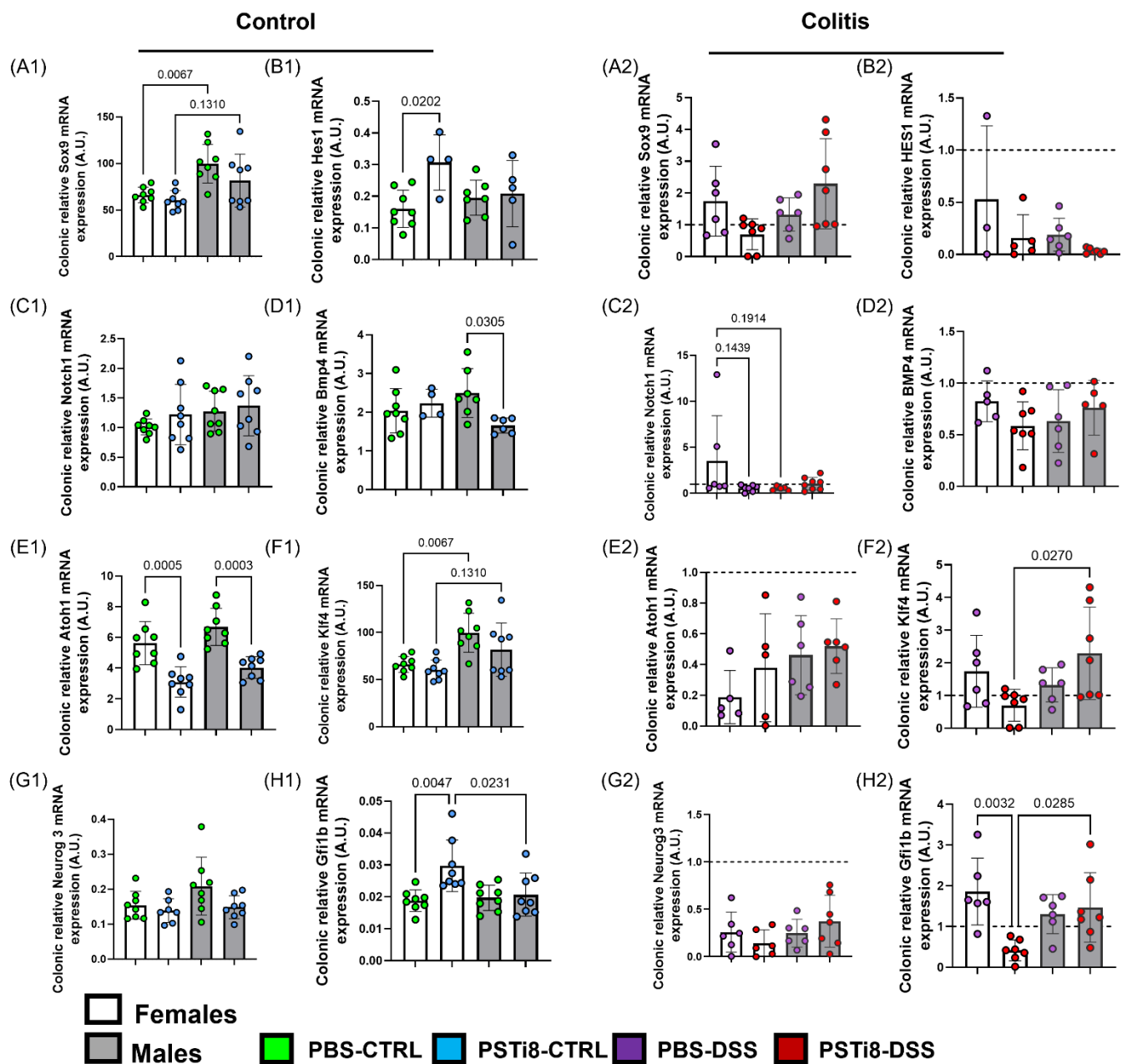


Figure 7. PST inhibition was associated with a change in the expression levels of several colonic epithelial cell lineage commitment markers between sexes at a steady state and colitic conditions. Males and females C57BL/6 (WT) (n = 4–8) were administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS to induce colitis. mRNA expression levels of markers specific for transcription factors mediating stem cell renewal *Sox9*, *Hes1* (A1–B2), absorptive progenitors (*Notch1*) (C1–C2), differentiation (D1–D2), secretory progenitors (*Atoh1*) along with differentiation of goblet cells (*Klf4*) (E1–F2),

enteroendocrine *Neurog* (G1–G2), and Tuft cells, *Gfi1b* (H1–H2) were measured by RT-qPCR. Data from DSS-treated mice were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. The histogram data represent the mean $\pm$ standard deviation. One-way and two-way ANOVA followed by a multiple comparison test were assessed. One-way ANOVA followed by Tukey's post hoc was performed between groups. The statistical analysis using robust regression and outliers was performed to identify outliers.

7.4.9. PSTi8 treatment correlates with changes in the expression of several UPR signaling markers in female mice at steady state and colitic conditions

Finally, we investigated whether PSTi8 regulated GRP78 downstream signaling and assessed activating transcription factor (ATF)6, X-box binding protein (XBP)1, C/EBP Homologous Protein (CHOP), glycogen synthase kinase (GSK)3 β , and mammalian target of rapamycin (MTOR) expression in colonic tissue from PSTi8- and PBS-treated male and female mice at a steady state and colitic conditions. qRT-PCR analysis revealed that *Atf6*, *Chop*, *Gsk3 β* , and *mTor* expression levels were significantly increased in PSTi8-treated female mice than PBS-treated female mice to reach the higher levels observed in all-male groups at a steady state (Figure 8(A1,C1–E1)). Although not significant, *Atf6* elevated expression in PSTi8-treated female mice was superior to levels observed in PSTi8-treated male mice, while *Xbp1* expression did not differ between both groups at a steady state (Figures 8(A1,B1)). No significant differences in *Atf6*, *Xbp1*, *Chop*, *Gsk3 β* , and *mTor* were observed in all-male groups at a steady state.

When evaluated in colitic conditions, PBS-treated females had significantly more *Chop* and a trend toward elevated *Gsk3 β* expression when compared to their female counterparts in non-colitic conditions ($p = 0.0004$ and 0.0532) (Figure S7(B1,C1)). By contrast, no significant changes in *Chop* and *Gsk3 β* were observed in colitic compared to in non-colitic PBS-treated male mice

(Figure S7(B2,C2)). No significant differences in the expression of all four transcription factors were measured between PST8-treated female mice in colitic and non-colitic conditions, whereas Atf6, Xbp1, Chop, Gsk3 β , and mTor expression levels were all significantly upregulated in PSTi8-treated male mice in colitic compared to in non-colitic conditions (for all transcription factors: $p < 0.05$). PSTi8 treatment correlated with altered mRNA expression levels of some of GRP78 downstream signaling proteins that differed in a sex-dependent manner, with significantly lower Atf6 and a trend toward reduced Xbp1, Chop, and Gsk3- β in PSTi8-treated females compared to in PBS-treated females and more marked compared to PSTi8-treated male mice, and no difference was observed across colitic male groups ($p = 0.01$ and 0.0005) (Figure 8(A2–E2)).

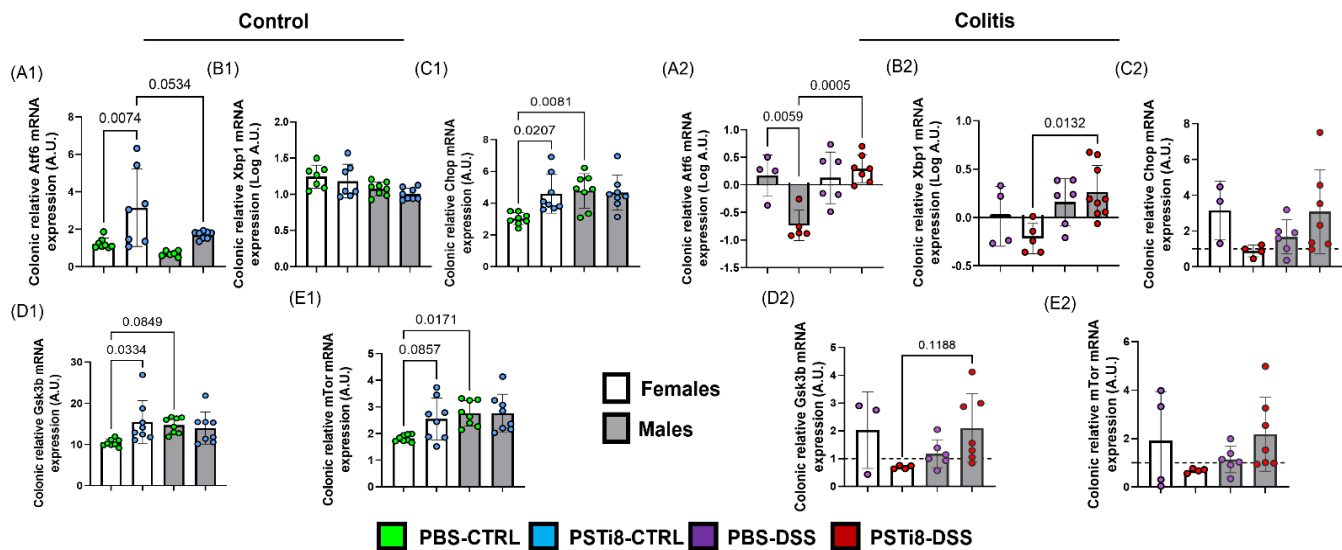


Figure 8. PST inhibition was associated with changes in the expression of several endoplasmic reticulum (ER) stress, mediators of apoptosis, and proliferation markers in a sex-dependent manner at a steady state. Colon tissues from males and females C57BL/6 (WT) (n = 3–8) administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS to induce colitis will be used. Biomarkers specific to ER stress (Atf6, Xbp1) (A1–B2), apoptosis (Chop) (C1–C2), and inhibition of cell growth (mTor, Gsk3b) (D1–E2) were assessed from colon samples by RT-qPCR. Data from DSS-treated mice were normalized to control conditions. The dashed line in each figure represents a ratio of 1, indicating no difference between DSS-induced colitis and control conditions. The histogram data represent the mean $\pm$ standard deviation. One-way ANOVA followed by a multiple comparison test was performed. One-way ANOVA followed by Tukey's post hoc was performed between groups. The statistical analysis using robust regression and outliers was performed to identify outliers.

7.5. Discussion

The colonic mucosa produces over 50 hormones and biologically active peptides, many of which are secreted by enteroendocrine cells (254). As part of the endocrine system, these peptide hormones can mediate diverse and contrasting physiological actions systematically by regulating the immune cells and the enteric nervous system or locally by regulating secretion, motility, insulin release, and nutrient uptake in the GI tract (358). Although a growing number of studies described a role for enteroendocrine peptide hormones in the context of metabolic physiological and pathological conditions such as obesity and diabetes, the effects of enteroendocrine secreted peptide hormones on colonic mucosal and epithelial cells integrity, profile, and functions in inflammatory disorders such as UC are yet to be fully understood. The current study demonstrated that the colonic mucosal inhibition of PST, an enteroendocrine peptide highly expressed in colonic tissues of patients with active UC and in a mice model of colitis, with PSTi8 treatment altered the epithelium repair process homeostasis and influenced moderately susceptibility to DSS-mediated colitis in a sex-dependent manner (18,359).

A few studies have recently reported that PST regulates intestinal mucosal permeability at a steady state, promotes inflammatory response and exacerbates disease severity in male mice with DSS-mediated colitis (18,265). Thus, PST treatment in male mice promoted the early onset of colitis and increased the colon mRNA expression of proinflammatory cytokines IL-8 and IL-18 by reducing the alternative activated macrophage activity and expression levels of TJ proteins CLD1, zonula occludens-1, occludin, and E-cadherin-1 during DSS-mediated colitis (18). Similarly, male mice treated with PSTi8 showed a trend toward delays in colitis onset. However, these results

differed in female mice as PSTi8 treatment with moderately worsened colitis progression, suggesting a role for sex-based differences in the response of colonic mucosa to PST.

The repair process of colonic mucosal is orchestrated through a series of events involving a complex interplay between several key players that regulate mucosal barrier integrity, such as cytokines, TJ proteins, and antimicrobial peptides (160,171,242,243,360,361). During colitis, the release of several proinflammatory mediators, such as IL-6, IL-18, IL-22, and RELM β , has been shown to enhance the intestinal epithelial cells proliferation, induce the release of antimicrobial peptides and anti-inflammatory mediators and promote the mucosa wound healing (226,229,239,242,243,362,363). Interestingly, PSTi8-treated groups displayed no significant differences in the production of proinflammatory mediators, PST, IL-6, IL-22, IL-18, and mRNA expression of TJ *Cld4*, goblet-derived bacterially induced *Relm β* , and antimicrobial peptides in colitic conditions. However, in homeostatic conditions, PST inhibition with PSTi8 correlated with changes in several markers associated with mucosal integrity in a sex-dependent manner. Thus, PST inhibition in females was associated with lower levels of IL-6 and IL-22, along with reduced *Relm β* mRNA expression, while promoting the production of PST and reducing *Cld4* mRNA expression in males in homeostatic conditions. Moreover, PSTi8 treatment did not alter the expression of antimicrobial peptides at a steady state, indicating a minimal influence of PST on colonic mucosa antimicrobial activities. These observations suggest a potential role of PST as a regulator of mucosa barrier integrity, whose effect is more marked in females than in males.

Cytokines are critical regulators of colonic epithelial barrier integrity as they can induce or restrict epithelial cell death, proliferation, and differentiation and consequently affect the colon's capacity for wound healing (300). The epithelial cell restitution, proliferation, and differentiation

are primordial mechanisms in the mucosa wound repair process critical to preserving the epithelial barrier function in colon homeostasis (186). The restitution process requires the migration of surrounding colonic epithelial cells to reseal the injury site and initiate healing without proliferation (186). The proliferation process increases the pool of colonic epithelial cells necessary to compensate for the cells shed or destroyed during injury. In contrast, differentiation and maturation help to preserve and reestablish the mucosal barrier integrity (360). Insufficient wound healing during colitis can lead to abscess and fistula, whereas excessive wound healing can cause intestinal fibrosis (183). Moreover, enhanced proliferation increases the risk for patients to develop colorectal cancer, which is more prevalent in males than females (364–367). Therefore, the altered mucosal differentiation observed in females could result in part from the diminished production of proinflammatory cytokines following PSTi8 treatment at a steady state. Along the same line, regulatory anti-inflammatory signaling reduces differentiation and enhances intestinal stem cell renewal (366). Moreover, we reported that PST decreased the alternatively activated macrophage (AAM) ability to produce anti-inflammatory mediators in colitic conditions. Therefore, PST inhibition may restore AAM and hence the production of anti-inflammatory cytokines (18). Further studies will be needed to investigate whether PST inhibition through enhanced production of anti-inflammatory cytokines can regulate colonic mucosal cell proliferation and differentiation at a steady state.

We further observed that the inhibitory effect of PSTi8 treatment in females' colonic mucosal differentiation was associated with substantial tuft cell reduction and a trend toward lower goblet cells at a steady state, indicating that PST likely regulates tuft and goblet cell homeostasis in females' colon. PSTi8 treatment in males correlated with a marked reduction in goblet,

enteroendocrine, and tuft cells, but no significant changes in colonocytes. The lack of changes in the colonocyte population could, therefore, explain the absence of changes in overall mucosal differentiation observed in males at a steady state. Our results showed that in colitis, PSTi8 treatment correlated with differences in the distribution of several differentiated epithelial cells in a sex-dependent manner. Thus, PSTi8 treatment was associated with reduced goblet and enteroendocrine cells but increased tuft cells in colonic epithelium, revealing tuft cells as one of the potential actors in the overall increase in mucosa proliferation during colitis in females (Figure 4C). A recent study reported a correlation between the presence of tuft cells with epithelial restitution (249). It would be of interest to investigate whether PSTi8-mediated increase in tuft cells could be a protective mechanism in response to exacerbated inflammation. By contrast, PSTi8 treatment in males correlated with a conserved number of enteroendocrine and colonocyte cell populations and increased goblet cells while displaying a not significant reduction of tuft cells during colitis. The distinct effect of PSTi8 treatment on the distribution of each specific differentiated colonic cell could explain the lack of changes in the mucosa differentiation observed in males following treatment during colitis. These results suggest that PST likely regulates the mucosa differentiation capacity by altering the profile of differentiated epithelial cell populations at a steady state and during DSS-mediated acute inflammation in a sex-dependent manner.

With a relatively short lifespan, the colonic epithelium requires constant self-renewal to replenish depleted differentiated cells and preserve the colonic mucosal homeostasis (192). The self-renewal of colonic epithelial cells is driven primarily by CBCs and DARSCs stem cells $LGR5^+$, $LY6A^+$, and $HOPX^+$ (192,195). Although dispensable, $Lgr5^+$ cells can give rise to all differentiated epithelial cells and, owing to their fast-cycling nature, promote rapid and daily

epithelium homeostatic regeneration (368). However, these stem cells are exquisitely sensitive to cell death during injuries such as radiation, ischemia, or colitis (194). The DARSCs LY6A⁺ and HOPX⁺ cells arise in response to severe colonic mucosa injury and LGR5⁺ cell depletion to restore the epithelium integrity (192,194,195). Moreover, all three stem cells can give rise to all terminally differentiated epithelial cells in the colon (192). Here, we found that PSTi8 treatment was associated with changes in the colonic epithelial stem cell populations differently at a steady state and colitic conditions between sexes. Thus, despite showing a trend toward increased HOPX⁺ cells, PSTi8 treatment did not significantly modulate the levels of all three stem cell populations in females at a steady state. By contrast, this treatment in males correlated with elevated HOPX⁺ cells, in line with the higher overall mucosa proliferation observed in Figure 4E, but lower LGR5⁺ and LY6A⁺ cells at a steady state. These results suggest that PST may regulate HOPX⁺ cell homeostasis in males and females. By contrast, in females, the lack of stem cell modulation associated with the increase in mucosal proliferative activity suggests that PST could regulate other DARSC subtypes, such as CLU⁺ cells shown to regenerate depleted LGR5⁺ cells and help restore the mucosa in acute and chronic DSS-induced colitis (192). PSTi8 treatment in colitic conditions was associated with a marked reduction in LY6A⁺ and HOPX⁺ cells. The heightened depletion of all three stem cells in females might be responsible for the colonic epithelial cells reduced proliferative capacity observed in Figures 3 and 4 and likely explains females' increased susceptibility to colitis. PSTi8 treatment in males preserved the Lgr5⁺ cell population while depleting HOPX⁺ and LY6A⁺ cells in colitis. Further studies will help decipher whether the ability of males treated with PSTi8 to maintain LGR5⁺ cells, which are fast-cycling stem cells, could increase the regenerative capacity of colonic epithelial cells in colitic conditions.

Several transcription markers promote colonic epithelial cells stemness (SOX9) (369), self-renewal and colonocytes (HES1 and NOTCH1) (206,370), differentiation (BMP4) (371), secretory phenotype (ATOH1), as well as goblet cells (Klf4) (372), enteroendocrine cells (NEUROG3) (373), and tuft cell (GFI1b) (374) lineage commitment. We demonstrated that PSTi8 treatment was associated with elevated *Hes1* and *Gfi1b* mRNA expression and reduced *Atoh1* levels in females in homeostatic conditions. Long thought as a marker of normal colon stem cells, HES1 expression has recently been associated with promoting stemness in a subset of the colonic epithelial cells, in addition to being proposed as a regulator of cancer stem cells in the colon (370). Given that *Hes1* mRNA expression in the current study does not correlate with the stem cell population regulating the colonic mucosal homeostasis, our data indicate that PSTi8 treatment may enhance the proliferation of unspecified stem cell population potentially associated with cancer stem cells in females at a steady state. GFI1b is a transcription factor specific to a subset of the tuft cell population (374). We showed that PSTi8 treatment correlated with enhanced *Gfi1b* mRNA expression in females at a steady state. However, this increase did not correlate with the expression of DCLK1⁺ cells, suggesting a potential difference between the population of cells detected by the DCLK1 marker and the one measured using *Gfi1b* mRNA expression. PSTi8 treatment was associated with the reduced mRNA expression of *Atoh1*, a master transcription factor that guides stem cells' commitment toward a secretory profile, in females and males at a steady state (192). This decrease in *Atoh1* mRNA expression was likely associated with the reduced goblet and enteroendocrine cell levels observed in females and males in homeostatic conditions. These results highlight a potential role of PST as a regulator of transcription factors that govern the colonic epithelial cell stemness, self-renewal, and differentiation, mediated in a sex-dependent manner.

PST and PSTi8 mechanisms of action occur through GRP78, a molecular chaperon that assists the folding and unfolding of proteins and prevents protein aggregation within the ER (352). As the master regulator of the UPR signaling induced in response to ER stress, triggered as a result of accumulated misfolded protein in ER, GRP78 controls the availability of ER transmembrane effector proteins inositol requiring enzyme 1 (IRE1) and ATF6 (375). IRE1 activities generate the potent transactivator of UPR target genes, XBP1, and with ATF6, upregulate GRP78 expression and prevent ER-stress-induced apoptosis mediated by the transcription factor CHOP (375). Moreover, GRP78 can also regulate cell survival, apoptosis, and systemic inflammation through the enzymes mTOR and GSK3- β (376,377). ER stress is a hallmark of UC pathophysiology, as it promotes dysregulated autophagy that causes inflammation and impaired intestinal barrier (378). Our results showed an enhanced UPR response in females treated with PSTi8 at a steady state. However, PSTi8 treatment was not associated with changes in GRP78 signaling during colitis, suggesting the effect of PSTi8 to be mainly mediated in homeostatic conditions in females. Given the critical and complex role of ER stress and UPR signaling in promoting colorectal cancer (CRC), further studies will help understand whether PST regulates CRC etiology in females. PSTi8 treatment did not alter the GRP78 downstream signaling in males at a steady state and during colitis. These results indicate that the effect of PSTi8 on the mucosa in males may not occur through GRP78 receptors and could potentially occur via the IRS, which is also a target receptor for PSTi8 inhibitory action (333,350–353). Several studies have shown that estrogen, a female sex hormone, can regulate GRP78 expression in various tissues. Thus, this may explain the dichotomy in the mucosal response to PSTi8 treatment observed between the sexes (354,379–381).

