SHIP deficiency is associated with the Crohn's disease susceptibility variant in ATG16L1 and leads to increased interleukin-1beta transcription and intestinal autoinflammation

by

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Abstract

Crohn`s disease (CD) is a polygenic immune-mediated disease of the gastrointestinal tract characterized by chronic inflammation. The SH2-domain-containing inositol polyphosphate 5′phosphatase (SHIP) is a hematopoietic-specific negative regulator of inflammatory cytokine production and plays an important role in regulating immune homeostasis. Using the SHIP deficient mouse model of intestinal inflammation, we found that IL-1β is increased in SHIP-/- mouse macrophages due to increased class I PI3K p110α activity. Macrophage depletion or treatment with an IL-1 receptor antagonist reduced development of intestinal inflammation in SHIP-/- mice.

To interrogate if SHIP is dysregulated in people with ileal CD, we demonstrate that subjects with ileal CD have reduced SHIP mRNA expression and enzymatic activity at sites of inflammation and in PBMCs, compared to control subjects. A single nucleotide polymorphism (SNP) in the gene encoding ATG16L1 (T300A) causes an autophagy defect and is associated with increased IL-1β production and susceptibility to CD. In all tissues from our patient cohort and in PBMCs from a second healthy control cohort, subjects, who were homozygous for the CD-associated ATG16L1 T300A encoding gene variant, had reduced SHIP mRNA expression and enzymatic activity, which correlated with increased IL-1β production. In addition, starvation-induced autophagy increased SHIP protein levels, which were reduced in the presence of the *ATG16L1* CD-associated risk allele.

Examining the effects of additional autophagy and CD-related gene variants, which may affect SHIP mRNA expression and enzymatic activity, on IL-1β production in PBMCs from a cohort of healthy control subjects, we found that the *NOD2* rs2066844 and the *XBP-1*

rs35873774 gene variants were associated with increased IL-1β production in response to specific PAMPs.

Collectively, these data identify SHIP up-regulation as a novel mechanism by which autophagy regulates IL-1β production and intestinal autoinflammation. Our findings also identify a subgroup of CD patients that could be amenable to treatment with therapy that targets IL-1β.

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. Collection of human samples used in the studies was approved by the University of British Columbia Research Ethics Boards, protocol numbers H09-01826, H04-0534, and H14-00622.

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 1.6 was reproduced with permission of Nature Publishing Group: Murthy A et al. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. Nature 2014; 506(7489).

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cell infiltration for the human samples. Dr. Laura M. Sly measured SHIP activity in the human samples.

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List of Abbreviations

ACTB	Gene encoding β-actin
AIEC	Adherent and invasive *Escherichia coli*
AIM2	Absent in melanoma 2
Akt	A serine/threonine protein kinase also known as protein kinase B (PKB)
ANOVA	Analysis of variance
AOSD	Adult onset Still's disease
Arg	Arginine
Arg I	Arginase I
AS605240	A class I PI3K p110γ selective inhibitor
ASC	Apoptosis-associated speck-like protein containing a CARD
ATG16L1	Autophagy related 16-like 1
ATG5	Autophagy protein 5
ATP	Adenosine triphosphate
BMMs	Bone marrow macrophages
Ca^{2+}	Calcium
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase recruitment domain
CCL2	Chemokine (C-C motif) ligand 2
CD	Crohn's disease
CD4	Cluster of differentiation 4

CGD	Chronic granulomatous disease
CINCA	Chronic infantile neurological syndrome
CLRs	C-type lectin receptors
CT	Computer tomography
CXCL8	Chemokine (C-X-C motif) ligand 8
DAMPs	Danger associated molecular patterns
DAPI	4´ 6-diamino-2-phenylindole
DCs	Dendritic cells
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCAS	Familial cold autoinflammatory syndrome
FCS	Fetal calf serum
FLICA	Fluorescent labeled inhibitor of caspases
FMF	Familial Mediterranean Fever
FSL	Pam2CGDPKHPKSF (TLR6 ligand)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gln	Glutamine
GM-CSF	Granulocyte-monocyte colony-stimulating factor

