COX-expressing tuft cells initiate Crohn's disease-like intestinal inflammation in SHIP$^{-/-}$ mice

by

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COX-expressing tuft cells initiate Crohn's disease-like intestinal inflammation in SHIP$^{-/-}$ mice

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Abstract

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is characterized by intestinal inflammation. Intestinal epithelial cells play a critical role in mucosal homeostasis and dysregulation of pro-inflammatory epithelial cell function could lead to the intestinal inflammation that characterizes IBD. However, we do not know the events that initiate inflammation or the cell types involved. One type of cell that may play a role is the tuft cell. Tuft cells are the only epithelial cells in the uninflamed intestine that express cyclooxygenase (COX)1 and COX2, the rate-limiting enzymes required for production of prostaglandins, like PGE2 and PGD2 which play important roles in immunity. In our research investigating the lipid phosphatase SHIP, it was discovered that tuft cells express SHIP. SHIP deficiency leads to increased PI3-kinase activity in cells resulting in increased cell proliferation, reduced apoptosis, and increased cell activation. SHIP expression is currently believed to be restricted to hematopoietic cells. However, using bone marrow transplantation, our laboratory found that tuft cells were not radiosensitive, suggesting that they are not bone-marrow derived and are not hematopoietic in origin.

In addition, SHIP-deficient mice develop spontaneous Crohn's disease-like intestinal inflammation. The onset of inflammation coincides with the developmental appearance of tuft cells. In wild type mice, tuft cells are found in the lung and ileum, both locations where SHIP-deficient mice develop spontaneous inflammation, and I found that tuft cell numbers were increased 6-fold in the inflamed ileum of SHIP-deficient mice. Based on this, I hypothesized that SHIP-deficient tuft cells may initiate or contribute to inflammation in the SHIP-deficient mouse. I found that SHIP-deficient mice had more

COX1 positive cells in the ileum, more COX activity, and more PGD2 and PGE2 in full thickness ileal tissue homogenates, compared to their wild-type littermates. Finally, prophylactic inhibition of COX activity with piroxicam reduced the development of intestinal inflammation in SHIP-deficient mice whereas therapeutic treatment had little effect. This suggests that tuft cells may be critical in the initiation of spontaneous intestinal inflammation in SHIP-deficient mice and help elucidate some of the basic biology involved in the inflammation present in patients with CD.

Lay Summary

My research investigates how a cell type in the gut, the tuft cell, may play a role in the inflammation related with Crohn's disease. Scientists know very little about functions of tuft cells and recently discovered that they share some features with immune cells. I have found that tuft cells express SHIP, a protein found only in hematopoietic cells. SHIP-deficient mice develop inflammation similar to Crohn's disease, and I found high numbers of tuft cells in inflamed sites. Tuft cells may play a role in the initiation of inflammation in these mice. When I tried to prevent inflammation using piroxicam (an anti-inflammatory drug), I found that tuft cell numbers remained low, and mice did not develop inflammation. Treating inflammation was not very effective, tuft cell numbers remained high, and inflammation was still present. Because of this work, we understand more about the basic biology and inflammation in this mouse model.

Preface

Animal studies were reviewed and approved by the University of British Columbia according to guidelines provided by the Canadian Council on Animal Care, protocol numbers A17-0071 and A17-0277.

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Chapters 2, 3, 4, 5. Jean Philippe Sauvé conducted all the experimental work, and data analysis described herein, with the exception of the following contributions: Hayley

Brugger performed the bone marrow transplant experiments and took the fluorescent photographs used in this thesis. Peter Dobranowski and Susan C. Menzies assisted with the counting of immune cell infiltrates, villus length, and goblet cells (Figures 3.5C and 3.6C). Annika Busch assisted with ELISA assays for PGD2 **(**Figures 3.7A and 3.8A). Susan C. Menzies did all of the genotyping for the mice.

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List of Abbreviations

5-ASA	5-aminosalicylic acid
AA	Arachidonic acid
Ab	Antibody
AIEC	Adherent and invasive *E. coli*
ATG16L1	Autophagy related 16 Like 1
c-PGES	Cytosolic PGE synthase
cAMP	Cyclic adenosine monophosphate
CARD15	Caspase recruitment domain-containing protein 15
CCC	Crohn's and Colitis Canada
CCL2	C-C Motif Chemokine Ligand 2
CD	Crohn's disease
CD4	Cluster of differentiation 4
CIHR	Canadian Institutes of Health Research
COX	Cyclooxygenase
CRTH2	Chemoattractant T-helper 2 receptor
Csk	C-Terminal Src Kinase
CT	Computed tomography
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DCLK1	Doublecortin-like kinase 1
DP	D prostanoid receptor
DSS	Dextran sodium sulphate

EE	Enteroendocrine
EGFR	epidermal growth factor recepto
Epac	Guanine nucleotide exchange protein activated by cAMP
FAE	Follicle-associated epithelium
FOXP3	Forkhead box P3
GI	Gastrointestinal
GPCRK	G protein-coupled receptor kinase
GPCR	G protein-coupled receptor
Grb	Growth factor receptor-bound protein
GWAS	Genome-wide association studies
H-PGDS	Hematopoietic PGD synthase
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
ISC	Intestinal stem cell
iTreg	Inducible Regulatory T cell
L-PGDS	Lipocalin-type PGD synthase
Lck	Lymphocyte-specific protein tyrosine kinase
LP	Lamina propria
m-PGES	Membrane-bound PGE synthase
MIP-1α	Macrophage inflammatory protein 1 alpha

MRI	Magnetic resonance imaging
MxIF	Multiplex immunofluorescence
NK	Natural killer
NLR	Nod-like receptor
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
p-EGFR	EGFR phosphotyrosine 1068
PAMP	Pathogen-associated molecular pattern
PDK1	Phosphoinositide-dependent kinase-1
PG	Prostaglandin
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PLA2	Phospholipase 2
PPARγ	Peroxisome proliferator-activated receptor gamma
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
SHIP	Src homology 2 domain-containing inositolpolyphosphate 5´-phosphatase
SMAD7	Mothers against decapentaplegic homolog 7
SNP	Single nucleotide polymorphism

SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
TA	Transit-amplifying
TGFβ	Transforming growth factor beta
TJ	Tight junction
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TxA2	Thromboxane A2
UC	Ulcerative colitis

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Dedication

To Priscila,
You have been with me in every moment, every obstacle, and this is the result of our determination. Thank you for being with me when I most needed it.

To my parents,
You have always supported me in every decision of my life, and I would not be the person I am without you. Thank you for giving me all the love and guidance I could ever wish for.
I love you all.

Chapter 1: Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic, or relapsing and remitting, idiopathic inflammatory disorder of the gastrointestinal tract that manifests in two main forms: Crohn's disease (CD) and ulcerative colitis (UC). The highest incidence reported in IBD is for people between 20-30 years old, but it can affect individuals of any age, in both sexes.[1] It is an incurable disease, and its most common symptoms include abdominal pain, diarrhea, weight loss, rectal bleeding, and impaired absorption leading to nutrient deficiency.[2] IBD incidence rates are similar in both men and women, but other factors, such as ethnicity, genetic profile, and diet have been shown to play significant roles.[1]

UC is a condition that typically affects the colon and the rectum, in which inflammation is restricted to the mucosal and epithelial layers.[1, 3] Ulcerations and rectal bleeding are usually associated with this disease, as well as edema, which is swelling resulting from fluid retention.[1] Common histological features of colonic tissue sections from UC patients are the presence of immune cell infiltration, crypt abscesses, reduced goblet cell numbers, and disruption of crypt architecture.[1]

CD, on the other hand, can affect any part of the gastrointestinal tract, from the mouth, tongue, esophagus to the colon and perianal region, which makes it clinically more complex than UC.[1] The inflammation in CD affects all layers of the intestine (transmural inflammation), and it tends to be discontinuous, with patches of inflamed tissue intercalated by areas of healthy tissue.[1] CD is classified according to the location, as being ileal, ileocolonic, exclusive colonic, or in other locations.[4] The distal ileum is the most common site of intestinal inflammation in CD.[1] Some of the common complications of CD include: fistulas, which are channels connecting the

intestine to surrounding organs; fibrosis, which is the excess accumulation of extracellular matrix (ECM) that leads to stiffening and/or scarring of the intestine; and transmural inflammation accompanied by the presence of immune cells and granulomas, as histological examination of ileal tissue sections reveals.[1, 5, 6]

The incidence rate of IBD is highest in northern Europe, the United Kingdom, and North America.[7, 8] Estimates from 2012 show Canada having the highest prevalence of IBD in the world, with an estimated 233,000 people with the disease.[9-11] Among these people, 104,000 were diagnosed with UC, and 129,000 were diagnosed with CD.[9-11] The incidence rate among children is rising, with estimates that 5900 children and teens younger than 18 years old have IBD in Canada.[12] This high incidence of IBD places a considerable burden on families as well as the Canadian healthcare system. In 2012, it was estimated that the direct costs of IBD in Canada, including hospitalization, surgery, medication, and laboratory tests amounted to 2.8 billion dollars annually.[9, 11]

IBD is associated with a social stigma, which can be reduced by increasing awareness of the disease in the general population.[11, 13] Preventive measures are still not available, and there is reason to believe that IBD prevalence and financial burden will continue to increase in the foreseeable future, as well as the psychological stress that affects the quality of life of patients and their families.[11, 13] The need for preventive measures is thus considered high, and prevention is an important goal.

Because of the chronic nature of IBD and its health, psychological, social, and economic effects, funding organizations such as the Canadian Institutes of Health Research (CIHR) and Crohn's and Colitis Canada (CCC) are important in order to identify new therapeutic

strategies to treat and better understand IBD, thus reducing the burden on the Canadian healthcare system and ultimately improving the lives of Canadians living with the disease.

With regards to these goals, this academic work focuses on characterizing mechanisms of inflammation in a murine model of Crohn's disease.

1.2 Clinical presentation and diagnosis

Patients with UC present with abdominal pain, cramping, and diarrhea containing blood mixed with mucus.[1] Patients with CD, on the other hand, may experience pain in the abdomen, diarrhea, perianal fistulas, and disease complications such as swelling, thickening of the intestinal wall, and blockage of the intestine.[1] UC and CD patients may also suffer from anorexia, diarrhea, and weight loss that result from inadequate nutrient absorption.[1,14] In children, IBD can result in delayed growth and even delayed sexual maturity.[14, 15] Diagnosis, therefore, includes assessment of symptoms such as diarrhea, the presence of blood and mucus in stool, abdominal pain, cramping, fever, weight loss; and if CD is suspected, perianal disease.[16] Diagnosis also includes review of the patient's medical history, recent use of medications, such as antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs), and a combination of tests and procedures to exclude pathology caused by the presence of enteric pathogens, such as *Clostridioides difficile*, *E. histolytica*, *Salmonella,* or diarrheagenic *Escherichia coli*.[1,16, 17] In addition, family history of IBD is an important consideration during diagnosis for IBD. Sigmoidoscopy or colonoscopy are performed to determine the presence of ulcers, bleeding, and inflammation.[16] Patients may also have X-rays, abdominal ultrasounds, CT scans, MRI or small bowel imaging, to determine the extent of disease and possible extraintestinal complications.[1,16] Between 25 and 33 percent of patients with IBD will develop extraintestinal manifestations or

complications.[1,16] The most common is peripheral arthritis, but may also include ankylosing spondylitis, sacroiliitis, osteoporosis, renal lithiasis, dermatological and ophthalmological and cutaneous manifestations, as well as thromboembolic, primary sclerosing cholangitis, and hepatobiliary manifestations.[1,16]

While IBD can limit quality of life because of pain, vomiting, diarrhea, and other harmful symptoms, it is rarely fatal on its own.[16] Fatalities due to complications such as toxic megacolon, bowel perforation, and surgical complications are also rare.[16] While patients with IBD, in particular UC, do have an increased risk of developing colorectal cancer, this is usually caught early due to routine surveillance of the colon by colonoscopy, and therefore, IBD patients diagnosed with colorectal cancer have better survival rates than the non-IBD population.[1,16]

1.3 Etiology and pathogenesis

Currently, the etiology of both UC and CD still remain unknown, despite considerable research being carried out, involving genetic, immunological, infectious, and environmental aspects that aim to elucidate biological aspects of these diseases.[17, 18] Similarly, the variables that determine onset and evolution remain unknown. These are characterized by exacerbation and remission outbreaks, common to both diseases.[5]

Although the etiology of IBD remains unknown, current thinking is that IBD occurs in genetically susceptible individuals due to an inappropriate initiation and/or perpetuation of immune responses to intestinal microbiota (Figure 1.1).[17, 18] This thesis focuses on an animal model of CD-like intestinal inflammation; therefore, the introduction of etiology is expanded to focus on CD.

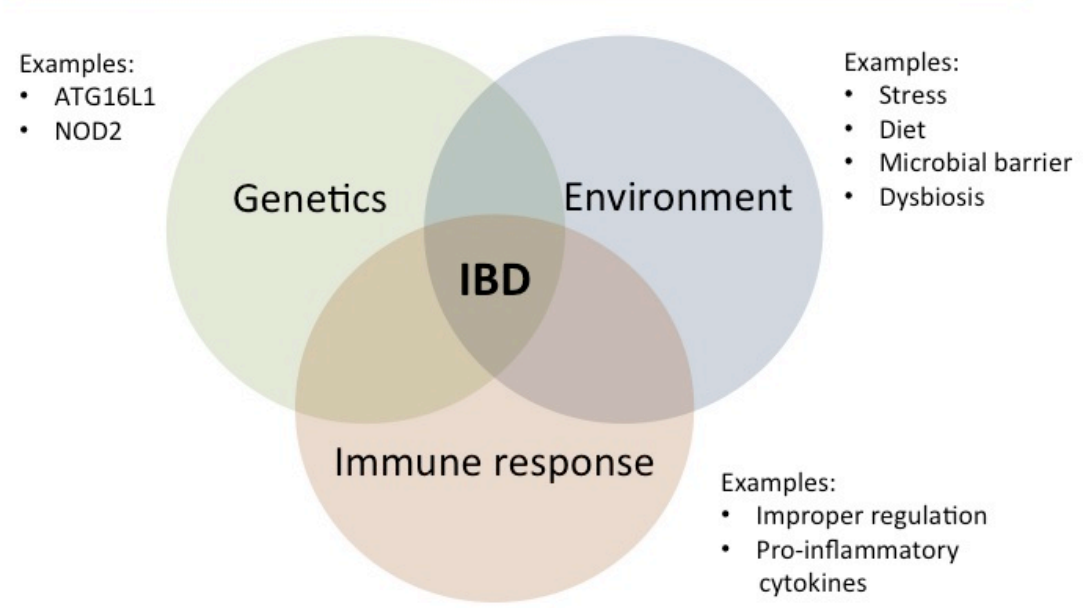

Figure 1.1 Etiology of inflammatory bowel disease (IBD).
IBD lies at the intersection of genetic, environmental, and immunologic factors. It is generally believed that IBD results from complex interactions between the intestinal microenvironment, the environment external to the host, and the immune response in genetically susceptible individuals. GWAS have identified 140 single nucleotide polymorphisms associated with CD, including those in ATG16L1 and NOD2. Modified and reproduced with permission of Nature Publishing Group: Xavier R.J. & Podolsky D.K, Nature 2007.[5]

1.3.1 The role of genetics in Crohn's disease

The first studies aimed at understanding the role of genetics in the onset and pathogenesis of CD were familial aggregation and twin studies, which revealed a consistently high prevalence of CD among relatives.[18-21] Studies in Sweden revealed the CD concordance rate in monozygotic twins was 50% whereas it was only 3.8% for dizygotic twins.[18-21] A similar study in Denmark showed that the CD concordance rate was 50% in monozygotic twins and 0% in dizygotic twins.[18-21] Among CD patients, between 2-14% have a family history of the disease.[22-25] However, very little is known about the effects of a positive family history on the severity and pathogenesis of CD in the individual.[26-31] The familial aggregation

and twin studies have been followed by genome-wide association studies (GWAS), which focus on identifying single nucleotide polymorphisms (SNPs) and candidate genes that may underlie disease susceptibility and pathogenesis.

Recent meta-analysis of GWAS identified 163 SNPs associated with susceptibility to IBD, of which 140 were susceptibility loci for CD.[30, 32] These included SNPs in *PTGER4* (encoding the prostaglandin E receptor 4)[33-35] and *MUC19*, both of which are associated with epithelial barrier function, as well as genes associated with the interleukin 23 (IL-23) signaling pathway, such as *IL23R* and *STAT3* (signal transducer and activator of transcription 3),[35, 36] which are critical in innate and adaptive immune responses.[37-39] SNPs in genes encoding ATG16L1,[35, 40, 41] NOD2,[42, 43] and IRGM[44] that are associated with CD, result in defective autophagy. Defects in autophagy result in enhanced bacterial persistence and intestinal inflammation, and have been associated with increased IL-1β production in both mouse and human cells.[45-47] Together, this suggests that CD may arise through distinct pathology-inducing mechanisms and thus, may be comprised of distinct pathological subsets of disease.[43, 48, 49] Continued characterization of these polymorphisms and pathways affected by them, may provide additional evidence that crosstalk between genetic, environmental, and immunological factors plays a critical role in the development of CD.

1.3.2 Environmental factors in Crohn's disease

The prevalence of CD has steadily risen in the past 50-60 years and this could be attributed, in part, to the fact that populations have migrated from areas with low incidence, such as East Asia, to areas with higher incidence, such as North America and Europe.[50] This, coupled with dietary changes, have implicated environmental factors as playing an important role in the

pathogenesis of CD.[32] Also, several environmental factors have been associated with increased risk for CD, including smoking, taking antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs), low vitamin D levels, and stress.[51, 52] Studies have shown that cigarette smoking increases the risk of developing CD by two-fold.[51, 52] It reduces cell proliferation and alters the ratio of regulatory T cells to T helper (Th) cells in the gut.[51-53] NSAID use is thought to exacerbate inflammation in IBD patients, possibly inducing flare-ups (discussed in detail further).[54-59] Finally, repeated use of antibiotics has also been associated with increased risk of developing CD in pediatric patients, which may act by altering the microbiota.[60, 61]

1.3.3 The microbiome in Crohn's disease

The gut lumen in humans is composed of a large, diverse population of different bacteria, with approximately 10^{12} microorganisms that are in close proximity to the intestinal epithelial barrier.[62, 63] Changes in the gut microbiota population may be due to changes in the external environment of the host.[62, 63] Dietary changes, for example the consumption of more sugars, as well as changes in behavior, such as reduced exercise, affect gut microbiota, which may contribute to the increased incidence of CD associated with these behaviors.[64-68] Indeed, dysbiosis in luminal bacteria, characterized by reduced diversity of the microbiota as well as increased adherent and invasive *E. coli* (AIEC), have been observed in CD patients (22%) compared to healthy controls (6.2%).[68-70] Viral infections have also been shown to alter the gut microbiota and have been implicated in CD pathogenesis.[71] Upon infection with norovirus, mice show abnormal Paneth cell structure and granules similar to those observed in CD patients.[71] Interestingly, the CD risk allele in *ATG16L1* has also been associated with changes in the composition of the intestinal microbiota.[72] Despite diverse mechanisms contributing to dysbiosis

in people with CD, these studies point to an important role for intestinal microbiota in the pathogenesis of CD.