This study has some limitations that need further investigation in the future. Firstly, we investigated the expressions of several markers at mRNA levels only. However, transcript levels do not always reflect the translated protein levels, as exemplified by data in Figures 5 and 6. These discrepancies can arise from several pre- and post-transcriptional mechanisms that may influence mRNA stability and impact protein translation, synthesis, stability, and degradation (382). Secondly, female mice's estrus cycle was not synchronized. However, studies have shown that the menstrual cycle and female sex hormones can influence intestinal function and exacerbate disease severity in UC (383). Third, we only investigated the impact of PSTi8 treatment mediated through the GRP78 signaling pathways. Given the lack of difference in GRP78 downstream signaling in males treated with PSTi8, it will be interesting to investigate the PSTi8-mediated effect on the mucosa through other PST receptors including IRS signaling pathways (354). Fourth, in the current study, we used an acute model of colitis to characterize the effect of PST inhibition on mucosal integrity. However, this model is associated with excessive inflammation, severe colonic epithelial destruction, and impaired mucosal functions. All these elements could mask the effect of PST on mucosa and impede the capacity to investigate the role of PST in the colonic mucosa. Therefore, evaluation of PST's role on the mucosa using a chronic DSS model of colitis with low to moderate mucosal destruction may provide more insights into the role of PST in colitis. Finally, gut microbiota plays a significant role in intestinal epithelial homeostasis and UC pathophysiology (326). Moreover, gut microbiota can also regulate sex hormones, which, in turn, can modulate intestinal integrity (344,384–386). Therefore, further studies will be necessary to confirm the interaction between the colonic epithelium and PST and to investigate the interaction between

PSTi8 effects, sex hormones, and microbiota on the colonic mucosa through GRP78 and insulin receptor.

7.6. Conclusion

In conclusion, the current study suggests that the colonic mucosa inhibition of PST with PSTi8 treatment is associated with changes in the epithelium repair process that differs in a sex-dependent manner at a steady state and could influence susceptibility to DSS-mediated colitis differently between female and male mice. (359). Altogether, this study suggested that PST inhibition with PSTi8 could alter the colonic mucosa integrity in females by limiting the differentiation while promoting the proliferation of colonic epithelial cells and increasing UPR signaling pathways in homeostatic conditions. These disturbances of the mucosa in homeostatic conditions could potentially explain the moderate increase in susceptibility to colitis associated with a trend toward worsened disease severity in female mice. By contrast, PSTi8 treatment in male mice resulted in the diminution of epithelial cell proliferation associated with a higher number of HOPX⁺ stem cells and a reduced number of most terminally differentiated epithelial cells goblet, enteroendocrine, and tuft cells at a steady state. However, unlike in females, during colitis, PSTi8 treatment may provide a protective effect on the colonic mucosa against DSS-mediated colitis in male mice as PST inhibition either preserved or promoted the proliferative and differentiation capacity of the colonic epithelium by increasing the number of goblet cells and preserving LGR5⁺ Stem cells and enteroendocrine cells, all of which were overall associated with delay disease onset.

7.7. Significance

This descriptive study provides new insights regarding the regulatory role of PST in the colonic mucosa integrity and epithelial properties and functions at a steady state and in inflammatory conditions and how its effect differs between sexes. Moreover, we describe the use of PSTi8 as a potential therapeutic tool that limits PST and regulates colonic mucosa repair in UC and could offer a novel therapeutic target for colorectal cancer.

7.8. Supplementary file

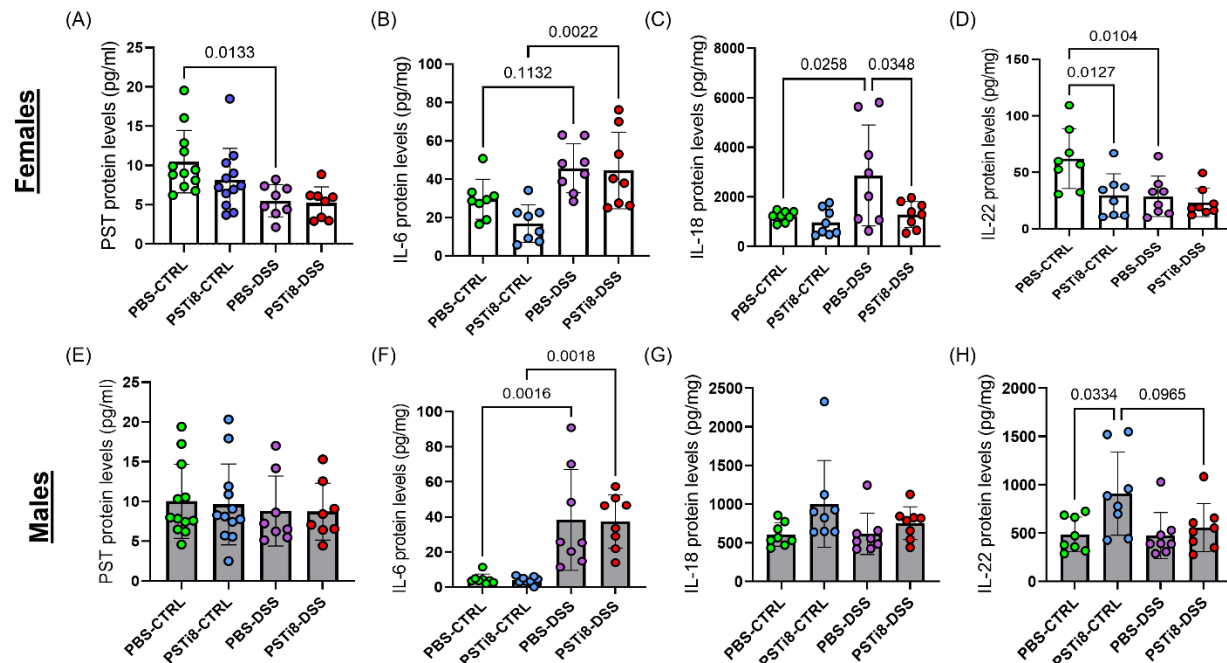


Figure S1. Effect of PSTi8-treatment on colonic mucosal cytokines profile in colitic compared to steady state conditions in male and female mice. Male and female C57BL/6 (WT) mice (n = 6-8) were administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS for five days to induce colitis (A–G). Colon tissues were collected and homogenized to extract proteins. Protein levels of PST (A, E) and mucosal regulatory inflammatory cytokines, Interleukin (IL)-6 (B, F), IL-18 (C, G), and IL-22 (B–D and F–H), were evaluated by ELISA. Data was expressed as the mean $\pm$ standard deviation. Statistical significance was determined using ordinary one-way ANOVA and Tukey's multiple comparisons test. Statistical analysis to determine outliers was performed using robust regression and outlier.

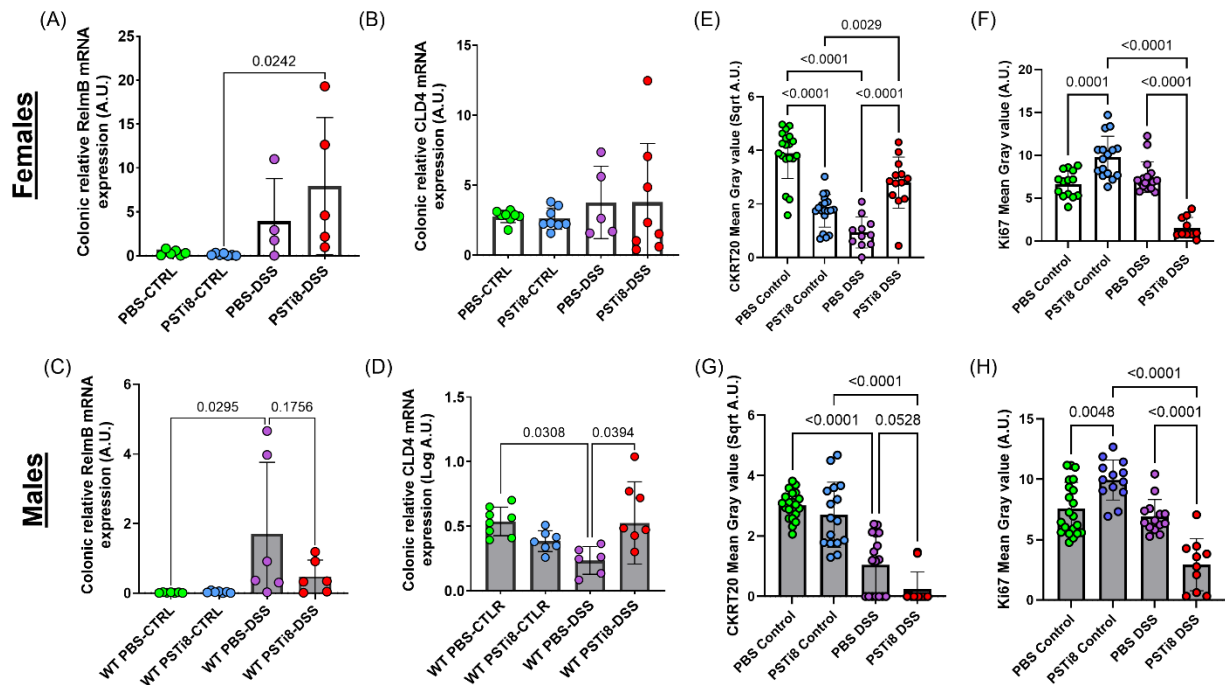


Figure S2. Effect of PSTi8-treatment on markers associated with the mucosal integrity in colitic compared to steady state conditions in male and female mice. (A-H) Colonic tissue collected from males and female C57BL/6 (WT) (n = 4–8) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used (control group, CTRL). mRNA expression of markers linked to microbiota mucosal colonization, *RelmB*, and tight junction, claudin (*Cld4*), were measured by qRT-PCR (A–D). Markers associated with epithelial differentiation (cytokeratin 20, CKRT20) and proliferation (Ki67) were measured by immunofluorescence (E–H). Results represented the means ± standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis to determine outliers was performed using robust regression and outlier.

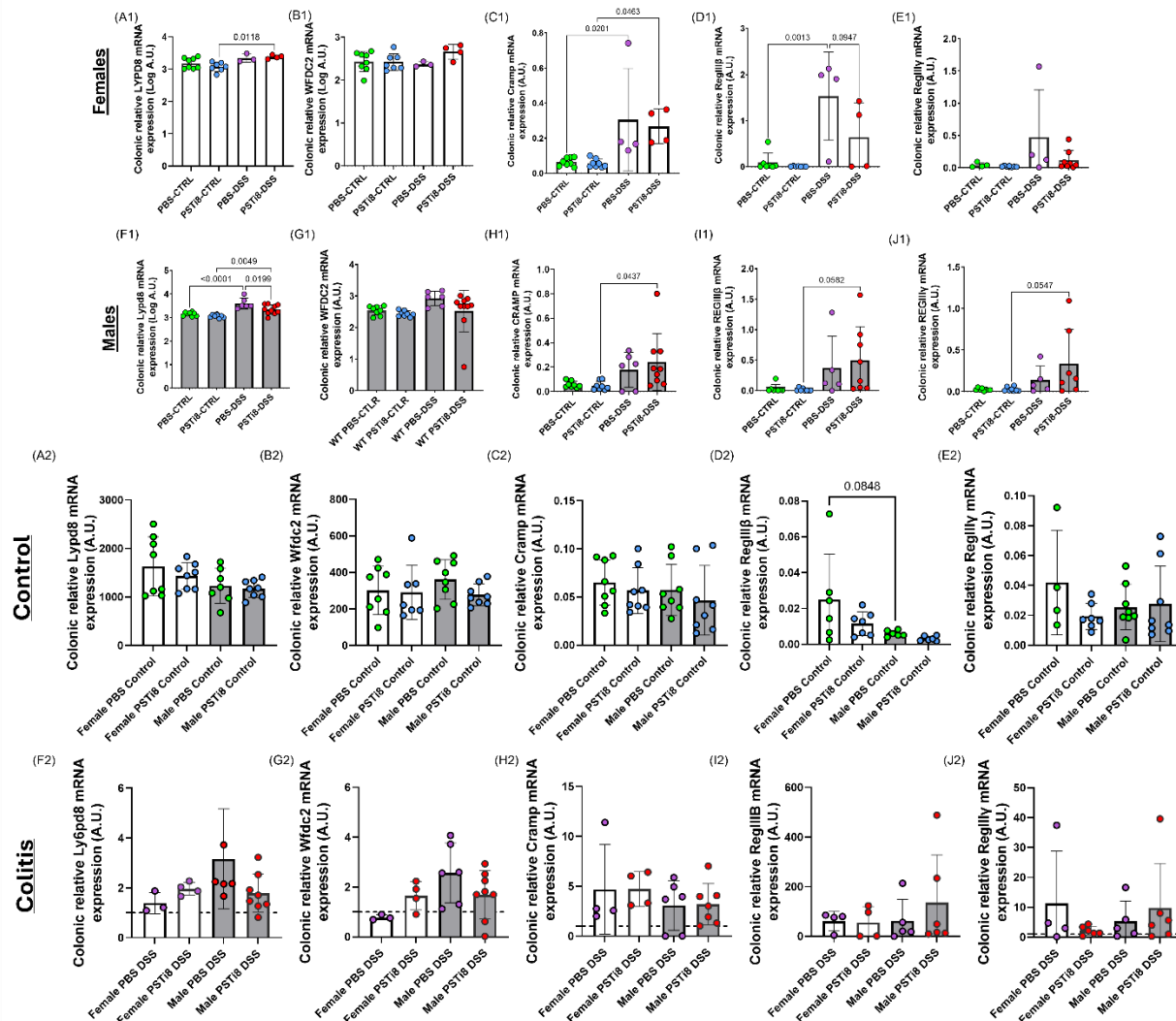


Figure S3. Effect of PST inhibition on colonic mucosal antimicrobial peptides expression in colitic compared to steady state conditions in male and female mice. Colonic tissue collected from males and female C57BL/6 (WT) (n = 4-8) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used (control group, CTRL) (A1–J2). mRNA expression of *Ly6p8* (containing 8), *Wfcd2*, cathelin-related antimicrobial peptide (*Cramp*), dysregulated expression of type III REG (*RegIIIγ*) and *RegIIIβ* were measured by qRT-qPCR. Data from DSS-treated mice was normalized to control conditions (F2–J2). The dashed line in each figure represents a ratio of 1, indicating an absence of difference between DSS-induced colitis and control conditions. Results represented the means $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis to determine outliers was performed using robust regression and outlier.

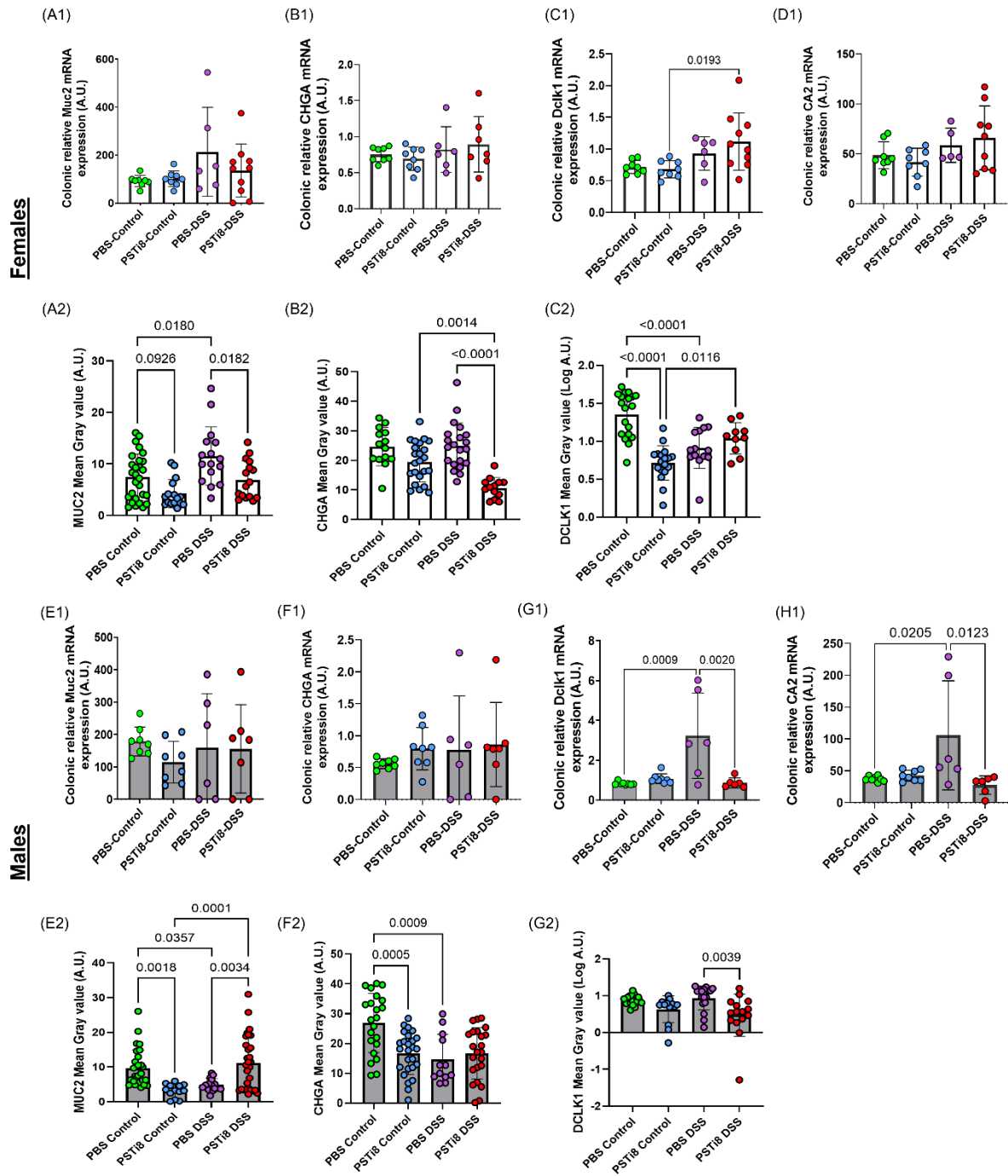
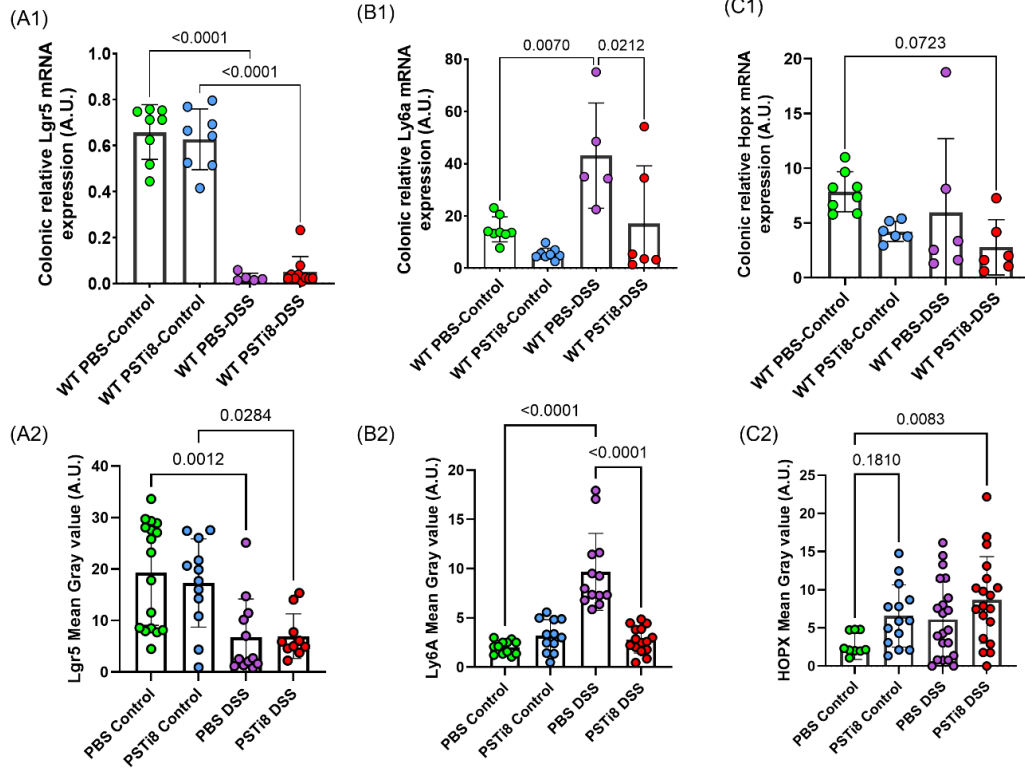


Figure S4. Effect of PST inhibition on terminally differentiated colonic epithelial cell markers in colitic compared to steady state conditions in male and female mice. Expression of markers specific to differentiated colonic epithelial cells was evaluated (A1–G2). Colonic tissues collected from males and female C57BL/6 (WT) (n = 5-8) treated intrarectally with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days concomitant with DSS or water for 5 days were used. mRNA and protein

levels of markers specific to goblet cells, mucin (MUC)2, enteroendocrine cells, chromogranin A (CHGA), and Tuft cells, doublecortin-like kinase (DCLK) 1, were characterized by RT-qPCR (**A1–H1**) and immunofluorescence (IF) (**A2–G2**). Results represented the means $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis evaluating using robust regression and outlier.