GWAS	Genome wide association studies
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HKLM	Heat-killed *Listeria monocytogenes*
hr	Hour
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IFI16	Gene-encoding interferon gamma-inducible protein 16 (human)
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL-1Ra	Interleukin 1 receptor antagonist
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-23	Interleukin 23
IL-23R	Interleukin 23 receptor
IMDM	Iscove's modified Dulbecco's medium
INPP5D	Gene encoding the SH2-domain-containing inositol polyphosphate-5´-phosphatase (SHIP) protein (human)
IRAK	Interleukin 1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
IRGM	Immunity-related GTPase family M protein
JAK2	Janus kinase 2

K^+	Potassium
KO	Knockout
LC3	Microtubule-associated protein light chain 3
LP	*Lamina propria*
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LY29	LY294002, a pan-PI3K inhibitor
LY30	LY303511, a non-inhibiting structural analogue of LY294002
MAMPs	Microbial associated molecular patterns
MAPK	Mitogen activated protein kinase
MCSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
MEFV	Gene-encoding Mediterranean fever
mg	Milligram
MHC	Major Histocompatibility complex
min	Minute
miR-155	microRNA-155
ml	Milliliter
mM	Millimolar
MMPs	Matrix metalloproteinase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MUC19	Mucin-19

MWS	Muckle-Wells syndrome
MyD88	Myeloid differentiation primary response gene 88
NACHT	A family of NTPase domains that mediate ATP-dependent oligomerization of NLRPs
NFκB	Nuclear factor kappa B
$NH4^{+}Cl^{-}$	Ammonium chloride
NK cells	Natural killer cells
NLRP3	Nod-like receptor family, pyrin domain containing 3
NLRs	Nod-like receptors
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
ORMDL3	Orosomucoid 1-like 3
Pam3CSK4	Pam3CysSerLys4, which is a TLR1/2 ligand
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphoinositide 3-kinase
PIK-90/PI-103	Class I PI3K p110α inhibitors
PMA	Phorbol 12-myristate 13-acetate
PolyI:C LMW	Low molecular weight Polyinosinic-polycytidylic acid, which is a TLR3 ligand

PRRs	Pattern recognition receptors
PTGER4	Prostaglandin E receptor 4
PTPRC	Protein tyrosine phosphatase, receptor type, C, which is the gene name for CD45 antigen
PVDF	Polyvinylidene fluoride
PYD	Pyrin domain
PYHIN	Pyrin and HIN protein domain
qRT-PCR	Quantitative real-time polymerase chain reaction
RLRs	RIG-like receptors
RNA	Ribonucleic acid
RNAi	RNA interference
RORt	Retinoic acid-related orphan receptor
ROS	Reactive oxygen species
Rplp0	Gene encoding the ribosomal protein, large, P0
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHIP	src homology 2 domain containing inositol polyphosphate 5'-phosphatase
siRNA	Small interfering RNA
SMAD7	Gene encoding the Mothers against decapentaplegic homolog 7 protein (A TGFβ signalling antagonist)
SNPs	Single nucleotide polymorphisms

SOCS	Suppressor of cytokine signalling
SOJIA	Systemic onset juvenile idiopathic arthritis
ssRNA	single stranded RNA
STAT3	Signal transducer and activator of transcription 3
SW18	A Class I PI3K p110γδ selective inhibitor
SW30	A Class I PI3K p110δ selective inhibitor
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TGX-221	A Class I PI3K p110β selective inhibitor
T_H	T helper cell
TL1A	TNF-like ligand 1A
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
TRAPS	TNF receptor-associated periodic syndrome
Tregs	regulatory T cells
TREM2	Triggering receptor expressed on myeloid cells 2
UC	Ulcerative colitis
UK	United Kingdom
UPR	Unfolded protein response
US FDA	United States Food and Drug Administration
UTR	Untranslated region
Wm	Wortmannin
XBP1	X-box binding protein 1

YVAD	A selective irreversible inhibitor of caspase-1
γδ T cells	gamma delta T cells

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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 of any age, with peak incidence reported amongst individuals in the third decade of life.[1] Patients usually suffer from recurring episodes of abdominal pain, diarrhea, rectal bleeding, and nutritional deficiencies.[2] No difference has been seen in the incidence rate of IBD between men and women, but other factors, such as race and ethnicity have been shown to play a significant role.

