PI3Kp110δ drives Crohn's disease-like intestinal fibrosis in SHIP$^{-/-}$ mice

by

Young Lo

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Abstract

Crohn's disease (CD) is an immune-mediated disease characterized by inflammation along the gastrointestinal tract. One in 3 people with CD will develop intestinal fibrosis requiring surgery within 10 years of diagnosis. Despite that, there are no treatments that target intestinal fibrosis directly. Our laboratory reported that mice deficient in the Src homology 2 domain-containing inositol polyphosphate 5´-phosphatase ($SHIP^{-/-}$) develop spontaneous CD-like intestinal inflammation with arginase-dependent fibrosis. We also reported that increased arginase I activity in $SHIP^{-/-}$ macrophages was dependent on increased Class IA phosphatidylinositol 3-kinase (PI3K) p110δ activity. Based on this, we hypothesized that $SHIP^{-/-}$ mice develop fibrosis due to increased PI3Kp110δ activity. $SHIP^{-/-}$ mice were crossed with mice deficient in PI3Kp110δ activity ($PI3Kp110\delta^{DA/DA}$). $SHIP^{-/-}$ $PI3Kp110\delta^{DA/DA}$ mice have reduced intestinal fibrosis compared to their $SHIP^{-/-}$ littermates including: reduced muscle thickening, $vimentin^{+}$ mesenchymal cells, collagen deposition, arginase activity, and IL-4 levels. $SHIP^{-/-}$ mice were also treated with a PI3Kp110δ isoform-specific inhibitor, IC87114. Pharmacological inhibition of PI3Kp110δ activity also reduced the above parameters that are associated with intestinal fibrosis in $SHIP^{-/-}$ mice. Surprisingly, PI3Kp110δ deficiency or inhibition also reduced ileal inflammation and IL-1β production in the $SHIP^{-/-}$ ileum suggesting that PI3Kp110δ, and/or fibrosis itself, may contribute directly to inflammation. Our data suggest that $SHIP^{-/-}$ mice develop intestinal fibrosis that is driven by PI3Kp110δ activity. Moreover, targeting PI3Kp110δ activity may be an effective strategy to reduce intestinal fibrosis in people with CD. Importantly, there are PI3Kp110δ isoform-specific inhibitors already licensed for use in people with certain

leukemias and lymphomas, so this research may be rapidly translatable into an effective therapy for intestinal fibrosis in people with CD.

Lay Summary

Canada has the highest incidence of inflammatory bowel disease (IBD) in the world, with 129,000 being diagnosed with Crohn's disease (CD). Furthermore, 1 in 3 people with CD will develop severe intestinal fibrosis and strictures requiring surgery within 10 years of diagnosis. Despite that, there are currently no treatments that target intestinal fibrosis directly. Our research has found that pharmacological inhibition of the enzyme PI3Kp110δ found in our immune cells has significant effects in treating mice suffering from CD intestinal fibrosis. It not only blocked the development of intestinal fibrosis, but also reversed severe fibrotic symptoms in these mice. Importantly, these results may be rapidly translatable into an effective therapy for reducing intestinal fibrosis in people with CD, as PI3Kp110δ inhibitor drugs are already being used in hospitals to treat diseases like leukemias and lymphomas.

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 A09-0027 and A09-0032.

Chapter 1. Figure 1.1 was modified and reproduced with permission of Nature Publishing Group: Xavier RJ & Podolsky DK. Unraveling the pathogenesis of inflammatory bowel disease. Nature 2007: 448(7152). Figure 1.2 was reproduced with permission of ELSEVIER LTD: Zaki MH, Lamkanfi M & Kanneganti TD. The NLRP3 inflammasome: contributions to intestinal homeostasis. Trends in Immunology 2011; 32(4). Figure 1.3 was reproduced with the permission of Johns Hopkins Medicine (Baltimore, Maryland). Crohn's Disease Diagnosis at Johns Hopkins. Figure 1.4 was reproduced with the permission of American Society for Clinical Investigation. The Journal of Clinical Investigation: Thomas A. Wynn. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. 117:542-549. 2007.

Chapter 2, 3, 4. Yeung Lo conducted all the experimental work, and data analysis described herein, with the exception of the following contributions: Jean Phillipe Sauve helped with the arginase I and Ym1 co-staining of sample slides and taking fluorescent photographs used in this thesis (Figure 3.6, 4.4). Sandy Xj Wu helped with the quantification of the inflammatory phenotype in mice. Susan C. Menzies did all of the genotyping for the mice.

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List of Abbreviations

AKT	Protein kinase B (PKB)
ANOVA	Analysis of variance
argI	Arginase I
CD	Crohn's disease
CD4	Cluster of differentiation 4
CTGF	Connective tissue growth factor
DAPI	4´ 6-diamino-2-phenylindole
DCs	Dendritic cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome wide association studies
H&E	Hematoxylin and eosin
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IPF	Idiopathic pulmonary fibrosis
INPP5D	Gene encoding the SH2-domain-containing inositol polyphosphate-5´- phosphatase (SHIP) protein (human)
LP	*Lamina propria*
MCP	Membrane cofactor protein
MIP	Macrophage inflammatory protein

MMP	Matrix metalloproteinase
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PDGF	Platelet-derived growth factor
PIP	Phosphatidylinositol phosphate
PI3K	Phosphoinositide 3-kinase
PRRs	Pattern recognition receptors
SEM	Standard error of the mean
SHIP	src homology 2 domain containing inositol polyphosphate 5′ phosphatase
SNPs	Single nucleotide polymorphisms
STAT6	Signal transducer and activator of transcription 6
TGF-β	Transforming growth factor beta
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
Tregs	regulatory T cells
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor

Acknowledgements

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I would like to thank my supervisor, Dr. Laura Sly, for providing continuous support and guidance throughout my training, for providing me with the tools to become a better scientist, for teaching me effective scientific and communication skills, and for her generosity and kindness. I will forever be grateful that you accepted me as a Master's student, and provided me with all the opportunities I have had in your laboratory.

I would also like to thank my supervisory committee members, Dr. Neil Reiner and Dr. Gregor Reid. You have all been extremely supportive and have provided excellent suggestions that have helped me throughout my training. I also want to thank the following staff at CFRI, who have assisted with my research: Baoping Song (Histology core), and Claire Harrison (Animal Care Facility). I am also grateful to the Vallance laboratory at CFRI for helping me with microscopy.

I would like to thank all past and present members of the Sly laboratory and my friends at BC Children's Hospital research institute for their help, support, and the wonderful memories that I will take with me. This includes Dr. Shelley Weisser, Dr. Keith McLarren, Dr. Eyler Ngoh, Roger Jen, Susan Menzies, Lisa Kozicky, Mahdis Monajemi, Peter Dobranowski, Jean-Philippe Sauvé, Tariq Vira, Saelin Bjornson, and Sandy Wu.

Personally, I would also like to thank all my friends for supporting me throughout this journey. Special thanks to Irene, Yeelin, Jane, and Vishwas for continuous encouragement, support, and motivation. I deeply appreciate all your support.

To my parents,

You have supported me throughout my years of education, and have made many sacrifices to give me every opportunity to pursue my dreams.

This success is yours too.

Chapter 1: Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic, relapsing, idiopathic inflammatory disorder of the gastrointestinal tract that manifests in two main forms: Crohn's disease (CD) and ulcerative colitis (UC). IBD can affect individuals at any age, with peak incidence reported among individuals occurring in their twenties.[1] Common symptoms that patients experience include abdominal pain, diarrhea, rectal bleeding, and nutritional deficiencies.[2] No differences have been reported in the incidence rate of IBD between men and women, but other factors, such as ethnicity and diet have been shown to play significant roles.[1]

Symptoms of UC are typically confined to the colon and the rectum.[1, 3] Inflammation in UC is restricted to the mucosal and submucosal (epithelial) layers.[2] It is also characterized by ulcerations, rectal bleeding, and edema, which is swelling resulting from retention of excess fluid.[1] Histological examination of colonic tissue sections from UC patients reveals the presence of immune cell infiltrates, crypt abscesses, reduced goblet cell numbers, and disruption of crypt architecture.[1]

On the other hand, symptoms of CD can affect any part of the gastrointestinal tract, from the mouth to the perianal region.[1] Inflammation in CD is discontinuous, with patches of inflamed tissue separated by areas of healthy tissue; and CD affects all layers of the intestine (transmural).[1] The most common site of intestinal inflammation in CD is the distal ileum. Histological examination of ileal tissue sections reveals transmural inflammation accompanied by the presence of immune cells and granulomas.[1, 4] Additional key features of CD include: fistulas, which are channels connecting the

intestine to surrounding organs; and strictures, which result from excess collagen deposition leading to narrowing of the intestine that may require surgery in severe cases.[5]

The incidence rate of IBD is highest in northern Europe, the United Kingdom (UK), and North America.[6, 7] According to estimates from 2012, Canada has the highest incidence of IBD in the world, with an estimated 233,000 people with the disease.[8-10] Among these people, 104,000 were diagnosed with UC, and 129,000 were diagnosed with CD.[8-10] The incidence rate of IBD among children is rising in Canada, with estimates that 5900 children less than 18 years old have IBD.[11] This high incidence of IBD places a huge burden on families as well as the Canadian healthcare system. In 2012, it was estimated that the costs of IBD, including hospitalization, surgery, medication, and laboratory tests amounted to 2.8 billion dollars in Canada.[8, 10]

The chronic nature of IBD and its financial strains cause psychological stress, which also affects the quality of life of patients and their families.[10, 12] IBD is associated with social stigma, which can be reduced by increasing awareness of the disease in the general population.[10, 12] Because of the health, psychological, social, and economic consequences of IBD, organizations like the Canadian Institute of Health Research (CIHR) and Crohn's and Colitis Canada are committed to funding research to identify new therapeutic strategies to treat IBD, and thereby reduce the burden on the Canadian healthcare system and improve the lives of Canadians living with the 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, muscle spasms in the region of the pelvis, 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, 13] In children, IBD can result in delayed growth and delayed sexual maturity.[13, 14] Diagnosis, therefore, includes assessment for symptoms such as diarrhea, the presence of blood and mucus, abdominal pain, cramping, fever, weight loss; and if CD is suspected, perianal disease.[15] Diagnosis also includes review of the patient's medical history, recent use of medications, such as antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs), and a combination of tests and procedures to exclude pathology caused by the presence of enteric pathogens, such as *Clostridium difficile*, *E. histolytica*, *Salmonella* Typhimurium, or *Escherichia coli*.[1, 15, 16] In addition, family history of IBD is an important consideration during diagnosis for IBD. Sigmoidoscopy or colonoscopy are performed to determine the presence of uclers, bleeding, and inflammation.[15] Patients may also have X-rays, abdominal ultrasounds, CT scans, MRI or small bowel imaging, to determine the extent of disease and possible extra-intestinal complications.[1, 15]

While IBD can limit quality of life because of pain, vomiting, diarrhea, and other deleterious symptoms, it is rarely fatal on its own.[15] Fatalities due to complications such as toxic megacolon, bowel perforation, and surgical complications are also rare.[15] 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 are actually more likely to survive.[1, 15]

1.3 Etiology and pathogenesis

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 an immune responses to intestinal flora (Figure 1.1).[16, 17] My thesis focuses on an animal model of CD-like intestinal inflammation, so I will expand my introduction of etiology focusing on CD.

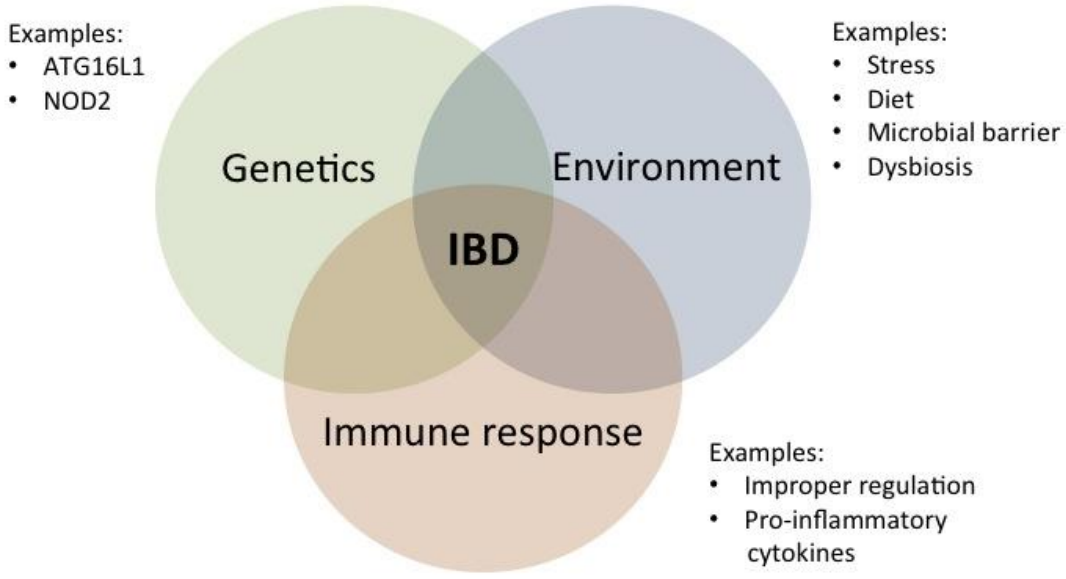

Figure 1.1 Etiology of Crohn's disease (CD). IBD lies at the intersection of genetic, environmental, and immunologic factors. It is believed that, IBD results from complex interactions between the intestinal microenvironment, the environment external to the host, and the immune response in genetically susceptible individuals. GWAS have identified 163 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.

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, 19] Studies in Sweden, revealed

the CD concordance rate in monozygotic twins was 50% whereas it was only 3.8% for dizygotic twins.[18, 19] A similar study in Denmark showed that the CD concordance rate was 50% in monozygotic twins and 0% in dizygotic twins.[18, 19] Among CD patients, between 2-14% have a family history of the disease.[20-23] However, very little is known about the effects of a positive family history on the severity and pathogenesis of CD in the individual.[24-29] 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) and *MUC19*, both of which are associated with epithelial barrier function,[33] as well as genes associated with interleukin 23 (IL-23) signalling pathway, such as *IL23R* and *STAT3* (signal transducer and activator of transcription 3),[32, 34, 35] which are critical in innate and adaptive immune responses.[30, 36, 37] Together, this suggests that CD may arise through distinct pathology-inducing mechanisms and thus, may be comprised of distinct pathological subsets of disease.[33, 38-40] 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.[41] This, coupled with dietary changes, have implicated environmental factors as playing an important role in the pathogenesis of CD.[41] As well, several specific environmental factors have been associated with increased risk for CD, including smoking, taking antibiotics and NSAIDs, low vitamin D levels, and stress.[42, 43] Studies have shown that cigarette smoking increases the risk of developing CD by two-fold.[42, 43] It reduces cell proliferation and alters the ratio of regulatory T cells to T helper cells in the gut.[41-44] 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.[45, 46] Patients diagnosed with IBD have also been reported to be deficient in vitamin D compared to healthy controls.[47]

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.[48, 49] Changes in the gut microbiota population may be due to changes in the external environment of the host.[48, 49] 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.[50-54] 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%).[54-56] Viral infections have also been shown to alter the gut microflora and have been implicated in CD pathogenesis.[57, 58] Upon infection with norovirus, mice show abnormal Paneth cell structure and granules similar to those observed in CD patients.[58] Interestingly, the CD risk allele in *ATG16L1* has also been associated with changes in the composition of the intestinal microflora.[57] 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 goblets cells, Paneth cells, enterocytes or colonocytes, and enteroendocrine cells, which separate the gut lumen from the *lamina propria* (LP) (Figure 1.2).[59] 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.[59-61] Goblet cells and paneth cells secret mucin and antimicrobial peptides that form the protective mucus layer between commensal bacteria and gut epithelial cells.[59-61] It is believed that one factor in developing CD is the result of damage to, or defects in, the epithelial barrier, which increases the epithelial permability.[62] Furthermore, in patients with CD, a dysregulated immune response to normal enteric microflora 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).[62-64]

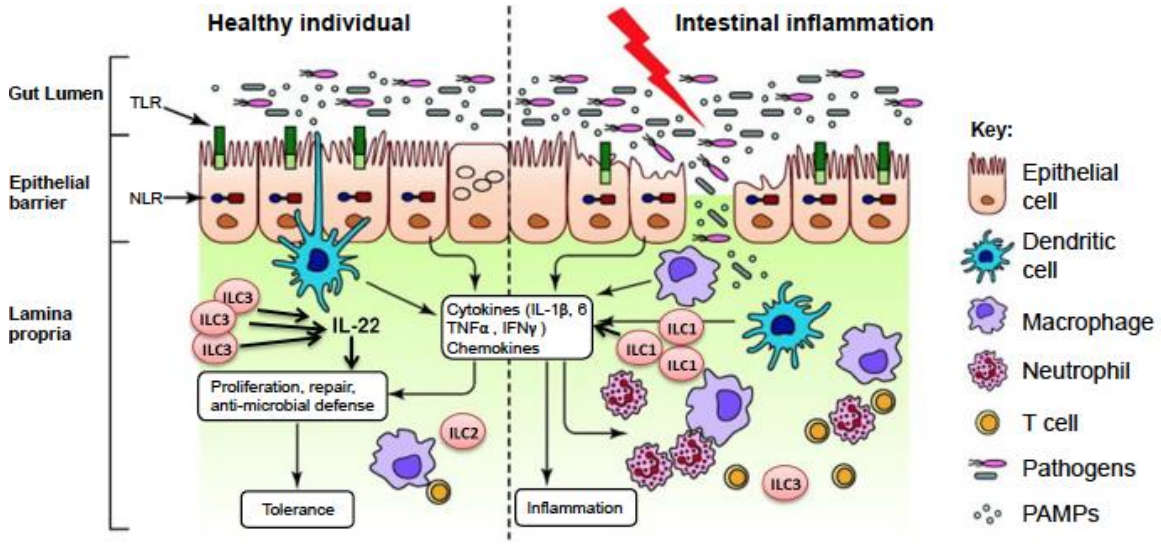

Figure 1.2 The epithelial barrier separates the gut lumen from the (LP). The intestinal epithelial barrier prevents LP immune cells from interacting with commensal microbes in the gut lumen. (Left) In a healthy individual, there exists a state of immune tolerance that allows commensal bacteria to live alongside immune cells in the gut. Epithelial cells, dendritic cells (DCs), and Paneth cells sample the gut lumen for microbes. DCs present microbial antigens to T cells, which in turn initiate a regulatory response to maintain immune homeostasis; epithelial cells release IL-18 that stimulates growth and proliferation of epithelial stem cells to repair damaged tissue; Paneth cells secrete anti-microbial host defense proteins to maintain homeostasis; while type 3 innate lymphoid cells (ILC3), which form the majority of ILCs in a healthy intestine, secrete IL-22, mucus, and antimicrobial peptides that mediate tissue repair and maintain tolerance. (Right) In a susceptible host, the intestinal epithelial barrier may be compromised allowing luminal bacteria and antigens to enter the LP where they encounter DCs and macrophages. These cells sense the presence of these microbes using their pattern recognition receptors (PRRs), and initiate an inflammatory response with production of pro-inflammatory cytokines, such as IL-1β, IL-18, IL-12, IL-6, TNFα, and IFNγ, resulting in inflammation. 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), which are the majority of ILCs present during an inflammatory state, to produce TNFα and IFNγ, which promote chronic inflammation. Modified and reproduced with permission of ELSEVIER LTD: Zaki, M.H. et al, Trends in Immunology 2011.

1.3.5 The immune response in Crohn's disease

The immune system is made up of the innate immune response and the adaptive immune response. Both 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.[65-69] Subepithelial DCs sample the gut lumen for the presence of non-pathogenic microbes as a regulatory response to provide tolerance.[65, 69] Studies in mice have shown that alterations in the proteins associated with immune responses can lead to intestinal inflammation.[70, 71] TLR2 and TLR4 expression in intestinal macrophages and DCs have been shown to be increased in CD patients compared to control subjects.[70, 72, 73] In addition, considerable evidence has confirmed a relationship between a polymorphism in the NOD2/CARD15 (Caspase recruitment domain) gene and CD, either alone or in combination with SNPs in TLRs, especially TLR4, or *ATG16L1*.[74, 75] As well, increased production of the pro-inflammatory cytokine, IL-1β, which has been linked to each of these gene variants,[68, 72] has been shown to play a critical role in CD pathogenesis, which is tightly regulated through TLRs via endogenous ligands

and/or danger-associated molecular pattern (DAMP) recognition.[66, 69, 72] 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.[61] CD4+ T helper cells are grouped into different classes including Th1, Th2, Th17, and regulatory T cells (Tregs).[76-79] Th1 cells are induced by IL-12 and IL-18 and produce high levels of IFNγ and TNFα.[79, 80] They respond to, and protect against, intracellular bacterial infections.[80] 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.[81] Th17 cells are induced by IL-6 and transforming growth factor beta (TGFβ) in mice, or IL-6 and IL-1β in humans.[77, 82] 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.[78, 79]

There is evidence suggesting that an imbalance of CD4+ T cells to Tregs is a major cause of intestinal inflammation.[76] CD has been commonly believed to be a Th1/Th17 mediated disease with increased levels of IFNγ, TNFα, IL-17A, and IL-2 reported in inflamed tissue taken from CD patients.[79, 83] Moreover, LP macrophages from CD patients produce high levels of IL-12 and IL-23, which drive Th1 and Th17

responses.[81, 83, 84] On the other hand, UC patients have increased IL-4 and IL-13 production suggesting a Th2 cytokine profile.[81, 83] IL-17 producing Th17 cells have been seen in both UC and CD in patients' inflamed tissue and are regulated by IL-23.[85, 86] In addition, SNPs in the *IL-23R* gene have been associated with IBD.[34] However, the IL-23R SNP, Arg381Gln (arginine381glutamine), has been reported to confer a two to three fold protection against development of pediatric CD, which suggests that it may actually reduce IL-23 responses.[87] 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 naïve T helper cells and aberrant immune responses to commensal bacteria and microbial antigens.[88, 89] 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.[90-93] 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.[18] The goal of treatment is to control inflammation,[15] but

every so often, "flare-ups" of acute symptoms may reappear, and depending on the circumstance, it may resolve on its own or require medication. 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.[15]

There is currently no cure for IBD. 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.[15] 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.[14, 15]

For treating mild to moderate UC and mild ileocolonic and colonic CD, 5-aminosalicylic acid (5-ASAs), such as sulfasalazine, mesalamine, olsalazine, and balsalazide, are used for local immunosupression.[1, 15] Corticosteroids, such as prednisone, are also used for moderate disease, but because of the side-effects of corticosteroids, long-term use is avoided.[14, 15] Another effective treatment option is exclusive enteral nutrition, which involves exclusion of normal diet for a period of time, which is replaced with liquid nutritional products.[14, 15]

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, 15] Biological

therapies are designed to target specific immune mediators of diseases, such as cytokines. These 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.[94, 95] Despite this, studies have shown that about 10% of CD patients are refractory to current anti-TNFα therapy and up to 30% of CD patients experience loss of treatment efficacy over time.[96] A new drug, vedolizumab, a mAb against the $\alpha_4\beta_7$ integrin, prevents 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.[97]

Severe cases of IBD may require surgery, such as bowel resection, strictureplasty, or a temporary or permanent colostomy or ileostomy.[1, 98] 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, 98]

1.4 Crohn's disease intestinal fibrosis

Intestinal fibrosis is a chronic and progressive process characterized by the excess accumulation of extracellular matrix (ECM), leading to stiffening and/or scarring of the intestine.[99] It develops through complex cell, cytokine, and growth factor interactions, with distinct cell types involved, including resident mesenchymal cells (fibroblasts, myofibroblasts, and smooth muscle cells), ECM-producing cells derived from epithelial and endothelial cells (through epithelial- and endothelial-mesenchymal transition),

stellate cells, pericytes, and local or bone marrow-derived stem cells.[99, 100] CD-associated intestinal fibrosis has often be attributed to a persistent immune-mediated intestinal inflammation, as evidence has shown that CD fibrosis follows the distribution and location of inflammation.[99, 100] It can involve the entire bowel wall of the gastrointestinal tract including the mucosa, submucosa, muscularis mucosa, muscularis propria and serosa layers (Figure 1.3).[99, 101] Approximately one in three people with CD develops strictures within 10 years of diagnosis and requires surgery to remove the diseased bowel, [98, 101] with the most common site of surgical resection being the distal ileum.[98, 101] Furthermore, patients who have undergone surgery for fibrosis frequently relapse, developing intestinal inflammation and fibrotic strictures at the same location as the previous resection.[101] Anti–TNFα therapy is effective at reducing inflammation in active CD, but the current thinking is that it is not effective at reducing fibrosis or fibrogenic gene expression.[101] Despite the urgent need to prevent or treat intestinal stricturing in patients with CD, little is known about the mechanisms underlying this process, in part because of a lack of suitable animal models for the study of intestinal fibrosis.