Females



Males

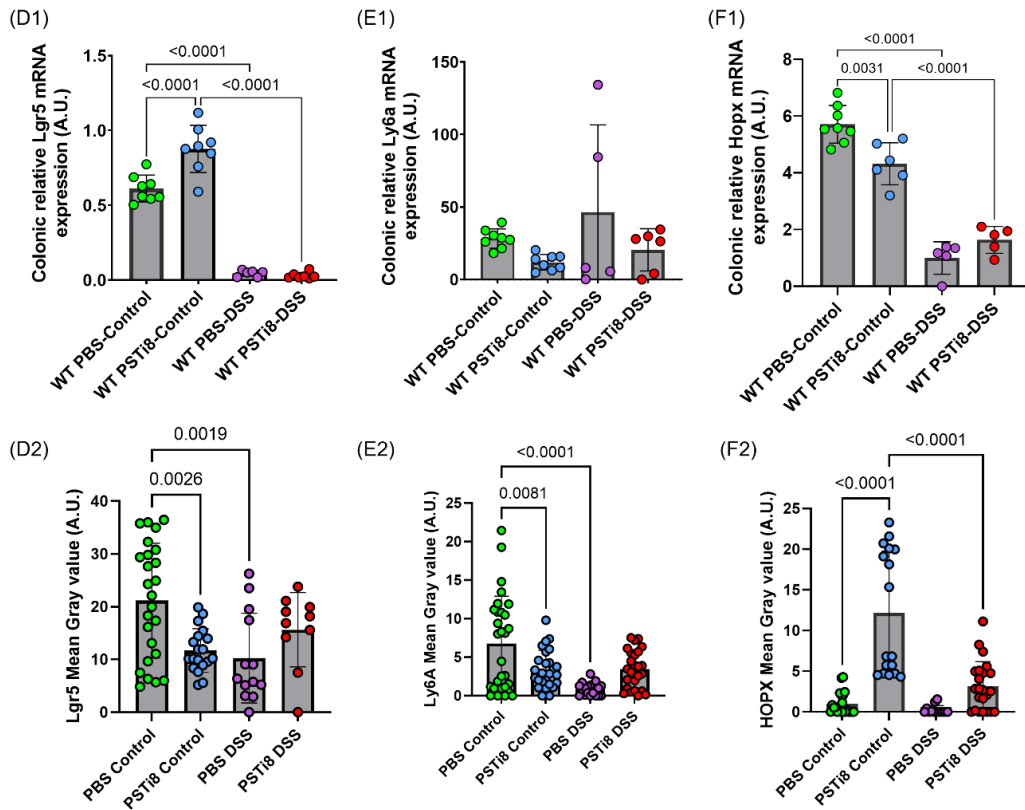


Figure S5. Effect of PST inhibition on colonic stem cells markers in colitic compared to steady state conditions in male and female mice. The expression of markers associated with colonic epithelial stem cells was evaluated (**A1–F2**). Male and female C57BL/6 (WT) (n = 4-8 mice per group) were utilized for this study. Mice were treated with intrarectal injection with 2.5 mg/kg/day PSTi8 or PBS daily for 6 days. One day after starting PSTi8 treatment, mice received for 5 consecutive days 5% dextran sulfate sodium (DSS) added in the drinking water to induce colitis or with water for the control group (CTRL) Colon tissue extracted at day five post-DSS treatment was analyzed using RT-qPCR (**A1–F1**) and immunofluorescence (**A2–F2**) (n=3). Biomarkers specific to crypt base columnar (LGR5), paligenosis (fetal-like) (LY6A), and damage-associated regenerative (HOPX), colonic stem cells were evaluated. Histogram data represent the mean $\pm$ standard deviation. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis to determine outliers was performed using robust regression and outlier.

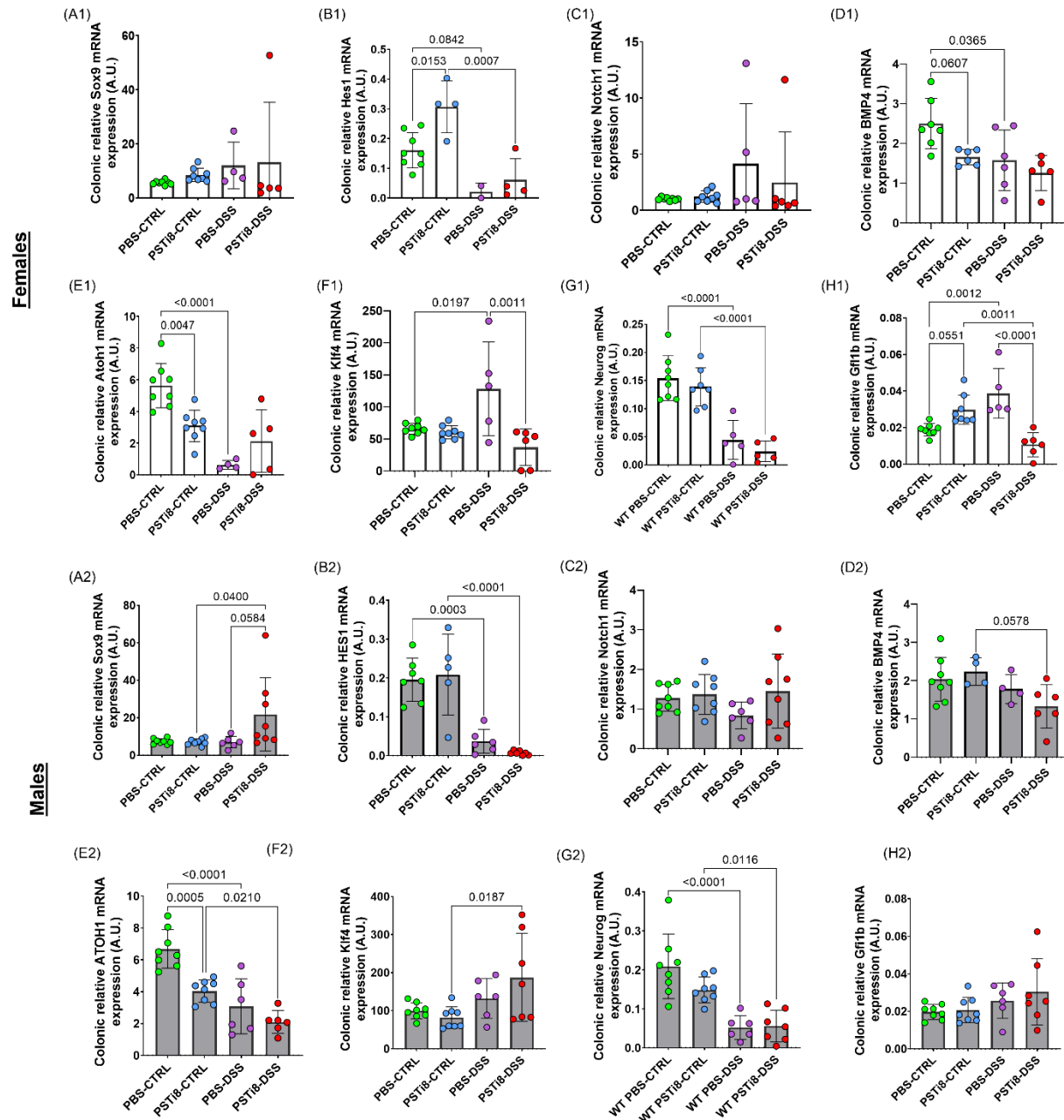


Figure S6. Effect of PST inhibition on colonic epithelial cell lineage commitment markers in colitic compared to steady state conditions in male and female mice. Males and females C57BL/6 (WT) (n = 4-8) were administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS to induce colitis. mRNA expression of markers specific for transcription factors mediating stem cell renewal and differentiation *Sox9*, *Hes1*, absorptive progenitors (*Notch1*), and *Bmp4*, secretory progenitors (*Atoh1*), along with differentiation of goblet cells (*Klf4*), enteroendocrine *Neurog.*, and Tuft cells, (*Gfi1b*) were

measured by RT-qPCR (A1–H2). Histogram data represent the mean $\pm$ standard deviation. One-way and two-way ANOVA followed by a multiple comparison test, were assessed. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis to determine outliers was performed using Robust regression and Outlier.

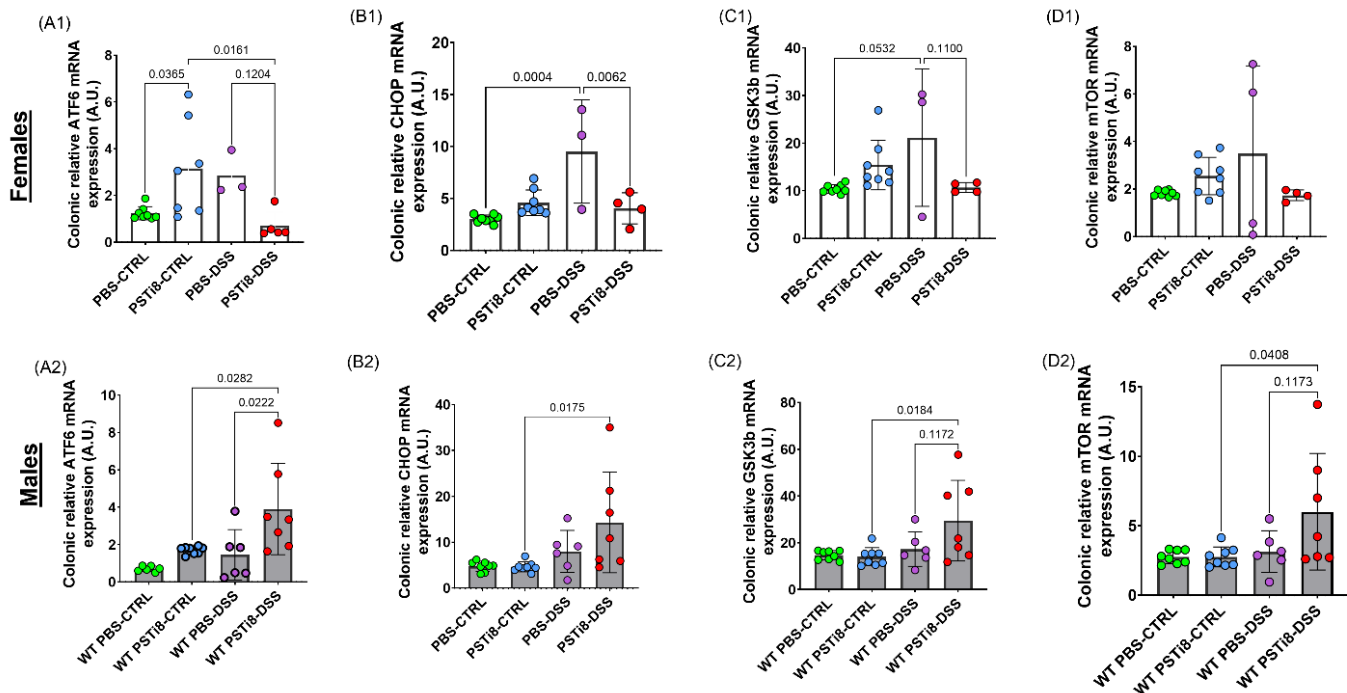


Figure S7. Effect of PST inhibition on mucosal endoplasmic reticulum (ER) stress, mediators of apoptosis, and proliferation markers in colitic compared to steady state conditions in male and female mice. Colon tissue from males and females C57BL/6 (WT) (n = 3-8) administered with PSTi8 (2.5 mg/kg/day) or PBS by intra-rectal route one day before treatment with 5% DSS to induce colitis will be used. Biomarkers specific to ER stress (*Atf6*, *Xbp1*) (A1–B2), apoptosis (*Chop*) (C1–C2), and inhibition of cell growth (*mTor*, *Gsk3 β*) (D1–E2) were assessed from colon samples by RT-qPCR. Histogram Data represent the mean $\pm$ standard deviation. One-way ANOVA followed by a multiple comparison test, was performed. One-way ANOVA followed by Tukey's post hoc was performed between groups. Statistical analysis to determine outliers was performed using robust regression and outlier.

CHAPTER VIII

GLOBAL DISCUSSION

This research aimed to investigate the impact of loss of CHGA and PST inhibition with PSTi8 on the colonic epithelium integrity and mucosal barrier function in the DSS-induced colitis model of UC. Moreover, we explored whether sex-related differences could influence the mucosal integrity and functions in the gut in the presence or absence of CHGA and PST at a steady state and during colitis.

In this thesis, our findings showed that females and males displayed distinct colonic epithelial cells and immune response profiles at steady state, which were altered differently in a sex-dependent manner in colitis. In brief, females displayed enhanced proliferative activity associated with elevated fast-cycling and reserve stem cell markers gene expression, hinting toward enhanced mucosal healing capacity in females than males at steady state.

Accordingly, females' higher colonic mucosal healing potential at steady state likely explains their subsequent improved resistance to DSS-induced colitis as it is accompanied by preserved epithelial stem cells population and enhanced differentiation toward the intestinal epithelial secretory lineage goblet and tuft cells, which promote epithelial barrier integrity by regulating the production of the mucus barrier and synthesis of various paracrine and endocrine effectors molecules that modulate epithelial cells proliferation and suppress gut inflammation (387). Moreover, females seem to respond to DSS-induced colitis by upregulating the mRNA expression of *Nkcc1* and *Cld4*, two components known to interfere with the formation of pore-forming *Cld2*, reduce the intestinal

cells' paracellular permeability and consequently promote mucosal integrity (172,283,284). In contrast, males' inability to preserve the colonic epithelial barrier function in DSS-induced colitis is demonstrated by lessened mucosal healing and increased mucosal bacterial infiltration exemplified by the amplified Tff3 and Relm β mRNA expression (49,241). Thus, the loss of mucosal barrier function in males with colitis likely caused colonic epithelial progenitors to differentiate into possibly immature colonocytes and paligenosis, key processes in the mucosal response to severe epithelial damage (195,285).

Sex-mediated differences in mucosal barrier functions may influence the colonic mucosal immune response. Therefore, we characterized the immune cell types and cytokine profiles along the large intestine of males and females under steady-state and colitic conditions. Our findings revealed that the colonic mucosal immune response differs substantially between sexes in both contexts. Thus, the homeostatic colon of females was enriched with innate immune cells DC, macrophages, and granulocytes as well as cells of adaptive immunity including Treg and Tfc, which correlated with higher levels of pro-inflammatory cytokines including TNF- α , IFN- γ , and IL-17A. Conversely, males exhibited a higher frequency of NK and CD8⁺ T cells and enriched Th17, and both anti- and pro-inflammatory cytokines, including IL-17C, IL-17F, IL-25, IL-10, TGF- β , and IL-6. These findings highlight a sex-specific immune response characterized by females exhibiting a stronger innate immune profile, while males show a predominant adaptive immune response at steady state.

Following the induction of acute colitis, both male and female colons showed an increase in macrophages and granulocytes, alongside a reduction in B cells. Furthermore, levels of IL-1 β , IL-6, TNF- α , and cecal IFN- γ were elevated, aligning with findings from previous studies. When analyzed by sexes, the colons of females displayed a higher frequency of NK cells, which regulate

neutrophil recruitment and function (320), as well as NKT and CD8⁺T lymphocytes. Moreover, females maintained CD11B⁺CD103⁺ DCs, which play a role in promoting immune tolerance in the gut. These variations in immune cell profiles correlated with a marked increase in IL-10 and TGF- β compared to males. By contrast, the colons of males displayed increased CD11B⁺CD103⁺ DCs, granulocytes, total macrophages, M1 and M2 macrophages, along with a higher prevalence of CD4 and CD8⁺T cells expressing PD-1, a checkpoint inhibitor that is highly expressed on activated lymphocytes in patients with active UC, likely indicating an exacerbated inflammatory response (388). Studies have shown that CD11B⁺CD103⁺ DCs, which are abundant in the colons of patients with UC, can promote the induction and expansion of Th17 cells in response to antigens and metabolites generated by commensal bacteria (389). Moreover, many studies reported altered intestinal microbiota composition and function in UC patients (326). In line with these findings, we demonstrated that males maintained elevated levels of Th17 cytokines in acute colitis, resembling the profile observed under steady-state conditions. However, there was a significant regional shift from the distal colon to the cecum, which correlated with an increase in the frequencies of CD11B⁺CD103⁺DCs in the cecum, suggesting regional changes in the composition of the microbiota between these two sections. The profiling of the immune system along the large intestine in colitic conditions revealed an increased inflammatory response associated with enhanced recruitment of myeloid cells, cecal expansion of Th17 cells, and a higher number of PD-1 expressing T lymphocytes, all of which are features of severe UC in males. Conversely, in females, despite the increased frequency of effector cells such as NKT and CD8⁺ T cells, known to trigger damage to the intestinal mucosa in colitis, we hypothesize that females exhibited milder

UC due to the increase in cells and cytokines known to downregulate inflammation and consequently improve colitis.

After characterizing the integrity, functionality, and immune response of the mucosa under both homeostatic and colitic conditions in males and females, we subsequently sought to investigate the impact of the lack of CHGA, a prohormone protein secreted by intestinal epithelial enteroendocrine cells, on the colonic mucosa of both sexes in steady-state and colitic conditions. Elevated levels of CHGA are associated with various pathologies, such as neuroendocrine tumors, chronic kidney disease, organ failure, and IBD (333). In previous studies, we reported that high CHGA expression in male colon tissue was correlated with increased inflammation, altered tight junctions, and impaired epithelial repair (390). Furthermore, we showed that CHGA depletion altered macrophage plasticity from an M1 to an M2 profile (17). Here, we expand on this knowledge by demonstrating that the absence of CHGA correlates with increased production of TGF- β , which likely reduces the abundance of hematopoietic cells, dampens mucosal inflammation, and limits intestinal mucosal damage. This also preserves the intestinal repair capacity and, as a result, affects colitis severity in males. However, the absence of CHGA did not provide females with resistance to DSS-induced colitis. Instead, it was associated with a loss of intestinal epithelial integrity and impaired mucosal barrier function, despite the increase in TGF- β levels within the colon. These findings underscore a potential mechanism where the inflammatory response preserves colonic mucosal integrity and function in females during intestinal inflammation, contrasting with males.

As a prohormone, CHGA can undergo proteolytic cleavage, resulting in numerous CHGA-derived peptides with various biological activities that affect multiple physiological processes, including

mucosal functions and immune responses (330). In the GI tract, PST, a peptide derived from CHGA that has a role in glucose homeostasis, is significantly elevated in patients with active UC. It has been shown to have a similar effect to CHGA and correlates with increased levels of inflammatory mediators, intestinal permeability, reduced tight junctions, and worsened colitis severity (18,265). Consequently, PST could be a potential molecular target in UC therapy (18).

Consequently, we examined if PST inhibition with the competitive inhibitor PSTi8 could alleviate the harmful effects of PST in the colon during colitis in both males and females.

In this study, PSTi8 treatment significantly affected the homeostatic mucosal barrier function but modified the mucosa in colitic conditions to a lesser extent. Consequently, both females and males treated with PSTi8 showed reduced epithelial differentiation, potentially due to decreased expression of the transcription factor Atoh1, which is known to regulate the commitment of intestinal progenitors towards a secretory lineage under steady-state conditions. However, the mechanisms of Atoh1 inhibition in response to PSTi8 treatment appear to vary between the sexes. Thus, the increased expression of Hes1 may primarily be responsible for Atoh1 suppression in females, whereas in males, it is likely mediated through the BMP4 signaling pathway, given the elevated BMP4 gene expression following PSTi8 treatment under steady-state conditions.

Consequently, the sex-mediated differences in the suppression of Atoh1 expression in colon tissues between sexes likely explain the contrasting profiles in homeostatic intestinal differentiated epithelial cells after PSTi8 treatment. This treatment revealed reduced goblet and tuft cells in both sexes, along with lower gene expression of enteroendocrine cells, which was primarily observed in the male colon. Conversely, PSTi8 treatment was associated with enhanced proliferation in both

sexes and elevated HOPX⁺ reserve stem cells yet resulted in reduced fetal-like and CBC stem cells in the male colon. There was no significant difference in the levels of all three stem cell populations in females, suggesting that PST inhibition with PSTi8 might influence the proliferation of a yet-to-be-identified population of proliferative intestinal cells in the female colon. Additionally, females treated with PSTi8 exhibited lower levels of cytokines mediating intestinal barrier function, including IL-6 and IL-22, alongside increased expression of ER stress/UPR downstream signaling proteins associated with the PST/GRP78 signaling pathway at steady state. This suggests that PST inhibition may have altered mucosal function in the colon of females, potentially through GRP78 receptors.

Interestingly, PSTi8 treatment increased susceptibility to colitis in females but had a protective effect in males. Thus, males treated with PSTi8 displayed preserved CBC levels associated with increased goblet and enteroendocrine cells and a lesser severity of colitis, suggesting that PST inhibition maintains mucosal integrity in the male colon. Conversely, the colonic mucosa of females exhibited reduced proliferation linked to lower levels of fetal-like and reserve stem cells, an altered differentiated cell profile, and decreased ER stress/UPR downstream signalling. This indicates impaired restoration of cellular protein homeostasis, diminished cell survival, enhanced cell death, and disease progression.

GLOBAL CONCLUSIONS

Overall, this thesis demonstrated sex-related differences in the epithelium integrity and mucosal barrier function at steady state and in response to DSS-induced colitis. These differences correlate, in part, with sex-related differences in immune response profiles along the large intestine and altered reaction to the loss of CHGA and neutralization of PST between males and females in healthy and colitic conditions.

CHAPTER IX

GLOBAL STUDIES LIMITATIONS

Further investigation will be necessary to address the limitations of the studies embodied in this thesis. Here, we did not characterize the profiles of sex hormones, including estrogens, progesterone, and androgens. However, studies have demonstrated that sex hormones can influence epithelial integrity and function while modulating both innate and adaptive immune responses, which in turn can affect disease severity in IBD (14,153,155,157). Additionally, sex hormones can modify the microbiota's composition, diversity, and abundance. This altered microbiota can indirectly affect intestinal mucosal barrier function and repair capability, influence the immune response, and ultimately, colon mucosal homeostasis and the pathophysiology of ulcerative colitis (326). Secondly, we did not assess the gut microbiota and microbial metabolites. Nonetheless, several studies have shown that microbiota can modulate the integrity and function of intestinal epithelial cells and regulate the distribution profile and response of immune cells in the GI tract, either directly or by affecting the integrity of the intestinal epithelium. Therefore, further studies are necessary to explore the interaction between sex hormones, microbiota, epithelial cells, and the distribution and response of immune cells along the LI.