1.3.4 The epithelial barrier in Crohn's disease

Intestinal immune homeostasis is maintained by the coordinated actions of intestinal epithelial cells and innate and adaptive immune cells. The intestinal epithelial barrier is composed of seven main types of cells including goblet cells, Paneth cells, enterocytes or colonocytes, enteroendocrine (EE) cells, tuft cells, M cells, and cup cells, which separate the gut lumen from the lamina propria (LP) (Figure 1.2).[73] This is a dynamic, physical barrier, which prevents the entry of microbes and foreign antigens into the LP but allows nutrients and water to pass into the circulation.[73-75] Goblet cells and Paneth cells secrete mucin and antimicrobial peptides that form the protective mucus layer between commensal bacteria and gut epithelial cells.[73-75] It is believed that one factor in developing CD is the result of damage to, or defects in, the epithelial barrier, which increases epithelial permeability.[76] Furthermore, in patients with CD, a dysregulated immune response to normal enteric microbiota has been shown to lead to increased mucosal secretion of pro-inflammatory cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), which can further exacerbate inflammation by increasing epithelial barrier permeability (Figure 1.2).[77, 78]

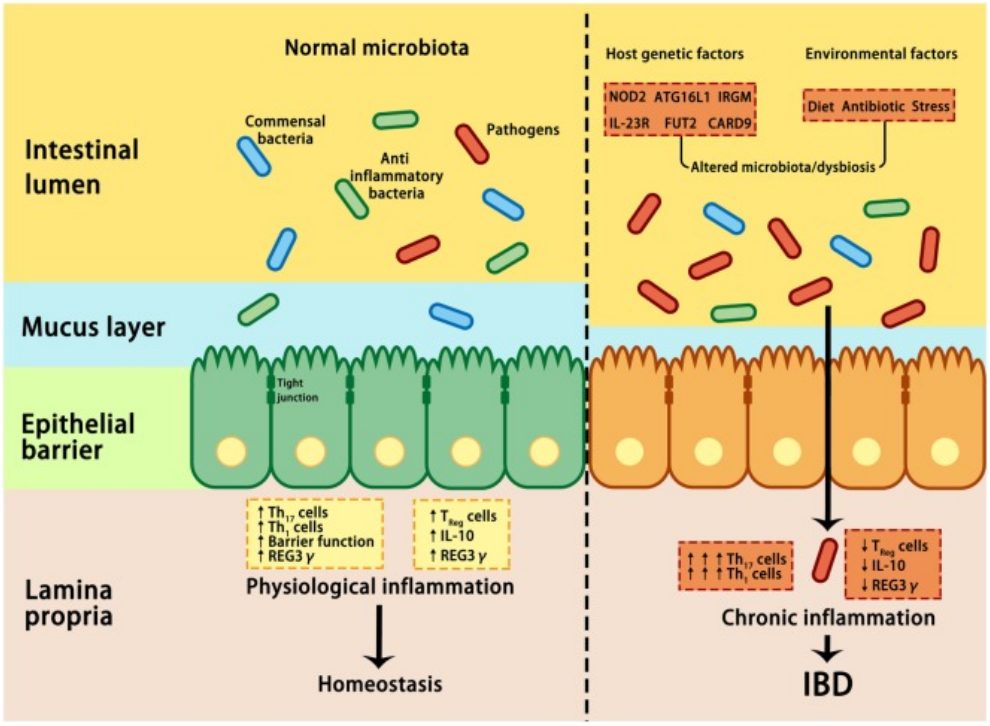

Figure 1.2 The epithelial barrier separates the lumen from the lamina propria.
The intestinal epithelial barrier prevents immune cells in the lamina propria (LP) from interacting with microbes present in the gut lumen. (Left) In normal, healthy conditions, there exists a state of immune tolerance that allows commensal bacteria to live alongside immune cells in the gut. The mucus layer limits the interaction between the microbiome and the underlying epithelial and immune cells. Epithelial cells, dendritic cells (DCs), and Paneth cells sample the gut lumen for microbes. Pathogens are suppressed by beneficial commensal bacteria through the induction of antimicrobial proteins, such as IL-10 and regenerating islet-derived protein 3 gamma (REG3γ), thus maintaining homeostasis. Epithelial cells release IL-18 that stimulates growth and proliferation of epithelial stem cells to repair damaged tissue. Paneth cells secrete antimicrobial host defense proteins to maintain homeostasis, mediate tissue repair, and maintain tolerance. (Right) In a susceptible individual (due to a combination of factors), the intestinal epithelial barrier may be compromised and breached, thus allowing microorganisms and antigens to enter the LP, where they interact with DCs and macrophages. These cells sense the presence of these microorganisms using pattern recognition receptors (PRRs), and trigger an uncontrolled chronic inflammatory response and hyper-activation of Th1 and Th17 cells, with production of pro-inflammatory cytokines, such as IL-1β, IL-18, IL-12, IL-6, TNFα, and IFNγ, as well as decrease in REG3γ and IL-10. Activated immune cells also produce chemokines, such as IL-8 and CCL2, which attract more immune cells (such as neutrophils) to the site of inflammation, where they encounter microbes and amplify the inflammatory response. IL-12 and IL-18 produced by macrophages and DCs stimulate type 1 innate lymphoid cells (ILC1) to produce TNFα and IFNγ, which further promote chronic inflammation. Reproduced with permission of Frontiers Media S.A. Ming, Z. *et al*., Frontiers in Immunology 2017.[79]

1.3.5 The immune response in Crohn's disease

Both innate and the adaptive immune responses have been shown to play critical roles in the pathogenesis of CD.

1.3.5.1 The innate immune response in Crohn's disease

The innate immune system is the first line of defense against invading microbes. It is comprised of cells including epithelial cells, leukocytes, such as monocytes, neutrophils, basophils, and natural killer (NK cells). These innate immune cells contain cell surface and endosomal PRRs, such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), that monitor the extracellular and intracellular compartments for pathogen-associated molecular pattern (PAMP) molecules.[80-83] Sub-epithelial DCs sample the gut lumen for the presence of non-pathogenic microbes as a regulatory response to provide tolerance.[80, 84] Studies in mice have shown that alterations in the proteins associated with immune responses can lead to intestinal inflammation.[85, 86] TLR2 and TLR4 expression in intestinal macrophages and DCs are increased in CD patients compared to control subjects.[87, 88] In addition, considerable evidence has confirmed a relationship between a polymorphism in the NOD2/CARD15 gene and CD, either alone or in combination with SNPs in TLRs, especially TLR4, or *ATG16L1*.[89, 90] As well, increased production of the pro-inflammatory cytokine, IL-1β, which has been linked to each of these gene variants,[89-91] has been shown to play a critical role in CD pathogenesis. IL-1β production is tightly regulated through TLRs via endogenous ligands and/or danger-associated molecular pattern (DAMP) recognition.[91, 92] Together, these data suggest a critical role for the innate immune response in the pathogenesis of CD.

1.3.5.2 The adaptive immune response in Crohn's disease

The adaptive immune system is made up of T and B lymphocytes and acts as the second line of defense to foreign microbes. It is highly specific in generating appropriate immune responses, and confers long-lasting immunological memory. Cluster of differentiation 4 (CD4+) T cells are key cells of adaptive immune responses that are important in defense against pathogenic microbes. CD4+ T helper cells are grouped into different classes including Th1, Th2, Th17, and regulatory T cells (Tregs).[93] Th1 cells are induced by IL-12 and IL-18 and produce high levels of IFNγ and TNFα. They respond to, and protect against, intracellular bacterial infections. Th2 cells are induced by IL-4 and produce high levels of IL-4, IL-5, IL-9, and IL-13, and protect against extracellular infections, such as parasitic helminths.[93] Th17 cells are induced by IL-6 and transforming growth factor beta (TGFβ) in mice, or IL-6 and IL-1β in humans. They produce high levels of IL-17A, IL-17F, IL-21, and IL-22, and are important for defense against extracellular pathogens and recruitment of neutrophils and macrophages.[94, 95]

There is evidence suggesting that an imbalance of CD4+ T cells to Tregs is a major cause of intestinal inflammation.[96-98] CD is widely believed to be a Th1/Th17 mediated disease with increased secretion of IFNγ, TNFα, IL-17A, and IL-2 reported in T cells from CD patients compared to those from control subjects.[99, 100] It has been shown that IFNγ and TNFα levels are increased in the inflamed mucosa of CD patients,[101, 102] while there are high levels of IL-12 produced by the cells in the lamina propria.[103] In UC patients, there is increased production of IL-4 and IL-13,[100] suggesting that Th1 and Th2 cytokines play an important role in the pathogenesis of CD and UC, respectively. IL-17 producing Th17 cells are also increased in the inflamed mucosa of IBD patients and are regulated by IL-23.[104, 105] LP macrophages from CD patients produce high levels of IL-23, which drives Th1 and Th17 responses.[106, 107] Also, SNPs in

the *IL-23R* gene have been associated with IBD.[34] However, the IL-23R SNP, Arg381Gln, has been reported to confer a 2 to 3-fold protection against development of pediatric CD, which suggests that it may actually reduce IL-23 responses.[34, 108] Together, these studies suggest that both IL-17 and IL-23 play an important role in the pathogenesis of both UC and CD, and targeting the IL-17/IL-23 pathway may serve as potential therapeutic strategy to treat IBD.

Tregs are critical for the maintenance of mucosal immune homeostasis. They exert their action by producing IL-10 and TGFβ, thus suppressing the proliferation of naive T helper cells and aberrant immune responses to commensal bacteria and microbial antigens.[109, 110] In CD patients, Treg numbers are significantly lower in blood compared to control subjects. In addition, Treg activity is also reduced in the intestinal mucosa of CD patients.[111-114] This suggests that one factor in CD development may be defects in Treg activity resulting in reduced anti-inflammatory cytokine production, and activation of Th1, Th2, or Th17 cells producing cytokines that promotes intestinal inflammation.

1.3.6 Therapeutic options

CD is characterized by relapsing and remitting inflammation, and it is estimated that approximately 90% of CD patients will experience a relapse. It is also reported that between 38-71% of CD patients will require surgery within 10 years of diagnosis of their disease, as complications from fibrosis and intestinal dysfunction arise.[19] The goal of treatment is to control inflammation, but every so often, flare-ups of acute symptoms may reappear, and depending on the circumstance, they may resolve on their own or require medication.[16, 19] The time between flare-ups can vary from weeks to years, and differs from patient to patient; in some cases, patients never experience a flare.[16, 19]

Despite the innumerable research that has been carried out, involving genetic, immunological, infectious, and environmental aspects that seek to clarify its etiology; IBD remains a group of diseases without a cure. In terms of medical management, it generally requires long-term treatment based on a combination of drugs designed to relieve patients of acute symptoms, provide long-term remission, and reduce the risks of complications. Treatments take into account the severity, location, and symptoms of disease, as well as an individual's tolerance.[16] Furthermore, patients' past disease course, medical history, and the duration and number of flares are also taken into consideration when considering disease management. Management of pediatric IBD also takes into greater account the age and pubertal status of the child.[15, 16] For treating mild to moderate UC, 5-aminosalicylic acid (5-ASAs), such as sulfasalazine, mesalamine, olsalazine, and balsalazide, are used for local immunosuppression.[1, 16] There is, however, limited evidence of 5-ASAs being useful for CD treatment, and most studies point to a modest to null effect (compared to placebo) of sulfasalazine, olsalazine, mesalamine in CD patients (reviewed in [115]). Corticosteroids, such as prednisone, are also used for moderate disease, but because of the side-effects of corticosteroids, long-term use is avoided.[15, 16] Budesonide is another important corticosteroid that has a potent local action and a reduced systemic activity due to limited resorption and important first-pass liver metabolism, without a significant suppression of plasma cortisol.[15, 16] It shows a better side effect profile and generally used for mild-moderate ileal and/or proximal colon disease.[15, 16] Another effective treatment option is exclusive enteral nutrition, which involves exclusion of normal diet for a period of time, being replaced with liquid nutritional products.[1, 116]

In patients with moderate to severe IBD, immunosuppressive drugs, such as azathioprine, 6-mercaptopurine, or methotrexate are used to suppress the immune response.

However, a disadvantage of using immunosuppressive drugs is that they are non-selective, so they reduce the patient's ability to fight infections.[1, 16] Biological therapies are designed to target specific immune mediators of diseases, such as cytokines. Those approved for IBD include infliximab, adalimumab, and certolizumab, which are monoclonal antibodies (mAb) directed against the pro-inflammatory cytokine, TNFα. These are effective at inducing and maintaining remission in patients, and have revolutionized the treatment for CD and UC.[1, 116, 117] Despite its relative success, many patients may experience primary non-response to biological therapy and a significant proportion may experience a loss of treatment efficacy or become intolerant to this kind of therapy.[118, 119, 120] Secondary non-responders who are switched to another anti-TNF drug may be less likely to clinically respond than patients who are anti-TNF naive.[121] As such, there is a strong need for biological agents targeting other inflammatory pathways and providing clinicians and patients with options to switch different classes of drugs.[118] Ustekinumab is a monoclonal antibody (ab) to the p40 subunit shared by pro-inflammatory cytokines IL-12 and IL-23, and is a suitable option with an alternative mechanism of action.[118] It was approved for adult patients with moderate to severe CD who have failed or were intolerant to treatment with immunomodulators, corticosteroids or at least one anti-TNF drug.[122] Others, such as secukinumab (human anti-IL-17A monoclonal antibody), have produced mixed results but have been shown to reduce moderate to severe CD in patients with the *TL1A* gene variant.[123] A new drug, vedolizumab, a mAb against the $\alpha_4\beta_7$ integrin, is used with the goal of preventing the recruitment of immune cells to the gut. It has been licensed for UC and CD treatment in the United-States, and for the treatment of UC in Canada.[101]

Severe cases of IBD may require surgery, such as bowel resection, strictureplasty, or a temporary or permanent colostomy or ileostomy.[1, 16] In CD, surgery would involve removing the

worst inflamed segments of the intestine and connecting the healthy regions, but unfortunately, it does not cure or eliminate the disease, as CD often recurs in the healthy part of the resected intestine.[1, 16] In UC, in most cases, colectomy will lead to full remission, but frequently at a cost for patients in terms of lifestyle. Complications arising from colectomy include infectious complications, faecal incontinence, and small bowel obstruction (reviewed in [124]).

This 'step-up' approach to treatment has the advantage of reserving drugs with higher levels of toxicity for those patients who are in need of more intensive therapy (Figure 1.3).[125] However, conventional therapies do not alter the development of disease complications or the need for surgery. Hence, paediatric gastroenterologists are moving toward an early aggressive approach, also known as the 'top-down'aproach, with the aim of changing the natural history of the disease.[126] However, there are no defined criteria from which a clinician can decide with a high degree of confidence which patients will benefit from this approach, and it generally comes down to the clinician's preference or previous experience.[127, 128] Identifying the genetic and clinical criteria for predicting which patients will have a disabling disease course is a challenge in current IBD research.

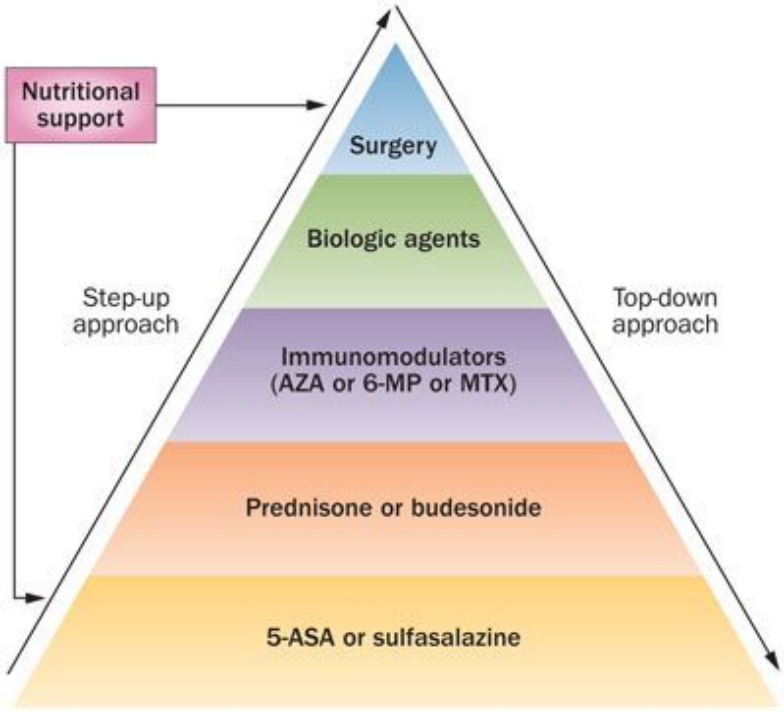

Figure 1.3 Approaches for IBD treatment.
Step-up approach: from mild to stronger and more toxic therapies. Top-down approach: early aggressive treatment with immunomodulators and biologic agents. Abbreviations: 5-ASA, 5-aminosalicylate; 6-MP, 6-mercaptopurine; AZA, azathioprine; MTX, methotrexate. Reproduced with permission of Springer Nature Publisher. Aloi, M. *et al.* Nat. Rev. Gastroenterol. Hepatol. 2013.[129]

1.4 Intestinal epithelial cells

The intestinal epithelial layer is functionally compartmentalized into multipotent intestinal stem cells (ISCs) residing predominantly near the bottom of the crypts,[130] transit-amplifying (TA) and lineage-primed progenitor cells, and differentiated cells such as enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and tuft cells (Figure 1.4). [131, 132]

Epithelial cells make crucial contributions to immunity. Of paramount importance, the epithelial layer forms a physical barrier between self and non-self and is often the site of first encounter between the host and a foreign microorganism or harmful substance. Post-mitotic

differentiated cells in the intestine are classified into two cell lineages (absorptive and secretory) based on their distinct functions and genetic differentiation programs. One type of absorptive cell (enterocyte) and four types of secretory cells (goblet, Paneth, EE, and tuft cells) comprise the small intestinal epithelium. In both small and large intestine, all post-mitotic differentiated cells are derived from stem cells that reside near the base of the crypts. Two additional cell types, cup cells and M cells, have yet to be definitively assigned to the absorptive or secretory classes of epithelial cells.[133, 134] Intestinal stem cells continuously self-renew throughout life and give rise to progenitors (transit amplifying cells) which undergo additional cell divisions prior to terminal differentiation and maturation.[132]

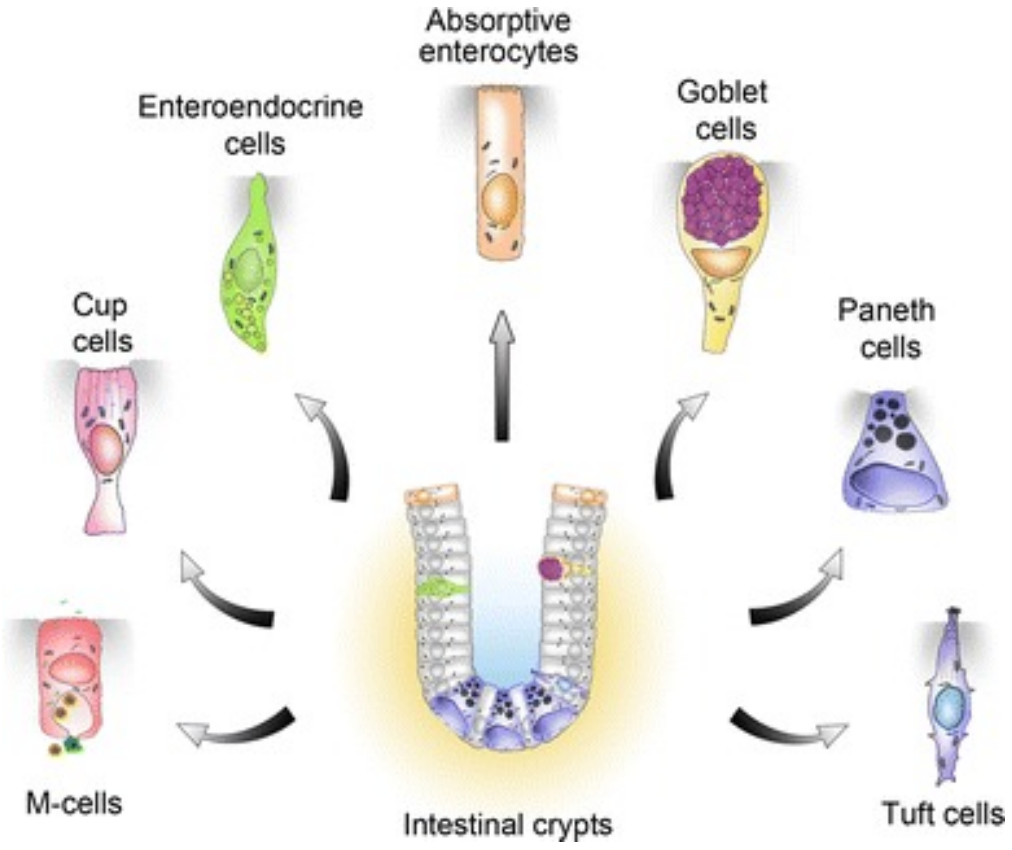

Figure 1.4 Representation of intestinal epithelial cell types generated from Lgr5-expressing crypt base columnar stem cells.
A single layer of epithelial cells separates the lumen from underlying lamina propria. Intestinal epithelial stem cells, at the bottom of the crypts, are responsible for the rapid renewal of the intestinal epithelium, and able to specialize into at least seven post mitotic differentiated cell types. Reproduced with permission of Springer Nature Publisher. Gerbe, F. *et al.* Cell. Mol. Life Sci. 2012.[135]