UC is typically confined to the colon and affects the rectum in 95% of patients.[1] Some UC patients also develop inflammation in the distal ileum, which is thought to result from backwash of cecal content.[3] Inflammation in UC is restricted to the mucosal and submucosal layers.[2] It is characterized by edema, which is swelling resulting from retention of excess fluid; ulceration, and rectal bleeding.[1] Histological analysis of tissue sections from UC patients reveals the presence of immune cell infiltrates and crypt abscesses, reduced goblet cell number, and alteration of crypt architecture.[1]

On the other hand, CD can affect any part of the gastrointestinal tract from the mouth to the perianal region.[1] Inflammation is usually discontinuous with patches of inflammation separated by areas of healthy tissue, and can involve the entire intestinal wall (transmural). The most common site of intestinal inflammation in CD is the distal part of the ileum.[4] Histological analysis of tissue sections from CD patients reveals transmural inflammation with the presence of immune cell aggregates known as granulomas.[1] Other key features

usually present in the affected areas of the gastrointestinal tract of CD patients include: fistulas, which are channels connecting the intestine to other sites; and strictures, which result in narrowing of the intestine and may require surgical intervention if the intestine becomes blocked.[5] The incidence rate of IBD is highest in northern Europe, the United Kingdom (UK), and North America.[6,7] It has been revealed that, Jewish populations have a threefold higher incidence of IBD compared to non-Jewish populations.[8] According to estimates from 2012, Canada has the highest incidence of IBD in the world, with an estimated 233,000 people having the disease.[9,10] Amongst these people, 129,000 were diagnosed with CD and 104,000 were diagnosed with UC.[9,10] The incidence rate of IBD among children less than 10 years old is rising in Canada. It is estimated that 5900 children less than 18 years of age have IBD, making Canada one of the countries with the highest incidence of pediatric IBD in the world.[11] The high incidence of IBD places a huge burden on the affected families and the Canadian healthcare system. The consequences of IBD on the Canadian economy cannot be over emphasized. In 2012, it was estimated that both direct and indirect costs of IBD in Canada, amounted to about 2.8 billion dollars. Direct costs include hospitalizations, surgical, and other procedures, such as colonoscopy, medications, laboratory tests, and services provided by physicians and other healthcare professionals.[10]

The chronic nature of IBD and the financial strain also causes psychological stress and affects the quality of life of patients and their families.[12] People with IBD have also been reported to have poor sleep.[13] IBD is also associated with social stigma, which can be reduced by increasing awareness of the disease amongst the population.[10] Because of the health, psychological, social, and economic consequences of IBD, organizations, such as the Canadian Institutes of Health Research and Crohn's and Colitis Canada are committed to

funding research aimed at improving the lives of Canadians living with IBD. A key research priority is to identify and validate new therapeutic strategies to treat the disease thereby reducing the burden of IBD on patients, their families, and the Canadian health care system.

In keeping with these goals, my doctoral work focusses on characterizing mechanisms and exploring novel interventions to treat intestinal inflammation in Crohn's disease.

1.2 Clinical presentation and diagnosis

Patients with UC present with abdominal pain, cramping, and diarrhea containing blood mixed with mucus.[1] CD patients, on the other hand, may experience pain in the right lower quadrant of the abdomen and disease complications may result in swelling, thickening of the bowel wall, and blockage of the intestine.[1] Patients also suffer from anorexia, diarrhea, and weight loss.[1] In children, IBD can result in delayed growth and delayed sexual maturation.[14] Diagnosis of IBD usually requires a review of the patient's history and a combination of different tests and procedures to exclude the pathological effects caused by the presence of enteric pathogens, such as *Clostridium difficile, Salmonella typhimurium, Shigella flexneri, Mycobacterium tuberculosis,* or *Escherichia coli.*[1,15] Review of patient's history include assessment for symptoms such as diarrhoea with blood and mucus, abdominal pain and cramping, fever, weight loss, and perianal disease, if CD is suspected.[15] Also, the patient's recent use of medications such as antibiotics and NSAIDs and the history of IBD in the family are important considerations during diagnosis of IBD. Various blood examinations, such as complete blood count; erythrocyte sedimentation rate and C-reactive protein (which negatively correlate with inflammation and disease severity); and liver function tests are also performed to help exclude other causes and confirm diagnosis.[15] If a