In this study, we used a systemic CHGA knockout mouse model to investigate the role of CHGA in the colonic epithelium. The systemic knockout mouse model has the advantage of providing more predictable, reproducible, and valuable insights into the role of CHGA in the gut. CHGA is secreted in various tissues and cells, including neuroendocrine tissues, where more than 80% of CHGA is stored, as well as in immune cells and the gut, which accounts for 5% of all stored CHGA

(391,392). CHGA is crucial during the formation of secretory granules involved in the packaging, storage, and secretion of hormones. Furthermore, as a pro-hormone, CHGA produces biologically active proteins that serve various functions. Thus, the complete deletion of CHGA, as observed in the CHGA^{-/-} mouse model, does not merely impact the gut but may also modulate other tissues and cellular functions. Therefore, we must exercise caution when interpreting the results obtained from this mouse model. The use of colonoid cultures to investigate the effects of CHGA deficiency on the colonic epithelium should partially address the potential issues related to exogenous factors. Additionally, a conditional CHGA knockout mouse model targeting the colonic epithelial enteroendocrine cells will help overcome the limitations associated with a global deletion of CHGA.

Finally, we employed an acute model of colitis in all studies to characterize the influence of CHGA deletion and the effect of PSTi8 inhibition on intestinal mucosa integrity, function, and immune response. However, this model is linked to excessive inflammation, severe colonic epithelial damage, and impaired mucosal functions. These factors could mask and hinder a comprehensive characterization of the roles of CHGA and PST in the colonic mucosa. Therefore, investigating the roles of CHGA and PST in the mucosa using a chronic DSS model of colitis with low to moderate mucosal damage may provide greater insights into their functions in chronic colitis.

GLOBAL SIGNIFICANCE

With an annual incidence rate estimated at 7.2% per year Canada-wide, ulcerative colitis (UC) can lead to severe complications, including delayed growth in children and increased risk for cancer in adults and severely negatively impact the quality of life of UC patients. However, the limited effectiveness of commonly available UC therapies for mucosal healing and long-term remission highlights the need to develop new and effective therapeutic strategies for UC. *Chromogranin A and pancreastatin could be exploited to create new unique and efficacious UC drugs that can induce and maintain complete mucosal healing and provide long-term remission for all patients with UC, and PSTi8 could be used as a novel targeted therapy for mucosal healing in UC.*

GLOBAL FUTURE RESEARCH DIRECTIONS

9.1. Short-term future directions

In Chapter IV, we discussed sex-based differences in colonic epithelial integrity and function at steady state and in response to DSS-induced colitis, which correlate with variations in epithelial cell proliferation and differentiation. However, the mechanisms underlying these differences are poorly understood. Therefore, future short-term directions should focus on profiling the expression of transcription factors that govern stem cell self-renewal (β -catenin and Notch1) and the differentiation and commitment of progenitors toward secretory or absorptive lineages (BMP4, Hes1, Atoh1) to investigate whether these signalling pathways differ between sexes. Moreover, assessing sex hormones in females (17 β -estradiol and progesterone) and males (testosterone) and their interaction with the colonic epithelial repair process and signalling pathways might provide insight into the role of sex hormones in mucosal healing at steady state and during inflammation.

In Chapter VI, we suggested that TGF- β likely plays a role in regulating inflammation and the mucosal healing process in the absence of CHGA. In addition, to identify the primary source of TGF- β , further research could explore whether TGF- β influences colonic epithelial proliferation and differentiation is mediated through the TGF- β /SMAD3 signaling pathway in the absence of CHGA in homeostatic and colitic conditions.

9.2. Long-term future directions

We recently demonstrated that elevated CHGA and PST are associated with increased markers of apoptosis and endoplasmic reticulum (ER) stress related to the autophagy mechanism. Given that dysregulation of autophagy contributes to the onset and progression of IBD, potential future directions for this project include investigating the role of CHGA, PST, and PSTi8 in ER-stress-mediated autophagy in UC.

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ANNEX I

Table 1. Narrative and sequence of selected genes

Target gene names		Primer sequences (5' to 3')	PCR temperature profile denature/anneal/extend.
TATA box protein (TBP)	Forward	ACCGTGAATCTTGGCTGTAAAC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	GCAGCAAATCGCTTGGGATTA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Mucosal integrity			
Trefoil factor 3 (Tff3)	Forward	TCT GGC TAA TGC TGT TGG TG	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	CTC CTG CAG AGG TTT GAA GC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Resistin-like molecule β (RelmB)	Forward	CCATTTCTGAGCTTTCTGG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	AGCACATCCAGTGACAACCA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Lymphocyte antigen 6 complex locus G6D (Ly6g)	Forward	GAGAGGAAGTTTTATCTGTGCAGC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	TCTCAGGTGGGACCCCAATA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Na-K-Cl co-transporter (Nkcc1)	Forward	CAGCCTGGACGAGCTGGTGG	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Reverse	TGACCAGTTGGGGTTCACAT	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
Cathelin-related antimicrobial peptide (Cramp)	Forward	CGAGCTGTGGATGACTTCAA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	CAGGCTCGTTACAGCTGATG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
WAP four-disulfide Core domain 2 (Wfdc2)	Forward	AGG TGG ACA GCC AGT GTT CT	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	GTGACCATAGCAAGACATGCA	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
Claudin 4 (Cld4)	Forward	GGCGTCTATGGGACTACAGG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	GAGCGCACAACCTCAGGATG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
LY6/PLAUR Domain Containing 8 (Lypd8)	Forward	GCCTTCACTGTCCATCTATT	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	GTGACCATAGCAAGACATGCA	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
Differentiated epithelial cells			

Carbonic anhydrases (Ca2)	Forward	CAAGCACAACGGACCAGA	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	ATGAGCAGAGGCTGTAGG	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
Mucin 2 (Muc2)	Forward	GAT GGC ACC TAC CTC GTT GT	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	GTC CTG GCA CTT GTT GGA AT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Chromogranin A (Chga)	Forward	CAGGCTACAAAGCGATCCAG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	GCCTCTGTCTTTCCATCTCC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Spi-B transcription factor (SPIB)	Forward	AGCGCATGACGTATCAGAAGC	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Reverse	GGAATCCTATACACGGCACAGG	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
Glycoprotein 2 (Gp2)	Forward	GATACTGCACAGACCCCTCCA	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Reverse	GCAGTTCCGGTCATTGAGGTA	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
Doublecortin Like Kinase 1 (DCLK1)	Forward	CTGGGTAAATGATGATGGTCTCC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	ACAGAAACTCCTGCTGCAGT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Stem epithelial cells			
Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)	Forward	CTTCCGAATCGTCGATCTTC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	AACGATCGCTCTCAGGCTAA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Homeodomain-only protein homeobox (HOPX)	Forward	TCTCCATCCTTAGTCAGACGC	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	GGGTGCTTGTTGACCTTGTT	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
Lymphocyte antigen-6 A (Ly6a/Sca1)	Forward	AGGAGGCAGCAGTTATTGTGG	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
	Reverse	CGTTGACCTTAGTACCCAGGA	95°C, 30 s / 52°C, 30 s / 72°C, 45 s
Lineage commitments			
Hairy and enhancer of split 1 (Hes1)	Forward	CTTATGAAAGTCAAGTAAAAGGACG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	ATAGGCTTTGATGACTTTCTGTG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Forward	GACAAATATCCCTGCACCCT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s

Atonal homolog 1 (Atoh1)	Reverse	CAGAGGCAGAGATACGACAT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Neurogenic locus notch homolog protein 1 (Notch 1)	Forward	CTGAGAGCTCCTGCTTCAAT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	AGTACCATAGCTGTCTTGGC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Bone morphogenetic protein 4 (Bmp4)	Forward	TTCCTGGTAACCGAATGCTGA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	CCTGAATCTCGGCGACTTTTT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Growth Factor Independent 1B (Gfi1b)	Forward	ATGCCACGGTCCTTTCTAGTG	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	GGAAGGCTCTGGTTCAGCAA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Krüppel-like factor 4 (Klf4)	Forward	CAGACCAGATGCAGTCACAA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	GTTTCTCGCCTGTGTGAGTT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
Neurogenin 3 (Neurog3)	Forward	TCTCGCCTCTTCTGGCTTTC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	AAGTCGGTGAAGAACGGACAA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
SRY-Box Transcription Factor 9 (Sox9)	Forward	CAAGCACAACGGACCAGA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	CAGCGCCTTGAAGATAGCATT	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
GRP78 signaling pathway			
Activating transcription factor 6 (Atf6)	Forward	CTGGGCTCGGTAGTTTGTATC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	AGACCTGAATGGCTGCTTAC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
X-box binding protein 1 (Xbp1)	Forward	CCTTCAGTGACATGTCTTCTCC	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
	Reverse	CCCAGTGTTATGTGGCTCTTTA	95°C, 30 s / 55°C, 30 s / 72°C, 45 s
C/EBP homologous protein (Chop)	Forward	GGAGGTCCTGTCCTCAGATGAA	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Reverse	GCTCCTCTGTCAGCCAAGCTAG	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
Glycogen synthase kinase-3 beta (Gsk3b)	Forward	GAGCCACTGATTACACGTCCAG	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Reverse	CCAACTGATCCACACCACTGTC	95°C, 30 s / 59°C, 30 s / 72°C, 45 s
	Forward	AGAAGGGTCTCCAAGGACGACT	95°C, 30 s / 59°C, 30 s / 72°C, 45 s

Mammalian target of rapamycin (mTor)	Reverse	GCAGGACACAAAGGCAGCATTG	95°C, 30 s / 59°C, 30 s / 72°C, 45 s

Table II: List of antibodies and gating strategy

Antibodies	Source	Identifier
		Catalogue numbers
LIVE/DEAD Fixable Aqua Dead Cell Stain	Life Technology Inc.	L34966
Fc Receptor Binding Inhibitor Polyclonal Antibody, eBioscience	Life Technology Inc.	14-9161-73
anti-mouse CD45 Pacific Blue	BioLegend	157212
anti-mouse CD45 APC/Cyanine-7		147717
anti-mouse CD45 Brilliant Violet 785™		103149
anti-mouse CD45 Alexa 700		103128
anti-mouse CD3 FITC	BioLegend	100204
anti-mouse CD4 PE/ Cyanine-7	BioLegend	100422
anti-mouse CD8 PE/ Cyanine-5	Cedarlane	10980-MM48-C
anti-mouse CD19 PE	BioLegend	115508
anti-mouse CD19 Alexa 700		152413
anti-mouse NK-1.1 APC/Cyanine-7	BioLegend	156510
anti-mouse/human CD11b Pacific Blue	BioLegend	101224
anti-mouse/human CD11b Brilliant Violet 421		101235
anti-mouse F4/80 FITC	BioLegend	123108
anti-mouse Ly6 PE	Cedarlane	E-AB-F1108D
anti-mouse CD103 APC/Cyanine-7	BioLegend	121432
Anti-mouse CD209/DC-SIGN Antibody (APC)	Cedarlane	10200-MM01-A-100
anti-mouse CD279 (PD-1) Brilliant Violet 605	BioLegend	135220
anti-mouse CD185 (CXCR5) Brilliant Violet 785	BioLegend	145523
anti-mouse CD25	BioLegend	102008
anti-mouse FOXP3	Cedarlane	20-0191-U100
anti-mouse CD209 APC	Cedarlane	10200-MM01-A-100

B220 Brilliant Violet 785	BioLegend	103245
GL7 FITC	BioLegend	144603
FAS PE-CY7	BioLegend	152617
Anti-mouse CD86-PerCP	Cederlane	CLX330PCP
CD80-APC	BioLegend	104713
Sca1- Brilliant Violet 421	BioLegend	108127
CD138 Brilliant Violet 650	BioLegend	142517
FAS PE-CY7	BioLegend	152617
IgA-PE	e-Bioscience	12-4204-82
IgG APC	BioLegend	405308
Major subset of immune cells	Gating profile	
Dendritic cells (DCs)	CD45 ⁺ F4/80 ⁻ CD103 ⁺	
Macrophages	CD45 ⁺ CD103 ⁻ CD11b ⁺ F4/80 ⁺	
Granulocytes	CD45 ⁺ CD103 ⁻ CD11b ⁺ F4/80 ⁻ Ly6g ⁺	
Natural killer (NK)	CD45 ⁺ CD3 ⁻ NK1.1 ⁺	
Natural killer T cells (NKT)	CD45 ⁺ CD3 ⁺ NK1.1 ⁺	
B lymphocyte cells	CD45 ⁺ CD3 ⁻ CD19 ⁺	
CD4 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺	
CD8 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD8 ⁺	
CD11B-CD103 ⁺	CD45 ⁺ F4/80 ⁻ CD11b ⁻ CD103 ⁺	
DCs CD11B ⁺ CD103 ⁺ DCs,	CD45 ⁺ F4/80 ⁻ CD11b ⁺ CD103 ⁺	
M1 macrophages	CD45 ⁺ CD103 ⁻ CD11b ⁺ F4/80 ⁺ CD86 ⁺	
M1 macrophages	CD45 ⁺ CD103 ⁻ CD11b ⁺ F4/80 ⁺ CD209 ⁺	
Regulatory T cells (Treg)	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD25 ⁺ FOXP3 ⁺	
Follicular T Helper cells (Tfh)	CD45 ⁻ CD3 ⁺ CD4 ⁺ CXCR5 ⁺ (393)	
Follicular cytotoxic T cells (Tfc)	CD45 ⁻ CD3 ⁺ CD8 ⁺ CXCR5 ⁺ (394)	
PD1 ⁺ CD4 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ PD1 ⁻	
PD1 ⁺ CD8 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD8 ⁺ PD1 ⁻	
Germinal center B cells	CD45 ⁺ CD19 ⁺ CD95 ⁺ GL7 ⁺	
Effector B cells	CD45 ⁺ CD19 ⁺ CD86 ⁺ CD80 ⁺	
Memory B cells	CD45 ⁺ CD138 ⁺ B220 ⁻	
Antibody-secreting plasma cells	CD45 ⁺ CD138 ⁺ B220 ⁺	
IgA-secreting plasma cells	CD45 ⁺ CD19 ⁻ CD138 ⁺ IgA ⁺	
IgG-secreting plasma cells	CD45 ⁺ CD19 ⁻ CD138 ⁺ IgG ⁺	

Table III. List of antibodies.

Antibodies	Sources	Locations	Identifier Catalogue Numbers
Rat Ki-67 Monoclonal Antibody (SolA15)	Invitrogen	Waltham, MA, USA	14-5698-82
Rabbit Keratin 20 (D9Z1Z) XP [®] antibody	Cell Signaling Technology	Danvers, MA, USA	13063S
Rabbit Anti-MUC2 (EPR23479-47) antibody	Abcam, Inc	Waltham, MA, USA	ab272692
Rabbit Chromogranin A antibody	Invitrogen	Winnipeg, MB, CA	PA5-16685
Mouse Doublecortin (E-6) antibody	Santa Cruz	San Diego, CA, USA	sc-271390
Rabbit LGR5/GPR49 antibody	Bio-Techne	Minneapolis, MN, USA	NBP1-28904
Rat LY-6A/E (D7) antibody	Santa Cruz	San Diego, CA, USA	SC-52601
Mouse HOP (E-1) (HOPX) antibody	Santa Cruz	San Diego, CA, USA	SC-398703
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Waltham, MA, US	A11006
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 647	Invitrogen	Waltham, MA, US	A21247
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Waltham, MA, US	A11034
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 647	Invitrogen	Waltham, MA, US	A21244
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor [™] 488	Invitrogen	Waltham, MA, US	A11001

ANNEX II

Tshikudi MD, Bernstein NC, Mishra S, Ghia J-E, Armstrong KH. Influence of biological sex in inflammatory bowel diseases. *Nat Rev Gastroenterol Hepatol*. 2025; (Update).

Influence of biological sex in inflammatory bowel diseases

Diane M. Tshikudi¹, Charles N. Bernstein^{2,3}, Suresh Mishra¹⁰, Jean-Eric Ghia^{1,4,5,11} and Heather K. Armstrong^{1-9,11†}

¹Rady Faculty of Health Sciences, Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

²Section of Gastroenterology, Department of Internal Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada

³University of Manitoba IBD Clinical and Research Centre, University of Manitoba, Winnipeg, MB, Canada

⁴Manitoba IBD Clinical and Research Centre, University of Manitoba, Winnipeg, MB, Canada

⁵Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg, MB, Canada

⁶Rady Faculty of Health Sciences, Department of Internal Medicine, Manitoba Center for Proteomics and System Biology, University of Manitoba, Winnipeg, MB, Canada

⁷Manitoba MS Research Centre, University of Manitoba, Winnipeg, MB, Canada

⁸Rady Faculty of Health Sciences, Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada

⁹Faculty of Agriculture, Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada

¹⁰Section of Endocrinology, Department of Internal Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada

¹¹These authors jointly supervised this work: Jean-Eric Ghia, Heather K. Armstrong

[†]E-mail:

Abstract

The incidence rate of inflammatory bowel disease (IBD) has risen in the past decades and has emerged as a global health issue. IBD is characterized by chronic inflammation of the gastrointestinal tract. There is compelling evidence for the role of biological sex in IBD epidemiology, pathophysiology, disease progression, symptoms and extraintestinal diseases. IBD disease course, management and therapies differ between men and women yet there is a paucity of analysis of sex as a factor. This Review discusses the known influence of sex-linked genetic factors, hormones, and hormone receptors in IBD incidence, prevalence, disease burden, and clinical manifestation. Furthermore, we review the mechanisms underlying these sex-dependent effects on the dysregulation of gastrointestinal mucosal immunity (immune, epithelial barrier, and microbiota in IBD. To support the progressive inclusion of sex in the study of IBD, we summarize the current standard research methodology that should be implemented to investigate sex as a biological variable in IBD studies. Enhanced comprehension of the influence of sex in IBD pathophysiology will advance the development of targeted therapies and improve patient care.

Introduction

Historically, biomedical research has lacked adequate representation of biological sex in preclinical and clinical research despite a growing body of evidence highlighting the role of sex differences in disease susceptibility, therapeutic strategies, and clinical outcomes (14,15,50,395–400). In current years, awareness of this issue has risen and led funding agencies and scientific journals to promote policies that require researchers to consider sex as a biological determinant to implement in the study design, data collection and analysis, and reporting of all preclinical and clinical research (401–403).

Inflammatory bowel diseases (IBD) include Crohn's disease, ulcerative colitis, and IBD unclassified (IBDU), which are chronic and severely debilitating gastrointestinal diseases of

unknown aetiology. Clinical studies have reported modest sex associations in IBD epidemiology, disease progression, symptoms, and response to therapy (50,397,404,405). Yet, the precise role of sex in IBD remains poorly understood (50,397,404,405). To better understand the effects of biological sex in IBD, research teams need to appropriately integrate sex considerations into study design, supporting the discovery of personalized therapeutic strategies (398,401). This Review discusses the current knowledge regarding the involvement of sex-related dysmorphisms in IBD, outlining the potential mechanisms involved and biases or differences associated with IBD pathology. Lastly, we discuss the current best practices of sex research methodology from study design, subject selection, and data collection to analysis, interpretation, and data presentation.

Defining sex and gender

With emerging evidence suggesting that sex and gender contribute to differences in IBD, it becomes imperative to define the terms sex and gender and to recognize that these definitions continue to evolve (50,397,404–406). Sex describes the biological attributes that account for physical and physiological characteristics of men, women, intersex, and individuals undergoing sex reassignment. Gender refers to socially constructed roles encompassing behaviours, identities, and expressions (407,408). Thus, biological sex is defined as the assigned sex, male or female, given to an individual at birth based on their biological sex attributes, including sex hormones, chromosomes, reproductive organs, and sexual anatomy. Typically, humans have forty-six chromosomes, of which two are sex chromosomes X and Y, which encode for individual biological sex features associated with the assigned sex at birth (409). In that context, the combination of two chromosomes X typically led to a female phenotype associated with the development of ovaries which produce sex hormones that regulate secondary sex determinants such as oviducts, uterus, cervix, clitoris, labia, vagina, and mammary glands. Meanwhile, an individual identified as male typically has one X and one Y chromosome and develops testes that produce sex hormones that promote the development of seminal vesicles and prostate gland (409). However, chromosomal, gonadal, or anatomical atypical development, termed disorder of sex development, can result in a mismatch between an individual chromosomal pattern, sex hormones profile, and reproductive system, leading to the birth of intersex individual with sex biological traits that do not fit the

dichotomic notions of female or male classification (410). Individuals with intersex traits, estimated to account for 0.018 to 1.7% of the global population, can be identified at birth owing to atypical sexual anatomy or later affected by post-natal virilization, delay or absence of puberty or infertility (410–412). Overall, these definitions emphasize the large spectrum associated with biological sex, increasing the complexity of investigating the sex-mediated difference in the biomedical field. Although complex, the attributes of biological sex are relatively well defined, therefore facilitating the investigation of their effect on IBD.

In contrast, the notion of gender is a more complex and fluctuating social construct that differs with culture, environment, age, and era, preventing the development of standardized gender scoring systems and limiting their general application to broad IBD research (407). As a multifaceted and fluid construct, the concept of gender is influenced by societal, cultural, and environmental factors to create gender norms which, in turn, affect individual behaviour, interaction with others, perception of oneself and others, along with gender distribution of power and resource in society (413). In that context, gender is often categorized along four constructs, including gender role, gender identity, gender relation, and institutionalized gender (413). Biological sex can affect the disease pathophysiology, manifestations, and response to treatment, whereas gender influences, healthcare-seeking behaviours, decision-making, and therapeutic response (414). Thus, poor integration of sex and gender in biomedical research can lead to false interpretation, misdiagnosis, delay in treatment, and increased risk for adverse drug reactions (414,415).