Intestinal stem cells give rise to all different types of epithelial cells in the gut, though the mechanisms by which this happens are still not fully understood. To reach a fully mature state, epithelial cells depend on expression of different growth factors (e.g. *Sox9* and *Spdef* for Paneth cells, *Klf4* and *Spdef* for goblet cells). Absorptive enterocytes are the most abundant cell type in the small intestine. Their primary function is to absorb nutrients apically and export them basally, leading ultimately to nutrition of the individual, as well as secrete water and electrolytes.[136] Goblet cells are the most abundant secretory lineage of the intestinal epithelia,

comprising ~10-15% of the small intestinal epithelium and up to 50% of the colonic epithelium. They produce and secrete mucus to provide the epithelial cells a protective layer against noxious contents in the lumen.[137] Paneth cells are secretory cells located at the bottom of crypts that produce and secrete antimicrobial peptides into the lumen. Paneth cells are unique in the sense that once cell fate is determined, they migrate toward the base of the crypts where they fully mature. In addition, Paneth cells are responsible for the regulation and maintenance of the stem cell niche in the crypt, most likely by expression of β-catenin and other factors.[136] EE cells comprise approximately 1% of the small intestinal epithelium, are scattered throughout the mucosa as individual cells, and produce and secrete hormones. There are more than 16 subtypes of EE cells identified in the mouse intestine, but the mechanisms for generating diversity and specificity are not known.[138] M (Membranous or Microfold) cells are microbial trafficking cells that are primarily found within follicle-associated epithelium (FAE) overlying Peyer's patches and lymphoid follicles. M cells contain unusual membrane structures which facilitate presentation of microbes to underlying lymphocytes, macrophages, and DCs.[134] Lastly, cup cells are, for the most part limited to the ileum, which suggests a specific but still undetermined function.[139]

1.4.1 Epithelial cell types involved in Crohn's disease

The epithelial cell layer prevents excessive contact of harmful antigens with the immune cells and thereby also protects the gut from unwanted immune reactions. This is achieved by the sophisticated organization of the intestinal epithelium, which establishes a tightly regulated barrier.[140] The intestinal epithelium forms a monolayer of columnar epithelial cells that are tightly connected by tight junctions (TJs).[141] Although TJs can be considered as a part of the

physical barrier, specialized intestinal epithelial cells, such as goblet cells and Paneth cells, take over miscellaneous functions of antimicrobial defense, which make them crucial parts of the innate immune system. Specifically, goblet cells secrete a variety of antimicrobial molecules, such as trefoil factors and mucins.[137] Mucin secretion creates a thick mucus layer to prevent direct contact of bacteria to the epithelial cell surface and thereby to protect against invasive pathogens. There are mouse models of IBD involving mucin depletion (e.g. $Muc2^{-/-}$ mice), demonstrating that defective Muc2 could predispose to IBD, likely by increasing microbe interactions with the intestinal epithelium and the mucosal immune system.[142]

Paneth cells are professional producers of antimicrobial peptides, which are secreted within the crypts of the small intestine.[143] However, controlled antigen delivery to immune cells plays an important role in the education of the gut immune system. For instance, specialized M-cells take up intestinal microbes and their antigens and forward them to resident immune cells in the gut-associated lymphoid tissue, supporting the maturation of the immune system.[144] This means that the intestinal epithelium does not constitute a strict barrier, but consists of a highly regulated gate controlling the admission of antigens to protect the host's health.[145] The epithelial barrier integrity is challenged by the high rate of cell turnover. The epithelium is completely renewed within only 4–5 days with cells shedding into the gut lumen at the surface and proliferation of stem cells within the intestinal crypt replacing the constant cell loss.[145] A failure of coordinated renewal can cause severe defects in barrier function that can lead to excessive invasion of foreign antigens and intestinal inflammation, such as seen in patients with UC or CD.

1.4.2 Tuft cells

While much attention has been drawn to the regulation and function of most epithelial cell types, only recently have intestinal tuft cells emerged as an anatomically and functionally distinct epithelial cell entity. Tuft cells (also called brush cells, mainly in the airways) are a rare and understudied epithelial cell type with a characteristic shape including long and thick microvilli that extend actin bundles deep into their apical cytoplasm.[146] Tuft cells are likely involved in chemical sensation of luminal contents, based on expression of proteins involved in taste sensation (α-gustducin, Trpm5), and secretion of opioids in response to luminal nutrients.[146-148]

1.4.2.1 Discovery and distribution of tuft cells

For almost a century, tuft cells (also known as brush or caveolated cells in the past) have been identified in many mammals as an unusual epithelial cell type in numerous hollow organs, including the gallbladder,[149-153] stomach,[149, 154-156] lung alveolus,[157-161] and intestine.[162-166] Depending on which morphological criterion was retained, they were named ‘‘peculiar’’, ‘‘fibrillovesicular’’, ‘‘caveolated’’, ‘‘brush’’, or ‘‘tuft’’ cells, all referring to epithelial cells endowed with a unique tubulovesicular system and apical bundle of microfilaments connected to a tuft of long and thick microvilli protruding into the lumen.

The first observations are usually attributed to Rhodin and Dalhamn, in 1956, who described cells with a well-developed apical brush border in the rat trachea,[167] and Järvi and Keyrilainen (also in 1956) who found similar cells in the mouse glandular stomach.[168] These observations and descriptions were possible due to the advent of electron microscopy and the unique morphology of the tuft cells, with a unique tubulovesicular system and an apical bundle

of microfilaments connected to a tuft of long and thick microvilli projecting into the lumen.[167-169] Decades of investigation have revealed little regarding the function of this mysterious cell type, until recently, when some light was shed on tuft cell function in the airways and gastrointestinal tract.[135, 169-171]

1.4.2.2 Origin and differentiation

Previously, tuft cells were thought to be a rare type of EE cell, based on their frequency in the epithelium and function in chemical sensation.[148] However, tuft cells were proposed to be a 4th secretory lineage based on the genetic program required for their differentiation, as reported by Gerbe and colleagues.[146] The authors reported that tuft cells were dependent on Atoh1 for their formation, thus classifying them as a secretory cell type.[146] However, differentiation of tuft cells was not affected by deletion of *Neurog3* (required for EE cell differentiation), *Sox9* (required for Paneth cell differentiation), *Gfi1* or *Spdef* (goblet/Paneth differentiation factors).[146] The differentiation factors required for tuft cells remain to be identified. Interestingly, the intestinal stem cell marker doublecortin-like kinase 1 (DCLK1 or DCAMKL1) was shown to be localized to tuft cells, and the relationship between tuft and stem cells remains to be established.[146]

1.4.2.3 Proposed functions

Several research groups have proposed different functions for tuft cells since their discovery in the 1950's: secretion, absorption and reception.[169] At first, the speculations were based solely on microscopic observations of the tubulovesicular systems of these cells.[169, 172, 173] As more techniques (e.g. Cytochemistry with PA-TCH-SP-PD and lectin; EFTEM-TEM

tomography) were used in tuft cell investigation, the continuity between the luminal membrane and the tubulovesicular system was confirmed. It was proposed that the spheres present along the microvilli seemed to originate from the cytoplasm through a form of apocrine secretion, possibly containing enzymes, but undetermined.[169, 172, 173] It was also believed that numerous granules known as glycocalyceal bodies among the microvilli were secreted from tuft cells occasionally, and also proposed that they arise in association with the Golgi apparatus.[174]

The tubulovesicular system also gave hints that it was possible that endocytosis was taking place in the area, despite tuft cells not being able to absorb HRP [166, 174] and cationic ferritin.[166, 175] It was speculated that, despite tuft cells not being able to absorb macromolecules, there was some (though limited) evidence that they might absorb particular molecules.[169, 174]

Several groups have shown evidence of the receptive function of tuft cells. Luciano and colleagues[154] showed that the unique arrangement of the apical cytoskeletal components including the lateral microvilli, resembles those of the Merkel cell, a type of mechanoreceptor. If this were the case, these cells might be singularly predisposed to tolerate mechanical stress, supporting the idea that they are sensory in nature.[154] Because of the observations related to preferential distribution,[152] α-gustducin (involved in taste signaling transduction) expression,[176, 177] and connection with neurons, [152, 174, 176] most investigators now consider these cells to be chemoreceptive in nature.[169]

1.4.2.4 Tuft cells and IL-25 involvement in disease

Type 2 immune responses are evoked strongly by parasitic helminths at mucosal barriers, but these responses also characterize problematic airway responses to inhaled aeroallergens. Three recent studies on tuft cells following parasite infections tie this cell type to a

new paradigm of type 2 immune responses.[178-180] Tuft cells were found to be the source of IL-25 (also known as IL-17E), an epithelial-derived cytokine, whose effects are mediated by the IL-25 receptor (IL-17RB), and have been implicated in the pathogenesis of allergic disease, airway viral responses and parasitic infections (type 2 responses).[178-182]

IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are epithelial-derived cytokines (collectively termed alarmins) which regulate type 2 immune responses to parasitic infection in the gastrointestinal tract and to aeroallergen exposure in the lungs.[181-187] IL-25 is a member of the IL-17 family, but unlike other IL-17 cytokines, promotes Th2-mediated inflammation.[181, 182] Chronic exposure to IL-25 alone is sufficient to induce asthma-like airway inflammation, remodeling, and hyper-responsiveness in mice.[188] Recent work suggests that IL-25 may act as a link between adaptive and innate immune responses through its ability to control Toll-like receptor 9 (TLR9) expression and TLR9 receptor-induced responses by plasmacytoid DCs, in the airways.[189] It is possible that such a mechanism involving the TLR9 receptor is also happening in the gut, but it has not been elucidated thus far.

Although tuft cells in the intestine are known sources of IL-25,[190] the details underpinning how IL-25 orchestrates type 2 immunity have only recently been uncovered.[178-180] In response to intestinal helminthes, IL-25 from tuft cells activate lamina propria group 2 innate lymphoid cells (ILC2s) to secrete IL-13, which feeds back on epithelial crypt precursors to skew differentiation of small bowel epithelia toward mucus-producing goblet cells and additional tuft cells.[178-180] Thus, as revealed in this model in the small intestine, tuft cells can serve as epithelial sensors that use IL-25, and possibly additional signals, to activate ILC2s to skew epithelial cell fates toward mucus-secreting goblet cells in response to luminal perturbations elicited by parasitic helminths, as well as tuft cell hyperplasia.[178-180]

Despite recent work elucidating tuft cell function, the relationships and mechanisms between IL-25 and other epithelial cytokines capable of eliciting type 2 immune responses, such as TSLP and IL-33, still need further investigation. Such studies may ultimately guide further mechanistic insights regarding how these mechanisms can be applied to ameliorate human disease.

1.4.2.5 Tuft cell hyperplasia as a marker of inflammation

Three independent and complementary studies[178-180] (discussed above) have recently revealed a critical function of tuft cells in the initiation of type 2 immune responses, which are typically involved during intestinal protozoa or helminth parasite infections, and which are deleteriously activated in allergies.[191] Type 2 responses require activation and recruitment of type 2 helper T cells and ILC2s by epithelial cell-derived cytokines, including IL-25, IL-33, and TSLP, as previously mentioned.[186, 192, 193] Production of IL-13 by Th2 cells and ILC2s causes the remodeling of the intestinal epithelium, including goblet cell hyperplasia and hypercontractibility of smooth muscle cells that peak at the time of worm expulsion.[178-180, 194, 195] For example, Howitt *et al.* found that the tuft cell population expands considerably during infections with helminths such as *Trichinella spiralis*, *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus*, in an IL-4/IL-13 signaling-dependent way.[178] These studies identified tuft cells as the trigger to the induction of the type 2 response following parasite infections.

Outside of the nervous system, DCLK1 (or DCAMKL1, an important tuft cell marker) was initially proposed to stain specifically for quiescent gastrointestinal stem cells.[196, 197] It was later understood that DCLK1+ cells in the GI tract are mostly tuft cells and predominantly post-mitotic.[198] However, a subset of intestinal and colonic DCLK1+ tuft cells are long-lived, largely

quiescent cells, that regulate and contribute to the stem cell niche.[198] DCLK1+ tuft cells are post-mitotic, fully differentiated cells, but a study using DCLK1-CreERT-BAC transgenic mice showed that a subset of DCLK1+ colonic and intestinal cells are long-lived and can function as powerful cancer initiating cells in the setting of APC mutation and inflammation.[199] The authors claim that DCLK1 seems to play a role in a variety of different cancers. Further research efforts are needed to clarify the underlying mechanisms before DCLK1+ cells can be used as a therapeutic target, although it appears to be a promising approach for the future.

An increase in tuft cell numbers has also been associated with gastric inflammation, hyperplasia, and metaplasia in mice.[200] In humans, the representation of tuft cells tends to increase in the inflamed stomach or the metaplastic intestine.[200] Together, the studies mentioned above reveal an exceptional level of functional integration and cooperation between the epithelial and hematopoietic compartments in mounting an efficient response against parasite infections and other malignancies, putting the tuft cell as an epithelial sentinel linking signals from the lumen to the immune system.

1.5 Src homology 2 domain-containing inositol polyphosphate 5´-phosphatase

1.5.1 Description and function

The src homology 2 domain-containing inositolpolyphosphate 5´-phosphatase (SHIP) is considered primarily a hematopoietic-specific lipid phosphatase that negatively regulates class I phosphatidylinositol 3-kinase (PI3K) activity. SHIP is also expressed in osteoblasts and mesenchymal stem cells.[201, 202] The human gene encoding the 145kDa SHIP protein (*INPP5D*) is located at chromosome 2q37.1.[203] Two other SHIP isoforms exist, the 150kDa SHIP2 that is similar in structure and biochemical function to SHIP,[204-206] and the 104kDa sSHIP, which lacks

the SH2 domain. SHIP2 is ubiquitously expressed and is seen in high levels in human skeletal muscles, placenta, and heart.[207] sSHIP is restricted to murine hematopoietic and embryonic stem cells.[204, 208]

PI3Ks are a family of enzymes that are critical in cellular processes including cell growth, differentiation, proliferation, and inflammation.[209, 210] PI3Ks can be grouped into three main classes, class I, II, and III, based on their substrates, molecular structures, and regulation within the cell.[209, 211] Class I PI3Ks are heterodimeric enzymes: Class IA is composed of 1 of 5 regulatory subunits, p50α, p55α or p55γ, p85α, p85β, and 1 of 3 catalytic subunits, p110α, p110β, or p110δ; and Class IB is composed of 1 of 2 regulatory subunits, p87 or p101, and the catalytic subunit, p110γ. p110α and p110β are ubiquitously expressed whereas p110γ and p110δ are mainly restricted to hematopoietic cells.[210] PI3Kp110 catalytic subunits have overlapping as well as unique functions downstream of specific receptor tyrosine kinases, growth factor, cytokine, and TLRs.[212] Class I PI3Ks phosphorylate the 3′ position of the inositol ring of phosphatidylinositol-4,5-bisphosphate PI(4,5)P_2 to generate PI(3,4,5)P_3, a critical second messenger.[209, 211] Class II PI3K is membrane bound, usually activated by tyrosine kinases and integrins,[213, 214] and is involved in cell migration.[215] Class III PI3K consists of a single catalytic subunit Vps34 and a regulatory subunit Vps15, and has been implicated in autophagy.[216] Class II PI3K catalyzes the phosphorylation of PI and PIP to PI(3)P and PI(3,4)P 2,[216] whereas class III PI3K only catalyzes the production of PI(3)P from PI.[211] Note that SHIP is one of the primary points of focus of this work.

1.5.2 SHIP enzymatic activity

To exert its action, SHIP is translocated from the cytoplasm, where it resides, to the inner leaflet of the cell membrane where PI(3,4,5)P_3 synthesis occurs.[203] This happens through the association with an adaptor (such as Shc) and scaffold proteins (such as the growth factor receptor-bound protein (Grb) family of proteins) and/or direct binding of its SH2 domain.[203] PI(3,4,5)P_3 recruits serine-threonine kinases, such as AKT/PKB and phosphoinositide-dependent kinase-1 (PDK1), to the plasma membrane, where it begins driving cellular processes,[209] such as growth, proliferation, differentiation, and immune activation.[217] SHIP antagonizes these actions by dephosphorylating the 5′ position of the inositol ring to form PI(3,4)P_2 (Figure 1.5).[218] Therefore, SHIP regulates the downstream cellular processes in immune cells, such as cytokine production and inflammatory responses (Figure 1.5).[219]

Furthermore, SHIP enhances neutrophil apoptosis,[220] decreases B cell proliferation, chemotaxis, and activation,[220-222] and promotes T cell survival and maintains innate immune balance at mucosal surfaces.[221] SHIP can be regulated either at the level of transcription or post-transcriptionally.[220] In macrophages, TGFβ has been shown to up-regulate SHIP mRNA expression in both human and mouse cells,[222] while Mothers against decapentaplegic homolog 7 (SMAD7), which blocks TGFβ activity, has the counter effect of reducing SHIP expression.[223] Post-transcriptionally, IL-4 has been shown to induce SHIP protein degradation in macrophages.[220] Studies have also shown that tyrosine phosphorylation of SHIP targets it for ubiquitination and proteasomal degradation.[224]

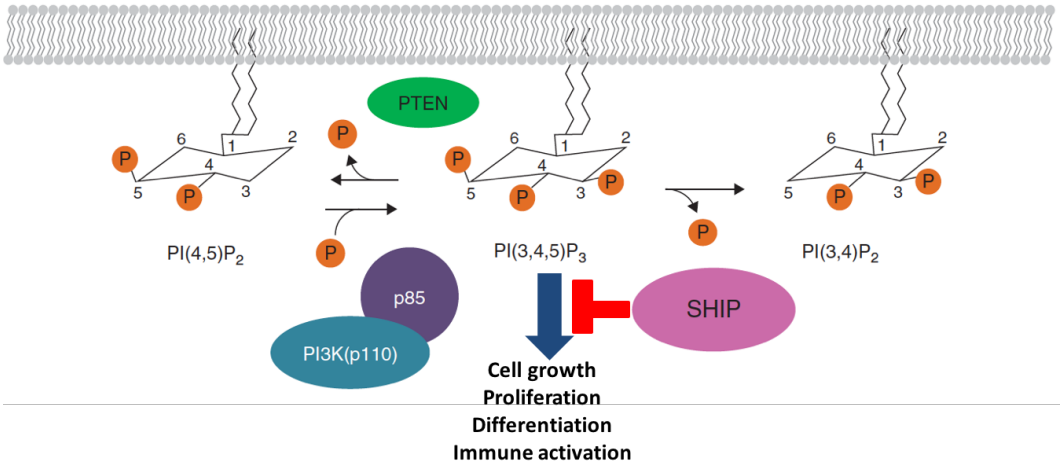

Figure 1.5 The phosphatase activity of SHIP.
Ligation of receptor tyrosine kinases (RTKs), cytokine receptors (cytokine Rs), growth factor receptors (GFRs), G protein-coupled receptors (GPCRs), and TLRs activate Class I PI3K, which is comprised of a p110 catalytic and a p85 regulatory subunit. Class I PI3K phosphorylates $PI(4,5)P_2$ to produce the second messenger $PI(3,4,5)P_3$. SHIP dephosphorylates $PI(3,4,5)P_3$ to form $PI(3,4)P_2$, and blocks cellular processes, such as growth, proliferation, differentiation, and immune activation. PI3K activity can be reversed by the tumor suppressor Phosphatase and tensin homolog (PTEN). Modified and reproduced with permission of: John Wiley and Sons Publisher - Dobranowski and Sly, J. Leukoc. Biol 2017.[225]

1.5.3 The SHIP-deficient mouse

The SHIP-deficient mouse (*Inpp5d*$^{-/-}$), which will be referred to as SHIP$^{-/-}$, is smaller in size than its wild-type counterparts and has a reduced lifespan, asthmatic lungs, splenomegaly, and a myeloproliferative disorder.[220] These mice were first developed in 1998 by targeting and deleting the first exon of SHIP, thus disrupting SHIP activity.[220]

SHIP$^{-/-}$ mouse macrophages are also hyper-responsive to IL-4.[221, 226, 227] Note that macrophages formerly known as M2 macrophages primed with IL-4 or IL-13 are now defined as M(IL-4) or M(IL-13), and have a wound healing and tissue remodeling phenotype, in order to repair tissue damage from the inflammatory response (reviewed in [228]). In addition, SHIP$^{-/-}$ mice have hyperactive, IL-4-secreting basophils, which expose macrophages to this M(IL-4)-skewing

cytokine.[220] This results in macrophages that constitutively express high levels of the M(IL-4) markers, argI and Ym1, and which also secrete high levels of the anti-inflammatory cytokines, IL-10 and TGFβ.[220, 229] Studies have shown that SHIP$^{-/-}$ mice have granulocytes that are less susceptible to apoptotic signals, and granulocyte-monocyte infiltrations can be found in various tissues in these mice, such as in the terminal ileum.[92, 230]

Recently, our research team, and Kerr's group,[92, 230] reported that the SHIP$^{-/-}$ mice spontaneously develop ileitis with several key features resembling CD, including both inflammatory and fibrotic components, that were restricted to the distal ileum.