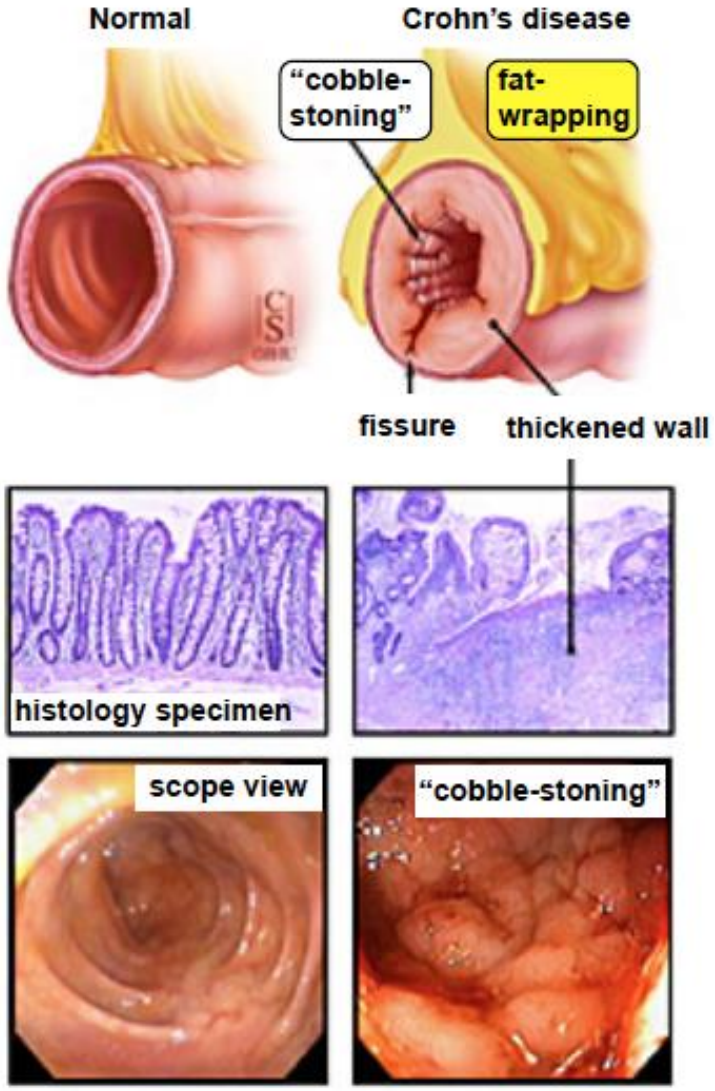

Figure 1.3 Gross pathological and histological appearance of Crohn's disease-associated intestinal fibrosis. In CD, intestinal fibrosis **(right)** is comprised of an excess buildup of collagen, ECM in the form of scar tissue, and can lead to thickened wall and strictures in the ileum. Discontinuous patches of transmural damage of the intestine as well as shortened villi are symptoms commonly found in CD patients. Reproduced with the permission of Johns Hopkins Medicine (Baltimore, Maryland). Crohn's Disease Diagnosis at Johns Hopkins.

In IBD, it is still unclear what factors lead to chronicity. In addition, once intestinal inflammation is chronic, we do not understand what sets the stage for the development of intestinal strictures. However, it is believed that chronic inflammation is

characterized by continuous events of injury and repair that may lead to the development of fibrosis (Figure 1.4).[99, 100] Yet, this process does not occur in all patients with CD.[102] Furthermore, chronic intestinal inflammatory diseases exist, such as celiac disease or lymphocytic colitis, that are not complicated by fibrosis and stricture formation.[103] These findings suggest that there are distinct mechanisms of inflammation and fibrosis, and, therefore, it is critical to explore these pathologies as separate targets, which would allow tailored treatment for wound healing abnormalities, like those which can cause fibrosis in people with CD.

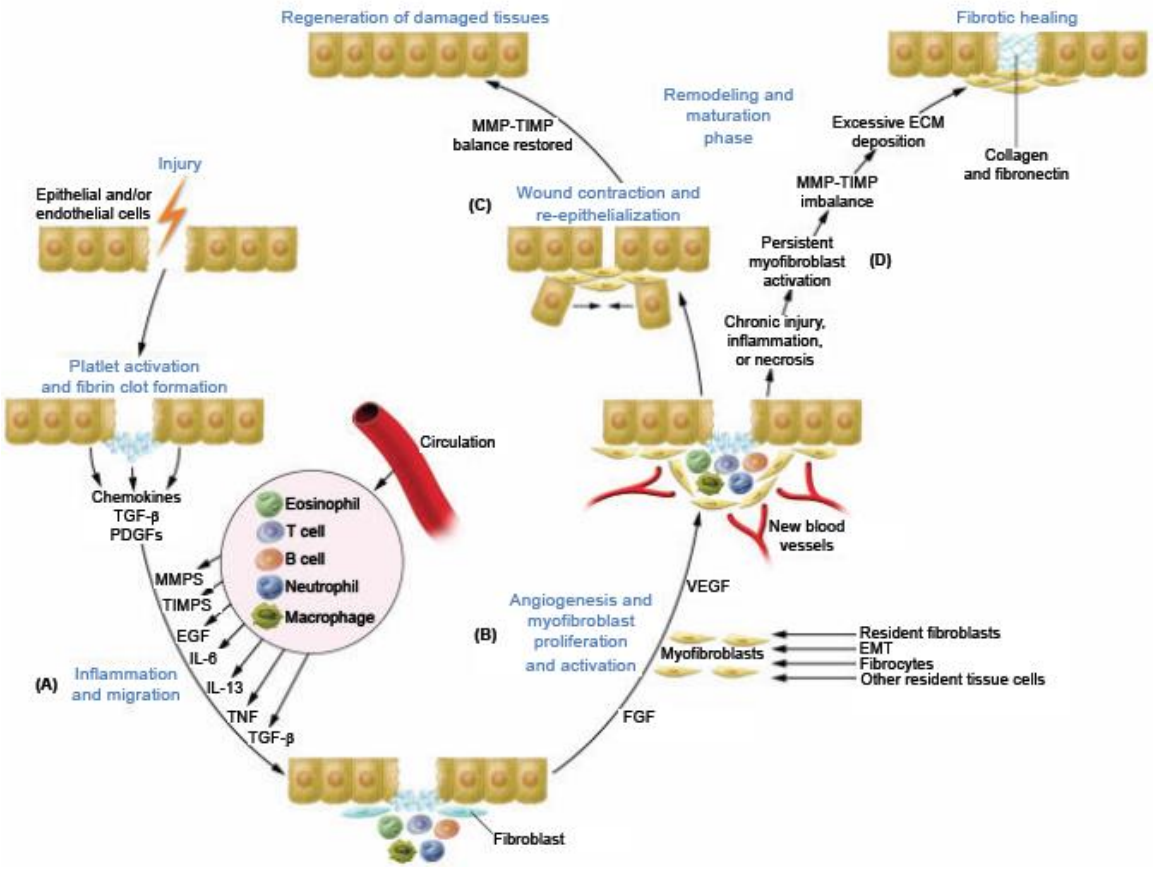

Figure 1.4 Fibrosis as a result of out of control wound-healing. The process of wound healing can lead to tissue regeneration but can also cause fibrosis. **(A)** In case of an injury due to infection, chemical, or physical damage, an inflammatory response is triggered. This includes the induction of cytokines, chemokines, and growth factors, as well as the infiltration of immune cells including macrophages, neutrophils, T cells and B cells. Activated macrophages and neutrophils clean up tissue debris and dead cells. **(B)** This stage is followed by the activation and proliferation of myofibroblast and production of ECM. **(C)** Normally, in response to tissue injury, the healing process includes the deposition of extracellular matrix proteins, the most abundant of which are members of the collagen family, which contributes to tissue restitution. **(D)** Fibrosis can be considered a pathological consequence of an excessive healing response downstream of chronic inflammation. This figure was reproduced with permission of American Society for Clinical Investigation. Thomas A. Wynn. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. 117:542-549. 2007

1.4.1 Overview of cell types involved in intestinal fibrosis

Fibrosis is a complicated, multistage, progressive process, which occurs differently in different organs, making fibrotic diseases like intestinal fibrosis difficult to study and perplexing to treat. However, it is generally believed that consistent involvement from mesenchymal-derived cells (fibroblasts, myofibroblasts, and smooth muscle cell), macrophages, and other immune cells regulate fibrotic diseases.[103, 104]

Many studies on the progression of fibrosis in CD have focused on the involvement of non-immune cell types, including mesenchymal cells, epithelial cells, nerve cells, and platelets.[104-107] In addition, there has also been evidence indicating that neo-angiogenesis, the growth of new blood vessels, is associated with the progression of fibrosis in CD.[106] Indeed, the microcirculation and endothelial cells perform crucial roles in maintaining intestinal immune homeostasis: endothelial cells regulate the type and the number of leukocytes that migrate from blood to the interstitial space and specialized vascular cells, such as the endothelium venules, can, thereby, selectively control the influx of specific immune cells to the site of injury.[105-108]

Neutrophils are the first cell type recruited after injury, and represent the most abundant inflammatory cells in the early stages of wound healing.[100] Activated neutrophils degranulate and release inflammatory as well as pro-fibrogenic cytokines, and subsequently recruit macrophages to the site of injury (Figure 1.4).[99, 109] During this leukocyte migration phase, the neutrophils and activated macrophages eliminate tissue debris, dead cells, and any foreign microbes.[99-101, 109] They also produce cytokines and chemokines, such as platelet-derived growth factor (PDGF) and TGFβ, which initiate and amplify the wound-healing response. [102, 103] These factors are also mitogenic and

chemotactic for endothelial cells, which surround the injury and form new blood vessels as they migrate towards the center of the injury (Figure 1.4).[103] Macrophages also promote inflammation by recruiting and activating additional monocytes and neutrophils, presenting foreign antigen to CD4+ T cells, and modulating T cell responses with co-stimulation and cytokines.[99-103, 109] T cells, in turn, coordinate the fibrotic response through enhancing neutrophil recruitment by IL-17A, activating macrophages with IL-4 and IL-13, and inducing collagen production by fibroblasts with IL-4, IL-13, and TGFβ (Figure 1.4).

1.4.1.1 Fibroblasts in intestinal fibrosis

Fibroblasts are a heterogeneous population of cells present in the interstitium of all tissues and organs, where they play critical roles in maintaining structural integrity, regulating matrix homeostasis, and in the healing process.[99, 100] Fibroblasts are also the predominant mucosal cells in the intestine that produce components of the ECM.[100] ECM consists of several molecular components including collagen (I-VI), elastin, glycoproteins, glycosaminoglycans and proteoglycans (Figure 1.4).[104, 105] ECM, itself, is an active structure, it has been shown to directly regulate the inflammatory response as well as the healing response and fibrotic progression by controlling focal adhesion with immune and non-immune cells, as well as other fibroblasts.[100, 101] Reactive oxygen species, and signs of stress, such as dying cells, presence of foreign microbes, and paracrine signals from neighboring cells (such as macrophages), stimulate fibroblast activation.[102, 109] Activated fibroblasts are essential in wound healing, but have been shown to execute the steps that initiate and perpetuate fibrosis.[102, 109] Not surprisingly, all

patients with CD experience some degree of mild subclinical fibrosis, with transmural accumulation of fibroblasts throughout the gut wall, and accumulation of ECM in the form of permanent fibrotic scars.[102]

1.4.1.2 Macrophages in intestinal fibrosis

Given their multifunctional capabilities and heterogeneous phenotypes, it is not surprising that macrophages can both promote and reduce fibrosis. Resident tissue macrophages are present at, and circulating precursors are recruited to, sites of injury, in response to chemokines, such as macrophage inflammatory proteins MIP-1α, MIP-1β, and monocyte chemotactic protein membrane cofactor protein-1 (MCP-1), which are produced by fibroblasts.[108, 110] Macrophage-derived TGFβ causes fibroblasts to produce interstitial fibrillar collagen as well as tissue inhibitors of metalloproteinases (TIMPs) to block ECM degradation.[110, 111] Macrophages can also stimulate fibroblast proliferation, survival, and migration by producing PDGF; a study has shown that alveolar macrophages recovered from idiopathic pulmonary fibrosis (IPF) patients spontaneously produce PDGF.[110-112] In addition, macrophages taken from IPF patients were also found to be high producers of IL-1β, which is also another activator of fibroblasts.[113, 114]

Monocytes and macrophages can be categorized into several distinct phenotypes based on their responses to inflammatory stimuli. Classically activated macrophages and alternatively activated macrophages and some key defining features of each of these phenotypes are illustrated in Figure 1.5. Pertinent to this thesis, macrophages treated with IL-4, M(IL-4) were formerly refered to as alternatively activated macrophages.[103] M(IL-4) macrophages are thought of as wound-healing macrophages. They secrete relatively

more anti-inflammatory cytokines, IL-10 and TGFβ, in response to inflammatory stimuli. They also express arginase I (argI) that leads to the synthesis of L-proline and polyamines, both of which have been linked to wound repair and fibrosis (Figure 1.5).[115]

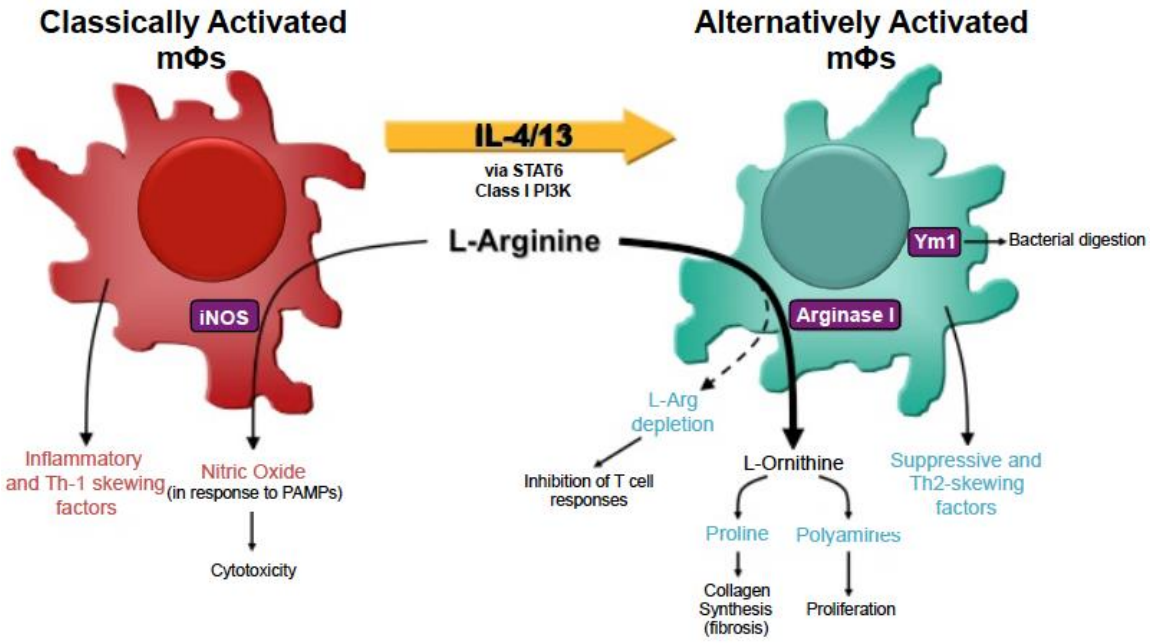

Figure 1.5 Macrophage activation. IL-4 or IL-13 skews macrophages to an M(IL-4) or alternatively activated phenotype. Signal transducer and activator of transcription 6 (STAT6) drives production of argI (found in M(IL-4)) uses L-arginine to catalyze the production of L-ornithine, a precursor of L-proline, which is essential for collagen synthesis. ArgI also leads to production of polyamines, like spermine and spermidine, stimulating growth and proliferation of fibroblasts. In competition with the argI enzyme is inducible nitric oxide synthase (iNOS), found in classically activated macrophages, which catalyzes the production of nitric oxide (NO).

1.4.1.3 T helper cells in intestinal fibrosis

CD4+ T cells adapt and amplify their responses to match different types of infections, and coordinate the many types of immune cells involved in fibrosis. There has been extensive evidence linking wound healing and fibrosis with the T helper type 2 (Th2) subset of immune cells, and the cytokines they produce, IL-4 and IL-13.[108, 116, 117]

IL-4 and IL-13 stimulate fibroblasts to synthesize collagen, and, additionally, skew macrophages to the alternatively activated macrophage phenotype previously mentioned.[117, 118] IL-13 has also been shown to be important in liver and lung fibrosis.[118]

1.4.2 Factors associated with collagen production

The key events that lead to the development of intestinal fibrosis are not only recruitment of immune and inflammatory cells, but also the exposure of mesenchymal cells to a variety of inflammatory mediators that are able to maintain these cells in a persistent state of trans- and de-differentiation between ECM producing fibroblasts, myofibroblasts, and smooth muscle cell phenotypes. Below is a highlight of the most important factors reported to be associated with collagen production.

1.4.2.1 TGFβ1

TGFβ1 has been shown to be one of the most potent pro-fibrogenic factors in the development of fibrosis. Not surprisingly, gene expression studies have demonstrated that TGFβ1 transcription levels are up-regulated in the intestinal mucosa of patients with CD intestinal fibrosis.[107] Although three isotypes of TGFβ exist (TGFβ1, TGFβ2 and TGFβ3), the β1 isoform is considered to be the main driver of fibrosis, and the predominant sources are macrophages and fibroblasts.[107] TGFβ1 can stimulate the migration and activation of potent ECM producing mesenchymal cells, such as smooth muscle cells, myofibroblasts, and fibroblasts.[115] In addition, it has been shown to stimulate the expression of other fibrogenic factors, including fibronectin, connective tissue growth factor (CTGF), IGF-I, and PDGF.[115] For instance, IGF-I stimulates

collagen production from subepithelial myofibroblasts, while PDGF increases fibroblast proliferation in the intestine of CD patients.[119-121] Increased levels of TGFβ1 found in CD patients have also been shown to disrupt ECM homeostasis by down-regulating MMP-1 and MMP-3 (matrix metalloproteinases) expression, while enhancing TIMP-1 (tissue inhibitors of metalloproteinase) expression, leading to a reduced ability to breakdown ECM proteins in CD patients.[115]

1.4.2.2 Arginase and L-arginine

The argI enzyme is expressed in M(IL-4) macrophages in mice, and has been shown to be linked to pathological wound healing and fibrosis in animal models of intestinal fibrosis.[122] ArgI sequesters L-arginine from iNOS and causes the production of ornithine and downstream L-proline, which contributes to collagen production. ArgI also contributes to the biosysnthesis of polyamines, such as spermine and spermidine, which promotes cell growth and proliferation of fibroblasts.[122-124]

1.4.3 Current animal models of CD-associated intestinal fibrosis

Despite the urgent need to prevent and treat intestinal structuring in patients with CD, little is known about the mechanism underlying the process of CD-associated intestinal fibrosis, in part because there is a lack of suitable animal models that recapitulate the features of pathology. Although animal models of IBD have been described, most models affect the large intestine, or do not correctly manifest CD-specific histopathological features.[125] There are three animal models that spontaneously develop chronic inflammation of the ileum, the SAMP1/YitFc mouse, the transgenic tumor

necrosis factor$^{\Delta ARE}$ (TNF$^{\Delta ARE}$) mouse, and the SHIP$^{-/-}$ mouse.[125] (see section 1.6.5). SAMP1/YitFc mice spontaneously develop terminal ileitis by 10 weeks of age, which is driven by Th1 and Th2 immune responses.[125] The TNF$^{\Delta ARE}$ mouse has a systemic increase in TNF levels that mediate chronic ileitis.[125] Both of these models recapitulate some features of CD, but have not been used as models for studying the treatment of intestinal fibrosis in part because the SAMP1/YitFc mouse is reported to develop fibrostenotic strictures much later in life (at the age of 40 weeks), while the TNF$^{\Delta ARE}$ mouse do not accumulate ECM components in the distal ileum.[126] For my studies, I am using a relatively new model of CD-like ileal inflammation with fibrosis, the SHIP$^{-/-}$ mouse. This animal model is described in detail in section 1.6.

1.4.4 Therapeutic options for intestinal fibrosis

In IBD, current therapeutic agents, such as corticosteroids, aminosalicylates, immunosuppressants, and even more recent biologic drugs, such as anti-TNFα antibodies, improve intestinal inflammation but do not significantly reduce the incidence of fibrosis in people with CD.[127] This underscores the idea that the mechanism(s) that regulate fibrosis may be distinct from those that regulate inflammation. In fact, several studies have suggested that anti-inflammatory drugs for IBD cannot prevent the evolution of fibrosis once the process of excess ECM production has started.[99] Therefore, surgical intestinal resection or stricturoplasty is necessary in up to 75% of CD patients during the course of their disease.[128] However, surgical resection is also associated with a high rate of recurrent stricturing disease, and the need for repeated surgery is high. In summary, we know that a large number of people with CD are going to develop fibrosis and we can

identify patients that have developed fibrosis and are likely to redevelop this pathological feature of disease, but anti-inflammatory strategies are not effective at targeting fibrosis. Hence, therapeutic approaches that target fibrosis directly are urgently needed.[128]

Pharmacological modulation of tissue ECM deposition, such as MMPs could be useful in the prevention and treatment of CD-associated intestinal fibrosis.[117] Currently, soluble factors that regulate the activation of ECM-producing cells are being investigated as potential targets for anti-fibrotic drugs, including: cytokines such as IL-6, IL-13, IL-17, IL-21; chemokines such as MCP-1, MIP-1, TGFβ1; growth factors such as CTGF, PDGF, insulin-like growth factor (IGF)-1 and 2, epidermal growth factor (EGF); angiogenic factors, such as vascular endothelial growth factor (VEGF), and products of oxidative stress.[99, 117] However, the lack of identification of specific cellular and molecular pathways leading to intestinal fibrosis has resulted in a shortage of well-tolerated anti-fibrotic drugs available to patients. Currently, there are no approved drugs used to treat intestinal fibrosis in people with CD.

1.5 Phosphoinositide 3-kinase

1.5.1 Description and function

Phosphoinositide 3-kinases (PI3K) are a family of enzymes that are critical in many cellular processes including cell growth, differentiation, proliferation, and inflammation.[129, 130] PI3K consists of three classes (Class I, II, and III), which have been characterized and grouped according to their structure and function. Class I PI3Ks are heterodimeric enzymes: Class IA consists of 1 of 5 regulatory subunits (p50α, p55α, p55γ, p85α, or p85β) and 1 of 3 catalytic subunits (p110α, p110β, or p110δ); Class IB is

composed of 1 of 2 regulatory subunits (p87 or p101), and the catalytic subunit p110γ.[129, 130] The catalytic subunits p110α and p110β are ubiquitously expressed, whereas the p110γ and p110δ are primarily found in hematopoietic cells.[129, 130] The class IA PI3Ks, which have been implicated in many human cancers, are activated downstream of receptor tyrosine kinases (RTK), G-protein coupled receptors (GPCR), or via interactions with activated RAS or Rho family of GTPases.[129, 130] Class II PI3Ks are membrane bound, and typically activated by tyrosine kinases and integrins,[131-133] and are involved in cell migration.[134] Class III PI3K consists of a single catalytic subunit Vps34 and has been implicated in autophagy.[129] Class I PI3Ks phosphorylate phosphatidylinositol phosphates $PI(3,4)P_2$ to generate $PI(3,4,5)P_3$ or PIP3.[129] Class II PI3K catalyzes the phosphorylation of PI and PI(4)P to PI(3)P and $PI(3,4)P_2$,[129] whereas class III PI3K only catalyzes the production of PI(3)P from PI.[129] PI3K is believed to be recruited to receptors by binding to phosphorylated residues that form the docking site for the SH2 domain of the PI3K regulatory subunit.[134] This phosphorylated regulatory-mediated translocation of PI3K helps position the catalytic subunit close to the membrane where the lipid substrate of PI3K is located.[134] However, the specificity for which regulatory subunit and its catalytic subunit is recruited to receptors is still unclear, as only receptor tyrosine kinase pathways have been explored so far.