patient presents without a history of blood in stool, colonoscopy and endoscopic examination may be performed with or without occult blood, with biopsies taken for histological analysis.[1,15] Sigmoidoscopy (used to see inside the sigmoid colon and rectum) or colonoscopy are used for examination of ulcers, bleeding, inflammation, and to determine the presence of multiple biopsies of the colon and terminal ileum.[15] Patients may also have X-rays, abdominal ultrasounds, CT scans (computer tomography scans), MRI (magnetic resonance imaging) or small bowel imaging, which is usually done for CD patients to determine the extend and severity of disease, the involvement of the terminal ileum and the rest of the small bowel, and also to look for extraintestinal complications.[1,15] In children in which upper GI disease (with symptoms such as nausea and vomiting) is more common, upper GI endoscopy may be perfomed.[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 or perpetuation of an immune response to intestinal flora (Figure 1.1).[16,17] CD is characterized by relapsing and remitting inflammation, and it is estimated that approximately 90% of CD patients will experience relapse at some point, while between 38-71% of CD patients will require surgery within 10 years of diagnosis for their disease.[18] Moreover, studies indicate that about 10% of CD patients are refractory to current anti-TNFα therapy and up to 30% of CD patients are predicted to become refractory to these therapies.[19,20] Identification of disease phenotypes and unique

molecular signatures of disease are essential to develop urgently needed novel strategies to effectively target intestinal inflammation in all patients with CD.

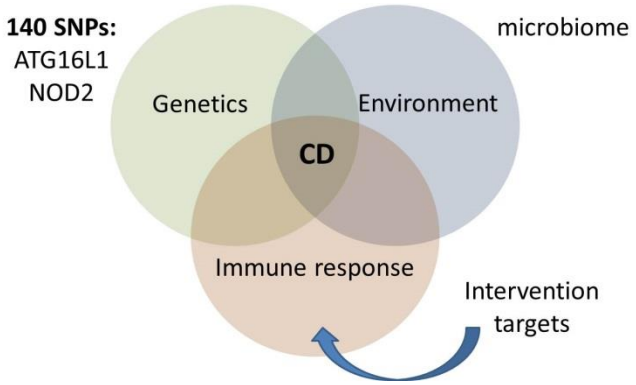

Figure 1.1 Etiology of Crohn's disease (CD)
The etiology of CD lies at the intersection of genetic, environmental, and immunologic factors. It is believed that, CD results from complex interactions between the intestinal microenvironment, including the microbiome and the environment external to the host, and the immune response in genetically susceptible individuals. GWAS have identified 140 single nucleotide polymorphisms associated with Crohn's disease, including those in ATG16L1 and NOD2. Current therapeutic intervention relies on non-specific suppression of the immune response. Modified and reproduced with permission of Nature Publishing Group: Xavier R.J. & Podolsky D.K, Nature 2007.[4]

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 was familial aggregation studies and twin studies, which revealed a steady and consistently increased prevalence of CD amongst relatives.[21] Studies in Europe revealed a very high CD concordance rate in monozygotic twins. In Sweden, the CD concordance rate in monozygotic twins was 50% whereas it was only 3.8% for dizygotic twins.[22] In Denmark, the CD concordance rate was 50% in monozygotic twins and 0% for dizygotic twins.[23] In the UK, the CD concordance rate was reported to be 33.3% in monozygotic twins and 9.9% for dizygotic twins.[24] Amongst CD patients,

between 2-14% have a family history of disease.[25-31] However, very little is now known of the effects of a positive family history on the severity and pathogenesis of CD. After the familial aggregation and twin studies, came the era of genome-wide association studies (GWAS), which focused on identifying single nucleotide polymorphisms (SNPs) and candidate genes that may underlie disease susceptibility.[32-42]