Sex factors on IBD clinical manifestation

Social determinants of health in IBD

Social determinants of health (SDOH) include socio-economic, the healthcare system, personal behaviours, and environmental conditions. Subdivided into intermediary SDOH, broadly categorized into food security, housing, transportation access, psychosocial, and socio-economic factors, SDOH is a determining factor that cause intrinsic societal stratification, creating and sustaining inequality across populations and shaping an individual and population health status (416,417). According to a population-based USA nationwide survey, 42% of patients with IBD

reported affected by food insecurity and reduced social support (418,419). In a population-based study from Canada using health administrative-based data with a cohort of 9,298 individuals with IBD, lower socio-economic status was linked to worsened health outcomes such as increased healthcare utilization and death for individuals with IBD (418,419). In a separate USA retrospective study using electronic medical records from 175 patients with IBD, increased risk for IBD-related hospitalization and surgery was associated with food insecurity, financial resources, and transportation, which affected more patients with Crohn's disease than ulcerative colitis (area under the receiver operating characteristic (AUROC): 0.86 and 0.68 for Crohn's disease and ulcerative colitis respectively), confirming the strong influence of SDOH in IBD outcome (420). Biological sex and gender interplay with other SDOH influencing the differential exposure to intermediary SDOH, which, in turn, affects the risk of exposure, use, and access to healthcare services and bias in healthcare systems, further shaping the development and implementation of health policies (420,421). Thus, sex and gender, along with other SDOH, have a strong influence on an individual or a community's experience of illness and health outcomes. Yet, data on sex and gender interaction with other SDOH in IBD outcomes are limited (418).

Sex differences in lifestyle behaviours in IBD

Numerous environmental factors, including lifestyle, pharmacologic agents, and ecological determinants, are sex and gender-specific and likely to contribute to IBD pathogenesis and severity. Men and women have considerable differences in substance exposure and lifestyle risk factors (55,422,423). Epidemiological and clinical studies strongly correlated poor diet, excessive alcohol consumption, smoking, and physical inactivity with a higher risk for chronic non-communicable diseases such as cardiovascular diseases, type 2 diabetes, cancer, and IBD and are strongly influenced by SDOH (423).

Studies across several countries worldwide report that women consumed more vegetables and fruit than men (423–426). By contrast, a few of these studies described a higher fat and meat consumption for men than women (425,426). Evidence associated dietary behaviours with IBD onset and disease severity: high consumption of dietary fibres from fruits and vegetables has been associated with a lower risk of developing Crohn's disease with no substantial changes in the risk

of ulcerative colitis (427). Conversely, high levels of total fat, saturated fat, polyunsaturated fatty acid, and omega-6 fatty acids intake increased the risk of developing ulcerative colitis and Crohn's disease. Some studies similarly reported an association between a high intake of meat and an increased risk for IBD (427). Despite the contradiction, several studies reported differences in macronutrient intake between patients with different IBD subtypes compared to the general population (428). Thus, patients with IBD had lower fibre consumption compared to health controls. When stratified by subtypes, A Dutch case-controlled study analyzing the dietary behaviours of 207 patients with ulcerative colitis stratified by sex reported a lower intake of total proteins, animal proteins, carbohydrates, and alcohol but increased fat consumption compared to control groups (429). These results were partially in line with several other studies (430,431). Similarly, patients with Crohn's disease had a lower intake of total protein, animal-based proteins, and alcohol (429). However, unlike in ulcerative colitis, the cohort of 286 patients with Crohn's disease consumed less plant-based proteins and reported a comparable fat intake to the control groups (429). The previously described Dutch case-controlled study of 493 patients with IBD reported negligible differences in dietary intake between men and women, in concordance with a German monocentric cross-sectional study with a cohort of 82 patient with IBD and age-matched cohort of 328 individuals representing the general German population, which described minor sex-related differences in the dietary behaviours of patients with IBD (429,432). By contrast, a systematic review with meta-analysis found a correlation between dietary protein intake and IBD risk in women but not in men in the Asian population from case-controlled studies performed in Iran, Israel and Japan (433). However, the study reported limited differences in IBD risk associated with elevated protein consumption in the European and North American population (433). In a USA cross-sectional population-based study with a cohort of 3 million individuals with IBD, men with IBD were more likely to consume vegetables once or more per day compared to men without IBD (84.9%, 95% CI 77.6–90.1; 76.0, 95% CI 75.0–77.0; $p<0.01$) and take any supplements (59.6%, 95% CI 46.8–71.3; 46.0, 95% CI 44.8–47.2; $P<0.05$) whereas women with IBD were more likely to consume sugar-sweetened beverages twice or more per day (26.8%, 95% CI 19.7–35.3; 17.8, 95% CI 17.0–18.7; $P<0.05$) and take vitamin D supplementation when compared to women without IBD (37.2%, 95% CI 27.9–47.5; 23.6, 95% CI 22.8–24.5; $P<0.01$) (434). No

significant differences in the intake of other vitamins were observed between women with and without IBD. These findings highlight the need to investigate and stratify data in macronutrient sources, dietary patterns, and geographical determinants.

According to the most current WHO 2019 report, an estimated 2.5 billion individuals 15 years and older consume alcohol worldwide (435). Although sex differences in alcohol consumption are universal, with a global prevalence rate of 52.2% for men and 35.4% for women, these differences vary markedly across geographical regions, countries, and cultures, with men to women ratio of current drinkers ranging, for example, from 1.29 in European Region to 2.68 in Eastern Mediterranean Region (435,436) (436). Moreover, current studies reported a rise in alcohol consumption for women but not for men, suggesting a narrowing in alcohol use gender gaps (437,438). However, women are more susceptible to alcohol use disorder (AUD), alcohol-related harms (memory blackout and hangover), and alcohol-associated medical consequences (alcohol-induced liver inflammation, cardiomyopathy, and breast cancer) than men (437–439). Although controversial due to insufficient and contradictory evidence, alcohol consumption might affect IBD pathophysiology, increase the risk of relapse, and enhance gastrointestinal symptoms potentially by altering the microbiota and promoting intestinal inflammation (440,441). Moreover, *in vitro* studies and clinical studies carried out on patients with autoimmune conditions suggested that alcohol consumption could interfere with the metabolism of IBD medications such as cyclosporin, Methotrexate, and 5-ASA by decreasing their efficacy or promoting liver disease (440,441). However, our understanding of the interplay between sex, alcohol consumption, and IBD is lacking and will require further investigation.

The Global Burden of Disease, Injuries and Risk Factors (GBD) Study reported proportion of adult smoking differs markedly between sexes, with a higher prevalence of smokers among men than women (32.6%, 32.2–33.1%, and 6.5%, 6.3–6.7%, respectively) (442). Tobacco smoking is the earliest and most extensively studied environmental risk factors that correlate with IBD natural history. In the past 40 years, several clinical studies have consistently reported a dichotomous relationship between smoking and the onset of IBD subtypes, as reports showed that smoking increased the risk of developing Crohn's disease (relative risk estimate (RR): 1.58, 95% CI 1.37–1.82, $P<0.001$) but has a protective effect in ulcerative colitis (RR: 0.55, 95% CI 0.48–0.64,

$P<0.001$) (443). Yet when stratified by biological sex, all data suggest that women with IBD are more susceptible to the harmful effects of smoking than men. A case-control study conducted in Sweden between 1984 and 1987 with 552 patients with IBD reported a five-fold increased risk for women to develop Crohn's disease in people who smoked compared to their nonsmoker counterparts (RR: 4.99, 95% CI 2.7–9.2; $P<0.001$) and unlike the 30% increased risk for Crohn's disease observed in men currently smoking (RR: 1.33, 95% CI 0.7–2.6, $P<0.001$) (444). In a population study with 1784 patients with IBD, smoking was also associated with an increased number of women with Crohn's disease diagnosed with more severe disease presentation (pancolitis: 46% compared to 32%, $P<0.01$) and an increased need for immunosuppressive therapy in people who smoked compared to nonsmokers ($58\pm4\%$ compared to $48\pm5\%$, $P<0.01$), whereas smoking did not significantly affect the disease course and severity in men with Crohn's disease (445). Moreover, in a small case-control study with 55 adults with Crohn's disease, the increased susceptibility to smoking was associated with higher levels of proinflammatory cytokines and decreased levels of regulatory T (T_{reg}) cells in women compared to men who smoked ($P=0.05$) (446). These findings corroborate the susceptibility of women with Crohn's disease to smoking and are consistent with observations from other studies (447,448). However, the effect of smoking on ulcerative colitis is unclear, as studies reported that women who currently smoked were less likely to develop ulcerative colitis compared to people who didn't smoke (444,449,450). Other studies find no significant differences in the development of ulcerative colitis between people who currently smoked and those who did not (447,451). The current findings result from studies conducted, for the most part, in a non-Jewish white population (452). Given the differential association between smoking in IBD and ethnicity, more studies will help to characterize the interaction between tobacco smoking in IBD and biological sex in other ethnic groups (452). Moreover, further studies will be required to clarify the underlying mechanisms that regulate the susceptibility of women with Crohn's disease and ulcerative colitis to smoking.

The World Health Organization considers insufficient physical activity as an emerging public health issue associated with increased risk factors for obesity, non-communicable diseases, mental health issues, and overall poor quality of life (453). In a population-based global survey conducted in 2016, insufficient physical activity was 27.5% (95% uncertainty interval (UI) 25.0–32.2) in

adults and 19% (95% UI 12.3–22.2) in adolescents (454,455). Moreover, across nearly all countries, girls and women (girls: 84.7%, 95% UI 83.0–88.2; women: 31.7%, 95% UI 28.6–39.0) were less active than boys and men (boys: 77.6%, 95% UI 76.1–80.4; men: 23.4%, 95% UI 21.1–30.7) with differences of prevalence of 7.1% for adolescents and 8.3% for adults (454,455). Despite the scarcity of studies investigating the effect of physical activity on IBD, most studies correlate positively physical activity with IBD onset and disease course. Several case-control and retrospective studies found that sedentary occupations increased the risk of developing IBD in men and women while increasing hospitalization and mortality rates in patients with (456–458). Additional case-control and prospective cohort studies associated infrequent physical activities in childhood with elevated risk of subsequently developing Crohn's disease and ulcerative colitis in adulthood (459). In contrast, increased physical activity in adulthood correlated with a reduced likelihood of developing Crohn's disease but not ulcerative colitis, regardless of ethnicity (460). Moreover, among patients with IBD, a higher level of physical activity correlated with a lower risk of active Crohn's disease, less extended ulcerative colitis, and reduced risk of relapse at six months in Crohn's disease and ulcerative colitis (461,462). These findings were consistent with two population-based prospective studies conducted with a cohort of 121,700 US women registered nurses and 240,984 Swedish men who underwent compulsory military conscription (463). Women who partake in vigorous physical activity had a lower risk of developing Crohn's disease than the less active women (HR: 0.63, 95% CI 0.42–0.95; $P=0.02$) (463). However, physical activity did not correlate with the likelihood of developing ulcerative colitis in women (463). Interestingly, in men, low fitness correlated with increased risk for Crohn's disease (HR: 1.62, 95% CI 1.31–2.00) and ulcerative colitis (HR: 1.36, 95% CI 1.17–1.59). Overall, these findings suggest a protective role for physical activity in IBD regardless of biological sex (464). Further investigations are needed to characterize the mechanism associated with the protective role of physical activity.

Sex differences in IBD epidemiology

In the past decade, the incidence and prevalence of IBD have steadily risen, becoming an emerging global health issue that affects 4.90 million individuals worldwide (465). Even though there are

conflicting results, mounting evidence points to sex biases in the epidemiology, pathogenesis, pathophysiology, disease course, and clinical outcomes of IBD (50,397,404–406). Sex bias refers to the underreporting or misreporting of demographic, social, and economic characteristics associated with one of the sexes. It is worth noting that sex differences in IBD seem to be an age-dependent relationship, further affected by factors such as geographical location (50,404). Moreover, the effect of sex differs between Crohn's disease and ulcerative colitis (50,404). Although IBD can develop at any age, the most common age for IBD diagnosis ranges from 10–30 years, accounting for >25% of all reported IBD cases (294). The known influence of sex in IBD epidemiology across age groups is visualized in **Figure 1**.

Sex differences in very-early onset IBD epidemiology

Very-early onset IBD (VEO-IBD) is a subset of paediatric IBD (pIBD) in which patients are diagnosed before 6 years of age (466,467). VEO-IBD accounts for 3–15.4% of all pIBD diagnoses and is sub-categorized by age into infantile-onset (<2 years) and neonatal-onset (by 28 days) IBD (466,468,469). Infantile-onset IBD is rare and equally distributed between Crohn's disease (36%), ulcerative colitis (31%), and IBDU (33%) (468–470). A multi-centre registry-based study with 1,370 patients reported that the proportion of diagnosed IBDU cases decreased markedly with age at diagnosis to ~18% for older paediatric patients (3–17 years) and 6% for adults (468). Ulcerative colitis is the most predominant form of IBD diagnoses between 3–5 years (47%; 35% Crohn's disease; 18% IBDU), contrasting with the higher prevalence of Crohn's disease (60–66%) in older paediatric patient age groups (468).

Although there is a higher prevalence of boys being diagnosed with infantile-onset IBD (63%) compared to girls, studies have not identified a significant difference in the distribution of sexes within each VEO-IBD age group (468,469). However, a Canadian population-based retrospective study with a cohort of 7,143 paediatric patients investigating the incidence and outcomes of pIBD suggested differences in clinical outcomes between boys and girls in the VEO-IBD age group (471). The researchers found that girls in the VEO-IBD age groups had lower outpatient physician visits (odds ratio (OR), 0.70, 95% CI, 0.50–0.98 Crohn's disease; 0.75, 95% CI, 0.57–0.97 ulcerative colitis) and lower hospitalization rates (HR, 0.56; 95% CI, 0.37–0.85; $P<0.006$; Crohn's

disease) compared to boys (HR, 1.07; 95% CI, 0.81–1.41; $P<0.65$). In contrast, boys diagnosed with ulcerative colitis in the VEO-IBD age groups had a lower risk of undergoing a colectomy (HR, 0.42; 95% CI, 0.21–0.85) compared with girls in the same age group (HR, 0.88; 95% CI, 0.47–1.63) (471). The limited number of VEO-IBD cases might hinder the investigation of the impact of sex within that population, highlighting the importance of conducting more extensive multi-centre and international cohort studies. However, when designing these studies, it is essential that teams recognize that environmental factors and geographical location also seem to influence sex associations in IBD (50,274,472).

Sex differences in IBD epidemiology in children and adolescents

Roughly 10-25% of all patients with IBD are diagnosed before 20 years of age, although the global paediatric incidence of IBD varies greatly between geographical locations (4,468,473–476). Most systematic studies since 1950 have reported rapid growth in the incidence and prevalence of pIBD (473,476). Age-stratified studies of Western and Korean patients with IBD showed that the highest prevalence of pIBD occurred during adolescence (10–17.9 years) (474,476–480). However, the age of referral from paediatric to adult care for IBD management differs substantially within various literature sources, therefore making the comparison of studies of patients with pIBD across diverse populations challenging (475).

Despite regional variability, the prevalence and incidence of Crohn's disease and ulcerative colitis consistently differ markedly between pediatric boys and girls (3). Large population-based studies in European, North American and East Asian pediatric populations reported a boys predominance in Crohn's disease prevalence (male: female, 1.34–6.5:1) and incidence rates (male: female, 1.32–1.91:1) compared to girls (474,476,477,480–483). In contrast, the incidence and prevalence of ulcerative colitis by biological sex in the pediatric population are ambiguous. Most studies reported a similar rate of paediatric ulcerative colitis between boys and girls (50,481,484). A retrospective population-based German study of pIBD in a cohort of 712 patients reported a similar incidence rate (male: female, 0.95:1) and a lower prevalence of boys with ulcerative colitis to girls (male: female, 0.7:1) (477). Subsequently, a Western study with a cohort of 236,181,614 men and 242,373,419 woman found an increased incidence rate of ulcerative colitis in girls aged 5–9 years

with a ratio of (female: male) 1.22:1,(50) supported by a multi-centre study with a cohort of 196 patients with IBD from Switzerland, which reported increased paediatric ulcerative colitis predominance in girls (female: male; 1:0.78) (482). In contrast, studies in Asian-Pacific pediatric populations reported a significantly ($P<0.05$) increased incidence of ulcerative colitis in boys (male: female, 3.1:1) compared to girls, beginning in mid-late adolescence (>15 – 19 years) (274,474). Factors including regional variability, relatively small cohort sizes, and the variability in the stratification of paediatric age groups might explain the discrepancy noted in the conflicting studies (475,480).

Sex bias in paediatric IBD extra-intestinal clinical manifestation

Cross-sectional studies using an administrative database from 44 affiliated children's Hospitals with a cohort of 5,782 hospitalized US patients with pIBD showed that adolescent girls are more likely to have anaemia (29–25% $P=0.012$), hypoalbuminemia (75–57% $P=0.01$), mood disorders (9–6%, $P<0.001$) and infection (12–8%, $P=0.001$) than boys. While adolescent boys with pIBD are affected by delayed in maturation including growth and sexual development more than girls (3% versus 1%, $P<0.001$) (294,483,485). One report with a cohort of 5782 adolescents with CD suggested that boys are affected by more malnutrition than girls with pIBD (18% versus 14%, $P=0.027$), although the investigators did not define malnutrition in that report ⁹⁷. In individuals who experience IBD onset before puberty, studies with a cohort of 87 and 34 individuals reported that the disease was commonly accompanied by delayed puberty, although this occurred more frequently in Crohn's disease than in ulcerative colitis (486,487). Hence, paediatric patients with Crohn's disease were more likely to present with delays in bone development, testicular enlargement, pubertal growth spurts, breast development, and menarche (486–488).

Sex differences in IBD epidemiology in adults

Most commonly diagnosed between the age of 15–30 years, the prevalence of IBD increases with age and peaks between age 50–54 years for women and 60–64 years for men (465). Comprehensive analyses of the global IBD trend revealed that women have a higher prevalence of IBD than men, accounting for 57% of all cases (465,489). Despite the discrepancy in the reported results, most studies described a sex bias in the epidemiology of Crohn's disease and, to a lesser extent,

ulcerative colitis (490). Investigations in Western countries observed a predominance of adult Crohn's disease in women compared to men (female: male, 1.20:1), (50,54,491) with a shift in the sex ratio of Crohn's disease incidence from male predominance (incidence rate ratio (IRR) for women to men, 0.83–0.70:1) from ages 5–14 years to a women predominance (IRR, 1.27–1.38: 1) from 25–75 years (50). On the other hand, epidemiology surveys across Asian reported a higher prevalence of Crohn's disease in male than female patients (female: male, 0.5:1) in all age ranges from 10–50 years (274,484,490).

Several European, American and Asian-based population studies reported a predominance of ulcerative colitis in men (women to men IRR, 0.74–0.8:1), but data are inconsistent with other studies demonstrating no significant difference (50,274,469,484,492–496). In contrast, studies in Macau with a cohort of 500,000 individuals and Hungary found a higher incidence rate of ulcerative colitis in women than in men (50,497). Taking age into account, the incidence rate of ulcerative colitis was comparable between both sexes from 10–45 years (IRR, 1.01–1.07:1) in patients with IBD from Western regions; however, the prevalence was higher in men than women (IRR, 0.83–0.92:1) after 45 years (50). Similar results were reported in Asian-Pacific patients with ulcerative colitis, indicating a higher incidence of ulcerative colitis in male individuals between the ages of 15–65 (274,404,484).

The shift in the biological sex ratio of Crohn's disease incidence from men to women predominance and of ulcerative colitis from women to men predominance occurs around puberty and menopause, hinting toward a role for sex hormones in the pathogenesis of both Crohn's disease and ulcerative colitis (50). Moreover, the sex distribution of IBD epidemiology differs markedly between different geographical regions, suggesting a potential role for environmental exposure in sex-mediated IBD pathogenesis (50,274,484). Consequently, this underscores the need to investigate unexplored countries and territories worldwide, focusing on well-powered cohort studies.

Sex bias in adult IBD extra-intestinal clinical manifestation

Extra-intestinal clinical manifestation and complications, a hallmark of IBD, can affect the musculoskeletal, ocular, cutaneous, hepatobiliary, renal, pulmonary, cardiovascular, and

hematological systems (498). According to several clinical studies, the prevalence of extra-intestinal clinical manifestation in patients with IBD ranges from 6-47%, although these prevalences differ in a sex-dependent manner (54,59,490,498–500). Thus, a Dutch IBD biobank study with a cohort of 2118 patients with Crohn's disease and 1269 patients with ulcerative colitis reported that extra-intestinal clinical manifestations are more common among women with IBD than men with IBD (54). Accordingly, cutaneous and musculoskeletal manifestations (arthritis and arthropathy) affect significantly more women with Crohn's disease and ulcerative colitis than men with both IBD subtypes. Conversely, men with Crohn's disease have a higher prevalence of osteopenia than women with Crohn's disease ($P<0.01$) (54). A population-based longitudinal study with a cohort of 4454 patients with IBD for 10 years or more found an increased association between Crohn's disease and the prevalence of erythema nodosum (1.9%-7.2%) (59). The same study found that primary sclerosing cholangitis and pyoderma gangrenosum are more prevalent in ulcerative colitis than in Crohn's disease. Iritis and uveitis have similar rates between Crohn's disease and ulcerative (59). When stratified by biological sex, women with ulcerative colitis are more likely to develop iritis/uveitis (women to men: 3.2% versus 0.9%, $P=0.01$) and erythema nodosum (women to men: 3.2–0.9%, $P=0.01$) than men with ulcerative colitis (women to men: 1.9% versus 0.7%, $P=0.02$) (59). By contrast, men with ulcerative colitis are more affected with primary sclerosing cholangitis compared to women with ulcerative colitis and men with Crohn's disease. While ankylosing spondylitis is more common in men with Crohn's disease than men with ulcerative colitis and women with either of both IBD subtypes (59).

Meta-analyses and population-based studies reported sex-based differences associated with IBD-mediated complications such as cancer and cardiovascular disorders (498). Thus, a large population-based Swedish study with a cohort of 7607 patients with IBD reported that both men and women with IBD had a higher risk of developing colorectal cancer compared to the general population (Men: RR, 2.6; 95% CI, 2.2–3.1, women: RR, 1.9; 95% CI, 1.5–2.4) (501). Although, men with IBD had a 60% higher risk of developing colorectal cancer than women with IBD (cumulative incidence 8.3% versus 3.5%) (498,501). Conversely, several studies reported that patients with IBD have an increased risk for cardiovascular disease compared to the general

population (498,502,503). Moreover, cardiovascular diseases are more common among women with IBD than men with IBD (498,502,503).