PIP3 functions to activate downstream signalling cascades, the most notable being the protein kinase B (AKT), which activates downstream signalling pathways required for cellular growth, survival, metabolism, and immune responses.[129] PIP3 can be dephosphorylated at the 5′ position, from PIP3 to $PI(3,4)P_2$, by the SHIP family of

inositol phosphatases; SHIP2, which is ubiquitously expressed, or SHIP, which is primarily found in hematopoietic cells (Figure 1.6).[130]

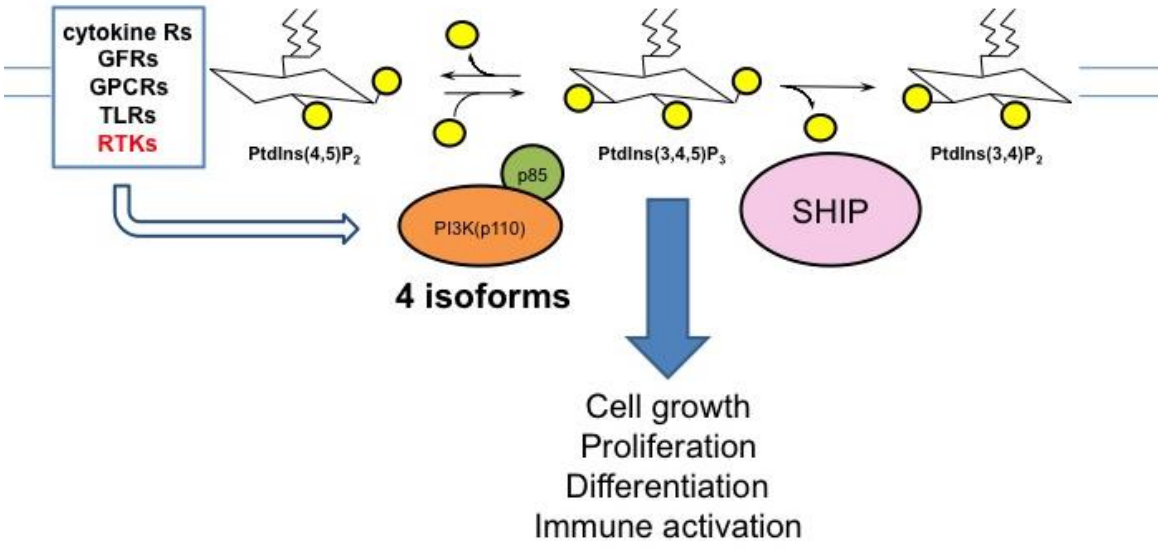

Figure 1.6 SHIP negatively regulates the immune response. Ligation of receptor tyrosine kinases (RTKs), cytokine receptors (cytokine Rs), growth factor receptors (GFRs), G protein-coupled receptors (GPCRs), and TLRs activate Class I PI3K, which is comprised of a p110 catalytic and a p85 regulatory subunit. 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.

The Class I PI3K catalytic isoforms have non-overlapping functions and are essential. Genetic ablation of p110α or p110β results in embryonic lethality in mice, indicating their essential and non-redundant roles during development and survival.[130] In this thesis, I will focus on the PI3Kp110δ catalytic subunit because our laboratory has found that it plays a critical role in the induction of the pro-fibrotic enzyme, argI, in macrophages and we hypothesize that it may play an important role in development of fibrosis in $SHIP^{-/-}$ mice.

1.5.2 Class IA PI3Kp110δ activity

The Class IA PI3K p110δ isoform is primarily expressed in the hematopoietic system, including myeloid cells, B- and T-cells, and plays key roles in leukocyte signalling, proliferation, differentiation, activation, and chemotaxis.[130] Recent evidence suggests a role of PI3Kp110δ in the generation and function of Tregs, myeloid-derived suppressor cells (MDSCs), mature T-cell activation, and in the chemo-attractant-mediated migration of macrophages and neutrophils to sites of injury.[130-132]

1.5.3 The PI3Kp110δ deficient mouse

PI3Kp110δ deficient ($PI3Kp110\delta^{D910A/D910A}$ or $PI3Kp110\delta^{DA/DA}$) mice are a kinase-dead, knock-in mouse model in which the p110δ catalytic subunit of Class IA PI3K, has been inactivated by mutating a single amino acid in the active site of the enzyme. $PI3Kp110\delta^{DA/DA}$ mice have no alteration in the expression levels or kinase activities of p110α and p110β.[133] This mouse model was first created and characterized by the Vanhaesebroeck laboratory, and has permitted discovery of key functions for PI3Kp110δ in immunity, inflammation, haematological malignancies and, most recently, cancer growth.[133] Deficiency in PI3Kp110δ activity has been shown to lead to mild colitis in mice, which begins at 8 weeks of age and progresses over time.[133-135] This has been attributed to macrophage function as these mice have shown to be defective in bactericidal activity and have increased inflammatory responses related to Toll-like receptor signalling, as well as elevated levels of M1 associated IL-12 and IL-23 production.[134]

Recent studies from our laboratory have demonstrated that the PI3Kp110δ isoform is required for IL-4 induced M(IL-4) skewing.[136] As a result, PI3Kp110δ deficient mice are impaired in their ability to induce argI in response to IL-4.[135, 136] Because M(IL-4) macrophages have been implicated in the pathological wound healing and fibrosis in animal models of IBD, PI3Kp110δ activity may have a role in the progression of CD-like intestinal fibrosis in mice.

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

1.6.1 Description and function

The SHIP (gene *Inpp5d*) is primarily a hematopoietic-specific, lipid phosphatase that negatively regulates class I PI3K activity.[137, 138] Two other SHIP enzymes exist; SHIP2 is similar in structure to SHIP,[137] and sSHIP, which lacks the N-terminal SH2 domain. SHIP2 is ubiquitously expressed and is in found in human skeletal muscles, placenta, and heart,[137, 139] whereas sSHIP is restricted to murine embryonic and hematopoietic stem cells.[137, 139, 140] Note that SHIP, which is sometimes referred to as SHIP1, is the primary focus of this work.

1.6.2 SHIP enzymatic activity

To exert its action, SHIP is translocated from the cytoplasm to the inner leaflet of the cell membrane where PI(3,4,5)P_3 synthesis occurs.[137, 138] 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.[138] PI(3,4,5)P_3 recruits serine-threonine kinases, such as AKT and

phosphoinositide-dependent kinase-1 (PDK1), to the plasma membrane, where it begins driving cellular processes,[138, 141] such as growth, proliferation, differentiation, and immune activation.[141] SHIP antagonizes these actions by dephosphorylating the 5′ position of the inositol ring to form $PI(3,4)P_2$ (Figure 1.6).[140] Therefore, SHIP regulates the downstream cellular processes in immune cells, such as cytokine production and inflammatory responses (Figure 1.6).[140]

Furthermore, SHIP enhances neutrophil apoptosis,[142] decreases B cell proliferation, chemotaxis, and activation,[142] and promotes T cell survival and maintains innate immune balance at mucosal surfaces.[143] SHIP can be regulated either at the level of transcription or post-transcripttionally.[142, 144] In macrophages, TGFβ has been shown to up-regulate SHIP mRNA expression in both human and mouse cells,[144] while mothers against decapentaplegic homolog 7 (SMAD7), which blocks TGFβ activity has the counter effect of reducing SHIP expression. Post-transcriptionally, IL-4 has been shown to induce SHIP protein degradation in macrophages.[142] Studies have also shown that, tyrosine phosphorylation of SHIP targets it for ubiquitination and proteasomal degradation.[140]

1.6.3 The SHIP deficient mouse

The SHIP deficient mouse (*Inpp5d*$^{-/-}$), which will be referred to as SHIP$^{-/-}$, are smaller in size than their wild-type counterparts and have a reduced lifespan, asthmatic lungs, splenomegaly, and a myeloproliferative disorder.[142]

In addition, SHIP$^{-/-}$ mice have hyperactive, IL-4-secreting basophils, which expose macrophages to this M(IL-4)-skewing cytokine.[136, 145] SHIP$^{-/-}$ mouse macrophages

are also hyper-responsive to IL-4.[136] 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.[135] 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.[125]

Recently, we, and Kerr's group,[125] 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.[142]

1.6.4 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.[126] Inflammation is characterized by abundant infiltrating Gr-1-postive immune cells (neutrophils), granuloma-like immune cell aggregates, multi-nucleated giant cells, goblet cell hyperplasia, and a mixed Th2 and Th17 cytokine profile.[126] 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.[125] Furthermore, both argI expression and activity are increased in the SHIP$^{-/-}$ mice ileum compared to that seen in wild type littermates.[126]

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

The SHIP$^{-/-}$ mice also develop findings of CD-like intestinal fibrosis, including increased collagen deposition, thickened muscularis layers, increased transmural accumulation of fibroblasts, and increased argI expression, which is inversely proportional to NO production, in the distal ileum.[126] In addition, SHIP$^{-/-}$ mice express a predominately an M(IL-4) population of macrophages in their ileum. In a recent study, SHIP$^{-/-}$ mice that were treated with arginase inhibitor *S*-(2-boronoethyl)-l-cysteine (BEC) did not show significant changes in the number of immune cell infiltrates in the ileum.[126] Rather, BEC reduced collagen deposition and muscle hyperplasia, suggesting that arginase activity is a potential target to limit intestinal fibrosis, like that seen in CD.

1.7 Thesis hypothesis and objectives

1.7.1 Summary of rationale

Previously, our laboratory reported that mice deficient in SHIP develop spontaneous CD-like ileal inflammation along with features of intestinal fibrosis, which were arginase-dependent. We also reported that argI expression was up-regulated in the $SHIP^{-/-}$ mice ileal tissues in multiple cell types, and that the p110δ catalytic subunit of PI3K was required for IL-4-induced argI expression in macrophages.

1.7.2 Hypothesis and objectives

Based on this, we hypothesized that $SHIP^{-/-}$ mice develop CD-like intestinal fibrosis that is driven by PI3Kp110δ activity. To investigate this hypothesis, I had two specific aims:

Aim 1: To determine whether genetic inactivation of PI3Kp110δ activity prevents the development of ileal fibrosis in $SHIP^{-/-}$ mice.

Aim 2: To determine whether pharmacological inhibition of PI3Kp110δ activity blocks or reverses ileal fibrosis in $SHIP^{-/-}$ mice.

Measurements of ileal fibrosis included those that have been reported for $SHIP^{-/-}$ mice: muscle thickening, accumulation of $vimentin^{+}$ mesenchymal cells, collagen accumulation visualized by Masson's trichrome stain for fibrosis and assayed by the Sircol assay for soluble collagen, increased arginase activity, and increased levels of IL-4 in full-thickness ileal tissue homogenates. Pro-fibrotic TGFβ levels in full-thickness ileal tissue homogenates were also measured.

1.7.3 Significance

These studies will contribute to our understanding of the role of PI3Kp110δ in ileal fibrosis in $SHIP^{-/-}$ mice. Importantly, this work may identify new therapeutic strategies to treat fibrosis in people with CD. Idelalisib, a PI3Kp110δ inhibitor, is already licensed for use in people with certain leukemias and lymphomas so results from these studies may be rapidly translatable into an effective therapy for people with CD-associated fibrosis.

Chapter 2: Materials and methods

2.1 Mice

Mice homozygous for SHIP deficiency (*Inpp5d*$^{-/-}$) on a mixed C57BL/6×129Sv background (F2 generation) were bred with PI3Kp110δ$^{DA/DA}$ (PI3Kp110δ$^{D910A/D910A}$) mice obtained from the Ludwig Cancer Research Institute and maintained on a C57BL/6 background. The PI3Kp110δ$^{DA/DA}$ mice have an aspartic acid (D) to alanine (A) substitution at amino acid number 910, which is in the active site of the enzyme. This renders the PI3Kp110δ enzyme catalytically inactive while maintaining its mRNA and protein expression level in cells.[131] Crossing SHIP$^{-/-}$ and PI3Kp110δ$^{DA/DA}$ mice generated mice heterozygous for SHIP (SHIP$^{+/-}$) and heterozygous for the PI3Kp110δ genotype (PI3Kp110δ$^{+/D910A}$). Subsequent matings were made from these mice to generate: SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3KP110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$ PI3KP110δ$^{DA/DA}$ mice that were used for experiments (F3 generation). Mice were assessed at 4, 8, and 12 weeks of age. In our animal facility, PI3Kp110δ$^{DA/DA}$ mice do not develop clinical or histological signs of disease at 8–9 weeks of age. Mice were housed under specific pathogen-free conditions in sterilized filter-top cages and received autoclaved food and water *ad libitum* at the Animal Care Facility at the BC Children's Hospital research institute (Vancouver, BC). Experiments were performed in accordance with Canadian Council on Animal Care guidelines (Protocol A09-0027 and A09-0032).

2.2 Oral gavage

8 week old SHIP$^{-/-}$ mice were administered 50 mg/kg IC87114, a purine-quinazoline-based PI3Kp110δ isoform-specific inhibitor, dissolved in 120-150 μl

PEG400 (Hampton Research, Aliso Viejo, CA) or an equal volume of PEG400, as a vehicle control, once daily by oral gavage. Treatment continued for 14 days, at which point the animals were euthanized, and their distal ileum were removed for analyses.

2.3 Cytokine measurements

Cytokine measurements were performed on clarified full thickness tissue homogenates from mice or tissue culture supernatants using the enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) was used for plotting cytokine levels. ELISA kits for mouse IL-1β and IL-4 were from BD Biosciences (Mississauga, ON). The mouse TGFβ1 ELISA kit was from eBioscience (San Diego, CA).

2.4 Nitric oxide assays

NO production was determined indirectly by measuring the nitrite/nitrate in tissue supernatants using the Nitrite/Nitrate Colorimetric Assay Kit (Invitrogen). Available nitrate in ileal samples is converted into nitrite with the addition of the Griess Reagents, which is then converted into a deep purple azo compound, and can be measured based on the absorbance (at 550 nm) due to the azo chromophore to accurately determine NO_2^- concentration.

2.5 Arginase assays

Fresh tissue samples were collected and homogenized in 1 mL arginase lysis buffer (0.1% Triton X-100, 25 mM Tris pH 8, aprotinin (40 μg/mL), leupeptin (8

μg/mL), PMSF (100 μM)) using a Polytron MR2100 bench top homogenizer. Homogenates were cleared by centrifugation at 16,000 × *g* for 10 minutes at 4°C. Arginase activity was then determined indirectly by measuring the concentration of urea generated by the arginase-dependent hydrolysis of L-arginine.

2.6 Sircol assays

Sections of fresh mouse distal ileum (30 to 100 mg) were minced with surgical scissors in 500 μL of 0.5 M acetic acid with 10 mg/mL pepsin, and agitated (at 1000 rpm on a VWR microplate shaker) overnight at 4°C. Tissue debris was removed and collagen was measured by the Sircol assay for soluble collagen (Biocolor Ltd, Carrickfergus, UK), according to the manufacturer's instructions. Briefly, Sircol dye reagent was used to bind to soluble collagen in the sample, and then separated from the supernatant by centrifugation. The collagen-containing pellet was resuspended in an alkaline solution and the absorbance of the solution measured at 540 nm. Collagen concentration in samples was determined by comparing it to the linear portion of a collagen standard curve.

2.7 Immunohistochemistry

For immunofluorescent detection of argI, Ym1, and vimentin, slide-mounted, 5 μm serial sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed by immersing the slides in 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0, at 95°C for 20 min and allowing slides to cool to room temperature. All slides were rinsed thoroughly in Tris-buffered

saline with 0.1% Tween-20. Endogenous avidin and biotin were blocked with an avidin-biotin blocking kit, according to the manufacturer′s instructions (Vector Laboratories, Burlingame, CA, USA). Primary antibodies, including mouse anti-argI (BD Biosciences, Mississauga, Canada), rabbit anti-Ym1 (StemCell Technologies, Vancouver, Canada), and mouse anti-vimentin (BD Pharmigen, San Jose, CA, USA), were used. Blocking buffers, secondary Alexa conjugated IgG antibodies (Molecular Probes/Invitrogen, Eugene, OR), and avidin-biotin-HRP were prepared and used from rabbit IgG, or mouse IgG detection kits, according to the manufacturer's instructions (Vector Laboratories). Sections were mounted in Vectashield mounting medium with 4′ 6-diamino-2-phenylindole (DAPI) (Vector Laboratories). Negative controls, an irrelevant isotype control and no primary antibody, were performed for all stainings. Images were acquired and analyzed using a Zeiss Axiovert 200 microscope, a Zeiss AxioCam HR camera, and the Zeiss AxioVision 4.0 software imaging system.

2.8 Histological analyses

Mice were euthanized and distal ileums were removed, cleared of ileal contents, and fixed in phosphate-buffered saline (PBS)-buffered 10% formalin. Tissue sections were embedded in paraffin, and 5 μm cross-sections were cut and stained with Hematoxylin and eosin (H&E) or Masson’s trichrome stain, as per manufacturers’ instructions (Sigma-Aldrich). Images were acquired using a Zeiss Axiovert 200 microscope, AxiocamHR camera, and Axiovision 4.0 software. The thickness of the muscularis externa from the serosa to the muscularis mucosa was measured at 6 points in 10 cross sections of distal ileum for each mouse. Villus length was determined by

counting epithelial cell nuclei from the crypt-villus junction to the villus tip, on uniform horizontal cross sections of crypts and villi. Goblet cells per crypt-villus were determined by counting from the base of crypts to the crypt-villus junction and from the crypt-villus junction to the villus tip. To count immune cell infiltrates, 6 representative fields of H&E-stained cross sections were obtained from each mouse at ×40 magnification; infiltrates were counted according to nuclear morphological features in the circular muscularis externa and submucosa.

2.9 Statistical analyses

One-way analysis of variance (ANOVA), were performed using GraphPad Prism version 5 software (version 5; GraphPad Software, Inc, La Jolla CA). For multiple comparisons, the Bonferroni correction was applied. Differences were considered significant at $P < 0.05$.

Chapter 3: PI3Kp110δ deficiency reduces ileal fibrosis in SHIP$^{-/-}$ mice

3.1 Introduction and rationale

Fibrosis is a serious complication for people with CD because it can lead to intestinal dysfunction and bowel occlusions. Intestinal fibrosis can involve the entire bowel wall of the gastrointestinal tract including the mucosa, submucosa, muscularis mucosa, muscularis propria, and serosa layers; with the most frequently affected area being the distal ileum.[99, 101] Approximately 1 in 3 people with CD develops strictures within 10 years of diagnosis and requires surgery to remove the diseased bowel.[98, 126] Furthermore, patients who have undergone surgery for fibrosis, frequently relapse and develop intestinal inflammation and fibrotic strictures at the same location as the previous resection.[126]

Currently, there are no treatments for CD-associated fibrosis. Despite the urgent need to prevent and treat intestinal strictures, little is known about the mechanisms underlying this process. This may be due, in part, to a lack of suitable animal models for the study of intestinal fibrosis.

Our laboratory recently reported that the SHIP$^{-/-}$ mouse spontaneously develop intestinal inflammation with several key features resembling CD. This includes both inflammatory and fibrotic components that were restricted to the distal ileum.[126] The intestinal fibrosis is characterized by thickened ileal muscle layers due to muscle hyperplasia, increased collagen deposition, and increased transmural fibroblast accumulation at the sites of collagen deposition.[126] In addition, SHIP$^{-/-}$ mice express a predominately M2 population of macrophages in the ileum because SHIP$^{-/-}$ mice have high systemic levels of basophil-derived IL-4,[135] and SHIP$^{-/-}$ macrophages are hyper-

responsive to IL-4 stimulation.[136] As such, SHIP$^{-/-}$ ileal macrophages have increased arginase I protein levels and arginase activity.[126]

Our laboratory also recently reported that the PI3Kp110δ isoform of Class IA PI3-kinase is required for IL-4 induced macrophage polarization to an M(IL-4) phenotype.[135] As a result, PI3Kp110δ deficient mice are impaired in their ability to induce the M(IL-4) marker, argI, in response to IL-4.[135, 136] Additional experiments from our laboratory have demonstrated that SHIP$^{-/-}$ mice treated with arginase inhibitor *S*-(2-boronoethyl)-L-cysteine (BEC) have reduced collagen deposition and muscle hyperplasia.[126] Taken together, these data suggest that PI3Kp110δ activity may drive intestinal fibrosis in SHIP$^{-/-}$ mice by increasing arginase activity. Based on these findings, we asked whether PI3Kp110δ drives intestinal fibrosis in SHIP$^{-/-}$ mice. To do so, we crossed germline SHIP$^{-/-}$ mice with mice that were deficient in PI3Kp110δ activity and measured fibrosis-related pathological features in double deficient mice and their control littermates.

3.2 Results

3.2.1 PI3Kp110δ deficiency improves gross pathology and reduces histological damage in SHIP$^{-/-}$ mice

To understand the contribution of PI3Kp110δ to intestinal fibrosis in SHIP$^{-/-}$ mice, we looked at ileum from 4, 8, and 12 week old mice that were healthy (SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$), expressed CD-like intestinal inflammation and fibrosis (SHIP$^{-/-}$PI3Kp110δ$^{+/+}$), and mice that were SHIP$^{-/-}$ and deficient in PI3Kp110δ activity (SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$). We found that, at 4 weeks of

age, the ileum from healthy control mice, the $SHIP^{+/+}$ mice and the $SHIP^{+/+}PI3Kp110\delta^{DA/DA}$ mice, all appeared healthy, with no signs of muscle thickening or redness (Figure 3.1). These control mice ($SHIP^{+/+}PI3Kp110\delta^{+/+}$ and $SHIP^{+/+}PI3Kp110\delta^{DA/DA}$) ileum also looked healthy at 8 weeks old and 12 weeks of age. However, the $SHIP^{-/-}$ mice ileum at 8 and 12 weeks of age did begin to show patches of redness and thickening. In contrast, the ileum from $SHIP^{-/-}PI3Kp110\delta^{DA/DA}$ mice, at 8 and 12 weeks old, showed intermediate levels of pathology (Figure 3.1).

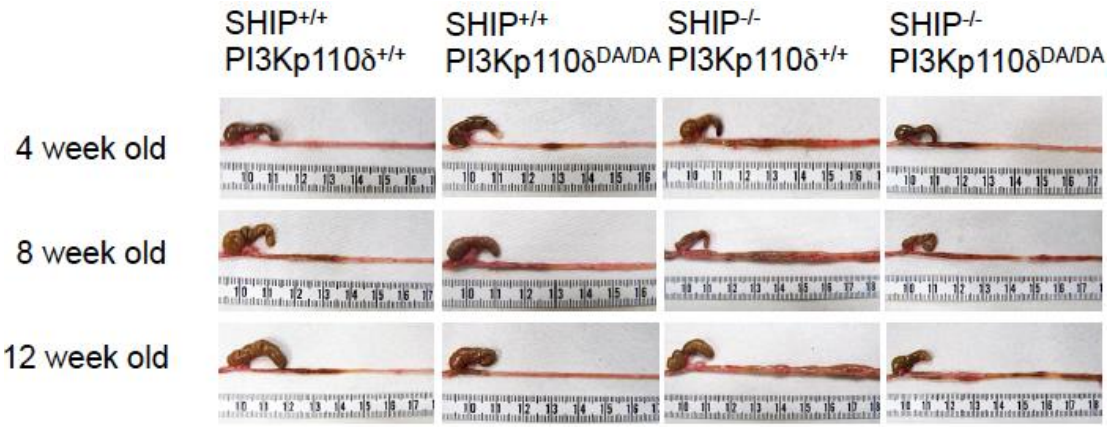

Figure 3.1 PI3Kp110δ deficiency reduces thickening of the ileum in $SHIP^{-/-}$ mice. Mice were euthanized at 4, 8, or 12 weeks of age and assessed for gross pathological features of CD-like intestinal fibrosis (for quantification of intestinal inflammation and intestinal fibrosis, please refer to Figures 5.1 and 3.3 respectively)**.** Gross anatomy of cecum and distal ileum from mice is shown. Columns indicate genotype and rows indicate age. Sections are representative of 6 individual mice per group.