1.5.4 The SHIP$^{-/-}$ mouse model of Crohn's disease-like intestinal inflammation

SHIP$^{-/-}$ mice develop spontaneous CD-like inflammation restricted to the distal ileum beginning at 4 weeks of age.[92, 230] Inflammation is characterized by abundant infiltrating Gr-1-positive immune cells (neutrophils), granuloma-like immune cell aggregates, multi-nucleated giant cells, goblet cell hyperplasia, and a mixed Th2 and Th17 cytokine profile.[92, 230] There is a paucity of T cells (CD4+ and CD8+) in the inflamed mucosa of SHIP$^{-/-}$ mice, suggesting that T cells might not play an important role in the onset of intestinal inflammation in this model.[230]

This mouse model is particularly relevant to humans because SHIP protein levels and activity have been found to be reduced in people with CD.[231-233] In fact, concentrations of SHIP mRNA, protein levels, and activity are reduced in immune cells within inflamed ileal tissues from newly diagnosed, treatment-naive pediatric patients with CD, as previously published by our research team.[231, 232] Somasundaram and colleagues have also found that SHIP protein levels are profoundly diminished in a subset of patients; however, SHIP activity and expression was not correlated to ATG16L1 SNP status in the adult cohort included in the study.[233] Evidence

suggests that aberrant SHIP activity can contribute to disease, at least in a subset of adult and pediatric CD patients. Although CD is typically considered a Th1-mediated disease, fibrosis in CD is mediated by IL-1β, TGFβ, IL-13 and other type II cytokines.[234] Further investigation is needed to determine whether the subset of CD patients with low levels of SHIP, are those that go on to develop intestinal fibrosis, one of the major complications that CD patients face.

1.6 COX enzymes and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a group of drugs with therapeutic applications as they have analgesic, antipyretic, and anti-inflammatory properties, which make them very attractive for both health professionals and patients.[235] The number of NSAIDs available on the market has increased over the past decades, and today it is estimated that 1 in 7 individuals with rheumatologic disorders are using NSAIDs.[236] Also, approximately 1 in 5 (50 million) US citizens report they use an NSAID for other acute complaints.[237] There is also an increasing trend in the use of NSAIDs, as the world population is aging, and chronic systemic diseases with painful symptoms appear to be increasing.[236, 238]

The major classes of NSAIDs (salicylate, diclofenac, naproxen, ibuprofen, acetaminophen, indomethacin and piroxicam) have a common feature, the inhibition of cyclooxygenase (COX) enzymes, which are responsible for the rate-limiting step in the synthesis of prostaglandin from arachidonic acid.[238, 239] The common anti-inflammatory drugs such as aspirin, ibuprofen, and naproxen block the action of both COX enzymes, COX1 and COX2. Different classes of NSAIDs preferentially inhibit COX2, such as celocoxib, meloxicam, carprofen, and nimesulide; or selectively inhibit COX2, such as rofecoxib, valdecoxib, etoricoxib, and lumiracoxib.[240, 241]

In the late 80's, increasing evidence of serious adverse gastrointestinal events, such as perforation, gastrointestinal ulceration and bleeding, led to a progressive decline in the use of conventional NSAIDs in the treatment of osteoarthritis and other diseases.[242] However, after 1998, the release of a new NSAID class with low GI toxicity, the selective COX2 inhibitors, particularly celecoxib and rofecoxib, modified the standard recommendation for analgesia in cases of osteoarthritis and rheumatoid arthritis.[242] Despite this gastrointestinal advantage, this safety profile of selective COX2 NSAIDs was affected and rofecoxib was withdrawn from the market in 2004 due to increased risk of myocardial infarction.[242, 243]

To study the action of NSAIDs on epithelial tissue is a way of recognizing the advantages and disadvantages of their use and potential application in the clinic. It is important to recognize the roles and mechanisms of action of these drugs in order to detect the changes caused in the physiological and pathophysiological processes.

1.7 Prostaglandin specification and function

Prostaglandins (PGs) are lipid mediators formed by the majority of cells in the body, and act in an autocrine and paracrine manner. As shown in Figure 1.6, the PGs originate from arachidonic acid (AA) released from membranes by phospholipases (PLA2), mainly group IV cytosolic phospholipase (cPLA2).[244] Released arachidonic acid is rapidly metabolized by COX1 and COX2 to form the intermediate prostaglandin, PGH2. While COX1 is a constitutive enzyme, responsible for the basal levels of prostaglandin production, the COX2 enzyme is induced at times of inflammation and acts to potentiate the production of PGs.[245] However, this view that constitutive COX1 exerts homeostatic functions and inducible COX2 exerts pathophysiological functions is oversimplistic and erroneous in some cases.[246] This notion has been challenged by

growing evidence indicating that both isoforms are present in normal tissues and can be up-regulated in various pathological conditions.[247] Both the expression and regulation of COX isoforms have been intensively investigated, and reviews about transcriptional regulatory mechanisms,[248] and the regulation of gene expression at the post-transcriptional level have been published.[249]

COX enzymes are inserted into the nuclear and endoplasmic reticulum membranes with their cytoplasmic-oriented substrate binding moiety.[245] The enzymes responsible for the metabolism of PGH2 will determine the end product, which can be PGI2, PGF2, PGD2, PGE2 or thromboxanes A2 (TxA2). The end product of the metabolism of PGH2 depends on the cell type in question. Prostaglandins produced are released by the cell predominantly through a prostaglandin transporter and, due to their short half-life, exert their function in an autocrine and/or paracrine manner.[250] In the specific case of PGE2, for example, PGH2 undergoes isomerization by three distinct PGE synthases, cytosolic PGE synthase (cPGES), and two membrane-bound PGE synthases, mPGES-1 and mPGES-2. While cPGES and mPGES-2 are constitutive enzymes, mPGES-1 is induced in response to various pro-inflammatory and mitogenic stimuli concomitantly with COX2. Thus, it is postulated that cPGES uses the PGH2 catabolized by COX1, while mPGES-1 uses PGH2 derived from COX2.[251]

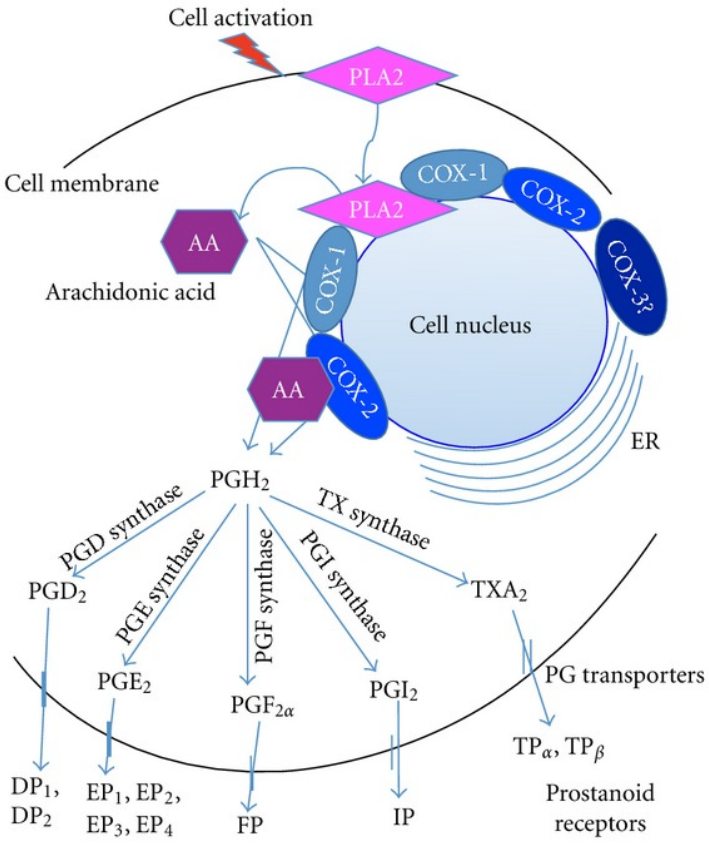

Figure 1.6. Prostanoid synthesis.
After cellular stimulation, PLA2 is activated and arachidonic acid (AA) is released from membrane phospholipids. Subsequently, AA is metabolized by the COX1 or COX2 enzymes in distinct cellular compartments and subsequently metabolized by specific synthases, which lead to the generation of synthase-specific prostanoids. Once the prostane is produced, they are transported out of the cell to bind to their respective receptors. Reproduced with permission of: Hindawi Publishing – Medeiros *et al.*, Mediators of Inflammation, 2012.[252]

1.7.1 PGE2 effects

PGE2 plays a well-established role as an inflammatory mediator in innate immunity. Its role in the induction of fever, pain, and vasodilation, and its involvement during the inflammatory process are well demonstrated by the use of cyclooxygenase inhibitors as potent anti-inflammatory agents.[253] Paradoxically, PGE2 also exerts anti-inflammatory actions on cells of the immune system, such as monocytes, neutrophils and lymphocytes.[254] cAMP can be

considered an important second messenger in cells of the innate immune system, acting most often as an inhibitor of the activation of these cells. Some functions of cAMP are well described in macrophages; PGE2 is the most important ligand in the context of innate immunity associated with increased intracellular cAMP.[255] Among the actions of PGE2 and cAMP are the inhibition of phagocytosis; inhibition of microbicidal activity; inhibition of the production of pro-inflammatory mediators such as TNFα, MIP-1α, and leukotriene B4, while increasing the production of anti-inflammatory IL-10 and suppressor of cytokine signaling 3 (SOCS3).[255]

In alveolar macrophages, the effector molecules protein kinase A (PKA) and guanine nucleotide exchange protein activated by cAMP (Epac) are responsible for the suppressive functions of cAMP. These effector molecules promote their actions independently or redundantly.[256] While PKA modulates the generation of pro-inflammatory and anti-inflammatory mediators, inhibiting the former and stimulating the latter, Epac promotes the inhibition of phagocytosis via FcR receptors, and both modulate the inhibition of microbicidal activity by decreasing the generation of reactive oxygen species (ROS).[256] However, the specificity of these effector molecules may vary from cell to cell, as demonstrated in DCs, in which both PKA and Epac act on the modulation of inflammatory mediators.[257]

PGE2 exerts its function through 4 receptor subtypes: EP1, EP2, EP3, and EP4. EP receptors are coupled to G protein (GPCRs) and vary in their molecular structure, PGE2 binding properties, tissue distribution, expression, and signal transduction (Figure 1.7).[258] Among these, EP2 and EP4 are expressed at high levels in monocytes and $CD4^{+}$ *naive* T cells in humans, while EP1 and EP3 are poorly or not expressed. Furthermore, the activation of human T cells promotes a 2- to 3-fold increase in EP2 and EP4 receptor expression.[259] On the other hand, in murine assays, in addition to the high expression of EP2 and EP4, the EP1 receptor is also present in

CD4^{+}*naive* T cells.[260] While EP1 is coupled receptor protein Gαq/p, both EP2 and EP4 are coupled to the α subunit of the stimulatory G protein (Gαs). The binding of PGE2 to these receptors promotes, respectively, the increase of intracellular Ca^{2+} and the increase of intracellular concentration of cAMP, an important second messenger that acts regulating diverse cellular functions.[261, 262]

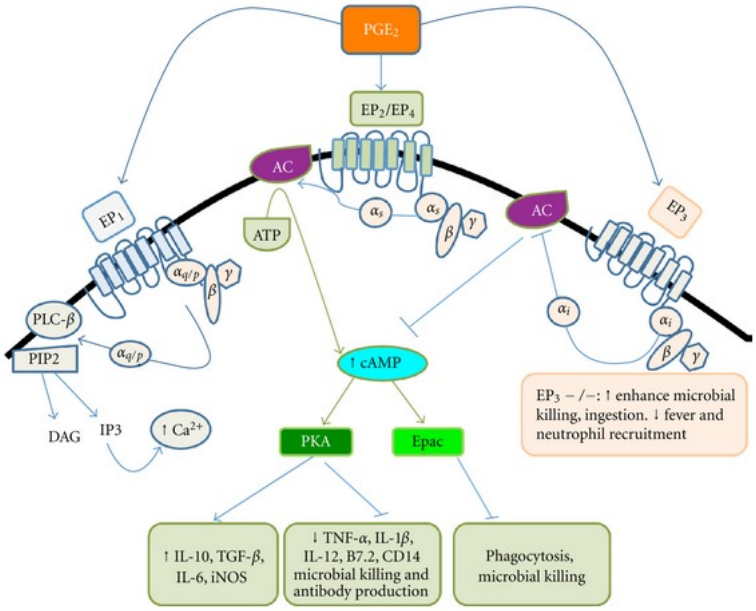

Figure 1.7. PGE2 receptors and their actions on macrophages.
PGE2 has four specific receptors: EP1, EP2, EP3 and EP4. All receptors are coupled to G protein, and EP2 and EP4 signaling are associated with release of the Gαs subunit of the Gβγ complex. EP3 signaling releases the Gαi subunit, while EP1 signaling releases the αq/p subunit. Release of the Gαq/p subunit promotes the increase of intracellular Ca^{2+}. The Gα subunit is able to bind to the adenylate cyclase and promotes the activation (Gαs) or inhibition (Gαi) of the generation of the cAMP enzyme product. In turn, cAMP signals through effector molecules PKA or Epac, which modulate the function of macrophages. Shown above are anti-microbial functions that are differentially regulated by effector molecules PKA and Epac in macrophages. Reproduced with permission of: Hindawi Publishing – Medeiros *et al.*, Mediators of Inflammation, 2012.[252]

The role of PGE2 in adaptive immunity, on the other hand, has been elucidated in recent years and, unlike the immunosuppressive functions previously described,[263, 264] recent studies demonstrate an important immune-activating function of this lipid mediator.[265, 266, 264] The suppressive role of PGE2 via EP2 was previously demonstrated by inhibition of T cell proliferative capacity in a mixed lymphocyte reaction.[267] This suppressor effect of PGE2 and 8-CPT-cAMP (cAMP-specific analogue that specifically activates PKA) in peripheral T cells is mediated by PKA-Csk, which acts by antagonizing TCR signaling, competing for Src family kinase activation (Lck).[268, 269] That is, while TCR stimulates the activation of this kinase, PGE2 stimulates inactivation.[268, 269] Recent studies also highlight an anti-inflammatory role of PGE2 because it plays a role in the differentiation of Treg cells.[264] Baratelli and colleagues[264] demonstrated that PGE2 enhances the expression of the forkhead box P3 (FOXP3) transcription factor in natural Treg cells (nTreg) and in $CD4^+$ *naive* T cells, promoting their differentiation into induced Treg cells (iTreg).[264]

Contrasting these direct or indirect suppressive effects of PGE2 on T cells, it has been reported that high concentrations of anti-CD3 (indirect TCR activation) outweigh the suppressive effect of PGE2.[265] In this context, in the presence of polarizing conditions, which promote differentiation of Th1 cells, PGE2 increases the percentage of IFNγ producing Th1 profile cells in a concentration-dependent manner.[265] Interestingly, this facilitating signaling of PGE2, although occurring via EP2 and EP4, was promoted by PI3K activation and not cAMP, and the Th17-expanding action of EP2 and EP4 is mediated by cAMP and not PI3K activation.[265, 266] This suggests that these two PGE2 actions are promoted through different signaling modules of EP2 and EP4.[265] It was demonstrated that EP2/EP4 signaling promotes immune inflammation through Th1 differentiation by inducing expression of the IL-12R subunit *Il12rb2* and the IFN-γ

receptor *Ifngr1*, thus facilitating IL-12 signaling.[265, 266] Notably, the EP2/EP4 signaling was also reported to synergize with IL-23 to facilitate Th17 cell expansion in murine and human T cells,[259, 265, 270] and it was suggested that EP4 antagonism may be therapeutically useful for various immune diseases.[265, 266]

Differentiated Th1 cells predominantly express the Ep1 receptor, which facilitates Th1 differentiation.[260] In Th17 cells, PGE2 acts via EP2 and EP4 receptors via the cAMP-PKA signaling pathway to aid in the differentiation of human $CD4^{+}$*naive* T cells into Th17 cells.[259] PGE2 enhances the expression of the receptors for IL-1 (IL-1R) and IL-23 (IL-23R) on differentiating T cells and, in combination with cytokines that promote differentiation of the Th17 subpopulation, increased the phosphorylation of STAT3 and induced a qualitative and quantitative change in Th17 function and phenotype to a more inflammatory/pathogenic pattern.[259] In mice, PGE2 acts via EP2 and EP4 receptors, and signals via cAMP/PKA facilitating the expansion of Th17 cells in conjunction with IL-23. In addition, PGE2 can increase IL-23 production by DCs and indirectly contribute to Th17 expansion.[265, 271] PGE2 also plays an important role in the recruitment of neutrophils to the joint cavity in a murine model of arthritis by increasing IL-17 synthesis and IL-12/IFNγ axis inhibition.[272] Therefore, PGE2 performs roles in both innate and adaptive immunity, acting as an immunosuppressive mediator and as an immunoactivator. Given its importance in gut immunity, PGE2 was measured as a downstream target of COX inhibition in this study.