GWAS have strengthened our understanding of the role that genetics play in the pathogenesis of CD. These studies have been facilitated by the completion of the Human Genome Project (2003) and the International HapMap Project (2005). The Human Genome Project completely mapped out the human genome and identified about 20,500 genes. The International HapMap project determined genetic variations in the human genome and generated a set of tools for researchers to utilize to investigate the effects of specific gene variants in disease.[43] Both projects have successfully generated a set of computerized databases containing genetic variants or single nucleotide polymorphisms (SNPs), some of which contribute to common diseases.[44] Linkage studies, which are used to identify inherited genetic markers associated with diseases in a large family, were the first studies used to identify a genetic locus on chromosome 16 strongly associated with CD, [44] which was later confirmed by GWAS as a SNP in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2).[32,33] A recent meta-analysis of GWAS identified 163 SNPs associated with susceptibility to IBD, of which 140 are susceptibility loci for CD.[16,42] The SNPs associated with disease often converge on specific biological processes, such as autophagy, innate and adaptive immune responses, suggesting that CD may comprise distinct pathological subsets of disease. GWAS have identified genes associated with increased susceptibility to CD including *PTGER4*

(encoding the prostaglandin E receptor 4),[45] and *MUC19* (encoding mucin 19),[39] both of which are associated with epithelial barrier function; and genes associated with the interleukin 23 (IL-23) signalling pathway, such as *IL23R, STAT3* (signal transducer and activator of transcription 3), and *JAK2* (Janus kinase 2).[34,46,47] GWAS analyses have also highlighted an unexpected but central role for autophagy in intestinal inflammation.[16] Autophagy-related SNPs that predispose people to develop CD include those in genes encoding *NOD2*, which is required for induction of autophagy in response to intracellular bacterial infection;[48,49] *XBP1* and *ORMDL3*, which are required for the unfolded response;[49-51] and autophagy genes *ATG5,*[16,52] *IRGM,*[38,53] and *ATG16L1;*[35,36] all of which have been implicated in autophagy-related defects in CD. Identification of these polymorphisms in key innate and adaptive immune genes associated with CD susceptibility provides more evidence that crosstalk between genetic, environmental, and immunological factors play a crucial role in the development of CD.

1.3.2 Environmental factors in Crohn's disease

The prevalence of CD has steadily increased in the past 50-60 years and part of this could be attributed to the fact that different populations have migrated from areas that had very low incidence to areas with higher incidence, such as North America and Europe.[54] This migration pattern coupled with the different genetic backgrounds and dietary changes of the populations, have demonstrated a role for environmental factors in the pathogenesis of CD.[30] Several environmental factors have been associated with increased risk for CD including smoking, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), oral contraceptives, diet, vitamin D, and stress. Cigarette smoking increases the risk of developing CD by two-

fold.[55,56] Studies have shown that, smoking reduces T helper cell proliferation and alters the ratio of regulatory T cells to T helper cells in the gut.[57] Interestingly, non-smokers are at an increased risk of developing UC.[55] Repeated use of antibiotics has also been associated with increased risk of developing CD in pediatric patients.[58] Antibiotics may alter the microbiota and thus may predispose people to the development of CD.[58] Oral contraceptives also increase the risk of developing CD, with studies revealing that the risk of CD is two-fold higher in women on oral contraceptives than control subjects.[59] NSAIDs are also associated with increased risk of developing IBD. This has been modeled using IL-10 KO mice, which develop spontaneous colonic inflammation when treated with NSAIDs.[60] Studies have also associated vitamin D deficiency with IBD. Patients diagnosed with IBD have been reported to be deficient or insufficient in vitamin D compared to healthy controls.[61] Stress is another environmental factor that has been associated with increased risk of developing IBD.[62,63] Both chronic and acute stress result in changes in the inflammatory response and may play a role in the development of intestinal inflammation during IBD. Chronic stress results in increased cortisol levels, which are associated with immunosuppression[64] and may lead to reduced intestinal inflammation. In addition, depression has been associated with reduced immune cell numbers (macrophages, NK cells) in the blood.[65] In middle aged to elderly patients, chronic psychological stress leads to subclinical increases in inflammation,[66] suggesting that, chronic stress could also enhance intestinal inflammation. Acute stress, on the other hand, leads to increased adrenaline and pro-inflammatory cytokines such as IL-6, TNFα, and IFNγ, which may enhance intestinal permeability and inflammation.[67]