To provide greater detail in characterizing the structural health of the distal ileum in each mouse, ileal sections were fixed for H&E staining. At 4 weeks old, the healthy control mice ($SHIP^{+/+}PI3Kp110\delta^{+/+}$ and $SHIP^{+/+}PI3Kp110\delta^{DA/DA}$) did not show evidence of muscle thickening, villus damage, or immune cell infiltration (Figure 3.2). Surprisingly, $SHIP^{-/-}$ mice (2 of 6) began to show signs of histological damage in their

ileum even at 4 weeks of age. However, the majority of these mice (4 of 6) did not have histological evidence of disease. In contrast, none of the 4 week old SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ showed histological damage or muscle thickening (Figure 3.2). At 8 and 12 weeks of age, the distal ileums from all of the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice exhibited thickening of the muscle layers, in particular the mucularis externa, accompanied by increased goblet cell hypertrophy, an abundance of immune cell infiltrates and aggregates throughout the gut wall, and damages to the crypt-villus architecture (Figure 3.2). Importantly, the 8 and 12 week old SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice ileum had thinner muscle layers, a lower number of immune cell infiltrates, and reduced damage to the intestinal architecture compared to SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice ileums (Figure 3.2).

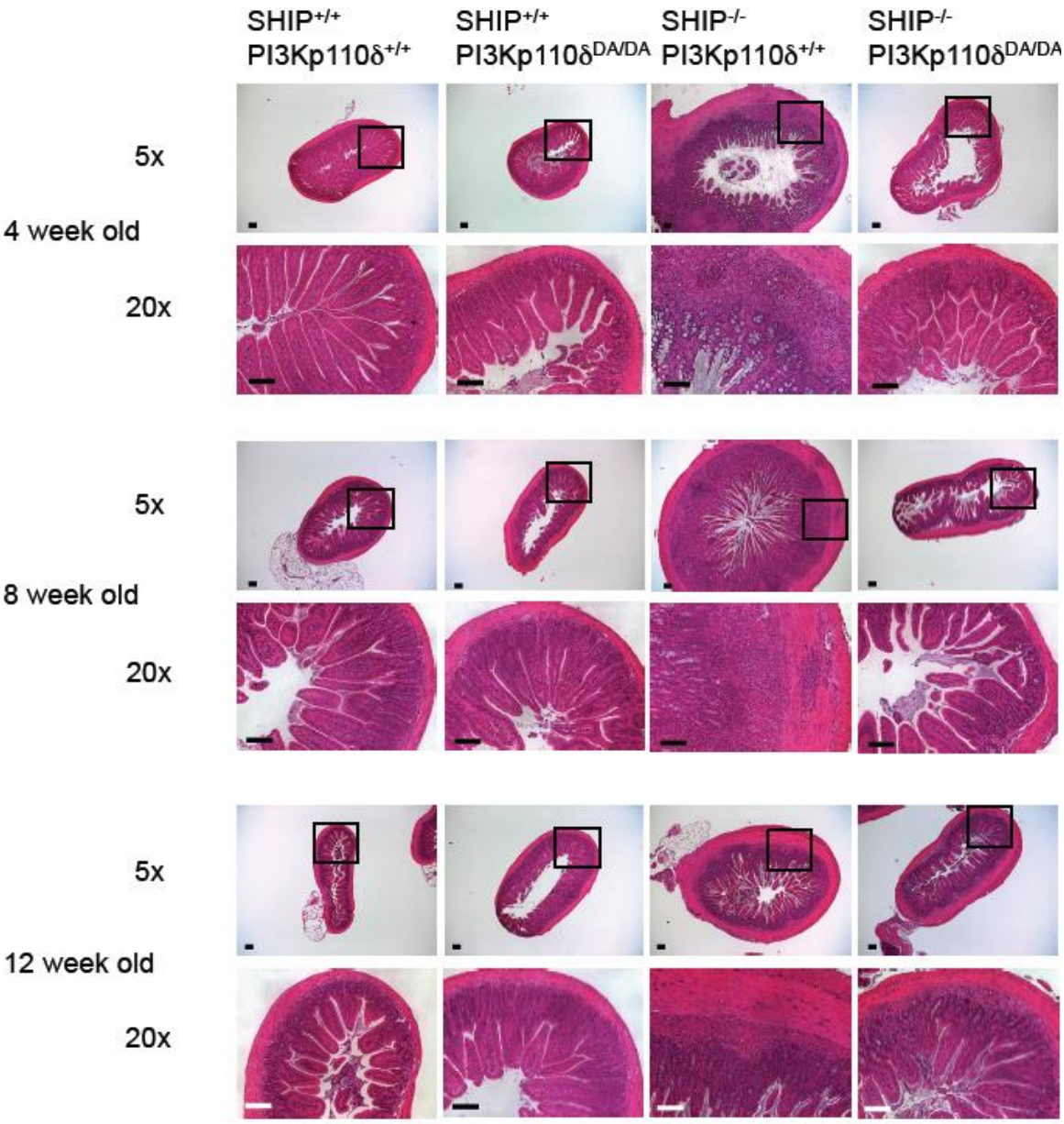

Figure 3.2 PI3Kp110δ deficiency reduces histological damage in SHIP$^{-/-}$ mice. H&E staining of ileal cross-sections from SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice at 4, 8, and 12 weeks of age. Magnifications 5x and 20x. Scale bars = 100 μm. A dramatic example of histological damage was chosen for 4 week old SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ as it shows that CD-like intestinal inflammation and fibrosis can also occur in young mice.

3.2.2 PI3Kp110δ deficiency reduces ileal fibrosis in SHIP$^{-/-}$ mice

We measured muscle thickness as a parameter of CD-like intestinal fibrosis and found that, as expected, healthy control mice, SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$ genotypes, had the thinnest ileal muscle layers (muscularis and serosa) at all ages, whereas the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice had the thickest muscle layers at all ages (Figure 3.3A). Most importantly, ileum from 8 week old SHIP$^{-/-}$ mice that were also deficient in PI3Kp110δ (SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$) had significantly thinner ileal muscle layers when compared to their SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ counterparts. This difference was not seen in mice at 12 weeks of age, which may be because the muscle is not as dramatically affected in SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice at 12 weeks of age.

To quantitate fibrosis, we also measured soluble collagen in ileal tissue using the Sircol assay for soluble collagen. We found that, at 4 weeks of age, there were no significant differences in soluble collagen between any of the 4 gentoypes of mice (Figure 3.3B). This is consistent with what has been previously reported, in that the majority of 4 week old SHIP$^{-/-}$ mice do not have signs of intestinal fibrosis.[126] At 8 weeks of age, the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice (disease control) had high levels of soluble collagen in their distal ileum, whereas the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice had significantly lower levels of soluble collagen (Figure 3.3B). These differences were maintained in the 12 week old mice (Figure 3.3B).

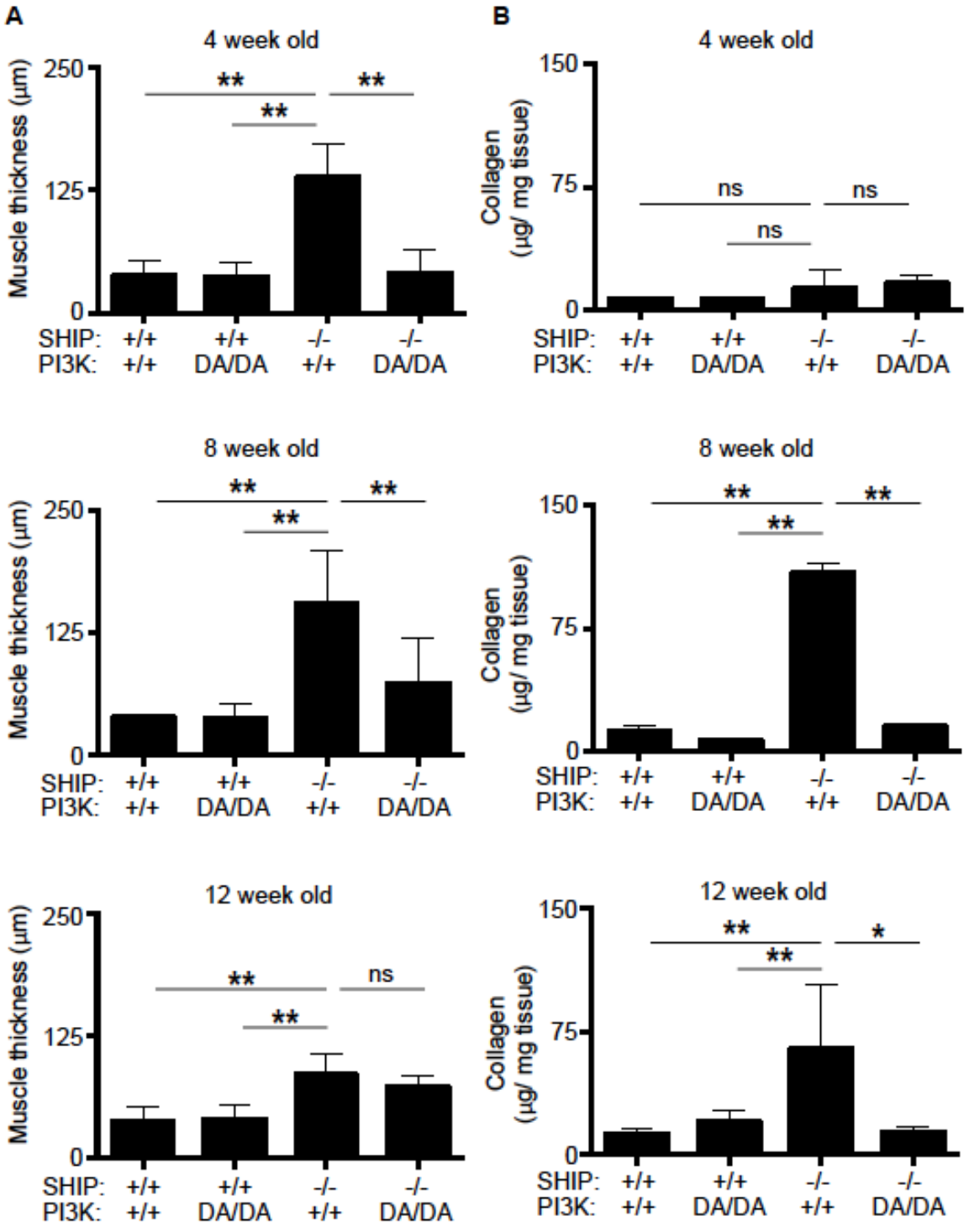

Figure 3.3 PI3Kp110δ deficiency reduces ileal fibrosis in SHIP$^{-/-}$ mice. (A) Muscle thickness and **(B)** soluble collagen in ileum from (left to right) SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice; at 4, 8, and 12 weeks of age. Data shown are the mean ± SD for 6 mice per group. *P < 0.05, **P < 0.01, and ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

As a second measure of collagen accumulation in the ileum of our mice, we performed Masson's trichrome staining for fibrosis on fixed ileal tissue sections. This stain distinguishes nuclei (black) and surrounding connective tissue and cytoplasm (red) from collagen (blue). We found that collagen was deposited mainly in the submucosa, but was also evident between muscle layers (muscularis and serosa layers), and was even present in the LP in the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ ileal sections (Figure 3.4). Similar to our findings for soluble collagen, the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice had the largest amount of collagen present (blue) at all ages, whereas PI3Kp110δ deficiency in SHIP$^{-/-}$ mice resulted in lower levels of collagen deposition in the distal ileum (Figure 3.4).

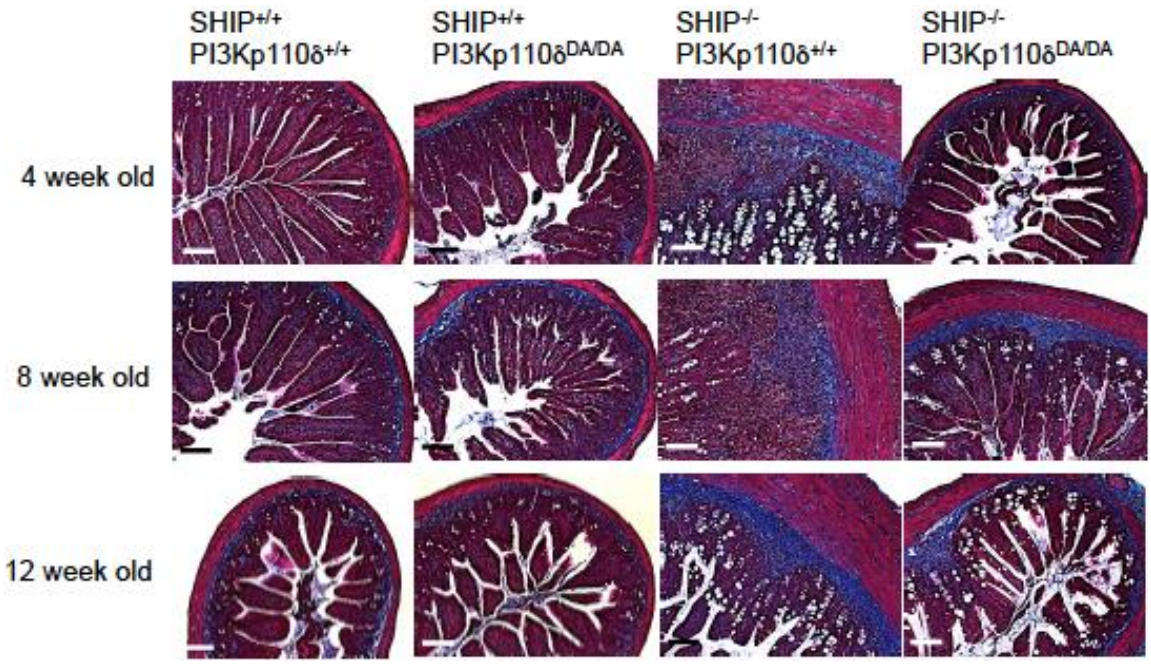

Figure 3.4 PI3Kp110δ deficiency reduces collagen deposition in SHIP$^{-/-}$ mice. Masson's trichrome staining for collagen deposition (blue) in the ileal cross-sections from SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice; at 4, 8, and 12 weeks of age. Photos were taken at 20x magnification. Columns indicate genotype and rows indicate age of mice. Scale bars = 100 μm. Sections are representative of 6 individual mice per group.

3.2.3 PI3Kp110δ deficiency reduces arginase activity in SHIP$^{-/-}$ mice

Given that argI is up-regulated in SHIP$^{-/-}$ mouse macrophages and is upstream of the L-proline required for collagen biosynthesis, and that PI3Kp110δ contributes to IL-4-induced argI gene expression in macropahges; we next asked whether PI3Kp110δ affects arginase activity and argI protein expression in the ileums from our cohort of mice.

Arginase activity was high in all genotypes of mice at 4 weeks of age (Fig. 3.5, left) and no significant differences were observed between SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice. Interestingly, arginase levels were significantly higher in the SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$ mice compared to the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice. At 8 weeks of age, the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice had high levels of arginase activity in ileal tissues, which was significantly higher than that found in either of the healthy control mice, SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ or SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$. Importantly, the SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice had significantly lower arginase activity in the ileum compared to the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice (Figure 3.5). This was also the case for ileal homogenates from 12 week old mice (Figure 3.5).

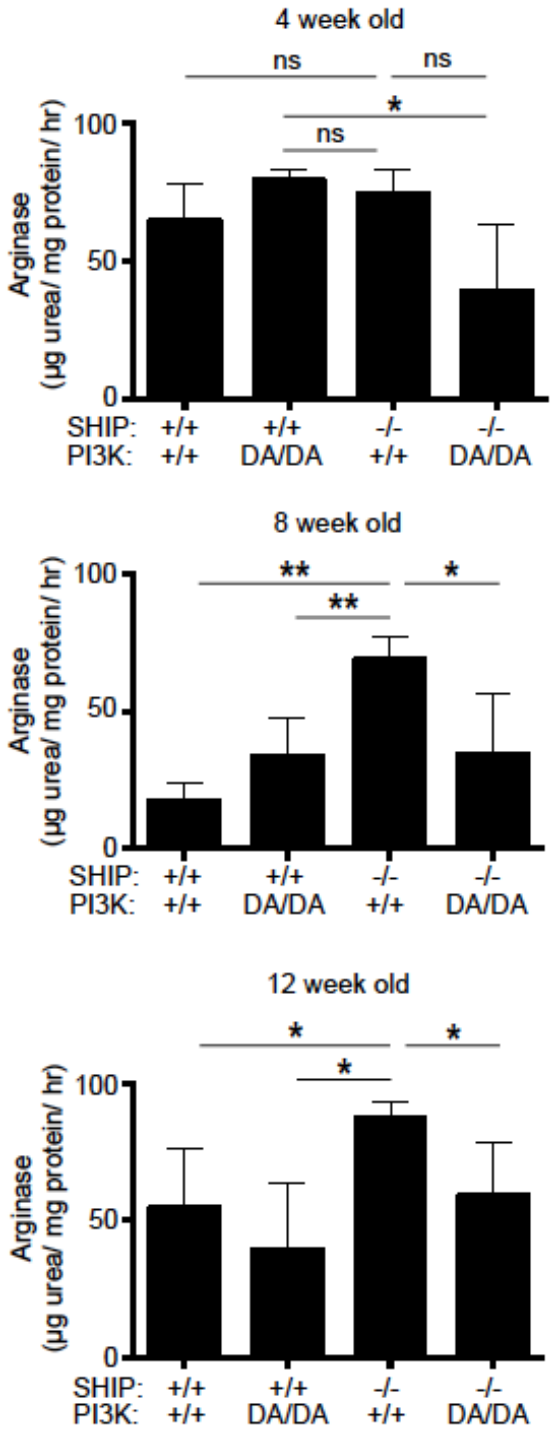

Figure 3.5 PI3Kp110δ deficiency reduces arginase activity in the ileum of SHIP$^{-/-}$ mice. Arginase activity assay of (from left to right) SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice; at 4, 8, and 12 weeks age. Data shown are the means ± SD for 6 mice per group. *P < 0.05, **P < 0.01, and ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

Immunofluorescence staining was then performed to investigate the number and location of cells expressing arginase in the distal ileum. ArgI (red) was co-stained with Ym1 (green), which is a marker for IL-4-treated murine macrophages, in ileal cross-sections. At all ages, healthy control mice expressed low baseline levels of argI primarily in the muscle layer and submucosa where collagen was typically found. In contrast, the SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice had more argI^{+} cells in the muscle layers and the submucosa at all ages (Figure 3.6). Finally, SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ had reduced numbers of argI expressing cells compared to SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ (Figure 3.6). It is interesting to note that argI^{+} cells were located in areas of high collagen deposition (according to Masson's trichrome staining; Fig. 3.4).

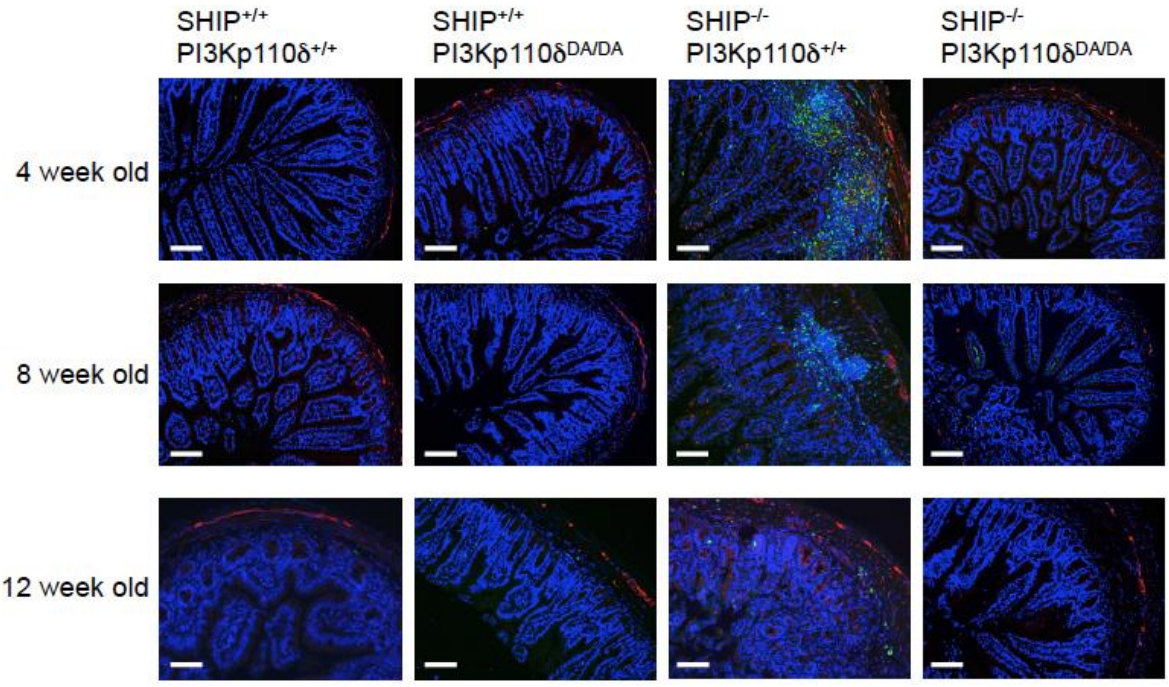

Figure 3.6 PI3Kp110δ deficiency reduces the number of argI^{+} cells in the ileum of SHIP$^{-/-}$ mice. Ileal cross-sections were co-stained with argI (red) and Ym1 (green), by immunofluorescence, and counterstained with DAPI (blue). Photographs were taken at 20x magnification. Scale bars = 100 μm. Sections are representative of 6 individual mice per group.

3.2.4 PI3Kp110δ driven fibrosis correlates with increased fibroblast numbers and IL-4 production, but not TGFβ production

Fibroblasts are key drivers of intestinal fibrosis, so we compared fibroblast numbers present in the distal ileum indirectly by immunofluorescent staining for vimentin^{+} mesenchymal cells (red). We found that at 8 weeks of age, SHIP$^{-/-}$ PI3Kp110δ$^{+/+}$ had more vimentin^{+} cells in their distal ileums than healthy control mice (SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$). Increased numbers of vimentin^{+} cells were located within the muscle layers, submucosa, and in the LP (Figure 3.7). Most importantly, we found that the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice had fewer vimnetin^{+} fibroblasts in their ileum than their SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ counterparts (Figure 3.7).

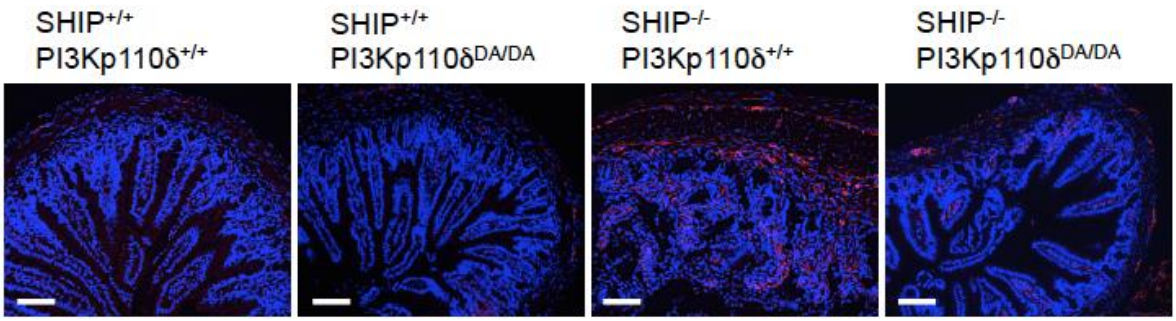

Figure 3.7 PI3Kp110δ deficiency reduces the number of vimentin^{+} mesenchymal cells in the distal ileum of SHIP$^{-/-}$ mice. Ileal cross-sections from 8 week old SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$ mice were stained by immunofluorescence for vimentin (red) and counterstained with DAPI (blue). Photographs were taken at 20x magnification. Scales bars = 100 μm. Sections are representative of 6 individual mice per genotype.