1.7.2 PGD2 and its dual role

Prostaglandin D2 (PGD2) and its metabolite 15dPGJ2 are prostanoids that have a role in pro- and anti-inflammatory responses, vasodilation, allergic responses, platelet aggregation, contraction of the airway smooth muscles, among other processes.[273-276] The enzymes that

synthesize PGD2 are hematopoietic PGD synthase (H-PGDS) and lipocalin-type PGD synthase (L-PGDS).[273] Beyond the nervous system, where it is better studied, L-PGDS also has an inhibitory effect on the progression of lung, ovarian, colorectal cancer, as well as some types of leukemia.[275, 277] H-PGDS is present in various cells of the immune system, which produce PGD2 as an allergic and inflammatory mediator. It is also characterized as a member of the glutathione S-transferase (GST) gene family, whose members are known to catalyze the binding of glutathione (GSH) to an electrophilic substrate.[278]

PGD2 exerts its functions through two receptors, named D Prostanoid (DP or DP1) and chemoattractant T-helper 2 receptor (CRTH2), also commonly known as DP2.[279] The activation of these receptors triggers several events. DP activation may increase cAMP concentration, thus inhibiting IL-12 production by DCs, inhibiting production of IFNγ by T cells, inducing IL-4 and IL-5 release by activated $CD4^+$ Th2 cells, inhibiting basophil migration and degranulation, and suppressing NK cell functions.[279-283] DP may have an overall anti-inflammatory role in the immune system, by antagonizing, and in fact limiting the effect of pro-inflammatory CRTH2 activation upon exposure to PGD2.[284]

Activation of the CRTH2 receptor can induce various biological responses, such as induction of migration in Th2 cells, basophils, and eosinophils, up-modulation of adhesion molecules such as CD11b in eosinophils, and induction of various features of cell activation in eosinophils including degranulation, actin polymerization, CD62L shedding, cell shape change, and mediator release.[279, 285, 286] CRTH2 is also present in DCs and participates in the migration of these cells.[287, 288] These observations indicate that CRTH2 signals are inherently pro-inflammatory and pro-stimulatory, and suggest the involvement of CRTH2 in various steps of

leukocyte pro-inflammatory activities such as endothelium adhesion, extravasation, chemotactic migration, and effector function.[251, 289]

The involvement of PGD2 in the pathophysiology of IBD is currently being debated. There are several strong arguments for a beneficial impact of PGD2 when interacting with its DP receptor. An experimental model of colitis in rats demonstrates that PGD2 induces a decrease in granulocytic infiltrate in the colonic mucosa.[290] This effect is also observed during the administration of a DP agonist, suggesting the involvement of this receptor.[290] A study based on the analysis of colonic biopsies of patients with ulcerative colitis shows the involvement of PGD2 and the DP receptor in the resolution of the inflammatory process and the persistence of remissions in this disease.[291] In fact, overexpression of DP associated with an increase in PGD2 production is observed in patients in remission compared to patients in the active phase of the disease.[291] Conversely, a deleterious effect of PGD2 has been observed in a model of trinitrobenzene sulphonic acid-induced colitis.[292] Other studies suggest the involvement of the COX/L-PGDS pathway in the pathophysiology of IBD.[293, 294] The study by Hokari *et al.*, also performed on biopsies of patients with ulcerative colitis, shows an increase in the expression of L-PGDS, correlated with the severity of the disease.[294] Also, it was demonstrated that L-PGDS has a possible involvement in dextran sulfate sodium (DSS)-induced colitis. L-PGDS$^{-/-}$ mice treated with DSS showed an attenuation of the inflammatory involvement when compared to DSS-treated wild type mice, adding to the complexity of the role played by PGD2 and L-PGDS in attenuating inflammatory symptoms of colitis and suggesting the potential usefulness of selective L-PGDS inhibitors for treatment of IBD.[293, 294]

With regard to DP, a specific signaling pathway involved in the induction of MUC5B mucin expression has been described.[295] An increase in expression of mucins MUC2 and

MUC5AC, via DP, has also been demonstrated on the epithelial intestinal cell line LS174T.[296] Since the main function of mucins is to form a mucous barrier that protects the mucosa, the beneficial role of PGD2 in IBD may be related to increased mucin secretion after activation of DP.[297] The mucins MUC5AC and MUC2 are indeed involved in epithelial repair during IBD, through their action on differentiation and cell growth.[298, 299]

The anti-inflammatory action of PGD2 is partly attributed to its product, 15d-PGJ2, which is a natural ligand of peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ is a key player in the maintenance of innate antimicrobial immunity in the colon.[300] Activation of PPARγ can cause an increase in eosinophil migration and actin polymerization, inhibit TNFα, IL-6 and IL-1β production, inhibit cellular proliferation, and induce apoptosis.[273] It also leads to inhibition of the transcription of pro-inflammatory cytokines by immunocompetent cells, and the arrest of proliferation and induction of differentiation of intestinal epithelial cells.[301, 302] In addition, it is already established that 15d-PGJ2 suppresses the activation of NF-κB by inhibiting IκB phosphorylation by the IκB kinase.[297, 303] Finally, a decrease in the expression of PPARγ is observed in the active phase of ulcerative colitis.[304] Genetic ablation of *PPARγ* was found to result in increased susceptibility to experimental colitis in rodents.[305] A lack of expression of the PPARγ anti-inflammatory signaling pathway could therefore be one of the elements that contributes to the pathophysiology of IBD.

In light of this growing knowledge base on the various functions of PGD2 in the immune response, and H-PGDS acting as a marker of tuft cells, PGD2 was measured as one of the targets downstream of COX inhibition in the present study.

1.8 Thesis hypothesis and objectives

1.8.1 Summary of rationale

- Previously, our laboratory reported that SHIP-deficient mice develop spontaneous CD-like ileal inflammation.
- In our research investigating the lipid phosphatase SHIP, it was discovered that tuft cells express SHIP, previously thought to be hematopoietic-restricted.
- SHIP deficiency leads to increased PI3-kinase activity in cells resulting in increased cell proliferation, reduced apoptosis, and increased immune cell activation.
- The onset of inflammation coincides with the developmental appearance of tuft cells, at 4 weeks of age in SHIP-deficient mice, a model of CD.
- Tuft cells are the only epithelial cells in the uninflamed intestine that express COX1 and COX2, required for prostaglandin production.
- In wild type mice, tuft cells are found in the lung and ileum, both locations where SHIP-deficient mice develop spontaneous inflammation.
- Tuft cell numbers were increased 6-fold in the inflamed ileum of SHIP-deficient mice, prompting the investigation presented herein.

1.8.2 Hypothesis and objectives

Based on this, I hypothesized that SHIP deficiency in intestinal tuft cells contributes to intestinal inflammation in the $SHIP^{-/-}$ mouse by increasing COX activity. To investigate this hypothesis, I had two specific aims:

Aim 1: To determine whether tuft cell hyperplasia was present before and/or after the onset of ileal inflammation in SHIP$^{-/-}$ mice.

Aim 2: To determine whether prophylactic or therapeutic treatments with piroxicam (a non-selective COX inhibitor) would be able to prevent or treat ileal inflammation in SHIP$^{-/-}$ mice.

I quantitated tuft cells numbers along the intestinal tract in SHIP$^{+/+}$ and SHIP$^{-/-}$ mice by immunohistochemistry (IHC). SHIP$^{-/-}$ mice were treated prophylactically or therapeutically with COX inhibitors. COX activity, PGE2, and PGD2 were measured. Measurements of inflammation included concentrations of pro-inflammatory cytokines IL-4, IL-13 and IL-1β and histological features of inflammation in the SHIP$^{-/-}$ mice that have been described previously: muscle thickening, immune cell infiltration, villus length, and goblet cell hyperplasia.

1.8.3 Significance

These studies will contribute to the understanding of the role of tuft cell-derived SHIP and COX in the spontaneous ileitis in SHIP$^{-/-}$ mice. Importantly, this work may help elucidate some of the basic biology involved in the inflammation present in CD patients, pointing to the connection between tuft cells, COX enzymes, and the underlying tissue, with tuft cells linking signals from the lumen to the immune system.

Chapter 2: Materials and methods

2.1 Mice

SHIP heterozygotes, an F2 generation of C57BL/6 x 129Sv mice, were bred to generate $SHIP^{+/+}$ and $SHIP^{-/-}$ littermates, which were co-housed after weaning. Mice were maintained in sterilized filter-top cages and fed autoclaved food and water under specific pathogen-free conditions at the Animal Care Facility at the BC Children's Hospital Research Institute (Vancouver, BC). Sentinel mice were routinely screened for pathogens using a comprehensive serological profile service (Radil, Columbia, MO). All mice used were between the ages of 4 and 10 weeks. Experimentation was performed in accordance with Canadian Council on Animal Care Guidelines and with approval from the institutional Animal Care Committee (Protocols A17-0071 and A17-0277).

2.2 Radiation and bone marrow transplantation

Mice were irradiated with a single dose of 550 Gy using a Rad Source S-2000 and administered 1.0×10^7 bone marrow cells prepared from $SHIP^{+/+}$ and $SHIP^{-/-}$ donor mice via tail vein injection. Chimeric mice were analyzed 16 weeks post-BMT.

2.3 Immunofluorescence

Paraffin-embedded sections were deparaffinized by heating to 60°C for 20 min, washed with xylene, followed by 3 ethanol washes (100% twice, 95%, 80%), and one final wash with water. Finally, sections were steamed for 20 min in 1mM EDTA buffer, pH 8.0, for antigen retrieval. Tissues were then treated with blocking buffer (goat or donkey serum in PBS

containing 1% bovine serum albumin [BSA], 0.1% Triton X-100, 0.05% Tween 20, and 0.05% sodium azide). The primary antibodies used were anti-SHIP (P1C1- Santa Cruz sc-8425), anti-DCLK1 (ab37994 – ABCAM), anti-HPGDS (160013 - Cayman Chemical), and anti-COX1 (M20 - Santa Cruz sc-1754). The secondary antibodies used were Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen #A11004), Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen #A11008), Alexa Fluor 488-conjugated donkey anti-goat (Invitrogen #A11055), and Alexa Fluor 568-conjugated donkey anti-rabbit (Invitrogen #A10042). 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen D3571) was used to stain DNA. ProLong gold anti-fade reagent was used to mount tissues. Negative controls, containing no primary antibody, were performed for all stainings. Tissues were viewed and images captured on a Zeiss Axiovert 200 microscope, AxiocamHR camera, and Axiovision 4.0 software. DCLK1+ tuft cells were counted manually and quantitated relative to total epithelial cells, in 6 sections per mouse separated by 50µm by two individuals blinded to experimental conditions.

2.4 COX activity assay

COX activity was measured using the COX Fluorescent Activity Assay Kit (Cat. No. 700200) from Cayman Chemical (Michigan, MI, USA). Briefly, fresh ileal samples were collected, rinsed with PBS and homogenized in 1.5mL lysis buffer (0.1% Triton X-100, 25 mM Tris pH 8, aprotinin (40g/mL), leupeptin (8g/mL), PMSF (100µM)) using a Polytron MR2100 bench top homogenizer. Homogenates were cleared by centrifugation at 16,000 ×*g* for 15 min at 4°C, and the supernatant was collected. Sample wells received 150µl of assay buffer, 10µl of Hemin, and 10µl of sample. Sample background wells received 160µl of assay buffer, 10µl of Hemin, and 10µl of sample. No COX inhibitors were used. Reactions were initiated by adding

10µl of arachidonic acid to the sample and positive control wells, but not the background wells. COX activity was determined by resorufin fluorescence (compared to a resorufin standard concentration between 0 and 10µM), analyzed with an excitation wavelength of 530-540 nm, and an emission wavelength of 585-595 nm on a Molecular Devices FilterMax F5 Multi-Mode Microplate Reader, using the proprietary Molecular Devices Multi-Mode Analysis software version 3.4.0.25.

2.5 PG and cytokine ELISAs

PG and cytokine ELISAs were performed on approximately 150 mg of clarified full-thickness ileal homogenates, normalized to gram of tissue, from mice using enzyme-linked immunosorbent assays (ELISAs) according to the manufacturers' instructions. ELISA kits for mouse PGD2 (Cat. No. 512031) and PGE2 Cat. No. 500141) were from Cayman Chemical (Michigan, MI, USA); ELISA kits for IL-1β (Cat. No. DY401-05) and IL-13 (Cat. No. DY413-05) were from R&D Systems (Minneapolis, MN, USA); and the ELISA kit for IL-4 (Cat. No. 555232) was from BD Biosciences (Mississauga, ON, Canada).

2.6 Piroxicam treatment

Piroxicam (10 mg/kg, Sigma, Cat. No. P5654) or PBS (vehicle) (DPBS, Gibco by Life BioSciences) was administered daily by IP injection to 4-week-old (prophylactic treatment) or 6-week-old (therapeutic treatment) $SHIP^{-/-}$ mice for 14 days. Mice were euthanized and tissues harvested for subsequent analyses.

2.7 Haemotoxylin and eosin (H&E) staining

Ileal tissue sections from $SHIP^{+/+}$ and $SHIP^{-/-}$ mice were fixed in PBS-buffered 10% formalin at 4°C for 24 hours. Tissue sections were embedded in paraffin, and 5 µm cross-sections were cut and stained with H&E by the histology core at the BC Children's Hospital Research Institute.

2.8 Histological analyses

Images of H&E stained tissue cross-sections were acquired using a Zeiss Axiovert 200 microscope, AxiocamHR camera, and Axiovision 4.0 software. Crypt/villus length was determined by counting epithelial cell nuclei from the crypt base to the villus tip on uniform horizontal ileal cross-sections. Goblet cells per crypt/villus were counted from the base of the crypt to the tip of the villus on uniform horizontal cross-sections. Immune cell infiltrates were counted in the circular muscularis externa and submucosa. In all cases, parameters were counted at 20× magnification in 6 H&E-stained sections separated by 50µm for each mouse, by two individuals blinded to experimental conditions.

2.9 Statistical analyses

Unpaired two-tailed Student's *t*-tests were performed as indicated, using GraphPad Prism version 6 (GraphPad Software Inc.). For multiple comparisons, the Bonferroni correction was applied. The Grubbs' test, or ESD method (extreme studentized deviate), was used to determine and exclude significant outliers. Outliers were identified only in PGD2 ELISAs (Figure 3.4B and Figure 3.7A). Differences were considered significant at $p < 0.05$.

Chapter 3: Results

3.1 The lipid phosphatase, SHIP, is expressed in intestinal epithelial cells

While staining murine ileal cross-sections for the lipid phosphatase, SHIP, our research team noted a strong expression of SHIP in a small population of cells within the epithelial cell layer. SHIP is considered to be restricted to hematopoietic cells.[201, 202] Thus, bone marrow transplants were performed to determine whether SHIP-expressing cells were intraepithelial lymphocytes (IELs) or epithelial cells. $SHIP^{+/+}$ or $SHIP^{-/-}$ bone marrow was transplanted into $SHIP^{+/+}$ or $SHIP^{-/-}$ mice and cross-sections from ilea were stained with SHIP, various epithelial cell markers (HPGDS is shown), and counterstained with DAPI 16 weeks post-transplant (Fig. 3.1). Upper panels show control transplants, i.e. $SHIP^{+/+}$ bone marrow into $SHIP^{+/+}$ mice and $SHIP^{-/-}$ bone marrow into $SHIP^{-/-}$ mice. SHIP expression in epithelial cells was retained when $SHIP^{-/-}$ bone marrow was transplanted into $SHIP^{+/+}$ mice (Fig. 3.1C), and there was no SHIP expression in epithelial cells when $SHIP^{+/+}$ bone marrow was transplanted into $SHIP^{-/-}$ mice (Fig. 3.1D). SHIP expression in cells within the epithelial cell layer maintained the recipient mouse genotype, demonstrating that SHIP expression is radioresistant and suggesting that SHIP is expressed in a subset of epithelial cells.

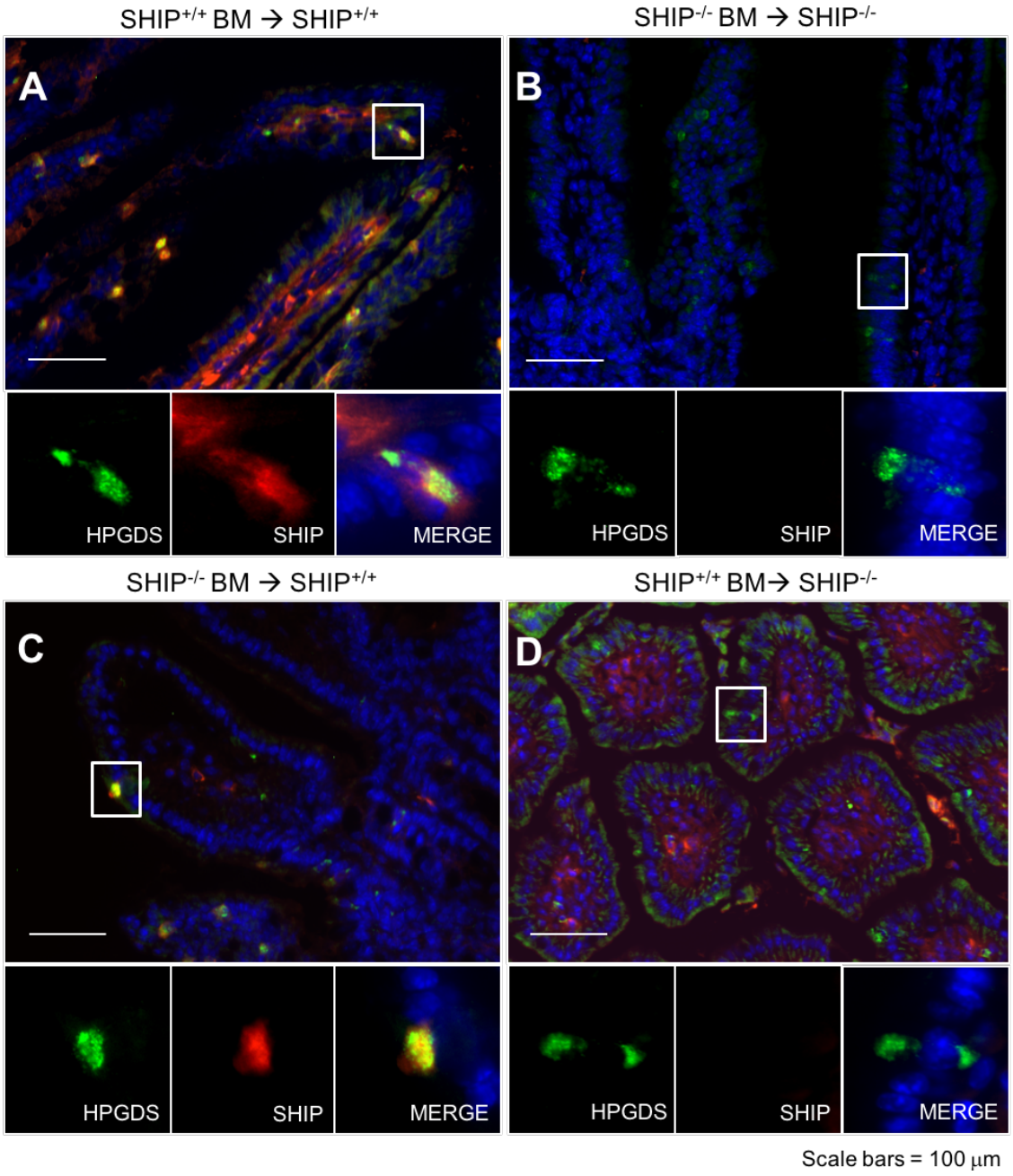

Figure 3.1. SHIP expression in intestinal epithelial cells is radioresistant.
Bone marrow transplants between SHIP$^{+/+}$ and SHIP$^{-/-}$ mice were performed to ensure that SHIP-expressing cells within the epithelium were not intraepithelial lymphocytes. Intestinal epithelium was assessed 16 weeks post-radiation treatment. Ileal cross-sections were stained for HPGDS (green), SHIP (red), and co-staining is shown (yellow). (A) Bone marrow from a SHIP$^{+/+}$ mouse transplanted into a SHIP$^{+/+}$ mouse. (B) Bone marrow from a SHIP$^{-/-}$ mouse transplanted into a SHIP$^{-/-}$ mouse. (C) Bone marrow from a SHIP$^{-/-}$ mouse transplanted into a SHIP$^{+/+}$ mouse. (D) Bone marrow from a SHIP$^{+/+}$ mouse transplanted into a SHIP$^{-/-}$ mouse. Photographs were taken at a magnification of 20× (top) and 40× (bottom). Scale bars = 100μm. Data shown are representative of 3 individual recipient mice per group, which yielded similar results.

3.2 Tuft cells express SHIP

Next, I asked which type(s) of intestinal epithelial cells express SHIP. Ileal cross-sections from wild type mice were co-stained with SHIP and various epithelial cell markers (data not shown). There was a nearly complete co-expression between SHIP and the tuft cell marker, HPGDS (Fig. 3.2A). There was also a nearly complete co-expression between SHIP and a second tuft cell marker, DCLK1 (Fig. 3.2B). Taken together, these data suggest that SHIP is expressed exclusively in tuft cells within the intestinal epithelium. Other epithelial cell markers used were mucin 2 (MUC-2) for goblet cells, alkaline phosphatase (ALP) for enterocytes, chromogranin-A (CgA) for EE cells, and lysozyme for Paneth cells.