1.3.3 The microbiome in Crohn's disease

The gut lumen in humans is composed of a plethora of different bacteria with approximately 10^{12} microorganisms that are in close proximity to the intestinal epithelial barrier.[68] Migration results in alteration of the gut microbiota due to: changes in the environment external to the host, dietary changes, changes in behaviour, and adaptation to new life styles, all of which could explain the increased incidence of CD in new populations, which have relocated.[69] In CD patients, dysbiosis in luminal bacteria, characterized by less diversity in the microbiota is very common.[70,71] CD is also associated with adherent and invasive *E. coli* (AIEC), with close to 22% of CD patients having AIEC in the lumen compared to 6.2% of healthy subjects.[72] Viral infections have also been implicated in CD pathogenesis and have been shown to alter the gut microflora. Evidence for this has been seen in mice harboring the homologue of the *ATG16L1* CD susceptibility gene found in humans.[73] Upon infection with norovirus, these mice show abnormal Paneth cell structure and granules similar to that observed in CD patients, who are homozygous for the *ATG16L1* gene variant.[73] Indeed, the CD risk alleles in *NOD2* and *ATG16L1* have also been associated with changes in the composition of intestinal microbiota.[74] These studies suggest 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 coordinated action of the intestinal epithelial cells and the innate and adaptive cells and responses. The intestinal epithelial barrier is a single layer of cells made up of four main types of epithelial cells: enterocytes

or colonocytes, goblets cells, Paneth cells, and enteroendocrine cells; separating the gut lumen from the *lamina propria* (LP).[75] It serves as a dynamic physical barrier preventing entry of microbes and other luminal antigens into the LP and also allows the passage of nutrients and water from the gut lumen into circulation.[75,76] Tight junctions seal the space between adjacent epithelial cells preventing entry of luminal bacteria into the LP.[77] Paneth cells and goblet cells secret mucin and antimicrobial peptides to form the protective mucus layer that limits contact between commensal bacteria and epithelial cells. It is believed that during CD, the epithelial barrier is defective, resulting in increased epithelial permeability.[78] In addition, MUC1 (a cell surface glycoprotein) mRNA expression is reduced in the inflamed ileal mucosa of CD subjects compared to the healthy mucosa.[79] Moreover, patients homozygous for the *ATG16L1* risk variant have abnormal Paneth cells, which may affect secretion of antibacterial peptides, such as defensins.[73,80] Furthermore, in patients with IBD, a dysregulated immune response to normal enteric microflora leads to increased mucosal secretion of IFNγ and TNFα, which have been associated with defective epithelial barrier permeability.[81]

In all, maintaining an intact intestinal epithelial barrier with fully functional Paneth and goblet cells, together with effective immunoregulatory mechanisms are crucial to prevent the onset of intestinal inflammation and CD (Figure 1.2).