Differences between sexes can also occur in response to IBD therapies such as corticosteroids, anti-inflammatory drugs, immunomodulators, and biologics (TNF blockers) (399,504). Cross-sectional studies and retrospective cohort studies have shown that women with IBD had an increased risk of developing intolerance to azathioprine (RR=1.56 $P=0.038$) (294,505). Moreover, women with IBD had increased hypersensitivity reactions to the biologic infliximab, reduced responsiveness to the biologic adalimumab, and increased side effects to TNF blockers, resulting in lower drug persistence to biologic therapy for women and more shift to corticosteroid treatment (294,506). Furthermore, a meta-analysis of observational studies on vedolizumab effectiveness and safety reported an increased clinical remission rate at maintenance (OR 1.021, 95% CI, 1.003–1.040, $P=0.043$), induction (OR 1.027, 95% CI, 1.006–1.049, $P=0.015$) and higher clinical response rate (OR 1.025, 95% CI, 1.006–1.046, $P=0.0022$) for men with Crohn's disease but, no significant difference between sexes in ulcerative colitis (504).

Sex differences in IBD disease severity

Managing the symptoms of IBD requires lifelong treatment, with severity being categorized as remission, mild, moderate, or severe (507). IBD severity is determined by evaluating the effect of the disease on the patient's symptoms and quality of life, inflammatory burden, and disease course using various grading scales (507–509). Although studies have found a potential sex bias in the presentation of IBD severity, the results have been contradictory (490). Phenotypic studies comparing the severity of IBD diagnosed in children and adults have shown that paediatric Crohn's disease and ulcerative colitis have a more severe phenotype characterized by a more extensive, severe, and aggressive disease course than those diagnosed as adults (481,510–512). When subdivided by sex, a Canadian population-based study derived from administrative health data with a cohort of 3,404 patients with IBD suggested that girls with IBD <21 years of age had a greater risk for hospitalization and emergency department utilization but no disparity in the rate of surgery in comparison to boys (513).

A retrospective cohort study of 989 pediatric patients demonstrated that girls with Crohn's disease had a more burdensome disease course defined by higher incidence (girls: 10 years cumulative incidence 40.2%; 95% CI, 27.9–55.4%; boys: cumulative incidence 20.6%, 95% CI, 15.5–27.0%) and increased risk for surgery than boys (H.R. 1.42; 95% CI, 1.004–2.02, $P<0.05$) (483,514). However, when age and sex were taken into account, a population-based study with 7143 patients with pIBD, only girls with VEO ulcerative colitis had lower rates of Crohn's disease-related outpatient visits, hospitalization, emergency department utilization, and surgery compared to older paediatric patients > 6 years; no differences were noted between boys with VEO Crohn's disease and older pediatric patients (boys) (471). Although children with Crohn's disease present predominantly with an ileocolonic disease distribution (469,482,483,515,516)(482,515), sex has not been associated with disease localization and behaviour (fistula, stricture) (482,483). Girls with VEO ulcerative colitis displayed lower rates of outpatient visits (OR 0.75; 95% CI, 0.63–0.94, $P<0.0001$), whereas boys with VEO ulcerative colitis displayed a lower risk for surgery (HR, 0.42; 95% CI, 0.21–0.85) than older paediatric patients with IBD (471). The hospitalization rate was higher in patients with VEO ulcerative colitis than in older paediatric patients and did not differ between the sexes (471). Although the phenotype of ulcerative colitis in paediatric patients has been shown to have a high bias toward extensive disease and pancolitis, no difference was noted between sexes across ages (481,482)(481,482,512). These findings suggest more limited sex bias in paediatric patients with ulcerative colitis compared to Crohn's disease. Limited population-based studies in paediatric IBD are stratified by age and sex. Hence, there is a need for further studies to understand the underlying mechanisms associated with sex bias in patients with VEO Crohn's disease and older paediatric patients.

Sex differences in disease location have not been reported in most adult IBD studies.(54,500,517) However, prospective Dutch studies with an overall 5,739 patients with IBD found that men with Crohn's disease had a significantly higher incidence rate of ileal disease (28%) compared to women with Crohn's disease (20%; $P<0.01$), whereas women with Crohn's disease were more likely to have colonic disease (33%) compared to male patients (26%; $P<0.01$) (54). In ulcerative colitis, proctitis was more frequent in women (10%) than in men (5%) (54,517).

Sex differences have also been reported in adult IBD activity, disease progression, and extraintestinal manifestations. Women with Crohn's disease are more likely to have more extraintestinal manifestations than men (52). Yet, studies show that men had a higher rate of surgery compared to women, with a greater frequency of procedures including stricturoplasty (6% versus 3%, $P=0.01$), small bowel resection (16% versus 9%, $P<0.01$), and ileocecal resection (40% versus 33%, $P<0.01$) (52,54). In contrast, there were no significant differences between men and women regarding disease activity, inflammatory burden, disease progression, risk of colectomy, and hospitalization for ulcerative colitis (52–54). Despite the clinical studies indicating sex differences in IBD, particularly Crohn's disease course, the underlying mechanism by which sex bias affects IBD severity has yet to be investigated.

Sex chromosomes and hormones in IBD

This influence of biological sex on innate and adaptive immune cell functions is attributed to genetic and physiological factors such as the sex chromosomes (heterologous XY for males and the homologous XX for females), reproductive organs (ovaries and testes), and sex hormones (such as estrogens, progestogens and androgens) (14). Hence, the effects of sex differences on the host immune response can substantially affect an individual's susceptibility to cancers, infectious diseases, autoimmune diseases, and other conditions influenced by dysregulated immune responses, such as IBD (398). Sex differences affect known IBD risk factors such as epithelial barrier, mucosal immunity, microbiota, and environmental triggers (140).

Sex chromosomes in IBD pathogenesis

In humans, the male chromosome Y contains approximately 100 genes, including genes that code for immune regulatory proteins involved in autoimmune and infectious diseases (14). Conversely, the human X chromosome contains more than 1,100 genes, including proteins related to pattern recognition receptors, cytokine receptors, transcription factors (elements of nuclear factor κ B, GATA1, and forkhead box 3), microRNAs, and long non-coding RNAs (14,398). Many of these genes are found to be altered in settings affected by dysregulated immunity, such as IBD (14,518).

Figure 2 visualizes the known links between sex chromosomes and hormones in IBD onset and progression across age groups.

Genome-wide association studies have identified approximately 300 genetic loci associated with monogenic and polygenic IBD (519). Of these loci, several have been linked to sex chromosomes (520). Most sex chromosome loci associated with IBD discovered so far include mutation in genes related to the X chromosome, also termed X-linked genes (for example, *IKBK* (also known as *NEMO*), *FOXP3*, *XIAP*, components of NADPH oxidase complex, *BTK*, *CD40L*) (520). Mutations of X-linked genes have been associated with the onset of various clinical features of IBD; for example, mutations in *NEMO* have been linked with epithelial barrier dysfunction (520,521). X-linked gene disorders are monogenic and primarily affect younger patients with IBD: 63.4% of patients with VEO-IBD, 17.4% of adolescents, and 10.9% of those over 18 years old (522). IBD X-linked gene disorders affect male and female patients, although female individuals harbouring only one X-gene copy might exhibit a milder phenotype (521). As mentioned earlier, the Y chromosomes encode genes that are shown to regulate the immune response (14); however, the exact contribution of these genes in IBD pathogenesis remains to be investigated.

Sex hormones in IBD pathogenesis

The sex hormones estrogens, progesterone, and androgens are primarily produced in the gonadal organs but also, in lesser amounts, in non-gonadal organs such as the placenta, skin, liver, heart, adipose tissue, adrenal glands, brain, and intestinal mucosa (65,66). The concentration and type of sex hormones vary substantially throughout the lives of both female and male individuals. The primary female sex hormones include progesterone and estrogens. There are four main types of endogenous estrogens, including estrone, 17 β -estradiol, estriol, and estetrol (65,67). Of these, 17 β -estradiol is the most potent and predominant estrogen in females during the reproductive years. Estrone, a weaker isoform of estrogens, is present in more marked amounts post-menopause, whereas estriol and estetrol are primarily found during pregnancy (67). Progesterone and estrogens are also found in male individuals but in lower amounts: Progesterone is synthesised mainly in the testes and adrenal glands, whereas 80% of circulating estrogens are produced within tissues by converting androgen testosterone into estrone and 17 β -estradiol (68,69). Androgens, such as testosterone and its more biologically active form, dihydrotestosterone, are the most abundant sex hormones in male persons, whereas they are produced in smaller amounts in female individuals (64).

Sex hormones vary substantially between sexes and across different age groups. Studies in prepubertal paediatric healthy individuals showed that girls had higher concentrations of 17 β -estradiol than boys, whereas testosterone concentrations were comparable in both sexes (76,77). These trends remain similar in ages 6 to 11 (78). As individuals progress through puberty (11–16 years) and transition into adulthood, sex hormone levels increase, shift between sexes, and peak between 20–29 years (78,79). However, as men and women age, the concentration of sex hormones gradually decreases (79). During menopause, women experience a drastic decrease in 17 β -estradiol and progesterone concentrations, similar to or lower than that observed in men (79–81). In healthy men with average body weight, the serum concentration of testosterone, 17 β -estradiol, estrone, and progesterone also decreased with age, although not to the same extent as in women (79–81). Sex hormones have been shown to affect gut function, IBD onset and disease severity (**Fig. 2**) (294). An observational study evaluating the relationship between sex hormones and systemic inflammation in 72 pubertal and post-pubertal patients with Crohn's disease reported that treatment with infliximab, an anti-TNF biologic, decreased inflammation and disease activity while rapidly increasing testosterone and 17 β -estradiol levels in boys and girls, respectively (488). Changes in the concentration of sex hormones promoted sexual maturation assessed using the Tanner scale, which grades the stage of sexual development according to the development of secondary sexual characteristics for both sexes within 10 weeks of initiating infliximab treatment (488). These data validated preclinical studies that suggested a regulatory role for systemic inflammation in promoting the production of sex hormones (523–525).

It is widely accepted that fluctuation of sex hormones related to the menstrual cycle can modulate gastrointestinal function and lead to gastrointestinal and non-gastrointestinal symptoms (82,83). Although menstrual changes in gastrointestinal function are common in healthy individuals, they are more pronounced in those with IBD and can result in symptoms such as constipation, nausea, vomiting, abdominal pain, flatulence, fatigue, irritability, and anxiety, among others (82,83). Further, in individuals from whom the onset of IBD occurred after puberty, the disease has been associated with changes in menstruation frequency, length of cycle, volume of flow, and level of pain (526). It is worth noting that in a cross-sectional study with a cohort of 1203 patients with IBD, female patients with ulcerative colitis had a higher risk of experiencing worse symptoms

compared to patients with Crohn's disease (84). Although still under investigation, research suggests that uterine prostaglandin might have a role in the gastrointestinal functional changes associated with menstruation (527). Preclinical and clinical evidence demonstrated that prostaglandin E₂, which is increased during menstruation, promoted inflammation in IBD by reducing microbiota-dependent intestinal T_{reg} cells (82,528). Yet, several studies found that hormonal contraceptives did not necessarily aggravate symptoms during menstruation for most patients with IBD. Indeed, 75–85% of 139 patients with IBD showed no changes in symptoms, 3–20% reported improvement, and only 5–7% reported worsening symptoms (82). In a case-control study investigating the association between circulating androgens and the risk of IBD, the researchers noted that elevated endogenous testosterone concentration was associated with a lower risk for Crohn's disease in women but not for ulcerative colitis (95).

Sex hormone therapy in IBD pathogenesis

Meta-analysis of 20 case-controlled and cohort studies reported a 32% higher risk for women using oral contraceptive pills (OCPs) to develop IBD than non-OCP users (87). When stratified by disease subtypes, women exposed to OCP were 24% and 30% more likely to develop Crohn's disease and ulcerative colitis, respectively, compared to the non-OCP exposed women (OR: Crohn's disease 1.24 $P=0.001$, ulcerative colitis 1.25–1.30 $P<0.05$) (85,87,88). Interestingly, studies have reported conflicting results on the relationship between exposure to OCPs and smoking in women and their effect on ulcerative colitis risk, with some reporting a lower risk and others showing a higher risk (85,88,89). A case-controlled study with a cohort of 4932 patients with IBD matched to 29 340 healthy individuals, published in 2022, investigating the effect of OCP formulation, dose, length of treatment, and method of delivery on the risk of developing IBD reported a higher risk for Crohn's disease and ulcerative colitis among combined OCPs (COCPs) users (estrogens plus progesterone) compared to non-users (89). These risks increased each month of COCP exposure per year, with a higher risk of 6.4% for Crohn's disease and 3.3% for ulcerative colitis compared to women not exposed to COCPs (89). The dose of OCP was low in contrast to standard estrogen pills and did not influence the risk for Crohn's disease or ulcerative colitis (89). In contrast, the formulation of COCPs influenced the risk for Crohn's disease, with a higher risk for women using second-generation COCPs compared to newer-generation COCPs, but it did not

influence the risk for ulcerative colitis (89). Notably, progesterone-only pills had no effect on Crohn's disease risk, whereas it was associated with a slight increase in ulcerative colitis risk (OR: 1.3) (89). Moreover, parenteral contraception did not affect the risk for IBD (89), suggesting a differential function for estrogens and progesterone in the onset of IBD subsets with a higher role for estrogen in Crohn's disease and a prominent role for progesterone in ulcerative colitis.

One large retrospective cohort study with 108844 postmenopausal US women found that hormone replacement therapy increased the risk of ulcerative colitis in healthy women after menopause but not for Crohn's disease (86). Research reported that 2–3.4% of patients with endometriosis, an estrogen-dependent condition, also had IBD, whereas 0–1% were reported in the general population (529). Moreover, a Danish nationwide study, 37 661 women hospitalised with endometriosis were more likely to develop IBD (standardized incidence ratio (SIR), 1.5 $P<0.05$), Crohn's disease (SIR, 1.6 $P<0.05$) and ulcerative colitis (SIR, 1.5 $P<0.05$) (530). In contrast, hormone replacement therapy taken during the first 2 years after the onset of menopause was associated with decreased IBD disease activity and more conservative treatment management (531). These studies suggest a potential role for elevated estrogen levels in the onset of IBD, and further investigation will be needed to understand the relationship between the level of estrogens and IBD pathogenesis.

Although the role of external hormones in women with IBD has been studied extensively, less is known about their role in men. Chronic states of low-grade inflammation have been shown to decrease testosterone levels (93). A cumulative prospective study that followed 92 patients with Crohn's disease and testosterone deficiency (≤ 12.1 nmol/L) who were treated with testosterone for 7 years, reported that normalizing testosterone levels led to a decrease in Crohn's disease activity index, inflammatory markers, and neutrophil infiltration (532). In contrast, a cohort study conducted on 31,842 newly diagnosed men with prostate cancer who were treated with androgen deprivation therapy reported a lower risk for ulcerative colitis (HR 0.52 $P<0.05$) and no significant risk for Crohn's disease in that population (HR 0.32) (94). This finding indicates a potential role for androgen in IBD pathogenesis that warrants further investigation. Despite an extensive number of studies investigating the role of estrogens in women with IBD, the role of estrogens and progesterone in IBD pathogenesis in men remains unclear and necessary for further investigation.

Gender dysphoria, which refers to a psychological distress one experiences as a result of divergence between one gender identity and sex assigned at birth, affects children, adolescents, and adults(533). Moreover, the prevalence of self-identified transgender and nonbinary (TGNB) individuals in Canada and US is estimated to be between 0.24 and 2.7%, of which a large number are youth (533–535). Gender-affirming hormone therapy (GAHT), which includes pubertal blockers and gender-affirming hormones (estrogen and testosterone), is commonly used to treat gender dysphoria in TGNB individuals (536). Pubertal blockers such as gonadotropin-releasing hormone analogues serve to suppress gonadotropins (follicle-stimulating hormone, Luteinizing hormone) and are used by adolescents primarily to prevent testosterone or estrogen production. Moreover, TGNB individuals can undergo feminizing hormone therapy, which utilizes estrogen (17β -estrogen) along with androgen receptor blockers to prevent masculinization, whereas masculinizing hormone therapy uses testosterone (536). GAHT in TGNB individuals is associated with an increased risk of poor bone development, abdominopelvic pain, long QT syndrome, thromboembolism, and infection, which are all potential risk factors for IBD (536). A current retrospective chart review study by Hassan and colleagues with 23 TGNB patients with IBD reported a trend toward a higher prevalence of Crohn's disease than ulcerative colitis (59.1% versus 40.9%, $P<0.23$). The incidence of flare was not associated with GAHT and did not differ significantly between TGNB individuals undergoing feminizing or masculinizing hormone therapy (537). Prospective studies with large populations will help understand the interaction between IBD and GAHT. Moreover, they will provide insight into the effect of known IBD risk factors in the TGNB population (537).

Fertility in IBD course of disease

Terminologies and definitions used to discuss reproductive capacity can be ambiguous as their usage often differs across research fields and medical settings as well as among various populations (538). The latest international glossary on infertility and fertility care defined several reproductive concepts such as fertility, fecundity, fecundability, infertility, and total fertility rate (539). In that context, fertility describes the capacity to establish a clinical pregnancy. Fecundity

corresponds to the capacity to have a live birth. Fecundability is the probability of pregnancy to occur in one menstrual cycle. Infertility is associated with a failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse (539). The total fertility rate corresponds to the average number of living births per woman (539). Parity corresponds to the number of offspring born from an individual. The fertility rate has several definitions, including the number of children per woman, the number of live births conceived in a year per 1,000 individuals of reproductive age, or the percentage of pregnant women in a population.

According to several population-based and referral centre-based studies across Western countries, IBD can marginally decrease fertility in women (540,541). Fertility assessment in a large population study of 27,331 women with IBD identified from the Swedish National Patient reported a reduced fertility rate of 1.52 ± 1.22 in women with IBD in comparison to 1.62 births per 1000 person-years in matched control individuals ($P < 0.0001$) (541). Fertility was decreased in Crohn's disease (HR 0.88, 95% CI 0.85–0.91) and IBDU cohorts (HR 0.86, 95% CI 0.83–0.89) and marginally reduced in the ulcerative colitis cohort (HR 0.96, 95% CI 0.93–0.98) compared to the matched control cohort (541). The same study reported a reduced parity progression ratio, defined as the probability of having additional children, in women with Crohn's disease (nulliparous to parity of 1, OR: 0.89, 95% CI 0.85–0.94, $P < 0.0001$) and IBDU (nulliparous to parity of 1, OR: 0.87, 95% CI 0.82–0.92; $P < 0.0001$) but not with ulcerative colitis in comparison to the control group (nulliparous to parity of 1, OR: 1.04, 95% CI 0.98–1.09; $P = 0.177$). Overall, these results corroborate a previous report from population-based and referral centre-based studies with a total of 946 patients, which described a decline of fertility between 17–44% for patients with Crohn's disease and no significant reduction in fertility for women with ulcerative colitis (540). However, the lower fertility rate in women with IBD resulted in most cases from psychosocial rather than physiological factors (540,542,543). Prospective international multi-centre and case-control studies reported that a higher percentage of women with IBD expressed concerns about the heritability of the condition, the risk of congenital anomalies during pregnancy, the potential teratogenic effects of medications, and the inability to support financially raising a child (542,544–547). As a result, some choose not to have children (544,545). However, extensive population-based studies have shown that the rate of involuntary infertility associated with fecundity issues

among women was similar to that of the general population (540–544). Reduced fecundity in IBD can result from active gut inflammation, pelvic infection, and surgical interventions (547). A systematic review and meta-analysis population-based study that included 10131 and 11335 patients with Crohn’s disease and ulcerative colitis, respectively, reported that patients with IBD refractory to medication or who present with complications have an increased risk of 46.6% (95% CI, 37.7–57.7%) for Crohn’s disease and 15.6% (95% CI, 12.5–19.6%) for ulcerative colitis to undergo abdominal surgical intervention ten years after diagnosis (548). Despite the scarcity and poor quality of existing studies investigating the effect of surgery in women with IBD fertility, a study published in 2021 by Lee and colleagues reported a lack of evidence to support the conclusion of the role of surgery in women with IBD involuntary infertility (549). This finding differs from several systematic reviews, meta-analyses, and retrospective studies which reported an increased risk for involuntary infertility in women with ulcerative colitis undergoing IBD-related surgeries with infertility rates that range from 43–63% for ileal pouch-anal anastomosis (IPAA) (550–552), and that increased from 12% to 36% and 46% to 69% after restorative proctocolectomy, and restorative proctocolectomy with IPAA respectively (551,553,554). The aetiology of involuntary infertility in women after IBD-related surgery results from structural dysfunction such as obstruction of the fallopian tubes and pelvic adhesion (552) (549,554–556).

Despite the scarce number of studies investigating fertility in men with IBD, several population-based and referral-centre studies reported a slight reduction of fertility in men with IBD compared to the general population. A nationwide population-based study of a cohort of 29,109 men from Sweden showed a lower fertility rate in men with IBD compared to matched control groups (1.28 ± 1.27 versus 1.35 ± 1.31 ; $P < 0.001$) (557). When stratified by IBD subtypes, most observational controlled studies report reduced fertility in men with Crohn’s disease of only 2–8% (HR 0.95, 95% CI 0.92–0.98) compared with matched control groups (540,557,558). By contrast, studies investigating fertility in men with ulcerative colitis had contradictory results. The population-based cohort study with 29,104 men with IBD and 140,901 matched individuals by Druvefors and colleagues showed a decreased fertility rate in men with ulcerative colitis (HR 0.93, 95% CI 0.91–0.96) and IBDU (HR 0.92, 95% CI 0.89–0.95), contrasting with previous population-based and referral-center studies which reported no reduced fertility in men with ulcerative colitis

(540,557,558). The minimally decreased fertility rate in men with IBD might, in part, be a consequence of psychosocial factors that lead to voluntarily childless and reduced fecundity mediated by poor nutritional status, reduced testosterone levels, sexual dysfunction, and altered semen quality in men with IBD (557). Possible reasons for impaired fertility in men with IBD are reduced testicular function due to zinc deficiency, decreased testosterone levels altered by the inflammation in more severe IBD activity, medical treatments, and surgical intervention (557,559). Two small comparative studies with 10 patients with IBD and 50 patients with ulcerative colitis reported the presence of anti-sperm antibodies, which were significantly ($P<0.05$) elevated in the semen and serum of men with IBD and ulcerative colitis compared to control groups (560,561). Interestingly, anti-sperm antibodies were also found in the cervical secretion collected during ovulation and serum of women with IBD and ulcerative colitis (560,561). These anti-sperm antibodies might contribute to the reduced fecundability observed in men and women with IBD.