To investigate the cytokines involved in fibrosis in SHIP deficient mice, we measured IL-4 and TGFβ levels in full thickness ileal tissue homogenates from each of the 4 genotypes of mice at 8 weeks of age. We found that SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice ileums (disease control) had higher levels of IL-4 compared to that found in the ileums of

SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice, but not significantly different compared to healthy control mice (SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$) (Figure 3.8). In contrast, there were no significant differences in TGFβ1 between SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ and SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice (Figure 3.8). That is, the levels of TGFβ1 did not correlate with the features of fibrosis in our cohort of mice. This reinforces the importance of IL-4/PI3Kp110δ in the development of CD-like intestinal fibrosis in the SHIP$^{-/-}$ mouse;[135] and suggests that fibrosis develops through a pathway independent of TGFβ in this model.

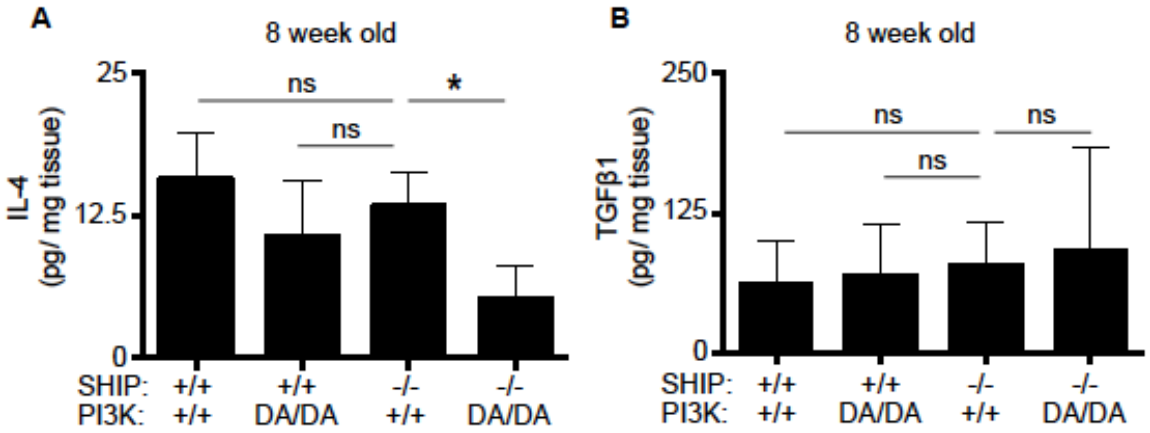

Figure 3.8 PI3Kp110δ driven fibrosis in SHIP$^{-/-}$ mice correlates with IL-4, but not TGFβ1, levels in the SHIP$^{-/-}$ ileum. (A) IL-4 and **(B)** TGFβ1 levels measured in full thickness ileal tissue homogenates from (left to right) SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice, at 8 weeks of age. Data shown are the means ± SD for 6 mice per group. *P < 0.05 and ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

3.3 Discussion

SHIP plays pleotropic roles in macrophage activation by limiting PI3K activity downstream of receptor stimulation. Thus, SHIP$^{-/-}$ macrophages are hyper-responsive to immune stimuli, and have been shown to contribute to the development of spontaneous CD-like intestinal inflammation in SHIP$^{-/-}$ mice. Inflammation in SHIP$^{-/-}$ mice is accompanied by intestinal fibrosis, which is dependent on kinase activity.[82, 126] ArgI expression is up-regulated in SHIP$^{-/-}$ ileal tissue, and the PI3Kp110δ catalytic isoform is required for IL-4-induced argI expression in M(IL-4)s. To investigate a potential role for PI3Kp110δ in SHIP$^{-/-}$ intestinal fibrosis, we crossed germline SHIP$^{-/-}$ mice with mice that have a germline deficiency in PI3Kp110δ activity.

Using these mice, we have found that PI3Kp110δ deficiency can effectively reduce features of ileal fibrosis in SHIP$^{-/-}$ mice. PI3Kp110δ deficiency improved gross pathology, reduced the fibrotic features of disease that we measured, and reduced molecular drivers of intestinal fibrosis. As we anticipated, PI3Kp110δ deficiency did indeed reduce arginase activity and argI expression in the ileum of SHIP$^{-/-}$ mice, demonstrating that PI3Kp110δ is important for the induction of argI *in vivo*. ArgI uses L-arginine in the production of ornithine and downstream L-proline, an amino acid, which is required in abundance for collagen production. Consistent with this, downstream collagen accumulation is reduced in SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice. In addition, we found that argI expression was closely localized to areas where collagen deposition occurred in the distal ileum of SHIP$^{-/-}$ mice in our IF stains; that is, in the mucosa, submucosa, muscularis mucosa, muscularis propria, and serosa layers (Figure 2.6).

ArgI is also critical in the biosynthesis of polyamines, such as spermine and

spermidine, which lead to stimulation of fibroblasts (Figure 1.5).[126] Indeed, our data suggests that SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice are experiencing reduced CD-like intestinal fibrosis through this pathway, as these mice had dramatically lower levels of fibroblasts, which likely contributes to the reduced levels of collagen in their distal ileums (Figure 3.7; 3.8A). Our data are consistent with a recent study from our laboratory, which demonstrated that treating SHIP$^{-/-}$ mice with the arginase inhibitor *S*-(2-boronoethyl)-l-cysteine (BEC) reduced collagen deposition in the ileum of SHIP$^{-/-}$ mice. Our data adds evidence to the notion that targeting arginase activity may be a viable strategy to limit intestinal fibrosis; and further, suggests that PI3Kp110δ may be an additional upstream target that we can use to limit intestinal fibrosis in people with CD.[126]

We also found that TGFβ1 levels in the distal ileum of 8 week old mice were not significantly different among genotypes in our cohort of mice (Figure 3.8B). TGFβ1 is up-regulated in the inflamed gut and is considered a critical player in intestinal fibrosis. One key mechanism by which TGFβ1 drives fibrosis is by increasing the fibroblast population (see section 1.3.8.1),[104] which has been implicated in executing the steps that initiate and perpetuate fibrosis (see section 1.3.7.1).[104] Despite finding no decrease in TGFβ1 levels in the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice ileum, we did find that fibroblast numbers were significantly reduced (Figure 3.7). This suggests that TGFβ1 is not absolutely required for increasing fibroblast numbers, or for the development of intestinal fibrosis. We did confirm that IL-4 levels were decreased in the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mouse ileum compared to the SHIP$^{-/-}$ mouse (Figure 3.8), and we found that they were. PI3Kp110δ is activated downstream of the IL-4 receptor and is required for argI induction in IL-4-treated macrophages.[136] Together, this suggests that IL-4, and not

TGFβ1, may be a key driver of ileal fibrosis in $SHIP^{-/-}$ mice.

Chapter 4: PI3Kp110δ inhibition reduces ileal fibrosis in SHIP$^{-/-}$ mice

4.1 Introduction and rationale

We have shown that a genetic ablation of PI3Kp110δ activity reduces features and drivers of ileal fibrosis in the SHIP$^{-/-}$ mouse. Thus, we next asked whether inhibiting PI3Kp110δ would be similarly effective because this strategy could be used to treat intestinal fibrosis in people with CD. We treated 8 week old SHIP$^{-/-}$ mice with the cell-permeable isoform-specific inhibitor of PI3Kp110δ, IC87114, which is purine-quinazoline-based and binds to the adenosine triphosphate (ATP)-binding pocket of the enzyme. We compared groups of IC87114 treated mice to mice treated with a vehicle control (PEG400) and to 8 week old SHIP$^{-/-}$ mice, as a positive control for disease.

4.2 Results

4.2.1 Inhibition of PI3Kp110δ improves gross pathology and reduces histological damage in SHIP$^{-/-}$ mice

We treated 8 week old SHIP$^{-/-}$ (SHIP$^{-/-}$PI3Kp110δ $^{+/+}$) mice with either PEG400, as a vehicle control, or IC87114, a PI3Kp110δ isoform-specific inhibitor. Mice were treated once daily by oral gavage for two weeks, and pathology was assessed in these mice. In addition, we assessed untreated 8 week old SHIP$^{-/-}$ mice, as a positive control for disease. We found that mice treated with PEG400 (vehicle control) were similar to the untreated SHIP$^{-/-}$ (SHIP$^{-/-}$PI3Kp110δ$^{+/+}$) mice (disease control) as they developed discontinuous patches of muscle thickening and redness in their distal ileums (Figure 4.1). In contrast, SHIP$^{-/-}$ mice that were treated with IC87114 had reduced signs of muscle thickening and redness in their distal ileums (Figure 4.1).

Figure 4.1 Inhibition of PI3Kp110δ activity improves gross pathology in SHIP$^{-/-}$ mice. Gross anatomy of the cecum and ileum of 8 week old SHIP$^{-/-}$ mice either untreated (left), or treated for 2 weeks with PEG400 (middle) or IC87114 (right). Mice were euthanized and cecums and ileums were photographed to visualize gross anatomical features. Results shown are representative of 6 mice per group.

Ileal tissue was also fixed for H&E staining, and we found that both disease control and vehicle control mice (PEG400 treated) had thick muscularis layers accompanied by goblet cell hyperplasia and hypertrophy, crypt-villus hyperplasia, and an abundance of immune cell infiltrates throughout the gut wall (Figure 4.2A). The SHIP$^{-/-}$ mice treated with IC87114, on the other hand, had reduced immune cell infiltration, reduced crypt-villus hyperplasia, and reduced muscle thickness compared to control mice (Figure 4.2B).

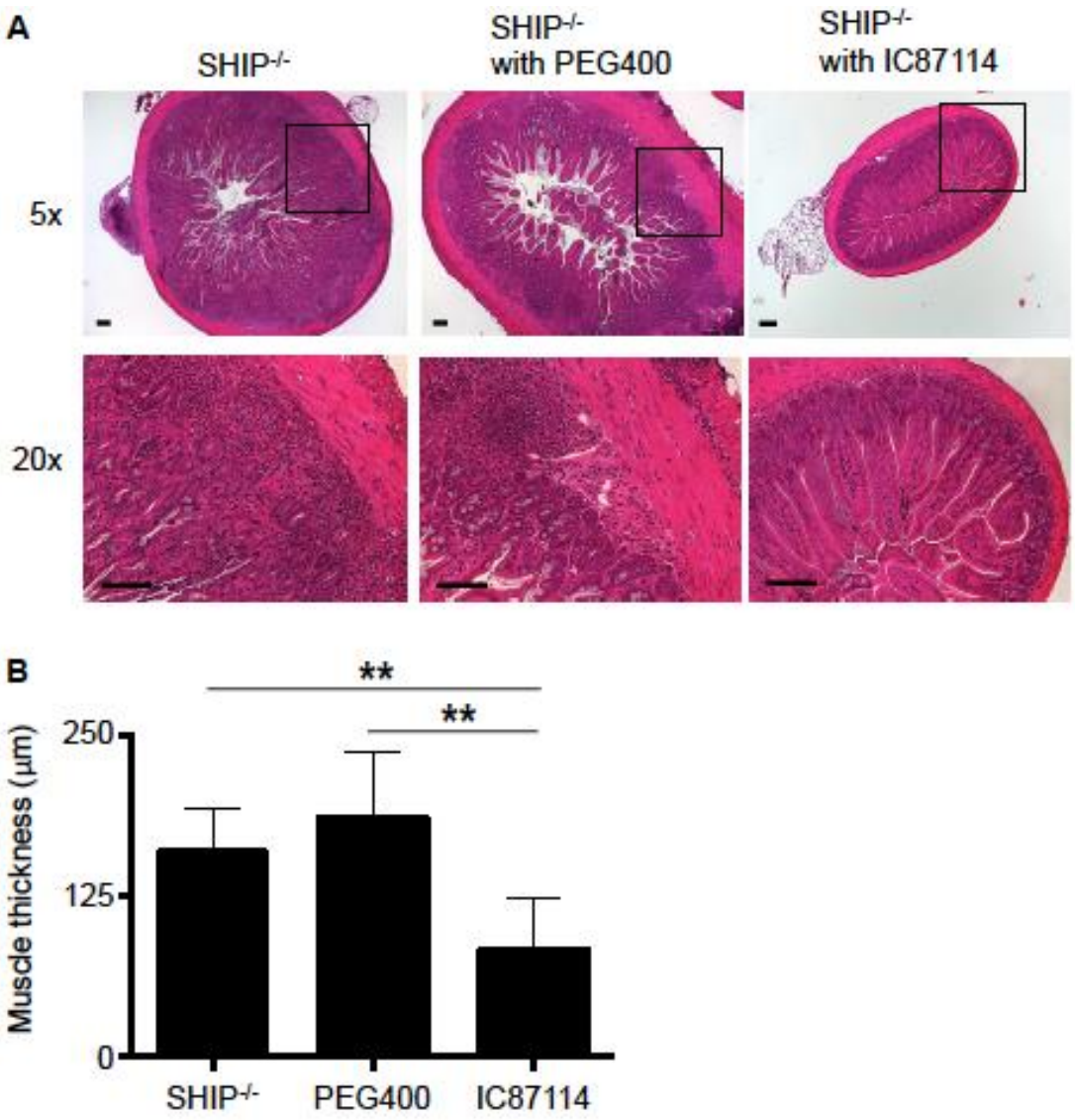

Figure 4.2 Inhibition of PI3Kp110δ activity reduces histological damage and muscle thickening in SHIP$^{-/-}$ mice. (A) H&E stained ileal cross-sections of 8 week old SHIP$^{-/-}$ mice either untreated (left), or treated for 2 weeks with PEG400 (middle) or IC87114 (right). Scale bars = 100 µm. Sections shown are representative of 6 similarly treated mice. **(B)** Muscle thickness of (from left to right) 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, 10 week old SHIP$^{-/-}$ mice treated with IC87114. Bars represent the mean ± SD for 6 mice per group. **P < 0.01 using a one-way ANOVA with Bonferroni correction for multiple comparisons.

4.2.2 Inhibition of PI3Kp110δ reduces ileal fibrosis in SHIP$^{-/-}$ mice

Next, we measured features of ileal fibrosis that we have described in the SHIP$^{-/-}$ mouse. We first performed Masson's trichrome staining and found that disease control mice (8 week old SHIP$^{-/-}$) had high levels of collagen deposition (blue) in the submucosa and muscle layers of their distal ileum, and SHIP$^{-/-}$ mice treated with PEG400 vehicle control showed no signs of recovery (Figure 4.3A). However, SHIP$^{-/-}$ mice were treated with IC87114, had significantly lower amounts of collagen deposition in their distal ileum (Figure 4.3A). This was also evident when we quantified soluble collagen using the Sircol assay. IC87114 treated mice had significantly lower amounts of soluble collagen in their distal ileum compared to both disease and vehicle treated control mice (Figure 4.3B).

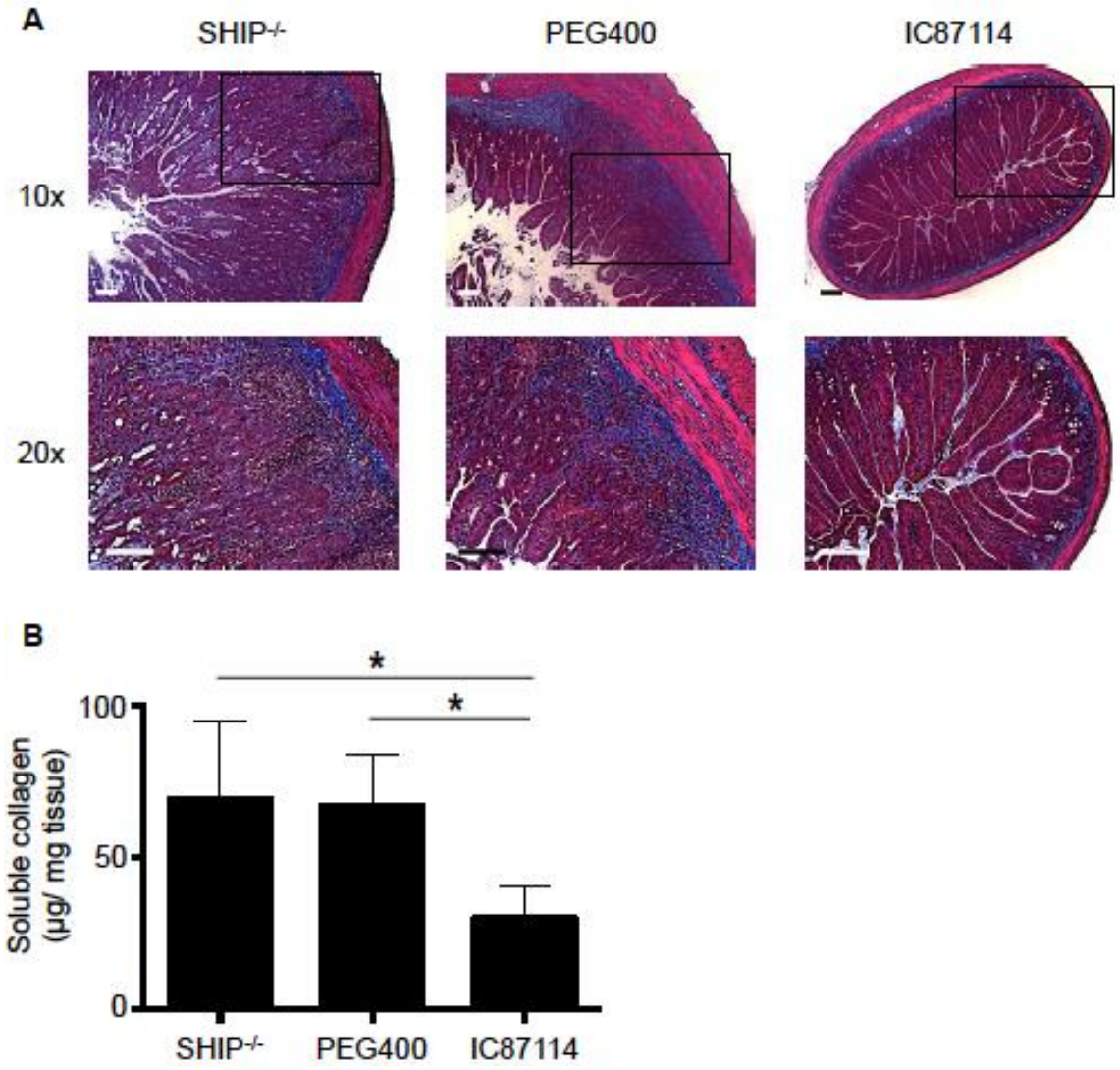

Figure 4.3 Inhibition of PI3Kp110δ activity reduces collagen deposition in SHIP$^{-/-}$ mice. (A) Masson's trichrome staining of ileal cross-sections from (left to right) 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, 10 week old SHIP$^{-/-}$ mice treated with IC87114. Scale bars = 100 µm. Results shown are representative of 6 mice per group. **(B)** Soluble collagen in the distal ileum of mice measured by Sircol assay. Bars represent the mean ± SD for 6 mice per group. *$P < 0.05$ using a one-way ANOVA with Bonferroni correction for multiple comparisons.

We then looked at argI and Ym1 expression in the distal ileum of these mice by immunofluorescent staining of ileal cross-sections. We found that, similar to our germline model of PI3Kp110δ deficiency in SHIP$^{-/-}$ mice, the SHIP$^{-/-}$ mice that were treated with IC87114 had significantly lower numbers of cells expressing argI (red) and

Ym1 (green) in the muscle layers and the submucosa of their distal ileums when compared to the disease control and vehicle control mice, which had an abundance of argI in the muscle layers and Ym1 in the submucosa and LP (Figure 4.4A). We also quantified arginase activity through an arginase assay and found similar results, in that PI3Kp110δ inhibition by IC87114 significantly reduced arginase activity in the distal ileum of the SHIP$^{-/-}$ mice when compared to both control groups (Figure 4.4B). Therefore, we show that PI3Kp110δ is able to regulate both argI expression in cells and arginase enzyme activity.

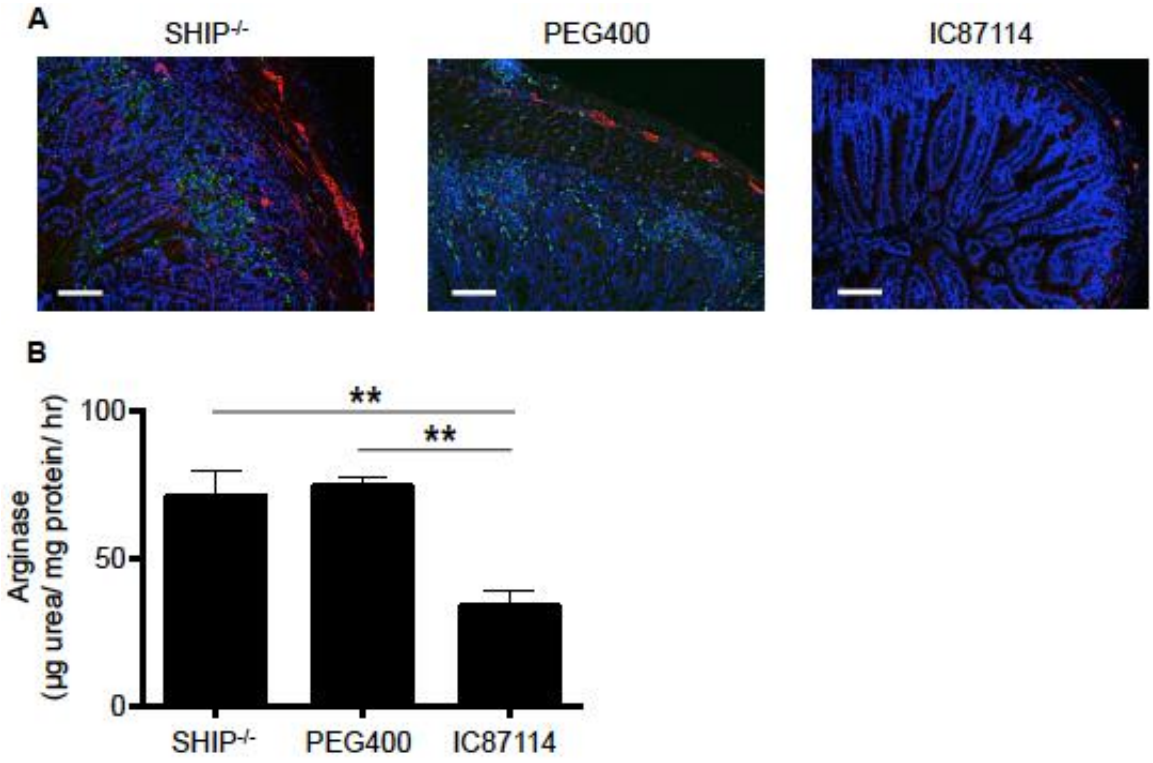

Figure 4.4 Inhibition of PI3Kp110δ activity reduces arginase and Ym1 expression and arginase activity in SHIP$^{-/-}$ mice. (A) Ileal cross-sections co-stained by immunofluorescence for argI (red) and Ym1 (green) and counterstained with DAPI (blue) taken at 20x magnification. Scale bars = 100 µm. Sections are representative of 6 individual mice per group. **(B)** Arginase activity in ileal tissue homogenates from (left to right) 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, and 10 week old SHIP$^{-/-}$ mice treated with IC87114. Bars represent the mean ± SD for 6 mice per group. **P < 0.01 using a one-way ANOVA with Bonferroni correction for multiple comparisons.

4.2.4 PI3Kp110δ activity correlates with IL-4 production, but not TGFβ production

Similar to our genetic model, we found that SHIP$^{-/-}$ mice treated with the PI3Kp110δ inhibitor, IC87114, had significantly reduced IL-4 levels in their distal ileums when compared to the disease control and vehicle control mice (Figure 4.5A). Also, TGFβ1 levels were not significantly different among treatment groups (Figure 4.5B). Mice that were given IC87114 still had significantly reduced numbers of vimentin^{+} fibroblasts in their distal ileum (Figure 4.5C). These findings further demonstrate that TGFβ1 levels do no correlate with the features of fibrosis in our SHIP$^{-/-}$ mice. Moreover, they reinforce the importance of IL-4/PI3Kp110δ in regulating the development of CD-like intestinal fibrosis in the SHIP$^{-/-}$ mouse.