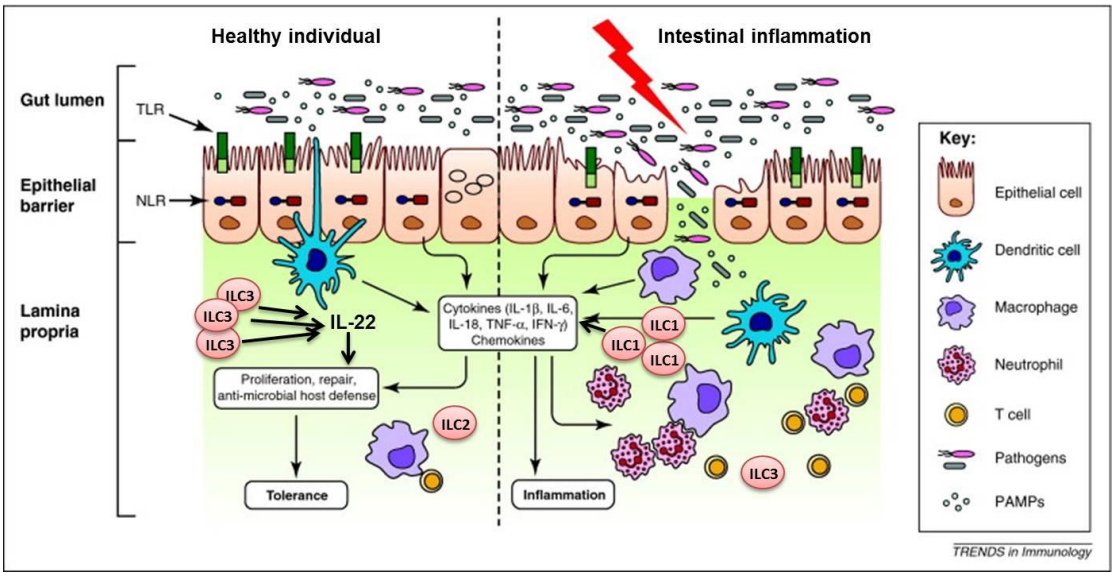

Figure 1.2 The epithelial barrier separates the gut lumen from the *lamina propria.* The intestinal epithelial barrier prevents LP immune cells from interacting with commensal microbes in the gut lumen. (Left) In a healthy individual, there exists a state of immune tolerance that allows commensal bacteria to live alongside immune cells in the gut. Epithelial cells, 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 sterile LP where they encounter DCs and macrophages. These cells sense the presence of these microbes using their 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, CCL2, which attract more immune cells to the site of inflammation, where they encounter these 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.[82]

1.3.5 The immune response in Crohn's disease

The immune system is made up of two arms: the innate immune system and the adaptive immune system. Both play critical roles in mucosal homeostasis and immune

dysregulation in CD. Below, I describe the roles the innate and adaptive immune systems play in the onset and pathogenesis of CD.

1.3.5.1 Innate immune response

The innate immune system is the first line of defense against invading microbes and is composed of cells including: epithelial cells, and leukocytes, such as monocytes, neutrophils, basophils, and eosinophils; macrophages, dendritic cells (DCs), and Natural Killer (NK) cells. Innate immune cells recognize pathogen-derived molecules in the intestinal microenvironment. Intraepithelial DCs sample the gut lumen for the presence of non-pathogenic microbes resulting in a regulatory response that provides tolerance.[82] Innate immune cells contain extracellular and endosomal pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-like receptors (RLRs),[83] and C-type lectin receptors (CLRs)[84] that monitor the extracellular and subcellular compartments for signs of infection or damage.[85] In humans, there are 10 TLRs, TLR1-10 (13 in mice), each recognizing specific microbial associated molecular patterns (MAMPs). Intestinal epithelial cells (IECs) on the other hand express TLRs basolaterally to limit interaction with MAMPs in the gut lumen.[86] NLRs and RLRs are intracellular PRRs that sense factors released during perturbations in tissue homeostasis known as damage-associated molecular patterns (DAMPs).[83] In healthy individuals, there is a steady-state induction of protective factors by TLRs when they come in contact with commensal bacteria, which results in immune tolerance.[87] Studies in mice have revealed that alteration in the proteins required for immune defense responses could lead to intestinal inflammation.[88] TLR4 expression in intestinal epithelial cells (IECs) and both

TLR2 and TLR4 expression in intestinal macrophages and DCs are increased in CD patients compared to control subjects.[89,90] Innate lymphoid cells (ILCs) are cells, which do not express the T-cell receptor (TCR) and serve as a source of cytokines, especially IL-23, at mucosal surfaces.[91] Patients with CD produce more IL-23[92] and express more of the transcription factor, retinoic acid-related orphan receptor (RORt)[93,94] in the LP compared to control patients. Thus the innate immune response is crucial to prevent the development of IBD since it serves as the first line of defense against microbes after they cross the intestinal epithelial barrier. Defects in this arm of the immune response may result in increased production of inflammatory mediators that drive or contribute to intestinal inflammation in CD.