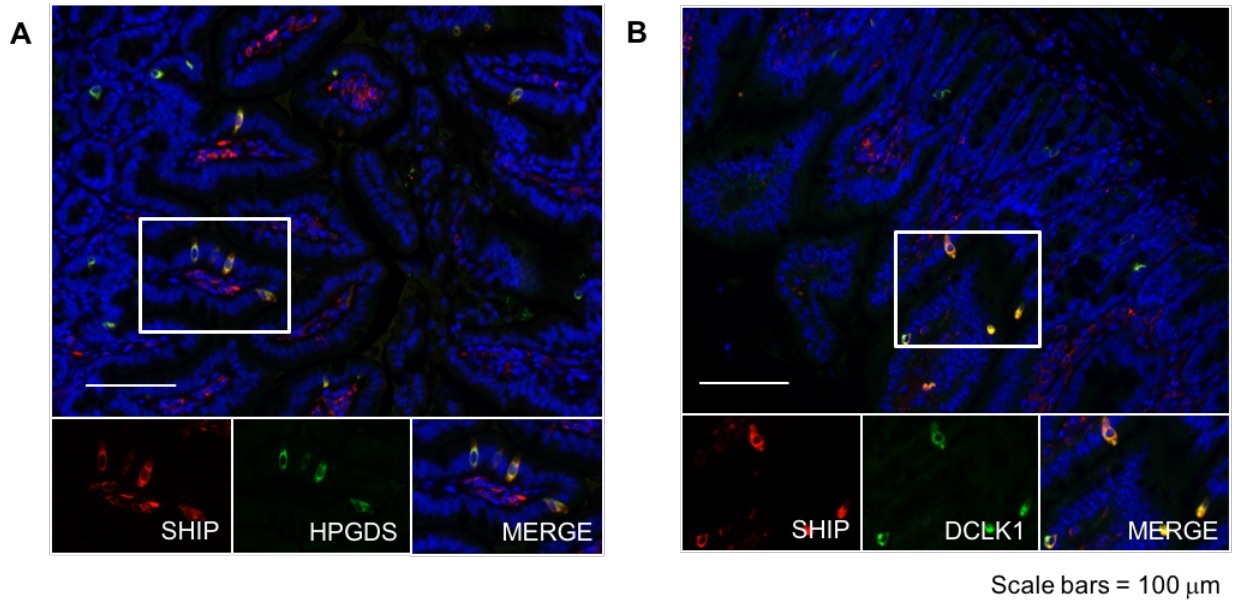

Figure 3.2. Tuft cells express SHIP, which was thought to be hematopoietic-specific. Ileal cross-sections from a SHIP$^{+/+}$ mouse were co-stained for SHIP and tuft cells markers. (A) Sections stained with SHIP (red), HPGDS (green), and co-staining (yellow). (B) Sections stained with SHIP (red), DCLK1 (green), and co-staining (yellow). Photographs were taken at a magnification of 20× (top and bottom). Scale bars = 100μm. Data shown are representative of 6 individual mice with similar results.

3.3 Inflammation, and not SHIP deficiency, drives tuft cell hyperplasia in SHIP$^{-/-}$ mice

It was noted that there were increased numbers of tuft cells in the ilea of SHIP$^{-/-}$ mice compared to SHIP$^{+/+}$ at 8 weeks of age. In order to determine whether SHIP deficiency or the inflammatory environment were driving tuft cell hyperplasia, tissue cross-sections from along the gastrointestinal tract of 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice were co-stained for SHIP and DCLK1 (Fig. 3.3A). Tuft cell hyperplasia was only evident in the inflamed distal ileum of 8-week-old SHIP$^{-/-}$ mice. Tuft cells were quantitated in tissue sections and no significant differences in tuft cell numbers were found between SHIP$^{+/+}$ and SHIP$^{-/-}$ mice at 4 weeks of age (Fig. 3.3B, left). Also, no significant differences were found in non-inflamed tissues of mice at 8 weeks of age (Fig. 3.3B, right). These results suggest that inflammation, rather than SHIP deficiency, drives tuft cell hyperplasia in the SHIP$^{-/-}$ mouse ileum.

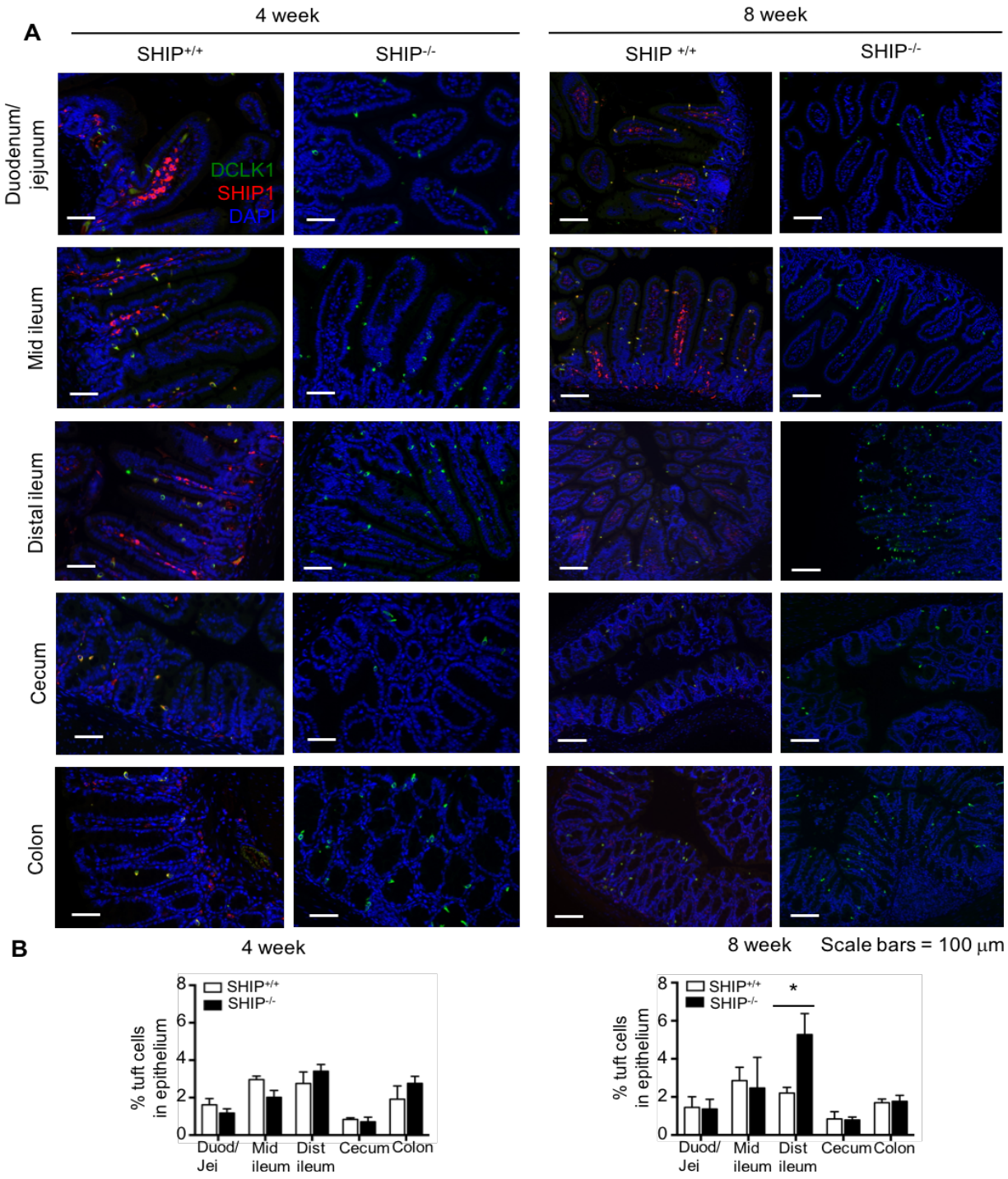

Figure 3.3. Tuft cell distribution along the intestinal tract.
(A) Duodenum/jejunum, mid ileum, distal ileum, cecum, and colon cross-sections of 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice co-stained with DCLK1 (green) and SHIP (red). Photographs were taken at a magnification of 20×. Scale bars = 100μm. Data shown are representative of 6 individual mice with similar results. B) Tuft cell quantification in 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice. *$p \leq 0.01$ comparing SHIP$^{+/+}$ with SHIP$^{-/-}$ mice tissues using a Student's *t*-test with Bonferroni correction for multiple comparisons.

3.4 COX1 expressing cells and COX activity are elevated in the SHIP$^{-/-}$ mouse ileum

Previous studies report that tuft cells are the only epithelial cells that express the cyclooxygenase enzymes, COX1 and COX2, in the absence of inflammation.[146, 190] Because COXs can contribute to inflammation, it was next asked whether COX1 expression and/or COX activity were elevated in the SHIP$^{-/-}$ mouse ileum. To do so, ileal and colonic cross-sections from 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice were stained for DCLK1 and COX1. There was a notable increase in COX1-expressing, DCLK1+ cells in the inflamed distal ileum of SHIP$^{-/-}$ mice, as well as other COX1-expressing cells in the lamina propria, which were most likely sub-epithelial immune cells (Fig. 3.4A, top right). Consistent with previous reports, tuft cells represent the vast majority of COX1-expressing epithelial cells in the absence of inflammation, in both 4-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ ilea, in 8-week-old SHIP$^{+/+}$ mice, and in colon cross-sections from both ages and genotypes (Fig. 3.4A).

Total COX activity and COX products, PGD2 and PGE2, were measured by ELISA in full-thickness tissue homogenates from the ilea of 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice and the colon of 8-week-old SHIP$^{-/-}$ mice, as an age-matched, non-inflamed control (Fig. 3.4B). COX activity, PGD2, and PGE2 were all significantly higher in the inflamed ileal tissues from 8-week-old SHIP$^{-/-}$ mice compared to their wild type littermates. Intriguingly, COX activity was also increased in ileal sections from 4-week-old SHIP$^{-/-}$ mice. This suggests that SHIP deficiency in tuft cells is sufficient to increase COX activity even in the absence of inflammation, and increased COX activity may contribute to the spontaneous development of ileal inflammation in the SHIP$^{-/-}$ mouse.

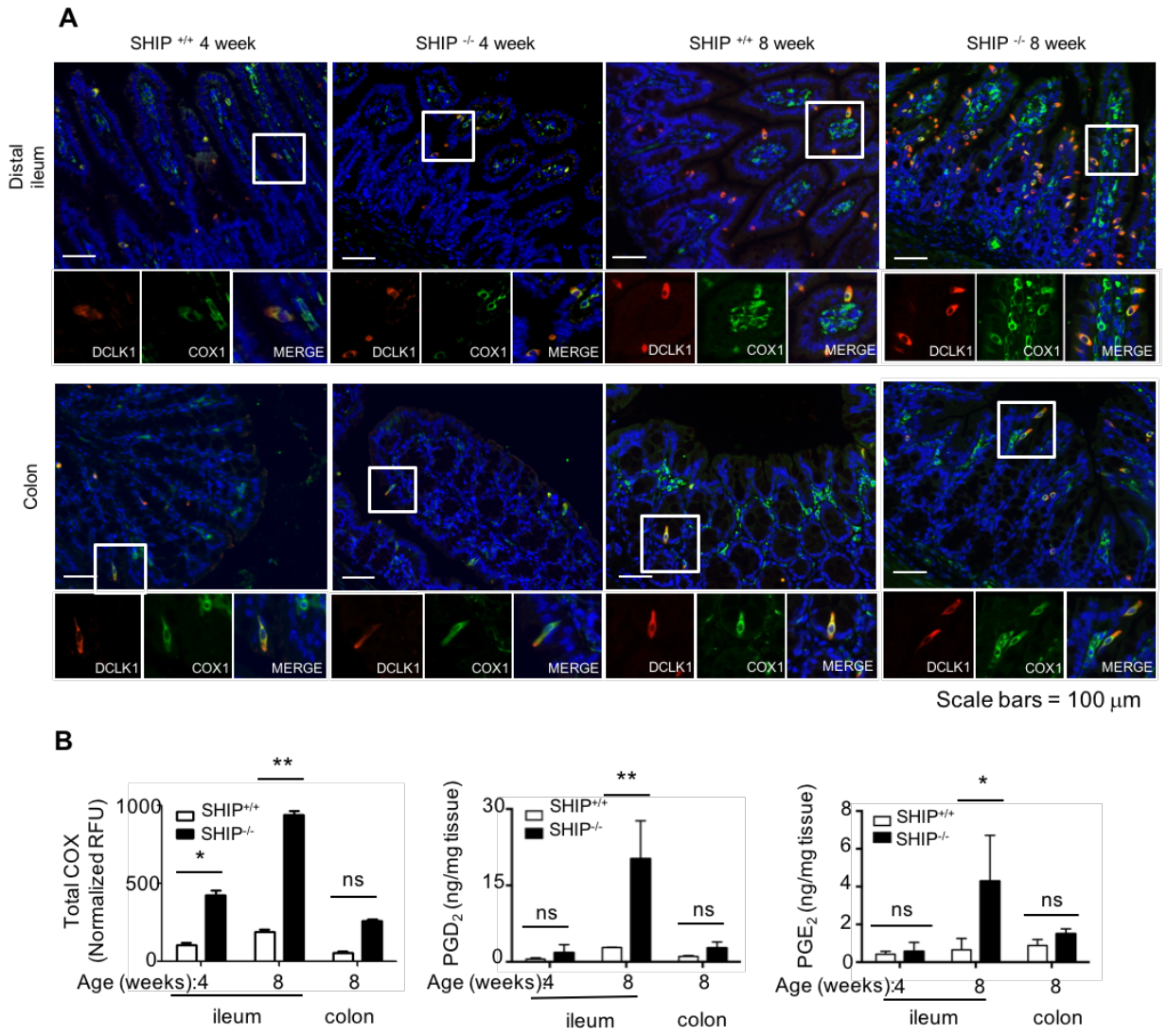

Figure 3.4. The inflamed SHIP$^{-/-}$ distal ileum presents more COX1-positive cells.
A) Immunofluorescent co-staining of DCLK1 (red), COX1 (green), and co-staining (yellow) of ileal and colonic cross-sections from 4- and 8-week-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice. Photographs were taken at a magnification of 20×. Scale bars = 100μm. Data shown are representative of 6 individual mice with similar results. (B) COX activity, PGD2, and PGE2 in ilea from 4- and 8-week-old mice and colons from 8-week-old SHIP$^{+/+}$ mice. n = 9 mice per group. *p ≤ 0.05, **p ≤ 0.01, ns = not significantly different comparing SHIP$^{+/+}$ and SHIP$^{-/-}$ mice using a Student's *t*-test with Bonferroni correction for multiple comparisons.

3.5 Piroxicam can be used prophylactically to prevent ileal inflammation in SHIP$^{-/-}$ mice

To determine whether increased COX activity in the 4-week-old SHIP$^{-/-}$ mouse ileum contributes to the development of ileal inflammation in the SHIP$^{-/-}$ mouse, SHIP$^{-/-}$ mice were treated with the COX inhibitor piroxicam. Piroxicam is one of the few NSAIDs that can be administered by parenteral routes.[306, 307] Thus, SHIP$^{-/-}$ mice received daily intraperitoneal (IP) injections of 10mg/kg piroxicam, or an equal volume of PBS as a vehicle and injection control, for 14 days, starting at either 4 weeks of age, before the onset of inflammation, for prophylactic treatment or 6 weeks of age for therapeutic treatment (after inflammation is evident). Prophylactic treatment with piroxicam reduced gross pathology associated with SHIP$^{-/-}$ ileal inflammation, including redness and size (Fig. 3.5A). Prophylactic treatment also reduced histopathology evident in H&E-stained ileal tissue cross-sections (Fig. 3.5B). Quantitation of histopathology demonstrated that SHIP$^{-/-}$ mice treated with piroxicam had significantly reduced crypt-villus hyperplasia, goblet cell hyperplasia, and reduced immune cell infiltration into the tissue, relative to sham treated controls (Fig. 3.5C).

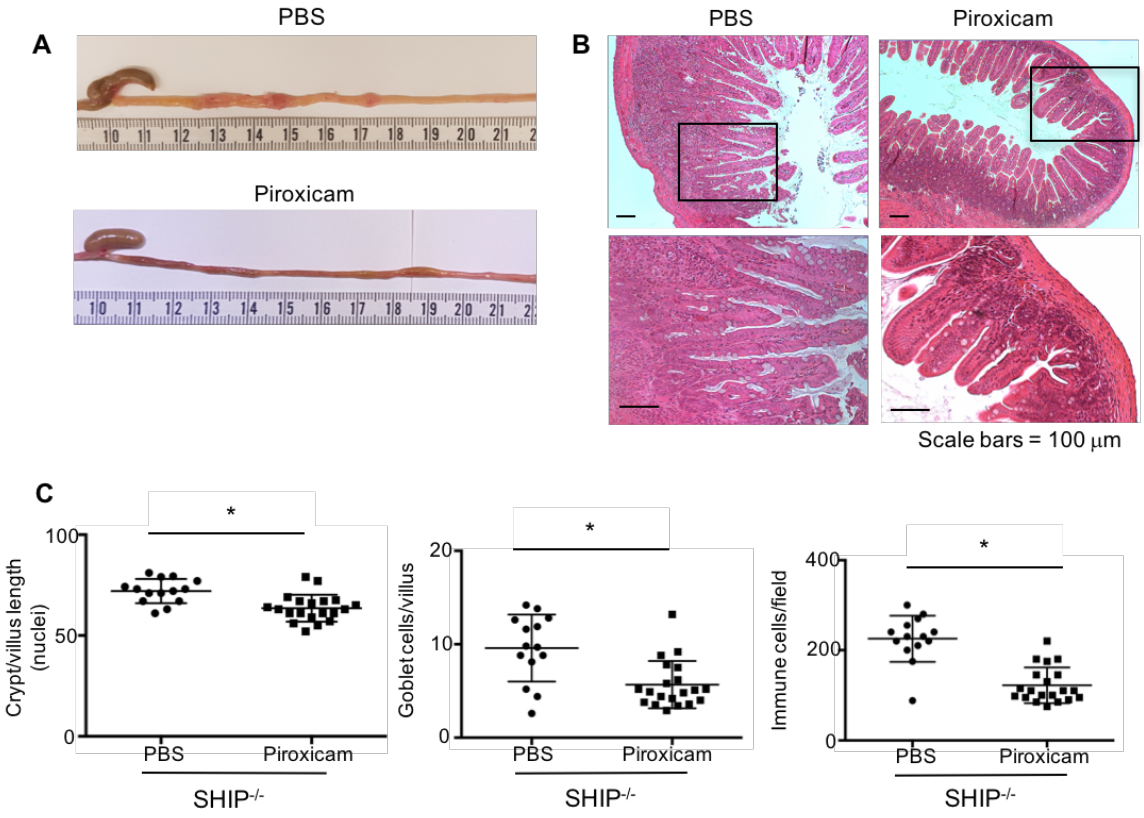

Figure 3.5: Piroxicam can be used prophylactically to prevent ileal inflammation in SHIP$^{-/-}$ mice.
A) Gross pathology of piroxicam-treated SHIP$^{-/-}$ mice compared to vehicle (PBS)-treated SHIP$^{-/-}$ mice. (B) H&E-stained ileal cross-sections of vehicle-treated and piroxicam-treated SHIP$^{-/-}$ mice. Photographs were taken at a magnification of 10× (top) and 20× (bottom). Scale bars = 100μm. (C) Crypt/villus length (left), quantification of goblet cells (middle), and quantification of immune cell infiltration (right) from SHIP$^{-/-}$ mice treated with vehicle or piroxicam. Points represent individual mice and lines show mean +/- SEM for each group. n = 14 mice treated with vehicle and 20 mice treated with piroxicam. *$p \leq 0.001$ comparing each histological feature of piroxicam- and vehicle-treated SHIP$^{-/-}$ mice using a Student's *t*-test with Bonferroni correction for multiple comparisons.

3.6 Piroxicam treatment is less effective at reducing ileal inflammation in SHIP$^{-/-}$ mice when used therapeutically

SHIP$^{-/-}$ mice were also treated with piroxicam therapeutically. Piroxicam was given to mice by IP injections beginning at 6 weeks of age, after inflammation was established. Mice were treated with piroxicam daily by IP injection for 14 days. After 14 days, mice were euthanized and tissues were harvested. In contrast to prophylactic treatment, therapeutic treatment with piroxicam was less effective at reducing inflammation in the SHIP$^{-/-}$ mice. Gross pathology and histopathology were only modestly reduced, with redness and swelling still present (Fig 3.6A and B). Though, crypt-villus and goblet cell hyperplasia were significantly reduced, immune cell infiltration was not reduced in the ilea of SHIP$^{-/-}$ mice treated therapeutically with piroxicam compared to vehicle-treated control mice (Fig 3.6C). This suggests that inflammation is still present, despite a modest decrease in crypt/villus length and goblet cell counts.