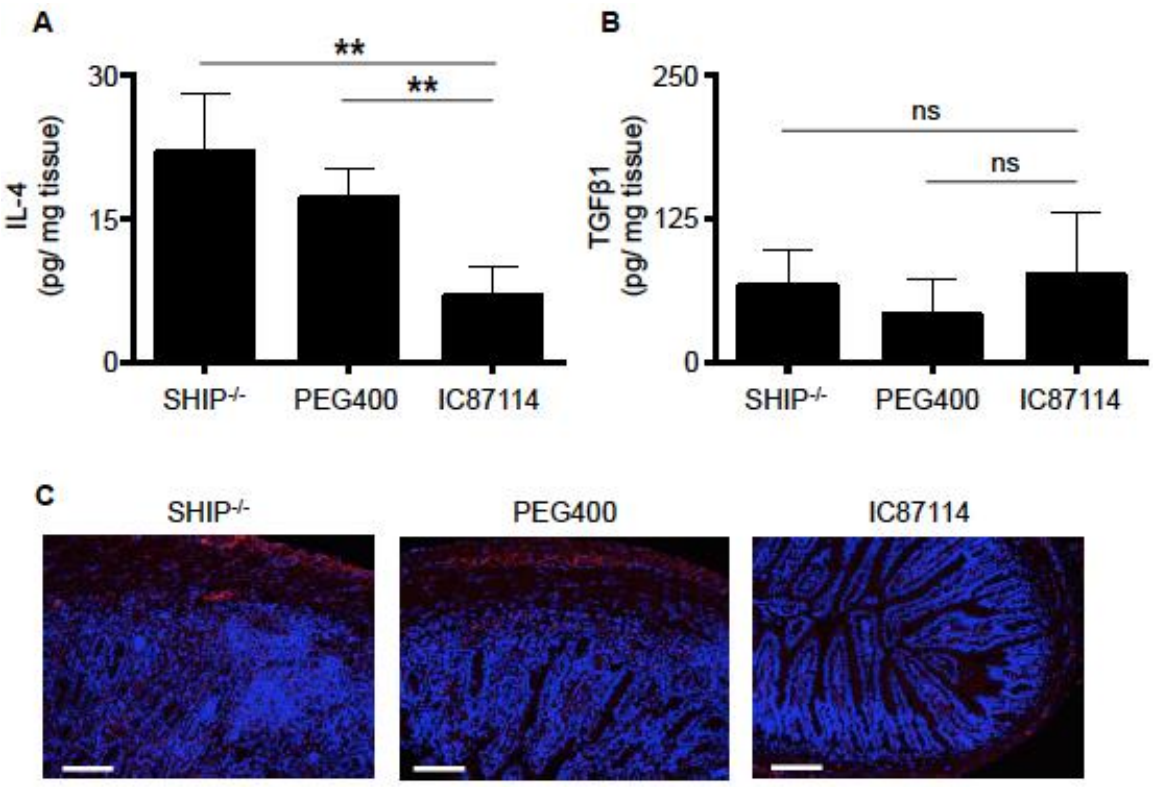

Figure 4.5 PI3Kp110δ inhibition in SHIP$^{-/-}$ mice correlates with decreased IL-4, but not TGFβ1, levels in the SHIP$^{-/-}$ ileum. (A) IL-4 measured in full thickness ileal tissue homogenates from (left to right) 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, and 10 week old SHIP$^{-/-}$ mice treated with IC87114. Bars represent the mean ± SD for 6 mice per group. **(B)** TGFβ1 measured from full thickness ileal tissue homogenates. Same order as (A). Bars represent the mean ± SD for 6 mice per group. **(C)** Immunofluorescent staining for vimentin (mesenchymal cells; red) counterstained with DAPI (blue) in ileal cross sections. Sections are shown at 20x magnification. Scale bars = 100 μm. Sections are representative of 6 individual mice per group. **P < 0.01, ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons**.**

4.3 Discussion

In chapter 3, we demonstrated that genetic deficiency in PI3Kp110δ activity can provide protection against the development of features of CD-like intestinal fibrosis in $SHIP^{-/-}$ mice. Based on these findings, we wanted to ask whether PI3Kp110δ inhibition could confer similar protection. To investigate this, we treated 8 week old $SHIP^{-/-}$ mice (with established inflammation and fibrosis) with an isoform-specific inhibitor of PI3Kp110δ, IC87114. IC87114 is a selective inhibitor with a 58 and 100-fold increased efficacy for PI3Kp110δ over the γ and α/β isoforms, respectively. Treated mice were compared to mice treated with vehicle control (PEG400) and to 8 week old $SHIP^{-/-}$ mice, as a positive control for disease.

Similar to our studies using the genetic, germline deficiency in PI3Kp110δ , we found that PI3Kp110δ inhibition can effectively reduce features of ileal fibrosis in $SHIP^{-/-}$ mice. PI3Kp110δ inhibition improved gross pathology, reduced fibrotic features of disease, and reduced molecular drivers of intestinal fibrosis. PI3Kp110δ inhibition reduced IL-4 levels (which will be discussed in Section 6), arginase activity, and argI in the distal ileum. As a consequence, IC87114-treated $SHIP^{-/-}$ mice had reduced muscle thickness, fibroblast numbers, and reduced collagen accumulation in the distal ileum (Figure 3.3-3.5). As in our genetic model, reduced ileal fibrosis in $SHIP^{-/-}$ mice treated with the PI3Kp110δ inhibitor did not correlate with reduced TGFβ1 levels in the distal ileum. These findings reinforce our previous assertion that IL-4/PI3Kp110δ and downstream argI induction drive intestinal fibrosis in $SHIP^{-/-}$ mice. It is interesting to note that PI3Kp110δ inhibition was equally effective at reducing ileal fibrosis in $SHIP^{-/-}$ mice (IL-4, arginase, soluble collagen, and muscle thickness) as germline deficiency in

PI3Kp110δ activity. This is particularly exciting because pharmacological inhibition of enzymatic activity *in vivo* is a viable treatment strategy that may be useful for people with CD accompanied by intestinal fibrosis.

Chapter 5: PI3Kp110δ deficiency or inhibition reduces intestinal inflammation in SHIP$^{-/-}$ mice

5.1 Introduction and rationale

CD is a chronic inflammatory disease characterized by intestinal inflammation that can occur anywhere along the gastrointestinal tract.[4] The most common site for inflammation is the distal part of the ileum.[4] Class I PI3K is critical in many cellular processes including immune activation, thus, SHIP deficient mice are hyper-responsive to immune stimuli, including increased IL-1β production in response to innate immune activation.[82] IL-1β acts as an alarm cytokine, initiating the inflammatory response, which can be produced by monocytes and macrophages and further amplify innate immune responses.[82]

We, and others, have reported that SHIP deficient mice develop spontaneous CD-like intestinal inflammation that is primarily restricted to the distal ileum,[126] along with features consistent with intestinal fibrosis. Indeed, CD causing intestinal fibrosis has often been attributed to a persistent immune-mediated intestinal inflammation, as evidence has shown that CD fibrosis follows the distribution and location of inflammation.[99]

We have demonstrated that PI3Kp110δ drives intestinal fibrosis in SHIP$^{-/-}$ mice, both through PI3Kp110δ germline deficiency and drug inhibition experiments. However, we also saw that the number of infiltrating immune cells seemed to be reduced in the ileums of SHIP$^{-/-}$ mice with genetic deficiency or pharmacological inhibition of PI3Kp110δ activity (Figure 3.2, 4.2A) Therefore, we wanted to ask whether PI3Kp110δ

also contributes to inflammation in the ileum of the SHIP$^{-/-}$ mice by quantifying the inflammatory features that we have described previously.[126]

5.2 Results

5.2.1 PI3Kp110δ deficiency or inhibition reduces histological features of ileal inflammation in SHIP$^{-/-}$ mice

First, we looked at the number of immune cell infiltrates in the distal ileum as a measure of inflammation, which we measured by counting immune cells per field in H&E stained cross sections. As expected, the healthy control mice (SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ and SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$) at 4, 8, and 12 weeks of age all had significantly lower numbers of immune cells in their distal ileum compared to their SHIP$^{-/-}$ counterparts (SHIP$^{-/-}$PI3Kp110δ$^{+/+}$), which had the highest (Figure 5.1A). We found that at 4 and 8 weeks old, the PI3Kp110δ deficiency in SHIP$^{-/-}$ mice (SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$) resulted in significantly lower numbers of immune cells in the distal ileum when compared to the SHIP$^{-/-}$ mice at these ages. However, at 12 weeks old, the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice did not show significantly lower numbers of immune cells compared to its SHIP$^{-/-}$ counterpart. This may be due to the lower number of immune cells present in the 12 week old SHIP$^{-/-}$ mouse ileums compared to that seen in the 4 and 8 week old SHIP$^{-/-}$ mice and is consistent with previous observations from our laboratory demonstrating that ileal inflammation in SHIP$^{-/-}$ mice is reduced from 12-20 weeks of age (Figure 5.1A).[126] Furthermore, when we counted goblet cell numbers (which is an indicator of goblet cell hyperplasia) in the crypt and the villi, we found similar results (Figure 5.1B). PI3Kp110δ

deficiency in SHIP$^{-/-}$ mice, at 4 and 8 weeks of age, caused reduced goblet cell numbers in the distal ileum.

We also measured ileal villi length in these mice as longer villi are an indication of inflammation in some mouse models (eg. dextran sodium sulphate (DSS)-induced colitis). However, in our mouse model, we did not see any discernible patterns in villi length related to SHIP or PI3KP110δ deficiency. Despite that, crypt-villus architecture appeared less damaged in the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice compared to the SHIP$^{-/-}$ mice in IHC stained slides (Figure 5.1C). This finding suggests that villus hyperplasia is not a sign of spontaneous inflammation in the SHIP$^{-/-}$ mouse.

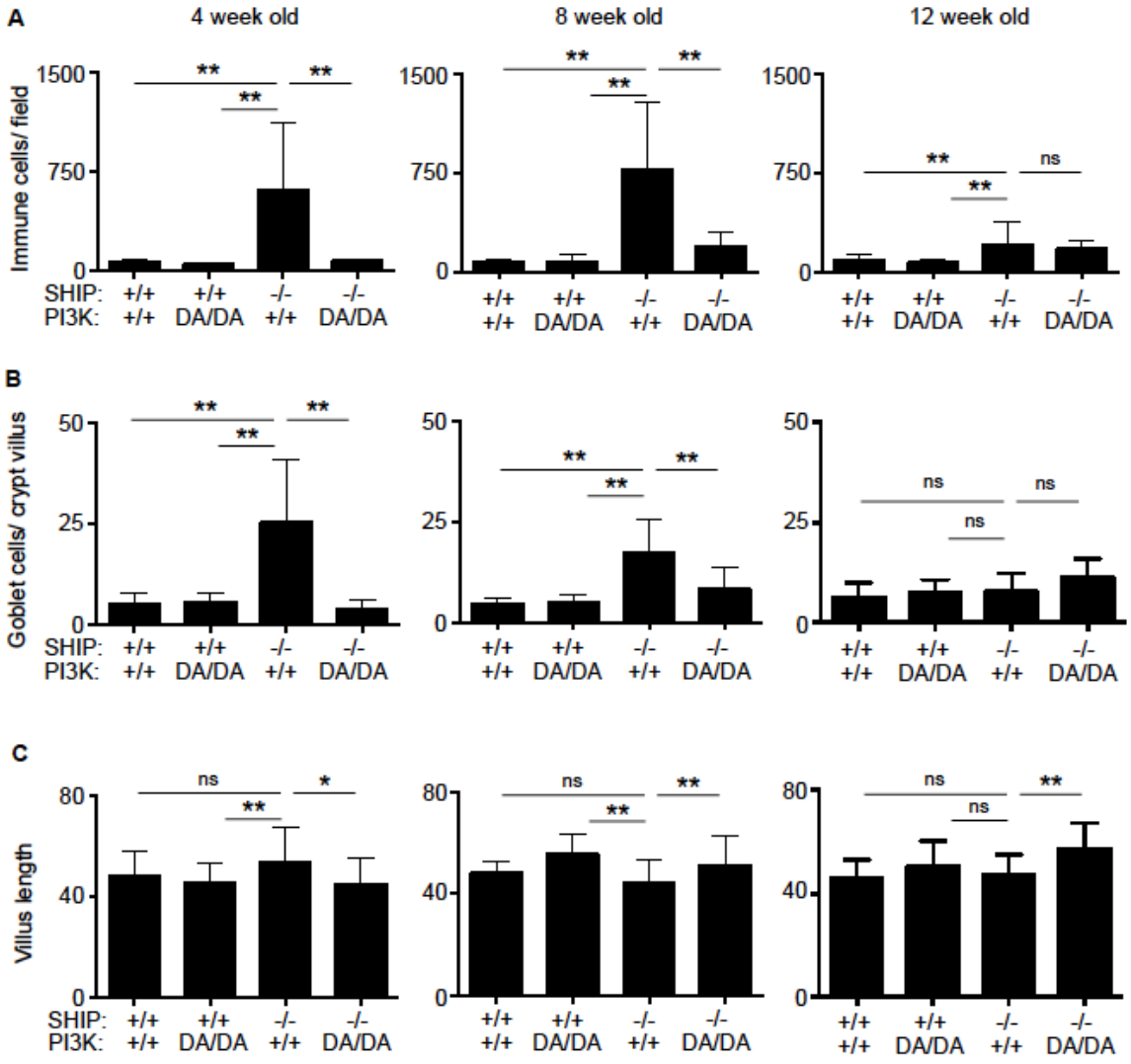

Figure 5.1 PI3Kp110δ deficiency reduces histological measures of intestinal inflammation in SHIP$^{-/-}$ mice. From left to right: SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice, at 4, 8 and 12 weeks of age. **(A)** Average number of immune cells per field counted from H&E-stained cross sections at 20x magnification. **(B)** Goblet cells per crypt-villus **(C)** Average villus length. Bars represent the means ± SD for 6 mice. *P < 0.05, **P < 0.01, ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

We also quantified intestinal inflammation in our PI3Kp110δ drug inhibition model. Perhaps not surprising based on our results so far, IC87114 drug inhibition of

PI3Kp110δ was equally effective at reducing the number of infiltrating immune cells and goblet cell hyperplasia as our germline PI3Kp110δ deficiency, as IC87114 treated mice had significantly reduced immune cell infiltrates and goblet cell numbers per crypt-villus in the distal ileum compared to disease or vehicle control mice (Figure 5.2). There was also a modest, but significant, reduction in ileal villus length in SHIP$^{-/-}$ mice treated with IC87114 compared to mice treated with vehicle control or 8 week old SHIP$^{-/-}$ mice. Together, these findings suggest that PI3Kp110δ does indeed play a role in regulating the inflammatory response in SHIP$^{-/-}$ mice.

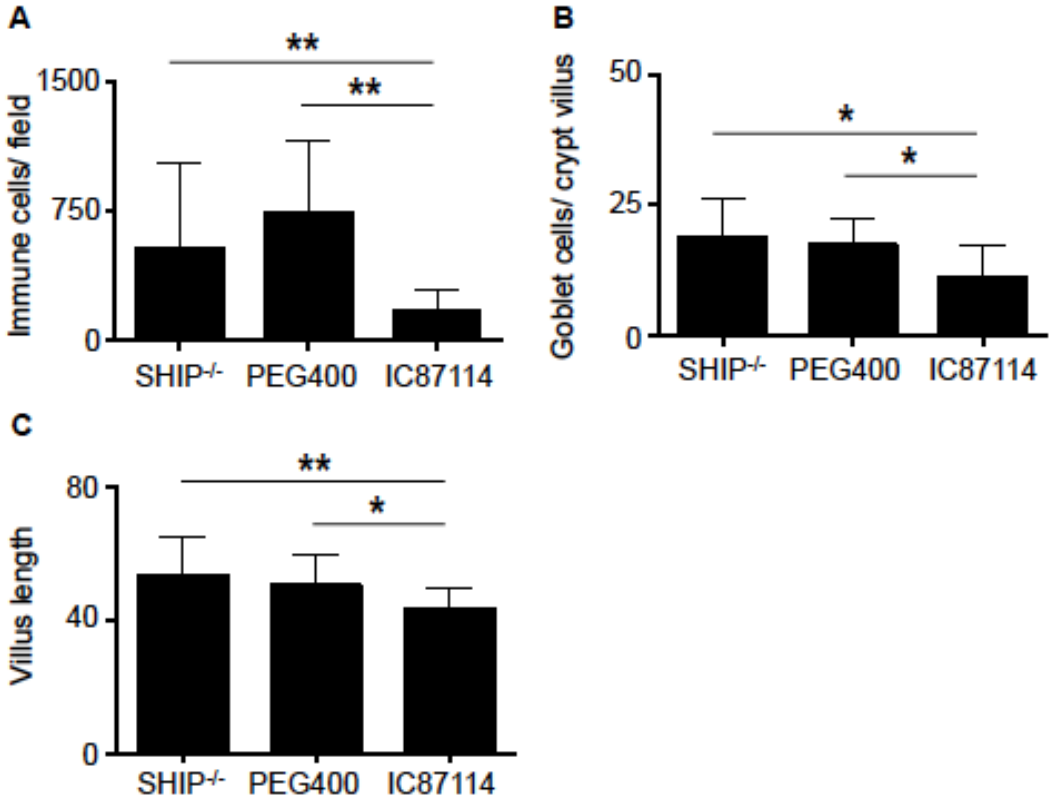

Figure 5.2 PI3Kp110δ inhibition reduces histological measures of intestinal inflammation in SHIP$^{-/-}$ mice. From left to right: 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, and 10 week old SHIP$^{-/-}$ mice treated with IC87114. **(A)** Average number of immune cells per field (number of nuclei found in the circular muscularis externa and submucosa) were counted from H&E-stained cross sections at 20x magnification. **(B)** Goblet cells per crypt-villus. **(C)** Average villus length. Bars represent the means ± SD for 6 mice per group. *P < 0.05, **P < 0.01 using a one-way ANOVA with Bonferroni correction for multiple comparisons.

5.2.2 PI3Kp110δ deficiency or inhibition may increase nitric oxide production in the distal ileum of SHIP$^{-/-}$ mice

Arginase competes with inducible iNOS for their common substrate, L-arginine. Because we found that loss or inhibition of PI3Kp110δ activity reduced argI expression and arginase activity in SHIP$^{-/-}$ mice, we predicted that PI3Kp110δ deficiency would increase pro-inflammatory NO production and increase inflammation. Therefore, to examine the impact of PI3Kp110δ deficiency on NO production, we measured nitrite/nitrate levels in ileal tissue homogenates using the Griess assay. In our genetic model, 4 week old SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice had significantly higher levels of nitrite/nitrate than the healthy SHIP$^{+/+}$PI3Kp110δ$^{+/+}$ mice (Figure 5.3A). However, this phenotype was not reduced by PI3Kp110δ deficiency, which is consistent with the arginase activity not being significantly different between these genotypes at 4 weeks of age (Figure 3.5). At 8 and 12 weeks of age, there were lower levels of nitrite/nitrate in the distal ileum of SHIP$^{-/-}$PI3Kp110δ$^{+/+}$ mice compared to healthy controls and the SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice, though the data were not statistically significantly different (Figure 5.3A). A similar result was present in the SHIP$^{-/-}$ mice treated pharmacologically (Figure 5.3B), as SHIP$^{-/-}$ mice treated with IC87114 had higher levels of nitrite/nitrate in ileal tissue homogenates than vehicle control and disease control mice but differences were not statistically significant. Despite the lack of statistical significance, these data are consistent with a model in which NO production is inversely related to arginase activity, as predicted. Importantly, our data suggest that NO production does not play an important role in reducing intestinal inflammation in SHIP$^{-/-}$ mice with PI3Kp110δ deficiency.

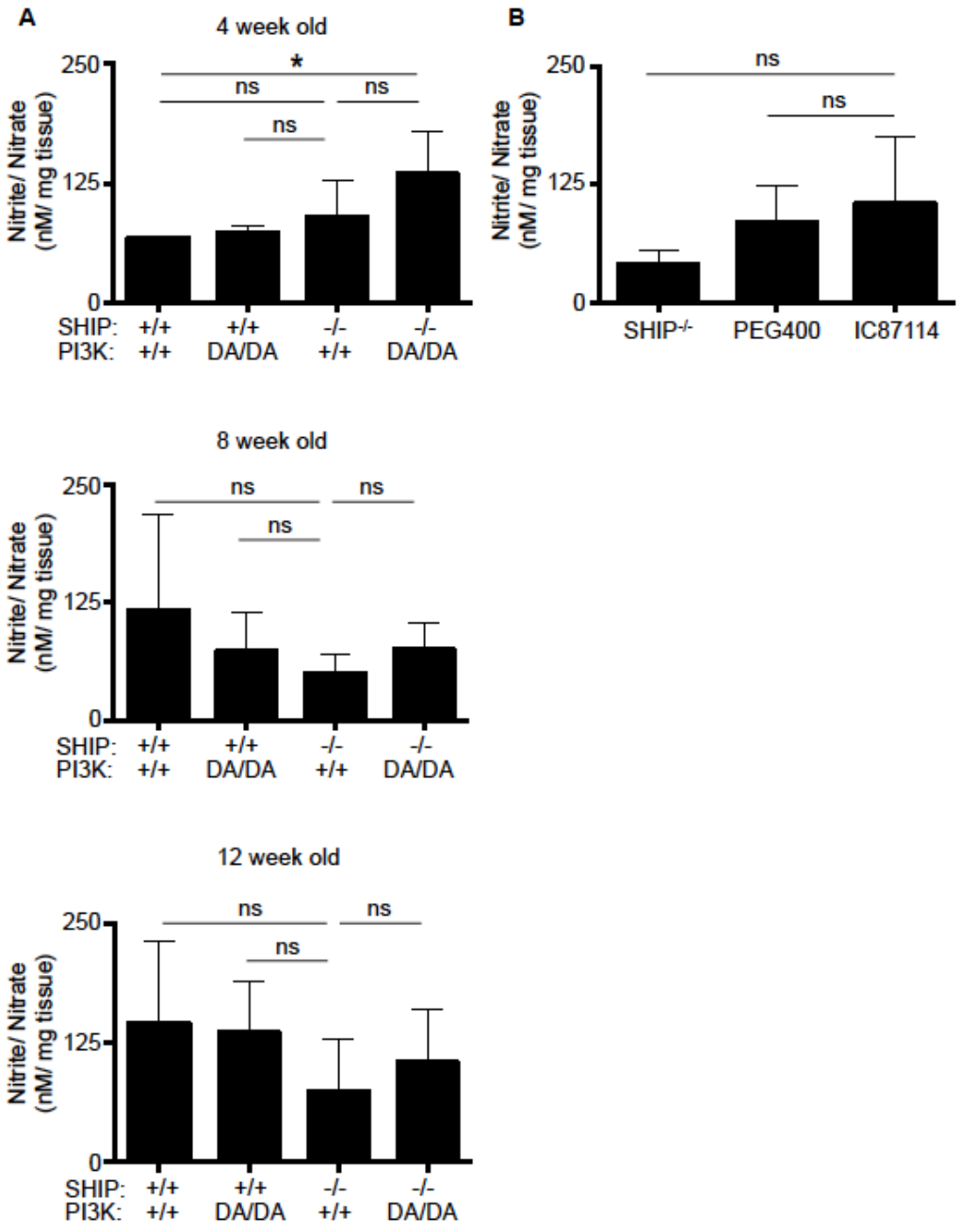

Figure 5.3 PI3Kp110δ deficiency or inhibition has no significant effect on NO in the distal ileum of SHIP$^{-/-}$ mice. NO production was determined indirectly by measuring the nitrite/nitrate in tissue homogenate. **(A)** Nitrite/nitrate levels in ileal tissue homogenates from SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice, at 4, 8 and 12 weeks old. **(B)** Nitrite/nitrate levels in ileal tissue homogenates from 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, and 10 week old SHIP$^{-/-}$ mice treated with IC87114. Bars represent the mean ± SD for 6 mice per group. *P < 0.05, ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

5.2.3 PI3Kp110δ deficiency or inhibition reduces IL-1β levels in the inflamed ileums of SHIP$^{-/-}$ mice

SHIP$^{-/-}$ mice are hyper-responsive to immune stimuli, and we have found that SHIP$^{-/-}$ macrophages produce high levels of IL-1β, which contributes to intestinal inflammation in SHIP$^{-/-}$ mice.[82] In contrast, we have also found that intestinal inflammation in SHIP$^{-/-}$ mice is independent of TNFα.[82] Therefore, we wanted to ask whether the reduced inflammatory phenotype in the PI3Kp110δ deficiency or inhibition experiments were related to IL-1β levels in the distal ileum of the SHIP$^{-/-}$ mice. Indeed, we found that at 4 and 8 weeks old, the SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice had significantly lower levels of IL-1β in their distal ileums compared to that present in their SHIP$^{-/-}$ PI3Kp110δ$^{+/+}$ counterparts (Figure 5.4A). PI3Kp110δ deficiency may also reduce IL-1β levels in the ileums of 12 week old SHIP$^{-/-}$ mice (compare SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ to SHIP$^{-/-}$PI3Kp110δ$^{+/+}$) but data did not reach statistical significance. Similar results were found in the SHIP$^{-/-}$ mice treated with IC87114, as these mice had significantly lower levels of IL-1β in their distal ileums compared to that found in the disease and vehicle control mice (Figure 5.4B).