Some drugs used to treat IBD have been shown to impair men's fertility. Sulfasalazine, a widely used treatment for ulcerative colitis, could lead to reversible infertility by affecting sperm count, morphology, and motility (555). Moreover, sulfasalazine was also associated with erectile dysfunction, which alters the fertility of men with IBD potentially by suppressing the synthesis of sperm acrosomal membrane proteins crucial for the spermatozoa fertilization of oocytes or by increasing oxidative stress by reducing the level of redox enzymes superoxide dismutase and glutathione reductase (542,562–564). Either a 5-aminosalicylate can be substituted, or men should be advised to discontinue the drug at least one month before trying to initiate pregnancy (542).

There are limited studies investigating fertility in men with IBD treated with immunomodulator drugs such as azathioprine, methotrexate and cyclosporine (559,565). Yet, several case studies studying methotrexate therapy in men with autoimmune disorders such as rheumatoid arthritis and severe psoriasis reported controversial findings, with some describing limited effects on male fertility. Others associated methotrexate with an increased risk for reversible infertility resulting from erectile dysfunction and reduced sperm count, likely as a result of decreased DNA synthesis rates and subsequent inhibition of cellular proliferation owing to its folate inhibitory action (566,567). However, in a systematic review and meta-analysis published in 2023, using ten studies with overall 268 patients with IBD, exposure to biologics (anti-TNF: infliximab, adalimumab,

golimumab, certolizumab; anti-integrin vedolizumab; JAK inhibitor: ustekinumab), azathioprine, and methotrexate, was not associated with impaired men fertility and adverse pregnancy outcomes (565).

Data regarding the effect of cyclosporine on male infertility are contrasting. A few studies on a limited number of men with autoimmune infertility or following renal transplantation found no association between cyclosporine and male fertility (568,569). By contrast, in preclinical studies, cyclosporine treatment is linked to damaged testicular tissue, impaired sperm physiology, diminished sperm count and motility, and reduced serum testosterone concentration in rat and mouse models (570). However, studies investigating the fertility of men with IBD using cyclosporin are lacking.

Patients with IBD frequently develop complications such as perianal abscesses, fistulas, stenosis, and dysplasia-associated lesions that can require surgical intervention (571). A population-based study of a cohort of 310 patients by Peyrin-Biroulet and colleagues reported a higher risk for major abdominal surgery (HR 1.6, 95% CI 1.02–2.4, $P<0.04$ for men with Crohn's disease (572). Moreover, results from a prospective Dutch cohort study with 2,118 patients with Crohn's disease and 1,236 patients with ulcerative colitis, respectively, showed that men with Crohn's disease had a higher prevalence of undergoing small bowel and ileocecal resection (16% and 40%, respectively) than women with Crohn's disease (9% and 33%, respectively; men versus women $P<0.01$ for both complications) (54). Similarly, male sex was associated with increased risk for colectomy in patients with ulcerative colitis (HR, 2.1; 95% CI, 1.3–3.5) (60). The most common surgical intervention for patients with IBD, proctocolectomy with IPAA, has been linked with sexual dysfunction in men. A large longitudinal study with a cohort of 1454 patients with chronic ulcerative colitis that underwent IPAA reported that 1% of men developed sexual dysfunction one year post-surgery, and this number doubled ten years following surgery (573). A meta-analysis of 43 observational studies reported a 3.6% increase in the prevalence of sexual dysfunction post-IPAA in men's fecundity (571,574). Although several other surgical interventions such as end-ileostomy, ileo-rectal anastomosis, ileo-anal anastomosis without a pouch, colectomy, and Koch pouch ileostomy can also serve as potential therapeutic strategies in IBD, studies of their effect on men fertility are limited and will require further research.

A successful pregnancy requires maternal immune regulation or modulation to develop a tolerance to the semi-allogenic fetus. During pregnancy, the concentration of progesterone and estrogens gradually peaks in the third trimester of pregnancy (575). Moreover, their levels are markedly higher than measured throughout the reproductive cycle and have been shown to mediate the immunological changes occurring during pregnancy (575,576). In healthy pregnant individuals, elevated progesterone promotes the differentiation of anti-inflammatory CD4⁺ helper T cells type 2 (T_H2) and immune suppressive T_{reg} cells while reducing natural killer cell activity (576,577). The skewed T_H2 response is associated with the secretion of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10, which dampen the release of inflammatory cytokines, IFN γ , and TNF, which are known to promote IBD pathogenesis (576). Moreover, this T_H2 environment correlates with increased production of blocking antibodies, lower effector antibodies, and potentially increased T_{reg} cell activity (576). Estrogens are thought to potentially affect all cells of the innate and adaptive immune system and have been shown in preclinical and clinical studies to exert a plethora of physiological and pathophysiological functions that can be pro-inflammatory or anti-inflammatory (578). During pregnancy, circulating estrogens can increase up to 60 times their non-pregnant levels, with estriol accounting for 90% of all produced estrogens (575,579). Estrogens, specifically estriol, are thought to have a similar effect as progesterone during pregnancy (575,580,581). Estrogens might promote a T_H2 cell immune signature, increase antibody levels, and improve immune tolerance by potentially boosting the number and function of T_{reg} cells (579,581). Therefore, this has led experts to speculate that pregnancy might improve pathology manifestations in patients with IBD. Yet, clinical studies investigating pregnancy in IBD have yielded conflicting results. Several studies reported fewer flares and a lower rate of relapse in women with Crohn's disease and ulcerative colitis measured at 3 and 10 years following pregnancy compared to pre-pregnancy rates (294,582). Moreover, in pregnant individuals with quiescent IBD, discontinuing anti-TNF therapy seems safe and does not lead to a relapse of either Crohn's disease or ulcerative colitis (582). Most clinical practice guidelines and consensus statements consider that biologic agents can be utilized safely by women with IBD during the first stage of pregnancy and advise discontinuation of the treatment between 22 and 26 weeks of gestation. However, some reports recommend prolonging the therapy with biologic agents until

30-32 weeks of gestation or throughout pregnancy for patients with active disease or at a high risk of relapse (583). In contrast, one study reported no significant differences in the progression of Crohn's disease during and after pregnancy compared to non-pregnant individuals. However, women with ulcerative colitis had a higher risk of experiencing a relapse during and after pregnancy than non-pregnant patients (582). It's worth noting that the progression of IBD activity during pregnancy might be linked to disease activity during conception. Research indicates that patients who conceived during active disease were twice as likely to experience a flare-up during pregnancy as those who conceived during remission (294,582).

Mechanisms underlying sex bias in IBD

In addition to reproductive organs, sex hormones influence a variety of tissues, including the gastrointestinal tract and numerous hematopoietic and non-hematopoietic cells through sex hormone receptors such as estrogen receptor isoforms (ER α , ER β , and G-protein coupled estrogen receptors (GPER, also known as GPR30)), progesterone receptor (isoforms PR-A, PR-B, and PR-C) and androgen receptor isoforms (66,96). Beyond the most common receptor isoforms, various alternative splice variants of genes encoding sex hormone receptors have been identified in different tissues. However, the functional role of these variants is still under investigation (97,98). Sex hormone receptors on the cell membrane can regulate multiple signal transduction pathways and interact with growth factor receptors to enhance specific cellular processes through non-genomic pathways (99). Therefore, sex-specific differences are mediated by the complexity of sex hormone-receptor signalling pathways. Differences in those pathways complicate interpretations of the role of sex hormones and their receptors in physiological and pathophysiological conditions. Currently, a growing body of literature has proposed a relationship between IBD and sex hormones, particularly estrogens, and their receptors with IBD pathophysiology (**Fig. 3**) (100–104). All three estrogen receptors have been reported in fibroblasts, vascular smooth muscle cells, endothelium of arterioles, enteric neurons, and epithelial cells of the gastrointestinal mucosa (90,103,107).

Estrogens' biological effects in mouse models of colitis

A mouse study investigating dextran sodium sulfate sodium (DSS)-mediated colitis found worsened histological outcomes in several male mice strains compared to their female counterparts, demonstrating a biological influence of sex in this setting, although results are conflicting between studies (**Box 1**) (43). Interestingly, the effects of estrogen supplementation were differentially associated with the experimental model of colitis utilized (63,90,91). In female C57Bl/6 DSS mice, treatment with a supraphysiological concentration of 17 β -estradiol, similar to those achieved during pregnancy, increased the disease severity and histologic score compared with untreated mice with DSS colitis (90). In contrast, another study reported that the C57Bl/6 female mice with colitis had reduced disease activity index, macroscopic and histological scores, and lower TNF concentration than C57Bl/6 male mice with DSS-mediated colitis (63). In the same study, 17 β -estradiol treatment in the ovariectomized female mice further improved female resistance to DSS-induced colitis without affecting the orchiectomized male mice (63). Further supporting these findings, treatment with a supraphysiological concentration of 17 β -estradiol reduced the macroscopic and histological scores in C57Bl/6 female mice with dinitrobenzene sulfonic acid (DNB)-induced colitis (90). This observation was accompanied by reduced neutrophilia and decreased levels of IFN γ and IL-13, resulting in reduced colitis severity in the female DNB mice (90). Similarly, in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis C57Bl/6 mice model, 17 β -estradiol treatment of the ovariectomized females reduced colitis severity resulting from decreased pro-inflammatory cytokines expression (91). Consistent with these findings, 17 β -estradiol treatment diminished the histological score in *Helicobacter hepaticus*-induced mouse colitis, HLA-B27 transgenic rats, and acetic acid-induced mice colitis (63). Although outcomes in males are consistently worse compared to females, 17 β -estradiol treatment in ICR male mice with colitis-associated colon carcinogenesis promoted the expression of genes associated with the mucosal barrier and tight junctions along with reduced cytokines gene expression (92). In addition to being model dependent, the discrepancies resulting from 17 β -estradiol treatment might also result from variations in 17 β -estradiol doses administered and might point toward the need to define physiologically relevant estrogen doses for the study of IBD.

Estrogen action is mediated through three estrogen receptor isoforms, namely, ER α , ER β , and GPER, and several ER α spliced variants, including ER α 36 and ER α 46 (113). Although all three

estrogen receptor isoforms are expressed concomitantly in the intestinal tissue, ER β is the most abundantly expressed in the colon (103). Despite the contrasting results, several preclinical and clinical studies have suggested a central role for dysregulated estrogen-receptor expression and signalling in the pathophysiology of IBD (112,113,156,584). Compared to healthy individuals, reduced mRNA levels of *Er β* have been noted in a single-centre study with 73 patients with ulcerative colitis or active Crohn's disease. Interestingly, when segregated by sex and age, no significant changes in ER β expression were detected in men with ulcerative colitis or Crohn's disease, whereas the expression of ER β was lower in women patients over 50 years with Crohn's disease but not those with ulcerative colitis (113). In preclinical studies, the level of *Er β* mRNA was reduced, concomitant with increased intestinal permeability, in male and female colitis animal models (IL-10 deficient mice and HLA-B27 rats) before the onset of colitis (112). Deletion of *Er β* was associated with sex differences in DSS-mediated colitis, including marked mucosal destruction in female mice only (114). Furthermore, in the absence of ER β , the expression of inflammatory markers most notably increased in male mice, along with an increasing number of colitis-driven tumours in males and tumour sizes in females (116). Beneficial responses were uncovered following activation of ER β in male DSS mice, promoting the transcription of NOD-like receptor pyrin family domain containing 6 (*Nlrp6*), resulting in pro-inflammatory cytokine IL-8 secretion, antimicrobial activities, mucus production, and increased autophagy (115). Furthermore, the ER β -NLRP6 complex inhibited IL-1 α and TNF production, promoted intestinal epithelial cell survival, and reduced mitochondrial oxidative stress caused by DSS-mediated colitis, reducing mitochondrial damage (115).

Elevated ER α and GPER protein levels are reported in intestinal tissues from 73 patients with Crohn's disease (n=31) and ulcerative colitis (n=42) regardless of sex and age (113). When segregated by sex, men with Crohn's disease or ulcerative colitis had increased levels of ER α and GPER protein expression compared to healthy individuals, whereas estrogen-receptor expression was age-dependent in women with IBD (113). In women under 50 years, the expression of GPER was elevated in ulcerative colitis compared to healthy individuals, whereas no significant changes were observed in the expression of ER α in both ulcerative colitis and Crohn's disease (113). Conversely, ER α protein expression was elevated in women over 50 years with ulcerative colitis

but not those with Crohn's disease, whereas GPER protein expression remained unaltered in both ulcerative colitis and Crohn's disease (113). Activation of GPER with specific agonist G1 and 17 β -estradiol improved male mice's resistance to TNBS-induced colitis, which was mediated through the activation of the ERK1 and ERK2 signalling pathway (109). Although *Er β* deletion induced mucosal damage in female DSS mice, deletion of *Er α* resulted in protection from colitis and reduced disease severity in several mouse models; these models included the ovariectomized *H. hepaticus*-induced colitis model, T cell-mediated colitis in recombination-activating genes (*Rag*)^{null} mice, as well as in intact DSS female mice (114,585). Unlike females, depletion of *Er α* in male mice enhanced males' susceptibility to DSS-mediated colitis (114). The discrepancy in disease severity between male and female mice in the absence of *Er α* was associated with a differential expression of estrogen-responsive genes *Socs3*, *Fos*, and *Ctsd* between sexes (114). In the absence of *Er α* , *Ctsd* gene expression was reduced in females with DSS-mediated colitis, whereas *Fos* was elevated in males but not females. In models where *Er α* was depleted, researchers found elevated *Socs3* gene expression in male and female DSS mice, with no significant sex-specific difference between the sexes (114).

In contrast to the extensive research investigating the relationship between estrogens and IBD, very few studies explored the effect of progesterone and androgens on IBD progression and chronicity. However, some studies have shown that testosterone and progesterone might be protective against IBD (**Box 1**) (95,586). A clinical study with a cohort of 83 and 91 patients with Crohn's disease and ulcerative colitis respectively found that women with increased testosterone levels had a lower risk of developing Crohn's disease compared to matched controls, but there was no correlation with ulcerative colitis (95). A preclinical study reported a lack of effect for testosterone treatment in orchietomized mice with DSS-induced colitis (63). Treatment with progesterone was associated with reduced inflammatory markers and disease severity in male rats with TNBS-induced colitis (586).

Sex bias in the gut's immune function in IBD

As the largest immune organ of the body, the gut houses both innate and adaptive immune cells involved in maintaining gut mucosal homeostasis (129). Therefore, any dysregulation of the

immune compartment in the gut can lead to pathology such as IBD (139,140). It is well documented that most immune cells express multiple sex hormone receptors, hinting at a potential modulatory role for sex hormones to differences in the immune response between males and females in pathological conditions such as IBD (148).

IBD is associated with a dysregulated innate immune response that leads to increased expression of pattern recognition (PRRs) sensing receptors such as toll-like receptor (TLR)2, TLR4, CD40, and chemokine receptor CCR7 (149). As a result, these receptors promote the recruitment of neutrophils and the production of inflammatory cytokines such as TNF IL-1 β , IL-6, and IL-18 (149). Androgen and estrogen response elements have been identified in the promoters of innate immune genes, several of which are part of the PRRs, IFN, JAK2, and STAT3 signalling pathways, which are known to have an important role in IBD pathogenesis (150–152). Men have higher levels of TLR4 expression and more robust inflammatory responses to bacterial endotoxin lipopolysaccharide in macrophages and neutrophils than women (14,153). Conversely, testosterone-sensitized macrophages display reduced TLR4 and TNF (154).

Crohn's disease is considered to evolve from a predominantly T_H1 cell response due to a high number of activated CD4⁺ T cells that secrete elevated levels of IL-2 and INF γ , as well as a T_H17 cell response that causes high production of IL-23, which induces the secretion of IL-17A and INF γ while inhibiting T_{reg} cell proliferation (157,587). In contrast, ulcerative colitis has primarily been associated with a non-conventional T_H2 cell response resulting in mucosal CD4⁺ T cell's elevated secretion of IL-5 and IL-13, which might impair intestinal barrier function (149). Estrogen receptors are expressed in all CD4⁺ T cell subsets, including T_H1, T_H2, T_H17, T_{reg}, and CD8⁺ cells (148). Although the classical nuclear progesterone receptor is not present in lymphocytes, membrane progesterone receptor (mPR) α and mPR β have been identified in CD4⁺ and CD8⁺ T cells (588). Additionally, mPR α protein expression on CD8⁺ T cells is sensitive to elevated progesterone levels and upregulated during the menstrual cycle's luteal phase, whereas mPRs on CD4⁺ remain unchanged (588). Mature T cells do not express androgen receptors, whereas their expression has been described in immature double-positive (CD4⁺ CD8⁺) and double-negative (CD4⁻ CD8⁻) T cell subsets, indicating a potential role for androgens in T cell development and maturation (148).

Patients with IBD display increased ER α and decreased ER β expression levels in their T cells compared to healthy patients (111). In patients with active IBD, T cells and T_{reg} cells expressing ER β were found to be low compared to healthy controls and patients in remission, whereas no significant difference was observed in T cell expression (111,155). The percentage of circulating T cells and CD4⁺ T cells expressing ER β was inversely correlated with disease activity and plasma concentration of inflammatory markers such as IL-6 and c-reactive protein in patients with Crohn's disease and ulcerative colitis (111,155). Moreover, T cells exposed to IL-6 expressed decreased levels of ER β (111). Interestingly, a small clinical study with a cohort of 19 patients with IBD undergoing anti-TNF treatment found that the peripheral blood T cells from patients responsive to anti-TNF treatment expressed higher protein levels of ER β than patients with IBD unresponsive to anti-TNF therapy ($P < 0.01$) (111). In contrast, no correlation was found between ER α anti-TNF therapy and proinflammatory cytokines (111). Together, these studies support a role for T cells expressing ER β in Crohn's disease and ulcerative colitis disease progression.

Preclinical studies have shown that although naive T cells respond to estrogen through ER α and ER β , mature T_{reg} cells mainly respond to estrogen through ER β (156). ER β activation has been shown to promote the expression of FOXP3 and the secretion of anti-inflammatory cytokines such as IL-10 and TGFB in T_{reg} cells (156). Dysregulation in the ER β signalling pathway in a spontaneous Crohn's disease-like ileitis SAMP–YitFC mouse model promoted intestinal inflammation in the female mice in contrast to the male mice (156). Deletion of *Er β* in these SAMP–YitFC mice resulted in mRNA overexpression of the glucocorticoid-responsive transcription factor *Tsc22d3*, which inhibited T_{reg} cell suppressive function in mature female T_{reg} cells and ultimately exacerbated intestinal inflammation (156,584). In a chronic DSS mouse colitis model, the exclusive activation of *Er β* could restore the population of CD4⁺ T expressing ER β and T_{reg} cells while also inhibiting the infiltration of effector CD4⁺ and CD8⁺ T cells in the colon mucosa (155). Interestingly, the deletion of *Er α* from the effector T cell population limited T cells-mediated colitis in the female Rag^{-/-} mouse model (157). More specifically, the deletion of *Er α* in effector T cells promoted the expansion of T_{reg} cells by enhancing the protein level of FOXP3 and T_{reg} cell-associated genes while suppressing the expression of *Foxp3* suppressor genes such as *Tcf7* and *Satb1* (157). Consequently, T_{reg} cell expansion limited effector T cell activation and expansion,

reduced inflammatory cytokines such as INF γ and IL-17A production, and ultimately alleviated colitis (157). These studies highlight the significance of maintaining a balance between estrogen receptors in IBD to preserve intestinal mucosa tolerance in the gut. Nonetheless, further investigation is necessary to understand the underlying mechanisms that initiate the distinct activation and uneven expression of estrogen receptors in T cells in patients with IBD. Moreover, it is essential also to investigate the role of progesterone and androgen receptors in immune cells in the context of IBD.

Sex bias on intestinal epithelium functions

Impaired epithelial barrier function resulting from elevated inflammatory cytokines is one of the hallmarks of IBD pathology (38). The sex hormone receptors, including ER α , ER β , GPER, and progesterone receptor, have been reported to be expressed in epithelial cells, whereas androgen receptors have only been reported on mesenchymal cells in the gut (100,109,118). In animal studies, inhibition of testosterone in male rats increased mucosal resistance to inflammation and promoted intestinal resistance to injury (589). In contrast, in the absence of ER β , the mouse colon presented pre-pathogenic features such as abnormal histology and disrupted tight junctions associated with IBD (66). The deletion of *Er β* led to IBD-like features such as hyperproliferation, loss of progenitor cell differentiation, decreased apoptosis, and increased shedding from differentiated cells (590). Female and male rats treated with estrogen displayed increased occludin and junctional adhesion molecule A, improving intestinal barrier function and resistance to inflammation (344,589). Women ulcerative colitis patient-derived organoids treated with estrogen increased the expression of tight junction proteins occludin, zonulin 1, claudin 1, and claudin 2 and improve the epithelial cell barrier functions (345). Moreover, estrogen treatment of organoids derived from women with ulcerative decreased the production of inflammatory cytokines, reduced endoplasmic reticulum stress, induced epithelial stem cell proliferation, and promoted gut mucosal wound healing (345). Male HLA-B27 transgenic rats treated with 17 β -estradiol or administrated with ER β agonist inhibited the KCNQ1 channels and chloride secretion, restoring the mucosa impermeability and reversing chronic diarrhoea (**Box 1**) (591). Moreover, mucus-producing cell lines HT29-MTX treated with estrogen enhanced mucin contents and promoted mucus viscosity and elasticity (294). Similarly, progesterone had a protective role in the intestinal epithelial barrier

function by upregulating the tight junction proteins occluding, claudin 1, and ZO-1 and inhibiting chloride secretion (294,345). In addition, progesterone has been shown to reduce the secretion of inflammatory cytokines and alleviate endoplasmic reticulum stress in organoids derived from women with ulcerative colitis (345). Although the role of androgens in regulating the intestinal epithelial barrier is poorly understood, a study has suggested that androgen hormones in male mice can promote intestinal epithelial stem cell proliferation while inhibiting their differentiation by downregulating the bone morphogenetic proteins signalling pathway (118).