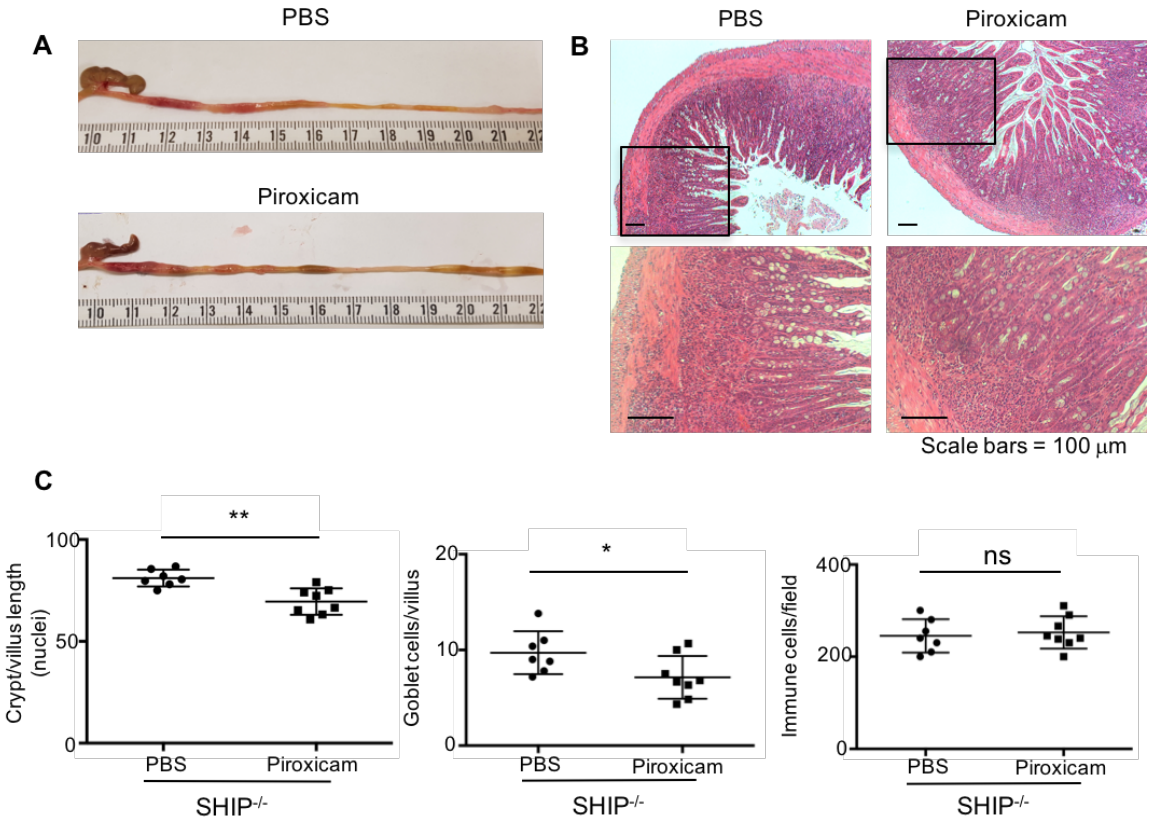

Figure 3.6: Piroxicam cannot be used therapeutically to reduce ileal inflammation in SHIP$^{-/-}$ mice.
(A) Gross pathology of piroxicam-treated SHIP$^{-/-}$ mice compared to vehicle-treated SHIP$^{-/-}$ mice. (B) H&E-stained ileal cross-sections of vehicle-treated and piroxicam-treated SHIP$^{-/-}$ mice. Photographs were taken at a magnification of 10× (top) and 20× (bottom). Scale bars = 100μm. (C) Crypt/villus length (left), quantification of goblet cells (middle), and quantification of immune cell infiltration (right) from SHIP$^{-/-}$ mice treated with vehicle or piroxicam. Points represent individual mice and lines show mean +/- SEM for each group. n = 7 mice treated with vehicle and 8 mice treated with piroxicam. *$p \leq 0.05$, **$p \leq 0.01$, ns = not significantly different comparing histological features of piroxicam- and vehicle-treated SHIP$^{-/-}$ mice using a Student's *t*-test with Bonferroni correction for multiple comparisons.

3.7 Increased COX activity in SHIP-deficient tuft cells may initiate IL-1β-driven autoinflammation in SHIP$^{-/-}$ mice

The COX enzymes are critical players in PG biosynthesis and the inflammatory response. Total COX activity as well as PGD2 and PGE2 levels, were assessed in distal ileal homogenates of vehicle- and piroxicam-treated SHIP$^{-/-}$mice. Prophylactic piroxicam treatment reduced COX activity by 72.2%, and PGD2 and PGE2 levels were reduced by 69.1% and 63.2%, respectively, compared to the vehicle-treated controls (Fig. 3.7A). Thus, piroxicam effectively lowered total COX activity and PG levels. Tuft cells present in the distal ilea of both piroxicam- and vehicle-treated mice were stained by immunofluorescence for DCLK1 and quantitated. Piroxicam treatment caused a 54.2% reduction in tuft cell numbers in the SHIP$^{-/-}$ ilea compared to vehicle-treated mice (Fig. 3.7B). Our research team had previously demonstrated that macrophage-derived IL-1β drives autoinflammatory ileitis in SHIP$^{-/-}$ mice.[232] Given that prophylactic piroxicam treatment effectively reduced inflammation in SHIP$^{-/-}$ ilea, IL-1β levels were examined in full thickness ileal tissue homogenates. Ileal IL-1β levels were reduced by 85.3% in SHIP$^{-/-}$ mice treated prophylactically with piroxicam compared to vehicle treated controls. Our research team had also reported that the Th2 cytokines, IL-4 and IL-13, are elevated in the inflamed SHIP$^{-/-}$ mouse ilea.[92] Thus, IL-4 and IL-13 were measured in clarified full-thickness ileal tissue homogenates. No significant differences in IL-4 or IL-13 levels were found in piroxicam-treated compared to vehicle-treated SHIP$^{-/-}$ mice (Fig. 3.7C).

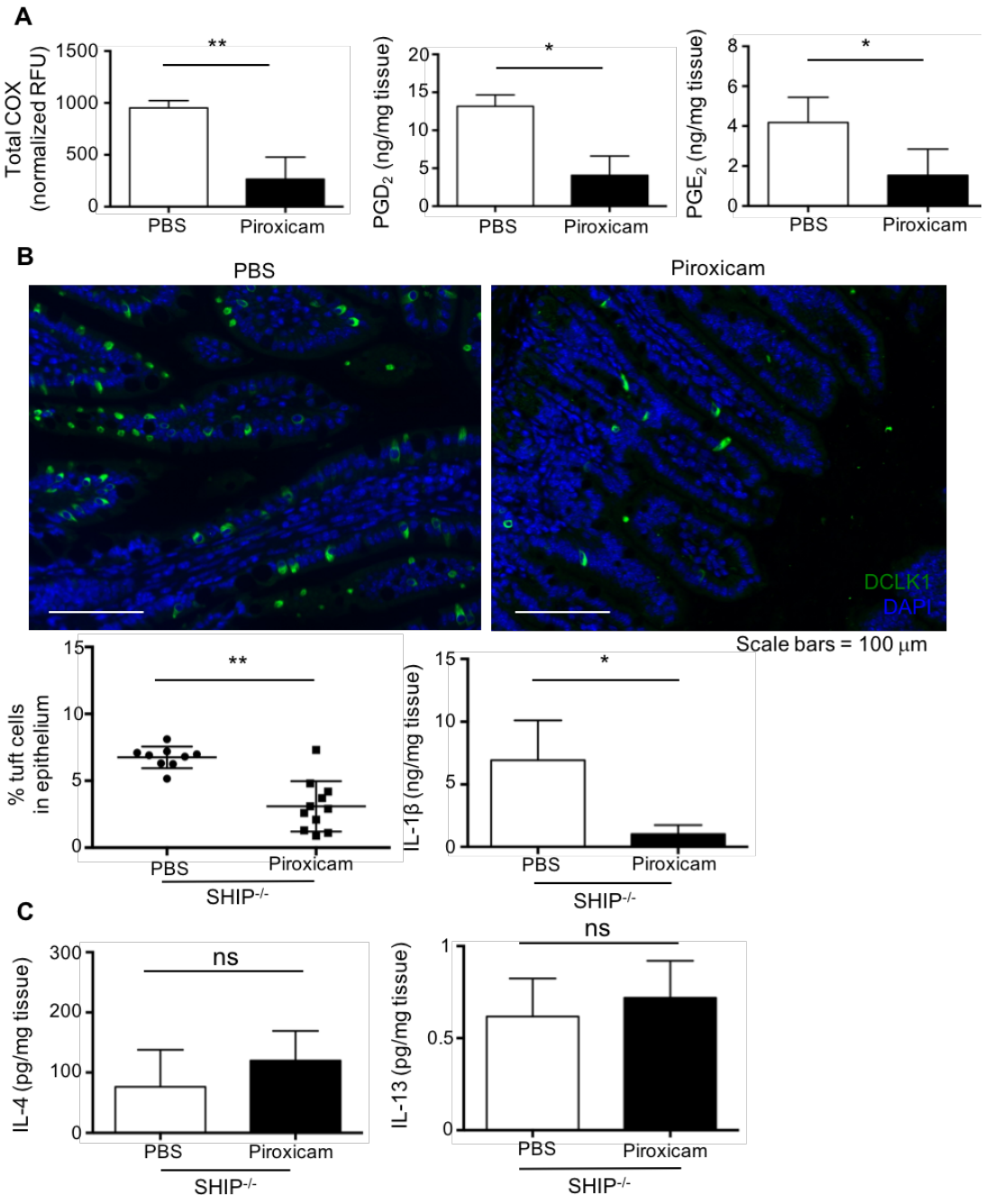

Figure 3.7: Piroxicam treatment reduces initiation of autoinflammatory response in SHIP$^{-/-}$ mice.

Increased COX activity in SHIP deficient tuft cells may initiate IL-1β-driven autoinflammation in SHIP$^{-/-}$ mice. (A) COX activity, PGD2, and PGE2 in distal ileum of vehicle- and piroxicam-treated SHIP$^{-/-}$ mice. (B) Immunofluorescent staining of DCLK1 in ileal sections (top), tuft cell quantification (lower left) and IL-1β concentration in full-thickness ileal tissue homogenates. (C) IL-4 (left) and IL-13 (right) concentrations in full-thickness ileal tissue homogenates. *p value ≤ 0.01, **p value ≤ 0.001, ns = not significantly different comparing piroxicam- and vehicle-treated SHIP$^{-/-}$ mice using a Student's *t*-test with Bonferroni correction.

3.8 Piroxicam treatment is less effective at reducing IL-1β levels in SHIP$^{-/-}$ mice when used therapeutically

Analysis of COX activity, PGD2, and PGE2 levels in the ilea of SHIP$^{-/-}$ mice that received therapeutic piroxicam treatment demonstrated that piroxicam reduced COX activity in SHIP$^{-/-}$ mice, as expected (Fig. 3.8A). However, therapeutic treatment did not cause a reduction in tuft cell hyperplasia in the SHIP$^{-/-}$ ilea compared to vehicle-treated mice (Fig. 3.8B). Though IL-1β levels were reduced by 35.6% by therapeutic treatment with piroxicam, this is much lower than the 85.3% reduction achieved when using piroxicam prophylactically (Fig. 3.8C). The relative levels of the Th-2 associated cytokines IL-4 and IL-13 were also not significantly different when comparing the two groups, suggesting that the overall Th2 response was not affected by COX inhibition (Fig. 3.8C). Taken together, these data demonstrate that prophylactic piroxicam treatment is more effective at reducing ileal inflammation in SHIP$^{-/-}$ mice than therapeutic treatment. This suggests that elevated COX activity may play an important role in the initiation of ileal inflammation in SHIP$^{-/-}$ mice.

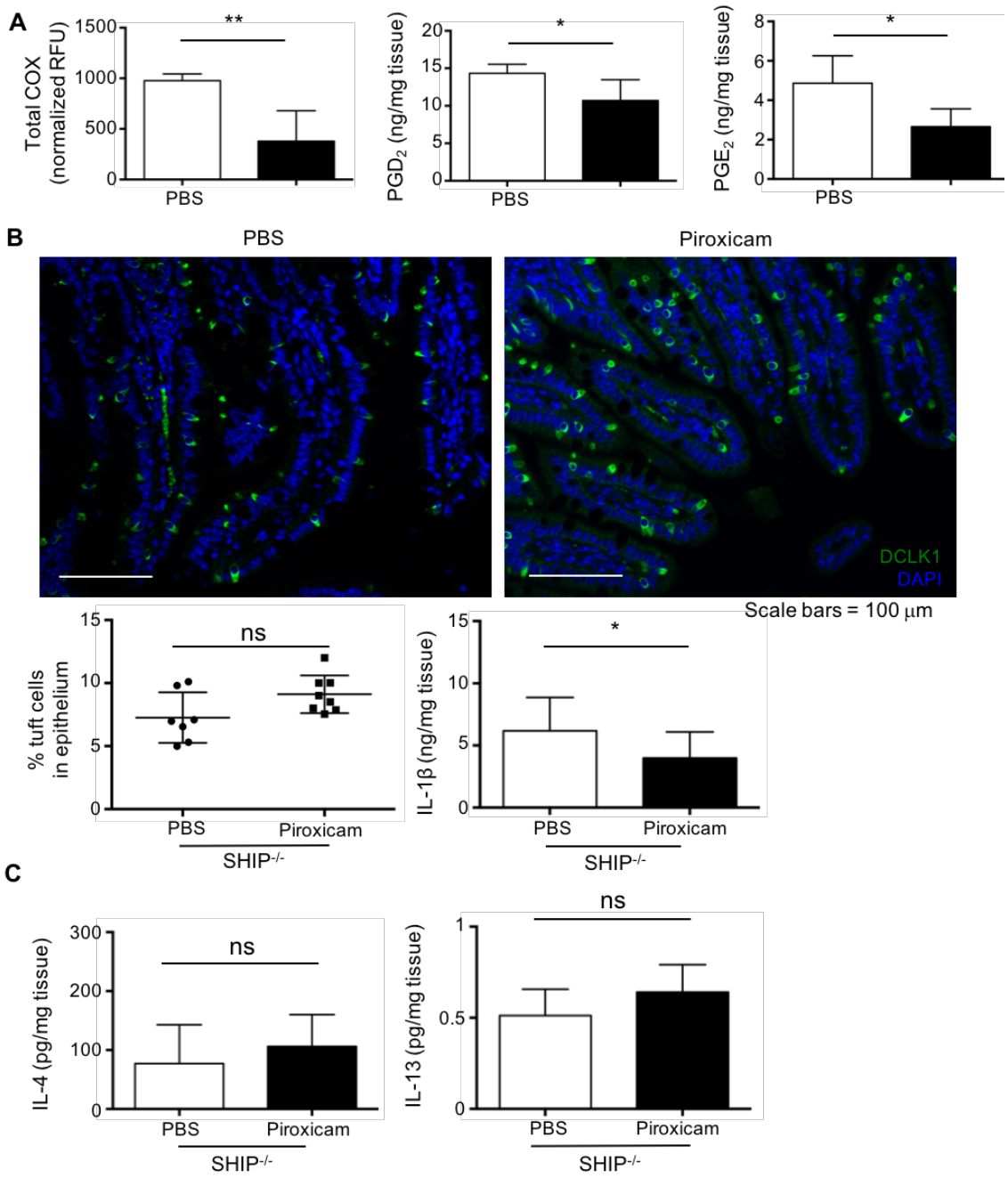

Figure 3.8: Therapeutic treatment with piroxicam is not sufficient to treat autoinflammatory response in SHIP$^{-/-}$ mice.
(A) COX activity, PGD2, and PGE2 in distal ileum of SHIP$^{-/-}$ mice treated prophylactically with vehicle or piroxicam. (B) Immunofluorescent staining of DCLK1 in ileal sections (top), tuft cell quantification (lower left) and IL-1β concentration in full-thickness ileal tissue homogenates (lower right). Photographs were taken at a magnification of 20×. Scale bars = 100μm. (C) IL-4 (left) and IL-13 (right) concentrations in full-thickness ileal tissue homogenates. For (A), (B), and (C); n = 7 mice treated with vehicle and 8 mice treated with piroxicam. *p ≤ 0.01, **p ≤ 0.001, ns = not significantly different comparing piroxicam- and vehicle-treated SHIP$^{-/-}$ mice using a Student's *t*-test with Bonferroni correction for multiple comparisons.

Chapter 4: Discussion

Herein, I show that DCLK1+ murine tuft cells express the lipid phosphatase SHIP, which was previously considered to be hematopoietic restricted. SHIP deficiency leads to increased COX activity in tuft cells. I also demonstrate that SHIP deficient mice have more COX1 positive cells in the inflamed ileum, as well as more COX activity, and higher PGD2 and PGE2 levels in full-thickness ileal tissue homogenates, compared to their wild type littermates. Finally, prophylactic treatment with piroxicam, a pan COX inhibitor, is effective at reducing the development of intestinal inflammation in SHIP-deficient mice, whereas therapeutic treatment had only minor effects.

The central paradigm for IBD is that CD is considered a Th1-mediated disease, whereas UC is Th2-mediated. However, both Th1 and Th2 cells are able to induce inflammation and aspects of IBD in animal models.[308] More recently, it has been reported that Th17 cells also contribute to pathogenesis in several animal models of IBD, previously considered to be driven by Th1 or Th2 cells.[308, 309] In humans, Th17 cytokines are highly expressed in the intestinal mucosa of people with both CD and UC,[104, 310-312] and results of GWAS demonstrate significant associations between genomic regions of the Th17/IL-23 pathway and IBD.[34] The SHIP-deficient mouse is an established model of CD-like intestinal inflammation that consistently recapitulates ileal localization, discontinuous inflammation, and the fibrosis that occurs in some patients with CD.[92] $SHIP^{-/-}$ mice ilea express a mixed Th2 and Th17 cytokine profile, with significantly increased production of IL-4, IL-13, and IL-23 compared to their $SHIP^{+/+}$ littermates.[92] The spontaneous inflammation is characterized by goblet cell hyperplasia, muscle thickening, increased collagen deposition, and immune cell infiltrates and aggregates.[92] Herein, I demonstrate tuft cell hyperplasia as another distinct feature of the inflammation present in these

mice. I have also demonstrated that piroxicam treatment was able to reduce IL-1β levels, which drives ileal inflammation in SHIP$^{-/-}$ mice.[232]

Tuft cells are the chemosensory cells of the GI tract and show a unique genetic signature, expressing genes previously associated with hematopoietic cell lineage.[171, 190, 313] A number of markers have been identified for intestinal tuft cells (reviewed in [135]) including structural (DCLK1, acetylated tubulin), taste-related (α-gustducin), and progenitor/stem (Sox9, Lgr5) markers.[314] Recently, McKinley *et al.* demonstrated the use of Multiplex immunofluorescence (MxIF) analytical tools for the characterization of intestinal tuft cells.[315] The team quantified tuft cell number and distribution throughout the mouse small intestine and colon, and identified two new intestinal tuft cell markers, Hopx and EGFR phosphotyrosine 1068 (p-EGFR).[315] Reports on tuft cell gene signatures using single-cell RNA sequencing have revealed different subsets of tuft cells that express genes related to immune regulation or neuronal development.[190, 313] Both subtypes of tuft cells express IL-25 but not IL-33, and also express receptors for the cytokines IL-4 (Il4ra), IL-13 (Il13ra1), and IL-25 (Il17rb), which could support autocrine signaling during Th2 cell responses.[313] One of the subtypes of tuft cells (tuft-2) distinguished by Haber *et al.* expresses the epithelial cytokine TSLP and CD45 (a pan-leukocyte marker), which was not previously associated with non-hematopoietic cells. The authors do not comment on tuft cell distribution.[313] These results point to a broad heterogeneity of tuft cells, within the intestinal compartment and colon, which probably indicates differential functions in a cell type previously considered a single homogenous population.[171, 190, 313-315] The gene signature of tuft cells also includes markers of the eicosanoid biosynthesis pathway, such as hematopoietic prostaglandin D synthase (H-PGDS), COX1 and COX2.[146, 190] Herein, I demonstrate that tuft cells are also the only epithelial cell type in the GI tract to express SHIP, which was previously

considered to be hematopoietic-restricted. The tuft cells in this study are most similar to the tuft-2 subset, since I have shown nearly complete co-expression of DCLK1 and SHIP, which is expressed exclusively in this subset.[313] These data are consistent with tuft cells being a unique epithelial cell in the gut, which express several markers previously associated with hematopoietic cell lineages, acting as a master regulator to integrate responses to mucosal stimuli.