Interestingly, we found that the number of immune cell infiltrates and goblet cells in the distal ileum corresponded with the levels we see in IL-1β results. When IL-1β levels were high, such as in the SHIP$^{-/-}$ mice, the mice also had significantly higher numbers of immune cell infiltrates and goblet cell hyperplasia, and when IL-1β levels were low or not significantly different, such as between 12 week old SHIP$^{-/-}$ and SHIP$^{-/-}$ PI3Kp110δ$^{DA/DA}$ mice, there were no significant differences in immune cell infiltration or goblet cell numbers. This data is consistent with our previous findings demonstrating that

IL-1β plays a critical role in intestinal inflammation in SHIP$^{-/-}$ mice,[82] and suggests that PI3Kp110δ plays a role in inflammation in SHIP$^{-/-}$ mice upstream of IL-1β production.

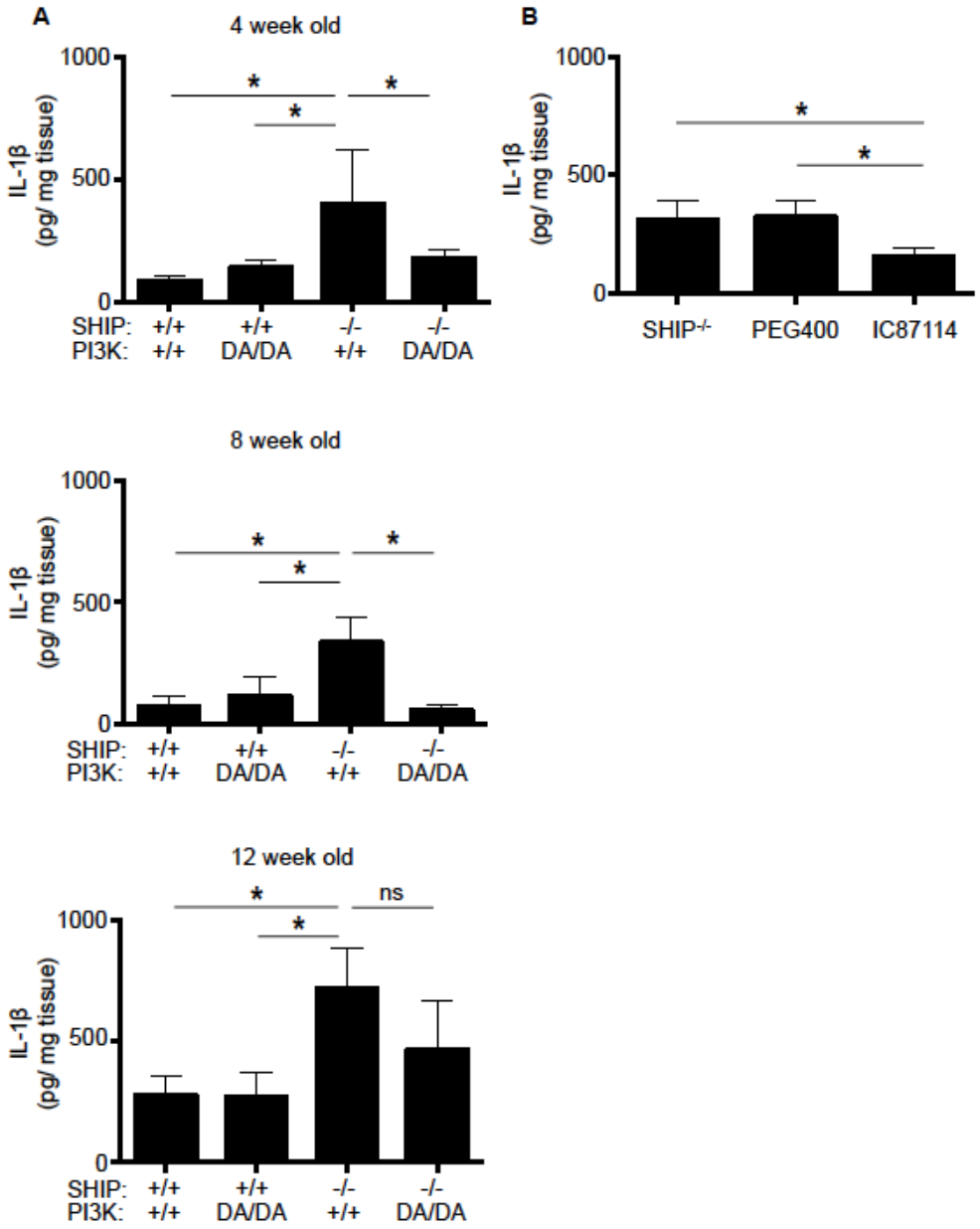

Figure 5.4 PI3Kp110δ deficiency or inhibition reduces IL-1β levels in the distal ileum of SHIP$^{-/-}$ mice. IL-1β was measured in full thickness tissue homogenates from mice. **(A)** From left to right: SHIP$^{+/+}$PI3Kp110δ$^{+/+}$, SHIP$^{+/+}$PI3Kp110δ$^{DA/DA}$, SHIP$^{-/-}$ PI3Kp110δ$^{+/+}$, and SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice, at 4, 8, and 12 weeks of age. **(B)** From left to right: 8 week old SHIP$^{-/-}$ mice, 10 week old SHIP$^{-/-}$ mice treated with PEG400, and 10 week old SHIP$^{-/-}$ mice treated with IC87114. Bars represent the means ± SD for 6 mice. *P < 0.05, ns = not significantly different using a one-way ANOVA with Bonferroni correction for multiple comparisons.

5.3 Discussion

Class I PI3K and SHIP are critical components in regulating proper immune responses. As a result, mice deficient in SHIP become hyper-responsive to immune stimuli. This includes increased IL-1β production in response to innate immune activation. We have reported that CD-like ileal inflammation in $SHIP^{-/-}$ mice is an autoinflammatory disease caused by macrophage-derived IL-1β. Though we have reported a direct role for the p110α subunit of Class I PI3K in auto-amplification of *il1b* mRNA transcription in macrophages; herein, we suggest a new role for the PI3Kp110δ catalytic subunit in affecting/regulating IL-1β levels in the $SHIP^{-/-}$ mice.

We found that in both of our model systems, germline deficiency and pharmacological inhibition of PI3Kp110δ activity, IL-1β levels in the distal ileum were significantly reduced when compared to their respective disease controls. This was accompanied by significant reductions in immune cell infiltration, and goblet cell hyperplasia (Figures 5.1 and 5.2). Indeed, CD-associated intestinal fibrosis in human patients has been attributed to a persistent immune-mediated inflammation in the intestine, as evidence has shown that the distribution of collagen deposition follows the locations of inflammation.[99] Thus, it is not surprising that the reduction in intestinal fibrosis is accompanied by, or begins with, a reduction in intestinal inflammation in the $SHIP^{-/-}$ mouse. Given that we have found that different Class I PI3K catalytic isoforms play direct and distinct roles in each of these processes, our findings do suggest an interesting question: Does PI3Kp110δ contribute to intestinal inflammation directly; or does fibrosis, itself, contribute to inflammation in the $SHIP^{-/-}$ mouse? This will be further discussed in Chapter 6.

Chapter 6: Concluding remarks and future directions

6.1 Concluding remarks

Normally, in response to tissue injury, the healing process includes the deposition of extracellular matrix proteins, the most abundant of which are members of the collagen family. Fibrosis can be considered a pathological consequence of an excessive healing response. It is described as a complicated, multistage, progressive process, which occurs differently in different organs, making fibrotic diseases like CD intestinal fibrosis difficult to study and perplexing to treat.

Many studies on the progression of fibrosis in CD have focused on the involvement of non-immune cell types, including mesenchymal cells (fibroblasts, myofibroblasts, and smooth muscle cells), epithelial cells, nerve cells, and platelets.[104, 105] Currently, pharmacological modulation of ECM-producing cells have been the focus of investigation as potential targets for anti-fibrotic drugs, including: growth factors like TGFβ1, CTGF, PDGF, insulin-like growth factor (IGF)-1 and 2, EGF; chemokines such as MCP-1, MIP-1; and angiogenic factors such as VEGF.[99, 108] However, the lack of identification of specific molecular pathways leading to intestinal fibrosis has resulted in a shortage of well-tolerated, anti-fibrotic drugs available to patients. This is also, in part, because there is a lack of tractable animal models that recapitulate the features of CD fibrotic pathology.

Herein, we have used the $SHIP^{-/-}$ mouse model of CD to address the two aims of this thesis: (1) To determine whether genetic inactivation of PI3Kp110δ activity prevents the development of ileal fibrosis in $SHIP^{-/-}$ mice, and (2) to determine whether pharmacological inhibition of PI3Kp110δ activity can block ileal fibrosis in $SHIP^{-/-}$ mice.

In doing so, we have been able to provide further insight into cell signalling pathways that can lead to the development of CD-like intestinal fibrosis *in vivo*, and build on past research mapping out the specific molecular pathways that may contribute to fibrosis (Figure 6.1). Ultimately, we hope to determine whether PI3Kp110δ may be a novel therapeutic target for treating intestinal fibrosis in people with CD.

There has been extensive evidence linking wounding healing and fibrosis with a T helper type 2 cytokine profile, specifically IL-4 and IL-13.[117, 118] Importantly for my thesis, SHIP$^{-/-}$ mice have been reported to develop a Th2-driven intestinal fibrosis, with high levels IL -4 and IL-13. IL-4 and IL-13 activate mouse ileal macrophages to the alternatively activated or M(IL-4) wound-healing phenotype (Fig. 1.5), including the induction of argI, via a STAT6- and PI3Kp110δ-driven process.[104, 117, 118] The argI enzyme found in M(IL-4) uses L-arginine to catalyze the production of L-ornithine, which leads to the production of L-proline, an essential amino acid in the collagen triple-helix structure; as well as polyamines, spermine and spermidine, which lead to fibroblast growth and production of interstitial fibrillar collagen.[104, 117, 118] Because we found PI3Kp110δ deficiency or inhibition in SHIP$^{-/-}$ mice leads to impaired ability to induce argI, each treatment resulted in reduced arginase levels, reduced number of fibroblasts, and reduced collagen deposition in the distal ileum of SHIP$^{-/-}$ mice.[123, 124] Together, our findings point to a critical role for PI3Kp110δ in the development of CD-like intestinal fibrosis in the SHIP$^{-/-}$ mice.

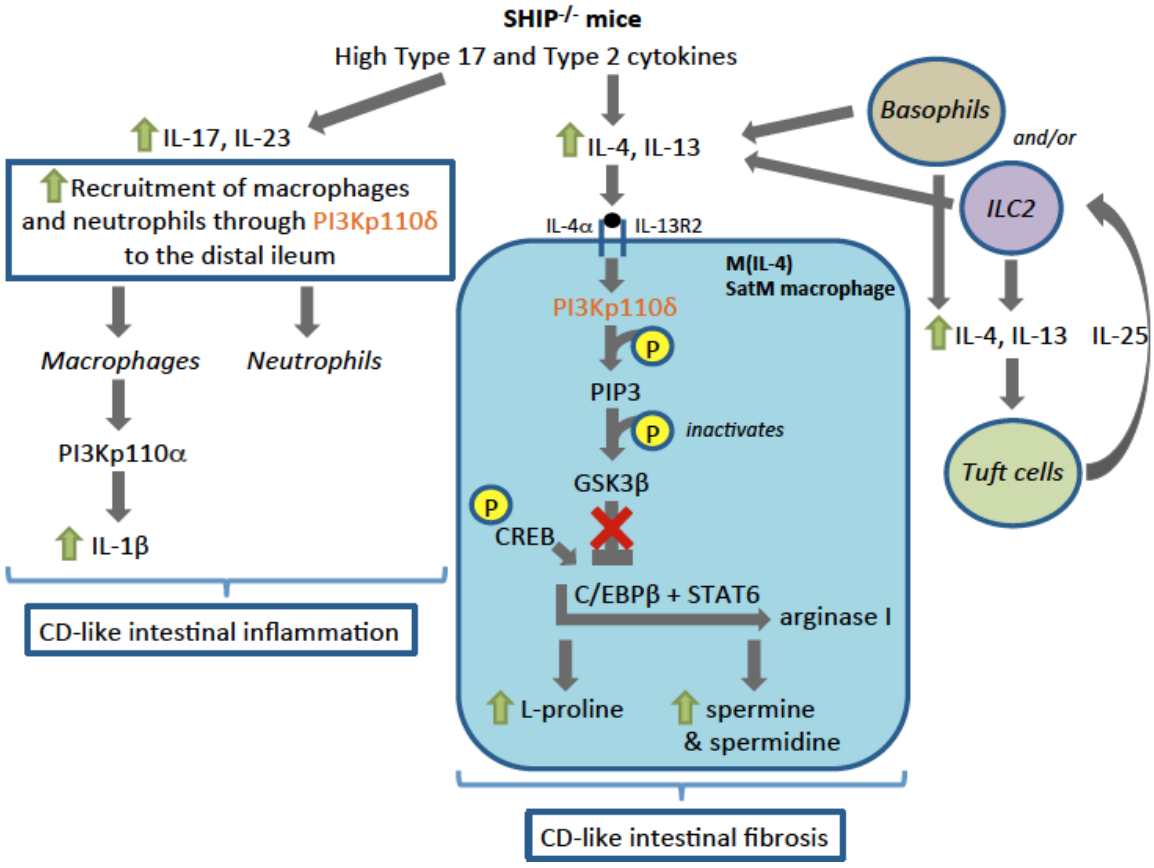

Figure 6.1 SHIP$^{-/-}$ ileal fibrosis is caused by PI3Kp110δ activity
SHIP$^{-/-}$ mice have a type 17 and type 2 cytokine profile. PI3Kp110δ may play a direct role in intestinal inflammation in SHIP$^{-/-}$ mice by facilitating neutrophil and macrophage recruitment to the SHIP$^{-/-}$ mouse ileum in response to high IL-17 and IL-23 levels. Basophils and ILC2s may contribute to high levels of IL-4 and IL-13 in the SHIP$^{-/-}$ mice, with IL-25 from tuft cells amplifying ILC2-produced IL-14 and IL-13. These type 2 cytokines skew macrophages to the M(IL-4) (or SatM phenotype). IL-4 ligation leads to downstream activation of the p110δ isoform of Class I PI3K. Its activity generates the second messenger PIP3. PIP3 phosphorylates and activates downstream Akt, which physically associates, phosphorylates, and inactivates GSK3β.[149] GSK3β inactivation allows the transcription factor C/EBPβ to cooperate with CREB and STAT6 in transcription of the argI gene. The resulting argI enzyme contributes to intestinal fibrosis by contributing to the production of L-proline, abundantly required for collagen biosynthesis; and polyamines, which contribute to cell growth. We have shown that PI3Kp110δ activity correlated with argI expression and ileal fibrosis in SHIP$^{-/-}$ mice using both genetic ablation and pharmacological inhibition of PI3Kp110δ activity.

Key events that lead to the development of intestinal fibrosis are not only the actions of immune cells, but also the exposure of mesenchymal cells (such as fibroblasts) to a variety of growth factors to produce ECM. TGFβ, in particular, has been found in high levels the inflamed gut of CD patients.[104] This growth factor can contribute to intestinal fibrosis, indirectly, by up-regulating PDGF receptors in fibroblasts; increasing fibroblast proliferation, survival, and migration to sites of injury; and/or directly by stimulating fibroblasts to produce interstitial fibrillar collagen (Figure 1.4).[119-121, 150] With this in mind, other cells types, including macrophages, have also been shown to produce PDGF, and a study has reported that alveolar macrophages recovered from idiopathic pulmonary fibrosis (IPF) patients spontaneously produce PDGF.[111] In addition, macrophages can cause an increase in the tissue inhibitors of metalloproteinases (TIMPs) that block ECM degradation.[110] Intestinal fibrosis may be due to increased pro-fibrogenic activity together with decreased ECM degradation resulting in pathology.

We have found that TGFβ1 levels in the distal ileum did not correlate with reduced fibrosis when PI3Kp110δ activity was genetically ablated or pharmacologically inhibited. Despite that, vimentin^{+} mesenchymal cell (e.g. fibroblast) numbers were significantly reduced in the absence of PI3Kp110δ activity. A newly identified subtype of macrophages recently reported in Nature also contributes to fibrosis, which is arginase-dependent, but TGFβ-independent. They described an atypical monocyte, which they termed segregated-nucleus-containing atypical monocyte (SatM), which is regulated by CCAAT/enhancer binding protein B (C/EBPβ).[149] They demonstrated that chimeric mice with C/EBPβ deficiency in hematopoietic cells failed to develop bleomycin-induced lung fibrosis despite developing inflammation.[149] Adoptive transfer of SatM into Cebpb$^{-/-}$ mice

restored bleomycin-indued fibrosis. Most notably, SatM did not produce TGFβ.[149] PI3Kp110δ and SHIP are upstream of C/EBPβ activation. In fact, our laboratory has speculated previously that STAT6-mediated transcription cooperates with the transcription factor C/EBPβ.[135] ArgI promoters have C/EBPβ binding sites, and C/EBPβ has been shown to be required for IL-4-induced argI transcription in macrophages via activation of CREB.[135] PI3Kp110δ activation leads to phosphorylation/inactivation of GSK3β and this positively regulates CREB, which in turn activates C/EBPβ driven transcription.[135] Our data are consistent with these reported results. PI3Kp110δ deficiency or inhibition may reduce fibrosis independent of TGFβ, as mice lacking PI3Kp110δ activity have lower levels of C/EBPβ-induced transcription of argI compared to $SHIP^{-/-}$ disease control mice, and may also have a reduced 'SatM' population, resulting in less intestinal fibrosis.

Our laboratory also reported that macrophages from $SHIP^{-/-}$ mice and from CD patients were found to be high producers of IL-1β, which is not only a key pro-inflammatory cytokine, but also an activator of fibroblasts.[82] In the $SHIP^{-/-}$ mouse, treatment with Anakinra, a synthetic IL-1 receptor antagonist, blocks macrophage-derived IL-1β production and reduces collagen deposition. Herein, we have shown that $SHIP^{-/-}$ mice, which are also deficient in PI3Kp110δ activity have reduced IL-1β production compared to the inflamed $SHIP^{-/-}$ control mice.[82] Thus, it is likely that the CD-associated intestinal fibrosis seen in $SHIP^{-/-}$ mice is a consequence of persistent macrophage-mediated IL-β production causing chronic intestinal inflammation.[99] However, it is possible that the pro-inflammatory IL-1β produced, also contributes directly to fibrosis by activating fibroblasts to produce collagen.

In our genetic model, we found that PI3Kp110δ deficiency in SHIP$^{-/-}$ mice was effective at reducing overt CD-like intestinal fibrosis. Mice at 8 and 12 weeks of age experienced significant reductions in soluble collagen and arginase, which were not evident in 4 week old mice (Figure 2.2, 2.3). This is somewhat expected as these pathological signs only begin at 4 weeks of age and build up in the mice over time. We did find that the majority of 4 week old SHIP$^{-/-}$ mice had some signs of intestinal inflammation, as we found elevated IL-1β levels and villus damage in the distal ileum of SHIP$^{-/-}$ mice compared to healthy control mice. Genetic ablation of PI3Kp110δ activity in 4 week old SHIP$^{-/-}$ mice (SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$), caused a significant reduction in these inflammatory symptoms and in muscle hypertrophy. Indeed, PI3Kp110δ has been shown to play a role in the chemoattractant-mediated migration of peripheral neutrophils and macrophages to sites of injury,[131, 151, 152] and neutrophils and macrophages comprise the vast majority of infiltrating immune cells in the SHIP$^{-/-}$ mouse ileum.[126] In addition, studies on *T. spiralis* infections in mice models have shown that inflammation, primarily in the mucosa and submucosa, is a powerful driver of muscle hypertrophy in the mouse small intestine.[153, 154] Therefore, it is plausible that PI3Kp110δ deficiency in younger mice prevents intestinal inflammation, and as a consequence, prevents the onset of intestinal fibrosis. Alternatively, PI3Kp110δ, like IL-1β may play a dual role contributing to both intestinal inflammation and fibrotic pathology in SHIP$^{-/-}$ mice.

Consistent with this idea, CD intestinal fibrosis has often been reported to follow the distribution and location of inflammation in human patients.[96] Furthermore, we saw significantly lower levels of Ym1 staining in SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ compared to SHIP$^{-/-}$ PI3Kp110δ$^{+/+}$ at 4 weeks old, with Ym1 being a novel marker for inflammation, and

M(IL-4) macrophages. Moreover, we saw reduced redness of the distal ilea in our gross pathological assessment of 4 week old SHIP$^{-/-}$PI3Kp110δ$^{DA/DA}$ mice compared to their SHIP$^{-/-}$ mice counterparts. Collectively, these findings suggest a role for PI3Kp110δ in regulating IL-1β in some manner, and will require further investigation.

Taken together, data in this thesis suggests that the p110δ catalytic isoform of Class I PI3K is a key driver of fibrotic pathology in SHIP$^{-/-}$ mice. As predicted, PI3Kp110δ activity correlated with argI expression in experiments using both genetic ablation and pharmacological inhibition of PI3Kp110δ activity. Loss of function of PI3Kp110δ may reduce argI expression by preventing phosphorylation and inactivation of GSK3β, which limits CREB and C/EBPβ-driven transcription of argI. ArgI may contribute to intestinal fibrosis by contributing to the production of L-proline, abundantly required for collagen biosynthesis, and polyamines, which contribute to cell growth. Figure 6.1 summarizes our model of how blocking PI3Kp110δ activity can be used to reduce CD intestinal fibrosis in SHIP$^{-/-}$ mice. We were surprised to find that deficiency in PI3Kp110δ also reduced intestinal inflammation in SHIP$^{-/-}$ mice. Though PI3Kp110δ is not likely to contribute to macrophage IL-1β transcription directly, which we have shown is dependent on the p110α catalytic isoform of Class I PI3K; PI3Kp110δ may play a direct role in intestinal inflammation in SHIP$^{-/-}$ mice by facilitating neutrophil and macrophage recruitment to the SHIP$^{-/-}$ mouse ileum.

Ultimately, our data builds on previous research done in our laboratory and supports a model in which macrophage-derived IL-1β contributes to ileal inflammation and PI3Kp110δ-driven argI expression contributes to intestinal fibrosis in SHIP$^{-/-}$ mice. The processes of inflammation and fibrosis are intimately linked; it is generally accepted

that fibrosis occurs downstream of chronic inflammation as a pathological consequence of dysregulated would healing. Furthermore, our data, and that of others, suggest that key molecular signaling molecules, PI3Kp110δ and IL-1β, play distinct roles and contribute to both of these processes. It is interesting to speculate that fibrosis, itself, may also contribute to, or exacerbate, intestinal inflammation in $SHIP^{-/-}$ mice, perhaps by providing a "danger signal" required for IL-1β production. Our data do not preclude the intriguing possibility that fibrosis may also contribute to chronic inflammation.

6.2 Future directions

PI3Kp110δ is activated downstream of IL-4 receptor engagement. Thus, the next question we hope to address is: Why were IL-4 levels significantly reduced in $SHIP^{-/-}$ mice that were deficient in PI3Kp110δ (Figure 3.8A, 4.5A)? $SHIP^{-/-}$ mice have been shown to have hyperactive, IL-4-secreting basophils, which drive STAT6- and PI3Kp110δ-dependent transcription, including transcription of argI, previously desribed.[136] The p110δ catalytic isoform of Class I PI3K is mainly expressed in hematopoietic cells.[136] This suggests that PI3Kp110δ may play a role in regulating IL-4 production by hematopoetic cells, like basophils.