Sex differences and gut microbiota

Gut microbiota can regulate host physiology through many actions that preserve the intestinal mucosa architecture, influence the epithelial barrier function, produce energy, protect against pathogenic microbiota colonization, and modulate the development and function of the immune system (592,593). On the other hand, dysbiosis and microbiota-produced carcinogenic metabolites and toxins can drive pathologies such as IBD (594).

Dysbiosis has been shown to have a crucial role in IBD pathogenesis, with well-documented marked differences in the mucosa-associated microbial communities between individuals with and without IBD (595–599). Patients with Crohn's disease often display a reduced relative abundance of Firmicutes and Bacteroidetes concomitant with an elevated relative abundance of Proteobacteria compared to patients with ulcerative colitis and individuals without IBD (599). The complexity of studying the microbiota in IBD rests heavily on the heterogeneity of the microbiota community between individuals and even within the same individual throughout the day (600). This diversity in the composition of gut microbiota is shaped by genetics, the host immune system, intestinal mucosa barrier function, and environmental factors, including geographical location, age, health, lifestyle, diet, and sex hormones (**Box 1**) (592,601). Although controversial, some preclinical and clinical studies reported sex-specific differences in gut microbiota composition (601,602). Moreover, microbiota communities have been shown in mice to metabolize sex hormones, consequently regulating the effect of sex hormones on gut mucosal functions (384,386,601,603). The diversity of the gut microbiota differs between sexes at the genus and species level (604). At the genus level, *Bifidobacteria* and *Blautia*, two genera associated

with IBD, were markedly more abundant in faecal samples from women compared to those of men, whereas *Veillonella* and *Methanobrevibacter* were more predominant in samples from men (604). At the species level, a higher abundance of *Bacteroides cacae* was uncovered in women's faecal samples, whereas *B. plebeius* and *Coprococcus catus* were elevated in men's samples (604). Moreover, marked differences in the microbial composition and diversity at the phylum level have been noted between sexes in 89 different strains of mice, which is influenced by several factors (601).

Several studies have described the importance of sex hormones in establishing sex-mediated differences in the gut microbiota (**Box 1**) (384,386,605). However, the gut microbiota difference mediated by sex is dependent on age (601,605). In a non-obese diabetic mouse model, gut microbiota diversity and composition were not different between male and female mice before puberty (606). Yet puberty was associated with lower microbiota diversity in the gut of males compared to females (606). Male nonobese diabetic (NOD) mice model of Type 1 diabetes housed in specific pathogen conditions were more protected than female NOD mice in the same conditions (384). Conversely, the microbiota of germ-free male NOD mice was similar to germ-free female NOD mice (384). Castration of male NOD mice results in a microbiota that resembles that of female NOD mice microbiota (606). These findings from mice studies suggest might act as one of the drivers of sex differences in gut microbiota composition (384,386,605). When investigating the effect of estrogens on gut microbiota, a clinical study reported a lack of correlation between the concentration of 17 β -estradiol in women and sex-mediated differences in gut microbiota (605). Yet, estrogens have been shown to inhibit the growth of *Escherichia coli*, *B. fragilis*, and *Fusobacterium nucleatum* in rat intestines (607). In contrast, a clinical study with a cohort of 91 pregnant women find that progesterone increased the abundance of *Proteobacteria* and *Actinobacteria* and decreased the abundance of *Faecalibacterium* (294,608). In addition, progesterone increased the abundance of *Bifidobacterium* in humans and mice during pregnancy (609). All these findings suggest a role for sex hormones in microbiota diversity and abundance (605). However, confounding factors such as genetic background, age, and environment can regulate gut microbiota, complexifying the data interpretation (601). Thus,

subsequent investigations are required to decipher the underlying mechanisms that regulate the role of the microbiota in sex bias in IBD.

The underlying mechanisms that govern sex-mediated influences in IBD are complex and involve a network of multiple players, such as sex hormones, sex hormone receptors, microbiota, immune cells, and epithelial cells, whose cumulative influences from each other determine the host's gastrointestinal resistance to insults and IBD outcome. Our current knowledge indicates that oestrogen supplementation reduced disease severity in male and conditionally in female mice by promoting the expansion of T_{reg} cells, production of anti-inflammatory cytokines, intestinal mucosa integrity, and barrier function (344,345,610). These effects seem to require the presence of *Erβ*, whose expression is susceptible to inflammatory factors and females' reproductive cycle (108,116). Similarly, although scarce, studies report a protective role for both progesterone and testosterone as they promote intestinal mucosal integrity (**Box 1**). Notably, although being regulated by the microbiota, testosterone can alter intestinal epithelial cell differentiation (118).

Inclusion of sex in IBD research

Sex as a biological variable must be accounted for in the research design, subject selection, sample size, data collection, statistical analysis, reporting, and dissemination in preclinical and clinical studies (407,611,612). Poorly designed biomedical research costs lives and resources. In basic biomedical research, failing to account for biological sex might lead to inaccurate conclusions, whereas in clinical studies, such shortcomings might delay timely diagnosis and treatment, and harm patients. Moreover, sex-combined analyses when sex differences exist might lead to erroneous conclusions or masking effects between sexes. Thus, an appropriate analysis of sex should be paramount in IBD research because of growing evidence in this field pertaining to sex differences in almost all aspects of this disease globally.

Despite the emphasis given in current years to use appropriately the terminology of sex and gender in research, the implementation of these terms into practice remains, for the most part, limited and approximate. The probable reluctance or confusion associated with these terms' utilization is likely from the poor understanding from researchers of what these terms entail. Therefore, to conduct

research that investigates biological sex and gender differences, it is crucial to explicitly clarify how sex and gender are defined while formulating the research question.

Research design to investigate sex effect in IBD

Two research strategies, namely, method and factorial designs, are used to investigate the effect of sex as a biological variable (612). The method design explores the difference in the main effect on both sexes separately (611). This approach has the advantage of using a smaller sample size to characterize the study effect and sample size in both sexes. However, the method design fails to assess the interaction between the main effect and the variable sexes (611,613). In contrast, data disaggregated by sexes can be integrated into a factorial design to test statistically the interaction between variables and whether sexes respond differently to an intervention (611,612). In addition, considering factorial designs in experimental studies, help to reduce the sample size required for sex-based comparisons (614). Monte Carlo simulation and ANOVAs can be suitable statistical analyses to compare correlation or difference between groups in the factorial research design (611,612).

Subject selection in clinical studies

Defining confounding factors during research design will help guide the data collection (611). Studies should account for confounding factors such as sex, gender, age, smoking status, age of disease onset, environment, reproductive stage and cycle, and hormonal therapy to reduce or eliminate the effect of these covariables on sex-mediated interaction in IBD and expand the research validity and generalizability (407). Overlooking confounding factors might lead to inaccurate data interpretation and conclusion.

Physiological and behavioural sex differences attributed to prenatal and perinatal activation have been demonstrated before puberty (615). At puberty, the reproductive maturation phase involves sex-specific changes in the central nervous system, sex hormone production, and secondary sexual characteristics such as the growth of testicles and facial hair, breast formation, and the menstrual cycle (616). Hence, studies should define the inclusion and exclusion criteria associated with reproductive maturation stages (407). In longitudinal research, accounting for

reproductive history should be an important consideration because of its potential to influence the cohort under consideration. For example, data acquisition might be affected if a patient gets pregnant during a longitudinal study. Moreover, pregnancy-related substantial changes in estradiol and progesterone levels might serve as a modulator for the association between other factors (617,618).

Model selection for basic and translational research studies

Animal models are crucial tools to investigate sex-dependent processes experimentally, examining genetic and sex hormone modifications such as gonadectomy with sex hormone treatments, transgenic animal models lacking sex hormone receptors, and four-core genotype (FCG) mouse models (14,619). Cell lines are also a potential alternative, although poor models for the study of sex hormone-mediated changes—most cell lines used in basic biomedical research are derived from one sex only. In addition, cell-line use raises the issue associated with a sample size of one patient from whom they are derived and limits the ability to distinguish between differences linked to sex, environmental, and genetic factors (407).

The use of explant biopsy samples, patient-derived cells, and peripheral blood mononuclear cell (PBMC) models such as organoids can help mitigate the limitation associated with using cell lines, as they mirror the pathophysiological changes observed in IBD and reflect the diversity in patients' population (620,621). However, these model systems do not recapitulate the complete gut microstructure as they lack the complex interaction between the microbiota, epithelium, immune, and stroma components, all known to differ between sexes (14,294,620,621). Moreover, despite being an excellent source for cells to investigate the immune response in IBD research, the immune cell phenotypes in PBMCs vary markedly from that of the intestinal mucosa, highlighting the need for caution when interpreting the data (620). The underlying cellular and molecular mechanisms associated with sex hormones and receptors in IBD pathology are still unclear (14). Clinical studies have investigated the effect of sex chromosomes and hormones by comparing the immune response of healthy individuals of both sexes to individuals with Klinefelter or Turner syndrome. Klinefelter and Turner syndrome are two inherited disorders that result from either supplementary

X chromosome (47, XXY) or X deletion (45, X or 45, X0) (14,518). These instances also raise an important point that biological sex exists in a spectrum.

The link between sex chromosomes, gonad-type development, and sex-steroid hormone production might pose a challenge to discern the influence of sex chromosomes and steroid hormones (and their potential interaction) depending on the intended aim or goal of a particular study. In preclinical studies, an excellent mouse model, the FCG model, can serve to investigate the distinctive effect of sex chromosomes and hormones (619). FCG mice are produced by either the deletion of the *Sry* gene from the Y chromosome (XY⁻), which leads to a female phenotype, or by the insertion of the *Sry* transgene onto an autosomal chromosome (XX) or XY⁻ FCG mice to produce the XX^{Sry} and XY^{Sry} FCG mice that are phenotypically male (619). FCG mice can be supplemented with sex hormones to investigate the interaction between sex hormones and the gonadal type or genetic component (that is, sex chromosomes) in the preclinical model of IBD.

Sample size

Sample size is an important consideration for robustly assessing sex differences because of its importance in determining substantial differences between the experimental groups under consideration, and this should reflect population prevalence. Researchers should aim to balance research validity, which requires narrowing subject selection, generality, and large population selection, by defining the appropriate sample size data (407). The statistical power necessary to detect sex differences is affected by the prevalence of the pathology in each sex, the outcome, and the strength of the interaction between sex and the main effect (622). Thus, it is crucial to plan studies with enough statistical power to detect the difference in the main effect between sexes (622). Thus, it is crucial to plan studies with enough statistical power to detect the difference in the main effect between sexes. This might be particularly complicated for the study of gender as it might be challenging to conduct prospective or randomized control trial studies that include gender-diverse individuals with a sample size large enough to detect gender differences owing to the small size of the known gender-diverse individuals in the general population and the relatively small number of IBD case worldwide (623,624).

Analysis and interpretation of sex differences in IBD

New government mandates mean that more studies have begun to address the role of sex as a biological variable and that more and more researchers will be collecting and analyzing data by sex (407,625). A simple search of ‘sex differences in IBD’ using PubMed (as of 30 June 2024) shows that the majority (>78%) of articles have been published in the past ten years. Growing evidence suggests that there is a need for thoughtful consideration of sex-related differences in IBD research, and lessons can be learned from other disciplines (407,625–627). Including sex as a biological variable not only has the potential to promote discovery and expand the relevance of biomedical research, but it also provides a foundation of knowledge on which to build future advances (407,627,628). To this end, we must utilize best practices to include sex considerations in study design, data collection, analysis, and interpretation to establish the rigour and reproducibility needed to inform clinical decision-making and improve outcomes (628). Without careful analysis and interpretation of the effects of sex differences on IBD, the pursuit of sex difference research, despite a mandate from funding agencies and scientific journals, will result in a body of literature of contradiction. There are many inconsistent and contradictory reports on the presence, magnitude, and importance of sex differences in various aspects of IBD, including prevalence and incidence (629), disease onset and progression during growth and ageing (483), and treatment outcomes (52,630–632). Some of them might be due to poor study design, analysis, and interpretation, as well as their subsequent perpetuation in review articles.

Criteria that need consideration are the intentional and accurate use of language and terminology when referring to sex and sex steroid hormones, an in-depth sex analysis incorporated into study designs and reporting, and the inclusion of sex considerations in the interpretation of study results (633,634). The use of proper terminology is essential in the study, analysis, and interpretation of sex-related differences in IBD, which are often overlooked. There are many examples of the imprecise use of even common terminology in the field of sex difference-based studies in IBD research in current literature. For example, the first deliberate investigation on sex-related differences in IBD was a study published in 1999 in which the body’s hydration and the distribution of the body’s water compartments were assessed compared with healthy individuals (635). Unfortunately, in this study, the term ‘gender’ was used incorrectly to describe biological sex. In addition, ‘gender’ has incorrectly been used in many studies with a focus on biological sex

(113,482,636), including a preclinical animal study published in 2013 on metabolic profiling (637). Notably, many of these articles have been published in journals in the field of gastroenterology and IBD (638–640), which is concerning. Moreover, in many articles, ‘gender’ and ‘sex’ have been used interchangeably (54,638,639). It is counterintuitive to describe sex differences using erroneous terminology. In addition, IBD is a complex, multifactorial, and multidimensional disease influenced by a variety of factors, including age, genetics, sex, gender, environment, and geography (641), which are difficult to capture comprehensively in any study. Thus, a description of ‘limitations’ is paramount in all studies and analyses pertaining to various aspects of IBD. Moreover, there is a need for caution in using the information gleaned from these studies and for further research to replicate the findings, which also requires consideration. For example, sex steroid hormones are an integral component in sex-related differences in physiology and pathophysiology at large, including various aspects of IBD (294,642). However, the modulatory effect of sex steroids on IBD might include their action on immune and non-immune cells of the gastrointestinal tract, as well as the gut microbiota and its potential interplay with the host, because all of these factors contribute to sex-related differences in various aspects of IBD (643–646). The relationship between sex steroids, gut microbiota, and IBD could be multifaceted (647). Thus, analysis and interpretation of data from an *in vivo* study on the modulatory role of sex steroids on immune cells of the gastrointestinal tract or microbiota alone are bound to have limitations and require a cautious approach in reporting sex differences in IBD. Together, these considerations, if implemented, are expected to reduce the perpetuation of incorrect or unclear information and provide better practices in the study of sex-related differences in IBD in the future.

Similarly, the imprecise, mixed use of terminology describing the male and the female sex steroid hormones (such as androgens, estrogens, and progesterone) are often found in scientific literature attempting to address the effects of sex differences. For example, estrogen versus estradiol and androgen versus testosterone have interchangeably been used in analysis and interpretation in many studies (294,648). These terminologies are also used in a mixed format (for example, estrogen and testosterone) (294,648). It is important to note that estrogens and androgens are a group of compounds, whereas 17 β -estradiol and testosterone are the most common hormones in each of these respective groups. Different forms of estrogens and androgens differ in their

potencies, and their concentration levels do not follow a uniform pattern during ageing (649,650). In addition, a simple description of sex steroid hormone levels is not sufficient because it is their free, biologically available form (and not a carrier protein-bound, biologically unavailable form), which is more important in their actions on cells and tissues (651,652). Also, the response of sex steroid hormones is regulated in different target cells by co-regulators (653,654) that need consideration in analysis and interpretation, or at least should be included in the limitations of the study. Progesterone concentration levels must also be considered.

Researchers from many disciplines are beginning to appreciate the importance of considering sex differences in the design, analysis, and interpretation of their studies (625,626,628,634), factors still underappreciated in the study of IBD. It is important to determine if there is a sex difference in the expression of the IBD trait in female and male patients. Then, one must consider whether the sex difference is attributable to the actions of sex steroids, sex chromosomes, or other factors. To answer the potential role of sex steroids requires knowledge of how to assess and manipulate the hormonal condition of the patients in the study appropriately (634). The analysis and interpretation of sex differences in IBD must pre-specify tests of sex differences to reduce the occurrence of a type I error, to account for confounding by factors associated with sex, and to rule out instances when sex differences were tested as post hoc exploratory analyses, and these analyses must interpret the apparent sex differences in the light of biological plausibility (407,625). Subgroup analyses from studies thoughtfully designed to query sex differences can provide sound evidence of the benefits of sex-based treatments to target IBD.

It is crucial to understand that there are different types of sex differences, such as qualitative (that is, the variables are not related to each other in the same way across sexes) and quantitative sex differences (407,625). Qualitative sex-difference analyses should be conducted independently by sex, whereas in quantitative sex differences, the average or mean of a dependent variable is different for female and male individuals, and analyses should be conducted with sex as a factor (407,625).

When defining key sex differences in translational and clinical research, studies must complete more than simple comparisons between male and female persons to avoid overemphasising sex

differences (407). The source of the observed sex differences is an important consideration, including environmental, genetic, hormonal and potentially other factors (14). Moreover, adjusting the possible intersecting and confounding factors, such as age, is essential to consider because sex differences in IBD seem to have an age-dependent relationship and sex distribution of IBD epidemiology differs between different geographical regions (50,274,484). In longitudinal studies, how sex differences evolve over time during different stages in life and how observed differences might vary by factors such as age, ethnicity, and socioeconomic status are important to examine (655). In addition, a lack of statistical power to test the interactions associated with sex differences can lead to overinterpretations of the findings in studies investigating sex and gender differences in IBD ¹⁹⁷. Thus, investigators should address the ability of the study to limit bias, eliminate confounding factors, and discuss surprise subgroup findings discovered through post-analysis ^{16,197}.

Big data encompasses a systematic analysis of large volumes of complex data to uncover trends, patterns, and correlations that generate valuable new insights, and has the potential to help address the sex-specific knowledge gap that currently exists in biomedical and healthcare research (656,657). In current years, the wide adoption of electronic health records combined with clinical information and public health records and the growing development and adoption of multi-omics approaches provided the scientific community with large repositories of structured, semi-structured, and unstructured data (658). Systemic and integrative analysis of big data using advanced machine learning and artificial intelligence techniques could help design effective patient-centric healthcare and disease management and provide real-world data for observational studies to extract real-world evidence to improve medical outcomes and population health (656). The use of big data in IBD research could help identify sex and/or gender differences in the disease risk predictors, manifestation, diagnosis, treatment, and prognosis, alleviating our knowledge gap associated with sex and gender disparity in IBD (656).

Reporting sex-specific differences in IBD

Finally, the dissemination of findings from experimental, clinical, and epidemiological studies also requires equal attention and adherence to the current mandate by different funding organizations and scientific journals in reporting sex differences in IBD. A few specifics that need consideration

are reporting the sex of the study participants (even in single-sex studies), sex distribution of the study participants (cells, animals, or humans), how information on sex was obtained, disaggregation of results by sex, are observed sex differences of practical importance, and reporting all results—positive, negative, and inconclusive (402).

Conclusion

Accumulating evidence shows differences in IBD pathogenesis, epidemiology, clinical course, and disease outcomes associated with sex (14,15,50,395–400). As several studies demonstrate that sex-mediated factors can differentially regulate IBD risk factors such as genetic predisposition, dysregulated immune response, impaired mucosal function, environmental exposure, and intestinal dysbiosis, it is crucial to integrate sex as a biological variable in IBD research (140). Thus, the appropriate integration of sex in research design and data analysis will reduce the inconsistencies observed between current studies, promote new insights into IBD pathophysiology and IBD management, extend the relevance of IBD research to all, and ultimately improve patient care.

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Author contributions

D.T. and H.A. researched data for the article. All authors contributed substantially to discussion of the content. D.T., C.N.B, JE.G., S.M., and H.A. wrote the article. All authors reviewed and/or edited the manuscript before submission.

Competing interests

Charles Bernstein has consulted to Abbvie Canada, Amgen Canada, Bristol Myers Squibb Canada, Eli Lilly, Janssen Canada, Pfizer Canada, Ferring Canada, Pendopharm Canada, Sandoz Canada, Takeda Canada. He has received unrestricted educational grants from Abbvie Canada, Boston Scientific, Fresenius-Kabi Canada, Bristol Myers Squibb Canada, Janssen Canada, Pfizer Canada, Organon Canada, Takeda Canada and research grants from Abbvie, Janssen, Bristol Myers Squibb, Pfizer, Sandoz, Takeda. He has been on speaker's bureaus of Abbvie Canada, Eli Lilly, Fresenius-Kabi, Takeda Canada, Pfizer Canada, and Janssen Canada.

JE.G has served as a member of the scientific medical advisory committee for Crohn's and Colitis Canada and as a member and chair of the research advisory committee for Research Manitoba.

The other authors have no potential competing interests to declare.

What is already known?: Sex differentially impacts IBD subtypes, ulcerative colitis and Crohn's disease epidemiology, disease course, and symptoms across ages.

What is new here?: This article summarizes current knowledge of the impact of biological sex on IBD, highlights research gaps, and recommends best practices research methodology to integrate sex in IBD research.

How can this study help patient care?: Improves integration and comprehension of the impact of sex in IBD, support the discovery of personalized therapeutic strategies, extend the relevance of pre-clinical and clinical research to a broader population.

Box 1: Sex hormones in preclinical IBD studies

17- β Estradiol (E2) Supplementation

- In female mice, the E2 effect is model, dose, and receptor-dependent and can reduce or worsen colitis severity.
- In male rats, E2 enhanced inflammation, gene expression of mucus, and tight junctions and restored mucosa impermeability.

Progesterone (P4) and testosterone supplementation

- In females' organoids, P4 reduces inflammation and improves the mucosa barrier integrity and function.
- In males, P4 reduces inflammation and colitis severity. Testosterone promotes intestinal stem cell proliferation in male mice.

Sex hormones and microbiota

- Gut microbiota can regulate the level of E2, P4, and testosterone. Inversely, E2 and P4 can alter the microbiota diversity and abundance in mice.
- Female mice display greater gut microbiota diversity than male mice.