Inflammation drives tuft cell expansion, which has been reported in the context of helminth infections.[171, 178, 179, 200] Howitt *et al.* demonstrated that infection of conventional and germ-free C57BL/6J mice with a diverse set of protozoa and parasitic helminths significantly increased the abundance of tuft cells from ~1% to 5-8% of total epithelial cells in the distal small intestine. This indicates that tuft cell expansion is a conserved response to parasite infection.[178] The data shown here are consistent with these studies and support tuft cell hyperplasia as a marker of inflammation. It has been shown that IL-4 or IL-13 are required to skew macrophages into an alternatively activated M2a phenotype.[227, 316] M2 macrophages are important during Th2 immune responses that mediate humoral immunity to defend against extracellular pathogens, such as parasitic worms.[317] It was demonstrated that $SHIP^{-/-}$ mice are Th2 skewed due to high levels of IL-4 produced by hyper-responsive $SHIP^{-/-}$ basophils.[318, 319] In the $SHIP^{-/-}$ mouse, SHIP deficiency in tuft cells is associated with an increase in COX activity, contributing to the exacerbated inflammation, which is expected from this mouse model. The elevated tuft cell numbers observed in this model are likely a conserved response to injury and/or type II inflammation, similar to that which occurs during parasitic helminth infections.

Tuft cells and tuft cell-derived IL-25 are protective in several mouse models of IBD.[320-323] In DSS-induced colitis, intestinal epithelial specific Dclk1-deficient mice ($Villin^{Cre}$;$Dclk1^{f/f}$) mice display exacerbated injury including higher damage scores, increased epithelial

permeability, higher levels of pro-inflammatory cytokines and chemokines, and dysregulated Wnt/b-Catenin pathway gene expression.[321] In addition, these mice exhibit increased gut permeability and higher IL-1β and IL-17 levels in the colon relative to wild type mice during DSS treatment.[321] This suggests that the tuft cell marker DCLK1 plays an important role in regulating colonic inflammatory responses and colonic epithelial integrity during DSS-induced colitis.[321] In addition, in an oxazolone-induced colitis model, administration of IL-25 improves the clinical symptoms, histopathological changes, and inflammation.[322] IL-25-mediated protection is associated with the induction of anti-inflammatory alternatively activated macrophages.[323] Th2 cytokines have been known to promote localized wound healing by enhancing alternatively activated macrophage activity that facilitates the production of proteins associated with accelerated tissue repair.[324] Consistent with these observations, tuft cell-generated IL-25 is reported to be significantly lower in the intestinal mucosa of people with IBD during active disease.[322, 325, 326] IL-25 levels were reported to be substantially lower during active disease than during remission, which may indicate that individuals with IBD have reduced tuft cell numbers or activity compared to healthy people.[326] This suggests that tuft cells and IL-25 may reduce the severity of intestinal injury and inflammation, and may be protective in IBD. In contrast, in this model, though tuft cells may be acting to dampen inflammation, SHIP deficient mice are hyper-responsive to innate and immune stimuli, and tuft cell hyperplasia alone is not sufficient to resolve the inflammation. Tuft cells are playing a role in Th2 inflammatory responses, and may be in fact leading to over-activation of Th2 cytokines and exacerbating inflammation in $SHIP^{-/-}$ mice.

The COX enzymes and their products play key physiological roles in various biological functions, and are associated with both promoting and dampening inflammation. COX1 is

considered a physiological 'housekeeping' enzyme whereas COX2 is induced in response to inflammation and specific signal transduction.[327] However, both enzymes contribute to the generation of autoregulatory and homeostatic prostanoids, and both can contribute to prostanoid release during inflammation.[245, 328] Prostaglandin production is generally low in uninflamed tissues, but increases rapidly during acute inflammation, prior to the recruitment of leukocytes and the infiltration of immune cells, in both mice and humans.[328] Here, I found that tuft cells were the only COX1 expressing epithelial cell type in the absence and presence of inflammation in SHIP$^{-/-}$ mice. Interestingly, COX activity was significantly increased in the uninflamed SHIP$^{-/-}$ ileum at 4 weeks of age. This suggests an early onset or build-up prior to the onset of overt intestinal inflammation, possibly leading to the inflammation present in SHIP$^{-/-}$ mice by 6 weeks of age.

NSAIDs inhibit COX1 and COX2 [329, 330] and exacerbate IBD, although there are conflicting reports about their association with IBD flares.[54-59] NSAIDs are generally the appropriate treatment for the arthropathies that are a common extra-intestinal complication of IBD.[331] COX-mediated disruption of the intestinal epithelial barrier associated with NSAID use can affect the interaction between the gut microbiome and immune cells in the intestinal epithelial layer, thus affecting risk for CD or UC. In addition, NSAIDs can alter platelet aggregation, release of inflammatory mediators, and microvascular response to stress, which may mediate CD and UC pathogenesis.[332-334] However, some studies report that there are no clear associations in flare-up events in IBD patients and NSAID use, and point to confounding factors and methodological shortcomings that may be in place when investigating such associations.[54-59] Overall, observational studies and clinical trials indicate that the majority of patients with quiescent IBD tolerate traditional NSAIDs, whereas about 20% of patients will experience a

clinical relapse;[335-337] however, NSAIDs are more frequently involved in aggravating pre-existing pro-inflammatory conditions.[336]

Though piroxicam is commonly used in NSAID-induced experimental colitis,[338-341] treatment during remission of IBD in humans is well tolerated and prevents the production of active prostanoids.[54, 55, 335] Indeed, 200 ppm piroxicam added to mouse chow causes toxicity in the gut and exacerbates colitis in IL-10$^{-/-}$ mice.[338, 339] Studies using other strains of mice are limited, but also point to piroxicam exacerbating a previous condition, when ingested with food.[340, 341] Such studies with NSAID-induced experimental colitis show that piroxicam, when taken orally, can damage the GI tract and increase inflammation. The irritancy caused by the "topical" effect is caused by the direct mucosal contact of the NSAID that occurs following oral ingestion and/or biliary excretion of the drug.[342] Considering these reports, I decided to administer intraperitoneal (i.p.) injections of piroxicam, in order to avoid the irritation caused by the "topical" effect on the intestinal mucosa. Indeed, I found that prophylactic i.p. injections of piroxicam are safe to use and did not exacerbate intestinal damage in this mouse model. Piroxicam reduced COX activity, PGD2, and PGE2 levels. Therapeutic administration showed that COX inhibition caused significant reduction in COX activity and PG levels, but these effects were not sufficient to dampen inflammation in the SHIP-deficient mouse model. Furthermore, prophylactic, but not therapeutic, treatment efficacy implicates COX enzymes in the onset of inflammation in the SHIP^{-} mice.

PGE2–EP receptor coupling may have a critical role in the onset of GI inflammation and/or tissue repair, but the available data is still limited. Even though PGE2 levels are significantly increased during IBD,[343] the functional role that PGE2 and EP receptors play in the pathogenesis of IBD remains undefined. PGE2 has been associated with intestinal protection,

attributed to the activation of EP3 and EP4 receptors, as well as protection of the gastric and intestinal mucosa.[344-346] The direct involvement of PGE2 in wound healing was demonstrated using mPGES-1 deficient mice, which exhibit delayed healing following acetic acid-induced gastric ulceration.[347] PGE2-EP4 is also protective during DSS-induced colitis[348-351] and may be beneficial in the treatment of gastric ulcers, duodenal ulcers, and certain forms of IBD.[346, 351, 352] In EP4 receptor knockout mice, a 7-day regime of DSS-induced colitis was reported to be more severe than that in wild type controls. This suggests that signaling via EP4 receptors may play a critical role in maintaining normal mucosal integrity and/or promoting healing.[351] It is possible that, in this model, PGE2 is acting to promote recovery and protection against injury, but the inflammation present in $SHIP^{-/-}$ mice does not resolve spontaneously, despite the high PGE2 levels reported herein. On the other hand, PGE2 may have an opposite effect, acting to promote and exacerbate the production of pro-inflammatory effectors. For example, studies *in vitro*, to address early responses of PGE2 in a variety of colonic epithelial cell lines clearly demonstrate that PGE2 couples via EP4 receptors to upregulate IL-8 mRNA expression and protein secretion confirming a pro-inflammatory role for PGE2.[353] As a pro-inflammatory PG, PGE2 has been implicated in regulation of the cytokine expression by DCs,[354] and plays a fundamental role in DC migration, permitting their homing to draining lymph nodes.[355, 356] Moreover, PGE2 potentiates Th1 and Th17 differentiation through PI3K and PKA , respectively, mediated by EP2 and EP4 receptors, and is associated with worsening TNBS-induced colitis.[265, 357, 358] PGE2 promotes the development and maturation of Th17 cells through activation of the EP2 receptor, while inhibiting IL-10 and IFN-γ synthesis through the EP4 receptor in human and mouse T cells, substantiating a role for PGE2 in regulation of Th17 responses.[259] Boniface *et al.* have shown that PGE2, in combination with IL-1β and IL-23, promoted differentiation of Th17

cells by upregulating the IL-1βR and IL-23R expression through the EP2/EP4-cAMP pathway.[259] The EP4 receptor is also capable of activating the PI3K signaling pathway by phosphorylation induced by G-protein-coupled receptor kinases (GRKs).[251, 359] In SHIP deficiency, this may ultimately result in the excess triggering of NF-κB-mediated transcriptional programs, as expected in this mouse model. Based on these observations, the exacerbated inflammation in SHIP$^{-/-}$ mice could be potentiated, in part, by the EP2-EP4 pro-inflammatory effects in T cells. However, there is a paucity of T cells (CD4+ and CD8+) in the inflamed mucosa of SHIP$^{-/-}$ mice,[92, 230] suggesting that T cells might not play an important role in the onset of intestinal inflammation in this model.

The anti-inflammatory effect of PGD2 in IBD patients and experimental models is thought to be mediated by activation of the DP receptor,[284, 290] while CRTH2 is considered to have pro-inflammatory effects.[360] PGD2 and the DP receptor have important anti-inflammatory effects in inhibiting the migration and activation of neutrophils, basophils, DCs, and T cells;[284] and are associated with ameliorating experimental colitis.[290] TNBS-induced colitis in rats results in a rapid increase in PGD2 synthesis via COX2 and a consequent reduction of granulocyte infiltration through activation of the DP receptor.[290] Levels of PGD2 in colon biopsies of patients during remission of UC are increased.[291] As reported in models of self-resolving inflammation[361] and experimental colitis,[290] the use of selective inhibitors of DP has been shown to abrogate the protective effects of PGD2, resulting in an increase in inflammatory cell infiltration and an imbalance in pro- and anti-inflammatory cytokines. Also, the increased susceptibility to chemically-induced colon cancer in rats that had recovered from TNBS-induced colitis was reversed by treatment with a DP receptor antagonist.[292] Interestingly, while PGD2 is regarded as anti-inflammatory in colitis, it promotes carcinogenesis after resolution of colitis.[293] The pro-

inflammatory effects of PGD2 and the CRTH2 receptor include T cell migration,[279] eosinophil chemotaxis,[360] and aggravation of asthma[362] and experimental models of IBD.[360] Other studies reported increased expression of L-PGDS[294] and infiltration of CRTH2-positive cells correlated with disease activity in UC patients.[363] The results herein are consistent with a model of IBD where PGD2 plays a pro-inflammatory role when it binds to its CRTH2 receptor.[360] The elevated levels of PGD2 found in the inflamed ilea of SHIP-deficient mice may indicate that PGD2 exacerbates the production of pro-inflammatory cytokines, and may contribute to the chemotaxis of eosinophils, basophils and monocytes to the site of inflammation.

It was also demonstrated that piroxicam was able to reduce IL-1β levels, which drives ileal inflammation in $SHIP^{-/-}$ mice.[232] IL-1β levels have also been correlated with disease severity in CD patients.[364-367] Low SHIP activity inversely correlates with elevated IL-1β production *ex vivo* in isolated macrophages in mice, in ileal tissues from mice, and in PBMCs from human subjects.[232] These data is consistent with the concept that high IL-1β levels play an important role in inflammation in SHIP-deficient mice and are associated with an inflammatory environment. Furthermore, no significant differences were found in the relative cytokine levels of IL-4 and IL-13 in $SHIP^{-/-}$ ilea with piroxicam treatment compared to vehicle-treated controls. The response observed is independent of the Th2 cytokines IL-4 and IL-13, suggesting that the mice treated prophylactically are still Th2-skewed, despite showing a healthier ileum.

In summary, the results identify DCLK1+ murine tuft cells as a unique cell in the gut, being the only epithelial cell type expressing the lipid phosphatase SHIP, and are consistent with tuft cell hyperplasia as a marker of inflammation. I have also focused on the role of tuft cells and COX inhibition in dampening inflammation in a prophylactic and therapeutic approach in this mouse model. Future studies investigating tuft cell properties and signaling pathways associated

with these sensory cells may possibly provide insight into ways of preventing inflammation and identify novel immunotherapeutic strategies to treat people with IBD.

Chapter 5: Concluding remarks and future directions

5.1 Concluding remarks

Intestinal epithelial cells play a critical role in mucosal homeostasis and dysregulation of pro-inflammatory epithelial cell function could lead to the intestinal inflammation that characterizes IBD. Tuft cells are a unique type of epithelial cell in the intestine that express COX1 and COX2, the rate-limiting enzymes required for production of prostaglandins, which play important roles in immune responses.[146, 190] In our research investigating the SHIP$^{-/-}$ mouse model of ileal inflammation, the research team discovered that tuft cells are the only epithelial cell type in the gut that expresses SHIP, currently believed to be restricted to hematopoietic cells. Additionally, these cells were found in high numbers in the inflamed distal ileum of these mice. Based on this, I hypothesized that SHIP deficiency in intestinal tuft cells contributes to intestinal inflammation in the SHIP$^{-/-}$ mouse by increasing COX activity. To investigate this hypothesis, I had two specific aims: 1. To determine whether tuft cell hyperplasia was present before and/or after the onset of ileal inflammation in SHIP$^{-/-}$ mice. 2. To determine whether prophylactic or therapeutic treatments with piroxicam (a non-selective COX inhibitor) would be able to prevent or treat ileal inflammation in SHIP$^{-/-}$ mice. To the best of my knowledge, this is the first set of experiments that focuses on the role of tuft cells in the context of COX inhibition in an IBD mouse model. In doing so, this work leads to a deeper understanding of the basic biology and some of the key players in inflammation in this mouse model of Crohn's disease.

The direct effects of tuft cells in human inflammatory bowel disease are presently unknown. These cells have been found to be protective in mouse DSS-induced colitis.[320-323] Also, tuft cell-generated IL-25 is reported to be significantly lower in the intestinal mucosa of

people with IBD during active disease.[322, 325, 326] These findings indicate that patients with active IBD may have fewer tuft cells than healthy controls. If the DSS model does translate to human disease, then, increasing tuft cell numbers might hypothetically dampen the pro-inflammatory response to IBD and possibly reduce the severity of intestinal injury. [320-323, 368] Taken together, these studies point to the protective role of tuft cells, triggering inflammatory processes leading to wound healing and increasing epithelial reconstitution in response to intestinal injury.[320-323, 325, 326] This may help explain the reasoning behind the exacerbated Th2 response, increased wound healing, most likely also leading to the fibrosis found in the SHIP$^{-/-}$ mice, as published by our lab recently.[369]

IL-1β is a pro-inflammatory cytokine critical in IBD pathogenesis. Our laboratory has previously demonstrated that the chronic ileitis in SHIP$^{-/-}$ mice is associated with elevated levels of macrophage-derived IL-1β, as well as the Th2 cytokines IL-4 and IL-13.[92, 232] In the present study, even when treating inflammation with piroxicam, Th2 cytokine levels remained high, and inflammation was still present, suggesting that other immune factors are at play. IL-1β is reduced following piroxicam treatment, both prophylactically and therapeutically. However, once inflammation has started, lowering COX activity and dampening IL-1β using piroxicam is not enough for inflammation to resolve completely. The inflammatory process is complex and, similar to other autoinflammatory diseases, COX inhibitors are not enough to treat inflammation therapeutically, often needing to be combined with an adjuvant therapy or replaced altogether.[370] Together, these findings contribute to the understanding of the role of tuft cells, tuft cell-derived SHIP and COX in the spontaneous ileitis in SHIP$^{-/-}$ mice.

5.2 Future directions

During intestinal infection with parasites, ILC2-derived IL-4 and IL-13 activate tuft cells to produce IL-25, which further amplifies type 2 cytokine secretion by ILC2s, creating a positive feedback loop, as described previously.[178, 371, 372] PI3Kp110δ is activated downstream of IL-4 receptor engagement and may play a role in regulating IL-4 production by hematopoietic cells, like basophils. I hypothesize that PI3Kp110δ deficiency in intestinal tuft cells leads to decreased IL-25 production and reduced IL-4 secretion and activation by ILC2s. To investigate this, our laboratory will use $SHIP^{+/+}PI3Kp110\delta^{+/+}$, $SHIP^{+/+}PI3Kp110\delta^{DA/DA}$, $SHIP^{-/-}PI3Kp110\delta^{+/+}$, $SHIP^{-/-}PI3Kp110\delta^{DA/DA}$ mice, already available in our animal facility. In collaboration with Dr. Lisa Osborne, an Assistant Professor at the University of British Columbia; Yvonne Pang, a Master's student in our laboratory, will infect mice (and assess uninfected controls) with *T. spiralis*, a helminth known to induce tuft cell hyperplasia.[178] Tuft cell and ILC2 numbers will be quantified by IHC/IF and IL-25 and IL-4 production will be measured by ELISA in full thickness tissue homogenates from healthy controls and infected mice. These studies will provide insight into the role of basophils, ILC2s, and tuft cells, in triggering intestinal inflammation in $SHIP^{-/-}$ mice, and whether cross-talk between these cell types is essential in the intestinal inflammation present in $SHIP^{-/-}$ mouse. Due to the tuft cell hyperplasia and the hyper-responsiveness of myeloid cells in SHIP-deficient mice, I hypothesize that these mice might be primed to clear such a helminth infection more efficiently than their $SHIP^{+/+}$ counterparts. However, it is also possible that they will fail to mount an appropriate response towards a worm infection since inflammation is already present in this mouse model, and adding another insult could worsen their condition. If this is the case, it is possible that SHIP

may be crucial in the overall signaling necessary for proper clearance of the helminth pathogens, leading to an inability of these mice to mount a proper immune response.

Recent studies suggest that tuft cells and tuft-cell-derived IL-25 are an important anti-inflammatory factor in the pathogenesis of IBD and a possible target to inhibit the Th1/Th17 inflammatory pathways, which are mediated by IL-12/IL-23.[320-323] It has been demonstrated that miR-31 can bind to the untranslated 3' region of IL-25 mRNA and directly regulate the expression of IL-25, in TNBS-induced colitis and IL-$10^{-/-}$ spontaneous colitis in mice. In the future, investigating whether administration of exogenous IL-25 and/or miR-31 inhibitors is also able to ameliorate inflammation in experimental models of ileitis may provide valuable clues for its effects in CD patients. IL-25 and miR-31 inhibitors may become new therapies for the treatment of IBD with potential benefits for patients and quality of life.

Despite recent work elucidating tuft cell function, the relationships and mechanisms between IL-25 and other epithelial cytokines capable of triggering Th2 immune responses, such as TSLP and IL-33, and the effects of tuft cell hyperplasia still need further investigation. Such studies may ultimately guide further mechanistic insights regarding how these mechanisms can be applied to ameliorate human disease, including IBD.

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