ILC2s are also hematopoeitc cells that produce Th2 cytokines, including IL-4 and IL-13.[155-157] During intestinal infection with parasites, ILC2-derived IL-4 activates intestinal tuft cells to produce IL-25, which further amplifies type 2 cytokine secretion by ILC2s.[156, 157] Tuft cells are specialized epithelial cells that are present in the hollow organs of the respiratory and gastrointestinal tracts.[157] We speculate that PI3Kp110δ deficiency in intestinal tuft cells leads to decreased IL-25 production and reduced activation and IL-4 secretion by ILC2s. To investigate this, our laboratory will use the mice that we created for this thesis; $SHIP^{+/+}PI3Kp110\delta^{+/+}$, $SHIP^{+/+}PI3Kp110\delta^{DA/DA}$, $SHIP^{-/-}PI3Kp110\delta^{+/+}$, $SHIP^{-/-}PI3Kp110\delta^{DA/DA}$. Parasitic infections are key triggers for ILC2s to produce the initiator cytokine IL-4,[156] which is a PI3Kp110δ-driven process (refer to Figure 6.1). In collaboration with Dr. Lisa Osborne, an Assistant Professor at the University of British Columbia; Jean Philippe Sauve, a Doctoral student in our laboratory, will infect mice (or assess uninfected controls) with the helminth, *T. spiralis*. Tuft cell and ILC2 numbers will be quantified by IHC/IF and flow cytometry and IL-25

and IL-4 production will be measured in full thickness tissue homogenates from uninfected and infected mice. These studies will provide insight into the role of basophils, ILCs, and tuft cells, in triggering intestinal inflammation in SHIP$^{-/-}$ mice, and whether cross-talk between these cell types is integral in the SHIP$^{-/-}$ mouse intestinal inflammation and fibrosis.

Type 2 cytokines are integral in our model of CD intestinal inflammation and fibrosis. We have, so far, suggested that SHIP$^{-/-}$ mice develop fibrotic-like histopathology in response to these cytokines. However, previous studies from our laboratory have also shown that SHIP$^{-/-}$ mice have elevated Th17 cytokines in their distal ilea, which may be caused by elevated IL-1β levels and the loss of mucosal barrier function.[126] Th17 cytokines, IL-17 and IL-23, play chemoattractant roles for neutrophils to infiltrate the distal ileum.[152] SHIP$^{-/-}$ ileal inflammation was characterized by abundant infiltrating Gr-1-positive immune cells.[126] In fact, data have shown a mixed population of macrophages and neutrophils in the submucosa and muscle layers of the SHIP$^{-/-}$ mouse ilea, that was reduced by PI3Kp110δ deficiency or inhibition. Furthermore, a previous experiment from our laboratory demonstrated that BEC (an arginase inhibitor) reduced arginase activity and expression in SHIP$^{-/-}$ mouse ilea macrophages. Importantly, blocking argI reduced collagen deposition and muscle hyperplasia, but had no impact on the number of immune cell infiltrates or aggregates in the ilea.[126] Together, this suggests that PI3Kp110δ may play an independent role in neutrophil recruitment to the inflamed SHIP$^{-/-}$ mouse intestine and we speculate that this may be linked to elevated Th17 cytokines. In future studies, we may investigate the role of IL-17 and IL-23 in CD-like intestinal inflammation, and specifically immune cell recruitment to the SHIP$^{-/-}$ ileum. This may be achieved by

crossing SHIP$^{-/-}$ mice with mice that are deficient in IL-17, IL-23, or their receptors, and by treating SHIP$^{-/-}$ mice with blocking antibodies to IL-17 or IL-23; similar to the experiments that I have performed in Chapters 3 and 4 of this thesis.

References

1. Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev 2002;15:79-94.
2. Rosenstiel P, Sina C, Franke A, et al. Towards a molecular risk map--recent advances on the etiology of inflammatory bowel disease. Semin Immunol 2009;21:334-45.
3. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 2007;369:1641-57.
4. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427-34.
5. Abraham C, Cho J. Interleukin-23/Th17 pathways and inflammatory bowel disease. Inflamm Bowel Dis 2009;15:1090-100.
6. Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 2012;142:46-54 e42; quiz e30.
7. Cosnes J, Gower-Rousseau C, Seksik P, et al. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology 2011;140:1785-94.
8. Bernstein CN, Wajda A, Svenson LW, et al. The epidemiology of inflammatory bowel disease in Canada: a population-based study. Am J Gastroenterol 2006;101:1559-68.

9. Fakhoury M, Negrulj R, Mooranian A, et al. Inflammatory bowel disease: clinical aspects and treatments. J Inflamm Res 2014;7:113-20.

10. Rocchi A, Benchimol EI, Bernstein CN, et al. Inflammatory bowel disease: a Canadian burden of illness review. Can J Gastroenterol 2012;26:811-7.

11. Benchimol EI, Guttmann A, Griffiths AM, et al. Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data. Gut 2009;58:1490-7.

12. Bernklev T, Jahnsen J, Aadland E, et al. Health-related quality of life in patients with inflammatory bowel disease five years after the initial diagnosis. Scand J Gastroenterol 2004;39:365-73.

13. Graff LA, Vincent N, Walker JR, et al. A population-based study of fatigue and sleep difficulties in inflammatory bowel disease. Inflamm Bowel Dis 2011;17:1882-9.

14. Kanof ME, Lake AM, Bayless TM. Decreased height velocity in children and adolescents before the diagnosis of Crohn's disease. Gastroenterology 1988;95:1523-7.

15. Bernstein CN, Fried M, Krabshuis JH, et al. World Gastroenterology Organization Practice Guidelines for the diagnosis and management of IBD in 2010. Inflamm Bowel Dis 2010;16:112-24.

16. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119-24.

17. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 2011;474:298-306.

18. Solberg IC, Vatn MH, Hoie O, et al. Clinical course in Crohn's disease: results of a Norwegian population-based ten-year follow-up study. Clin Gastroenterol Hepatol 2007;5:1430-8.

19. Halme L, Paavola-Sakki P, Turunen U, et al. Family and twin studies in inflammatory bowel disease. World J Gastroenterol 2006;12:3668-72.

20. Halfvarson J, Bodin L, Tysk C, et al. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology 2003;124:1767-73.

21. Orholm M, Binder V, Sorensen TI, et al. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand J Gastroenterol 2000;35:1075-81.

22. Thompson NP, Driscoll R, Pounder RE, et al. Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ 1996;312:95-6.

23. Yang H, McElree C, Roth MP, et al. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. Gut 1993;34:517-24.

24. Bayless TM, Tokayer AZ, Polito JM, 2nd, et al. Crohn's disease: concordance for site and clinical type in affected family members--potential hereditary influences. Gastroenterology 1996;111:573-9.

25. Carbonnel F, Macaigne G, Beaugerie L, et al. Crohn's disease severity in familial and sporadic cases. Gut 1999;44:91-5.

26. Orholm M, Munkholm P, Langholz E, et al. Familial occurrence of inflammatory bowel disease. N Engl J Med 1991;324:84-8.

27. Peeters M, Nevens H, Baert F, et al. Familial aggregation in Crohn's disease: increased age-adjusted risk and concordance in clinical characteristics. Gastroenterology 1996;111:597-603.

28. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol 2015;12:205-17.

29. Probert CS, Jayanthi V, Hughes AO, et al. Prevalence and family risk of ulcerative colitis and Crohn's disease: an epidemiological study among Europeans and south Asians in Leicestershire. Gut 1993;34:1547-51.

30. Rioux JD, Xavier RJ, Taylor KD, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 2007;39:596-604.

31. Parkes M, Barrett JC, Prescott NJ, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet 2007;39:830-2.

32. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008;40:955-62.

33. Libioulle C, Louis E, Hansoul S, et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. PLoS Genet 2007;3:e58.

34. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-3.

35. Wang K, Zhang H, Kugathasan S, et al. Diverse genome-wide association studies associate the IL12/IL23 pathway with Crohn Disease. Am J Hum Genet 2009;84:399-405.

36. Imielinski M, Baldassano RN, Griffiths A, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. Nat Genet 2009;41:1335-40.

37. Franke A, Balschun T, Karlsen TH, et al. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. Nat Genet 2008;40:713-5.

38. Ramjeet M, Hussey S, Philpott DJ, et al. 'Nodophagy': New crossroads in Crohn disease pathogenesis. Gut Microbes 2010;1:307-315.

39. Fritz T, Niederreiter L, Adolph T, et al. Crohn's disease: NOD2, autophagy and ER stress converge. Gut 2011;60:1580-8.

40. Michallet AS, Mondiere P, Taillardet M, et al. Compromising the unfolded protein response induces autophagy-mediated cell death in multiple myeloma cells. PLoS One 2011;6:e25820.

41. Loftus EV, Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology 2004;126:1504-17.

42. Rubin DT, Hanauer SB. Smoking and inflammatory bowel disease. Eur J Gastroenterol Hepatol 2000;12:855-62.

43. Mahid SS, Minor KS, Soto RE, et al. Smoking and inflammatory bowel disease: a meta-analysis. Mayo Clin Proc 2006;81:1462-71.

44. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002;2:372-7.

45. Virta L, Auvinen A, Helenius H, et al. Association of repeated exposure to antibiotics with the development of pediatric Crohn's disease--a nationwide, register-based finnish case-control study. Am J Epidemiol 2012;175:775-84.

46. Godet PG, May GR, Sutherland LR. Meta-analysis of the role of oral contraceptive agents in inflammatory bowel disease. Gut 1995;37:668-73.

47. Mouli VP, Ananthakrishnan AN. Review article: vitamin D and inflammatory bowel diseases. Aliment Pharmacol Ther 2014;39:125-36.

48. Levenstein S, Prantera C, Varvo V, et al. Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. Am J Gastroenterol 2000;95:1213-20.

49. Collins SM. Stress and the Gastrointestinal Tract IV. Modulation of intestinal inflammation by stress: basic mechanisms and clinical relevance. Am J Physiol Gastrointest Liver Physiol 2001;280:G315-8.

50. Fortes C, Farchi S, Forastiere F, et al. Depressive symptoms lead to impaired cellular immune response. Psychother Psychosom 2003;72:253-60.

51. Goebel MU, Mills PJ, Irwin MR, et al. Interleukin-6 and tumor necrosis factor-alpha production after acute psychological stress, exercise, and infused isoproterenol: differential effects and pathways. Psychosom Med 2000;62:591-8.

52. Bamias G, Corridoni D, Pizarro TT, et al. New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation. Cytokine 2012;59:451-9.

53. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559-63.

54. Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe 2014;15:382-92.

55. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology 2014;146:1489-99.

56. Darfeuille-Michaud A, Boudeau J, Bulois P, et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. Gastroenterology 2004;127:412-21.

57. Cadwell K, Patel KK, Maloney NS, et al. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 2010;141:1135-45.

58. Frank DN, Robertson CE, Hamm CM, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. Inflamm Bowel Dis 2011;17:179-84.

59. Podolsky DK. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. Am J Physiol 1999;277:G495-9.

60. Blikslager AT, Moeser AJ, Gookin JL, et al. Restoration of barrier function in injured intestinal mucosa. Physiol Rev 2007;87:545-64.

61. Wallace KL, Zheng LB, Kanazawa Y, et al. Immunopathology of inflammatory bowel disease. World J Gastroenterol 2014;20:6-21.

62. Salim SY, Soderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. Inflamm Bowel Dis 2011;17:362-81.

63. Buisine MP, Desreumaux P, Debailleul V, et al. Abnormalities in mucin gene expression in Crohn's disease. Inflamm Bowel Dis 1999;5:24-32.

64. Madsen KL, Malfair D, Gray D, et al. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. Inflamm Bowel Dis 1999;5:262-70.

65. Zaki MH, Lamkanfi M, Kanneganti TD. The Nlrp3 inflammasome: contributions to intestinal homeostasis. Trends Immunol 2011;32:171-9.

66. Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol 2006;27:352-7.

67. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. FEBS J 2005;272:6179-217.

68. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol 2013;13:397-411.

69. Lavelle EC, Murphy C, O'Neill LA, et al. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. Mucosal Immunol 2010;3:17-28.

70. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.

71. Elson CO, Cong Y, McCracken VJ, et al. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. Immunol Rev 2005;206:260-76.

72. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000;68:7010-7.

73. Hausmann M, Kiessling S, Mestermann S, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology 2002;122:1987-2000.

74. Prescott NJ, Fisher SA, Franke A, et al. A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5. Gastroenterology 2007;132:1665-71.

75. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.

76. Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. Immunol Rev 2006;212:256-71.

77. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204:1849-61.

78. Hue S, Ahern P, Buonocore S, et al. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J Exp Med 2006;203:2473-83.

79. Noguchi M, Hiwatashi N, Liu Z, et al. Enhanced interferon-gamma production and B7-2 expression in isolated intestinal mononuclear cells from patients with Crohn's disease. J Gastroenterol 1995;30 Suppl 8:52-5.

80. Fuss IJ, Neurath M, Boirivant M, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996;157:1261-70.

81. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest 2007;117:514-21.

82. Ngoh EN, Weisser SB, Lo Y, et al. Activity of SHIP, Which Prevents Expression of Interleukin 1beta, Is Reduced in Patients With Crohn's Disease. Gastroenterology 2016;150:465-76.

83. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterology 2011;140:1756-67.

84. Monteleone G, Biancone L, Marasco R, et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997;112:1169-78.

85. Fujino S, Andoh A, Bamba S, et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003;52:65-70.

86. McGovern D, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. Gut 2007;56:1333-6.

87. Dubinsky MC, Wang D, Picornell Y, et al. IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. Inflamm Bowel Dis 2007;13:511-5.

88. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. Nat Med 2004;10:801-5.

89. Valencia X, Stephens G, Goldbach-Mansky R, et al. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253-61.

90. Fantini MC, Becker C, Tubbe I, et al. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut 2006;55:671-80.

91. Chamouard P, Monneaux F, Richert Z, et al. Diminution of Circulating CD4+CD25 high T cells in naive Crohn's disease. Dig Dis Sci 2009;54:2084-93.

92. Maul J, Loddenkemper C, Mundt P, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005;128:1868-78.

93. Saruta M, Yu QT, Fleshner PR, et al. Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. Clin Immunol 2007;125:281-90.

94. Day AS, Burgess L. Exclusive enteral nutrition and induction of remission of active Crohn's disease in children. Expert Rev Clin Immunol 2013;9:375-83; quiz 384.

95. Hazlewood GS, Rezaie A, Borman M, et al. Comparative effectiveness of immunosuppressants and biologics for inducing and maintaining remission in Crohn's disease: a network meta-analysis. Gastroenterology 2015;148:344-54 e5; quiz e14-5.

96. Lichtenstein GR. Comprehensive review: antitumor necrosis factor agents in inflammatory bowel disease and factors implicated in treatment response. Therap Adv Gastroenterol 2013;6:269-93.

97. Colombel JF, Sands BE, Rutgeerts P, et al. The safety of vedolizumab for ulcerative colitis and Crohn's disease. Gut 2017;66:839-851.

98. Chang CW, Wong JM, Tung CC, et al. Intestinal stricture in Crohn's disease. Intest Res 2015;13:19-26.

99. Speca S, Giusti I, Rieder F, et al. Cellular and molecular mechanisms of intestinal fibrosis. World J Gastroenterol 2012;18:3635-61.

100. Schultz GS, Wysocki A. Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen 2009;17:153-62.

101. Van Assche G, Geboes K, Rutgeerts P. Medical therapy for Crohn's disease strictures. Inflamm Bowel Dis 2004;10:55-60.

102. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest 2007;117:524-9.

103. McCartney-Francis NL, Chan J, Wahl SM. Inflammatory joint disease: clinical, histological, and molecular parameters of acute and chronic inflammation and tissue destruction. Methods Mol Biol 2003;225:147-59.

104. Barron L, Wynn TA. Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. Am J Physiol Gastrointest Liver Physiol 2011;300:G723-8.

105. Danese S. Nonimmune cells in inflammatory bowel disease: from victim to villain. Trends Immunol 2008;29:555-64.

106. Chidlow JH, Jr., Shukla D, Grisham MB, et al. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. Am J Physiol Gastrointest Liver Physiol 2007;293:G5-G18.

107. Danese S. Inflammation and the mucosal microcirculation in inflammatory bowel disease: the ebb and flow. Curr Opin Gastroenterol 2007;23:384-9.

108. Holt AP, Salmon M, Buckley CD, et al. Immune interactions in hepatic fibrosis. Clin Liver Dis 2008;12:861-82, x.

109. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert Rev Mol Med 2011;13:e23.

110. Karlmark KR, Weiskirchen R, Zimmermann HW, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology 2009;50:261-74.

111. Martinet Y, Rom WN, Grotendorst GR, et al. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. N Engl J Med 1987;317:202-9.

112. Rosenbloom J, Castro SV, Jimenez SA. Narrative review: fibrotic diseases: cellular and molecular mechanisms and novel therapies. Ann Intern Med 2010;152:159-66.

113. Gasse P, Mary C, Guenon I, et al. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. J Clin Invest 2007;117:3786-99.

114. Weng HL, Liu Y, Chen JL, et al. The etiology of liver damage imparts cytokines transforming growth factor beta1 or interleukin-13 as driving forces in fibrogenesis. Hepatology 2009;50:230-43.

115. Burke JP, Mulsow JJ, O'Keane C, et al. Fibrogenesis in Crohn's disease. Am J Gastroenterol 2007;102:439-48.

116. Henderson NC, Iredale JP. Liver fibrosis: cellular mechanisms of progression and resolution. Clin Sci (Lond) 2007;112:265-80.

117. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008;214:199-210.

118. Sandler NG, Mentink-Kane MM, Cheever AW, et al. Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for Th1 and Th2 responses in tissue repair. J Immunol 2003;171:3655-67.

119. Rieder F, Fiocchi C. Intestinal fibrosis in inflammatory bowel disease - Current knowledge and future perspectives. J Crohns Colitis 2008;2:279-90.

120. Pucilowska JB, Williams KL, Lund PK. Fibrogenesis. IV. Fibrosis and inflammatory bowel disease: cellular mediators and animal models. Am J Physiol Gastrointest Liver Physiol 2000;279:G653-9.

121. Lawrance IC, Maxwell L, Doe W. Altered response of intestinal mucosal fibroblasts to profibrogenic cytokines in inflammatory bowel disease. Inflamm Bowel Dis 2001;7:226-36.

122. Wermuth PJ, Jimenez SA. The significance of macrophage polarization subtypes for animal models of tissue fibrosis and human fibrotic diseases. Clin Transl Med 2015;4:2.

123. Morris SM, Jr. Arginine metabolism: boundaries of our knowledge. J Nutr 2007;137:1602S-1609S.

124. Ding J, Tredget EE. The Role of Chemokines in Fibrotic Wound Healing. Adv Wound Care (New Rochelle) 2015;4:673-686.

125. Kerr WG, Park MY, Maubert M, et al. SHIP deficiency causes Crohn's disease-like ileitis. Gut 2011;60:177-88.

126. McLarren KW, Cole AE, Weisser SB, et al. SHIP-deficient mice develop spontaneous intestinal inflammation and arginase-dependent fibrosis. Am J Pathol 2011;179:180-8.

127. Sales-Campos H, Basso PJ, Alves VB, et al. Classical and recent advances in the treatment of inflammatory bowel diseases. Braz J Med Biol Res 2015;48:96-107.

128. Lewis RT, Maron DJ. Efficacy and complications of surgery for Crohn's disease. Gastroenterol Hepatol (N Y) 2010;6:587-96.

129. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002;296:1655-7.

130. Vanhaesebroeck B, Leevers SJ, Ahmadi K, et al. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 2001;70:535-602.

131. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, et al. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol 2010;11:329-41.

132. Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. Cancer Treat Res 2003;115:145-67.

133. Kok K, Geering B, Vanhaesebroeck B. Regulation of phosphoinositide 3-kinase expression in health and disease. Trends Biochem Sci 2009;34:115-27.

134. Qin S, Chock PB. Implication of phosphatidylinositol 3-kinase membrane recruitment in hydrogen peroxide-induced activation of PI3K and Akt. Biochemistry 2003;42:2995-3003.

135. Weisser SB, Kozicky LK, Brugger HK, et al. Arginase activity in alternatively activated macrophages protects PI3Kp110delta deficient mice from dextran sodium sulfate induced intestinal inflammation. Eur J Immunol 2014;44:3353-67.

136. Weisser SB, McLarren KW, Voglmaier N, et al. Alternative activation of macrophages by IL-4 requires SHIP degradation. Eur J Immunol 2011;41:1742-53.

137. Kerr WG. A role for SHIP in stem cell biology and transplantation. Curr Stem Cell Res Ther 2008;3:99-106.

138. Franke TF, Kaplan DR, Cantley LC, et al. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 1997;275:665-8.

139. Peng Q, Malhotra S, Torchia JA, et al. TREM2- and DAP12-dependent activation of PI3K requires DAP10 and is inhibited by SHIP1. Sci Signal 2010;3:ra38.

140. Kerr WG. Inhibitor and activator: dual functions for SHIP in immunity and cancer. Ann N Y Acad Sci 2011;1217:1-17.

141. Ma K, Cheung SM, Marshall AJ, et al. PI(3,4,5)P3 and PI(3,4)P2 levels correlate with PKB/akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P2 levels determine PKB activity. Cell Signal 2008;20:684-94.

142. Helgason CD, Damen JE, Rosten P, et al. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. Genes Dev 1998;12:1610-20.

143. Ghansah T, Paraiso KH, Highfill S, et al. Expansion of myeloid suppressor cells in SHIP-deficient mice represses allogeneic T cell responses. J Immunol 2004;173:7324-30.

144. Nakamura K, Kouro T, Kincade PW, et al. Src homology 2-containing 5-inositol phosphatase (SHIP) suppresses an early stage of lymphoid cell development through elevated interleukin-6 production by myeloid cells in bone marrow. J Exp Med 2004;199:243-54.

145. Rauh MJ, Ho V, Pereira C, et al. SHIP represses the generation of alternatively activated macrophages. Immunity 2005;23:361-74.

146. Sly LM, Hamilton MJ, Kuroda E, et al. SHIP prevents lipopolysaccharide from triggering an antiviral response in mice. Blood 2009;113:2945-54.

147. Sly LM, Rauh MJ, Kalesnikoff J, et al. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. Immunity 2004;21:227-39.

148. Weisser SB, Brugger HK, Voglmaier NS, et al. SHIP-deficient, alternatively activated macrophages protect mice during DSS-induced colitis. J Leukoc Biol 2011;90:483-92.

149. Satoh T, Nakagawa K, Sugihara F, et al. Identification of an atypical monocyte and committed progenitor involved in fibrosis. Nature 2017;541:96-101.

150. Simmons JG, Pucilowska JB, Keku TO, et al. IGF-I and TGF-beta1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. Am J Physiol Gastrointest Liver Physiol 2002;283:G809-18.

151. Yang Q, Modi P, Newcomb T, et al. Idelalisib: First-in-Class PI3K Delta Inhibitor for the Treatment of Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia, and Follicular Lymphoma. Clin Cancer Res 2015;21:1537-42.

152. Ali K, Soond DR, Pineiro R, et al. Inactivation of PI(3)K p110delta breaks regulatory T-cell-mediated immune tolerance to cancer. Nature 2014;510:407-11.

153. Tanovic A, Fernandez E, Jimenez M. Alterations in intestinal contractility during inflammation are caused by both smooth muscle damage and specific receptor-mediated mechanisms. Croat Med J 2006;47:318-26.

154. Blennerhassett MG, Vignjevic P, Vermillion DL, et al. Inflammation causes hyperplasia and hypertrophy in smooth muscle of rat small intestine. Am J Physiol 1992;262:G1041-6.

155. Pelly VS, Kannan Y, Coomes SM, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. Mucosal Immunol 2016;9:1407-1417.

156. Zhu J. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine 2015;75:14-24.

157. Gronke K, Diefenbach A. Tuft cell-derived IL-25 activates and maintains ILC2. Immunol Cell Biol 2016;94:221-3.