1.3.5.2 Adaptive immune response

The adaptive immune system is made up of T and B lymphocytes and acts as the second line of defense to invading pathogens. It is highly specific, recognizes 'self' from 'non-self' antigens, and generates appropriate immune responses. It also confers long lasting immunological memory. CD4+ T cells are important effector cells of the adaptive immune response that are important in defense against pathogenic microbes and excessive entry into the gut lumen of commensal bacteria. They can be grouped into different classes including: helper T cells (Th1, Th2, or Th17), or regulatory T cells (Tregs). Th1 cells are induced by IL-12 and IL-18 and produce high levels of interferon-γ (IFNγ) and TNFα. They protect against intracellular bacterial infection. Th2 cells produce high levels of IL-4, IL-5, IL-9, and IL-13, and protect against parasitic helminthes infections.[95] Th17 cells on the other hand, are

induced by IL-6 in the presence of transforming growth factor beta (TGFβ) in mice,[96] or both IL-6 and IL-1β in humans,[97] to produce high levels of IL-17A, IL-17F, IL-21, and IL-22.[98] Th17 cells are important for defense against extracellular pathogens via recruitment of neutrophils and macrophages to the site of infection.[99] IL-23 promotes the expansion of Th17 cell responses.[98]

An imbalance or overreaction of CD4+ T cells relative to Tregs is believed to cause intestinal inflammation.[100-103] CD is widely believed to be a Th1/Th17 mediated disease with increased secretion of IFNγ, TNFα, IL-17A, and IL-2 reported in T cells from CD patients compared to those from control subjects.[104,105] It has been shown that, IFNγ and TNFα levels are increased in the inflamed mucosa of CD patients,[106,107] while their LP cells produce high levels of IL-12.[108] In UC patients, there is increased production of IL-4 and IL-13,[105] suggesting that Th1 and Th2 cytokines play an important role in the pathogenesis of CD and UC, respectively. IL-17 producing Th17 cells are also increased in the inflamed mucosa of IBD patients and are regulated by IL-23.[109,110] LP macrophages from CD patients produce high levels of IL-23, which drives Th1 and Th17 responses.[111,112] In addition, SNPs in the *IL-23R* gene have been associated with IBD.[34] In fact, the *IL-23R* SNP, Arg381Gln (arginine381glutamine), has been reported to confer a 2-3 fold protection against development of pediatric CD.[34,113] These studies suggest that both IL-17 and IL-23 play an important role in the pathogenesis of IBD and consequently, targeting the IL-17/IL-23 pathway may serve as a therapeutic option in IBD. Monoclonal antibodies directed against the p40 subunit of IL-12/23 (ABT-874) and IL-23 (ustekinumab) have been tested as treatments for CD patients, but have failed in phase two clinical trials[114]; the human anti-IL-17A monoclonal antibody (secukinumab) induced modest clinical response in patients, who

were not responsive to anti-TNFα therapy.[115] Indeed, secukinumab has been shown to reduce moderate to severe CD in patients with the TNF-like ligand 1A (*TL1A)* gene variant.[115] The *TL1A* gene encodes a cytokine that belongs to the TNF ligand family, and drives pathogenic T cells in inflammatory processes[116]

IL-21 is another cytokine that is increased in intestinal tissues from IBD patients and enhances IFNγ production and Th1 signalling.[117] Moreover, IL-22 expression is increased in the gut of CD patients compared to control patients and drives epithelial cell migration and pro-inflammatory gene expression.[118]

In mice, IL-17 has also been shown to play an important role in different models of intestinal inflammation. IL-17F enhances dextran sodium sulfate (DSS)-induced colitis, whereas IL-17A protects mice against DSS-induced colitis.[119] Natural killer (NK) cells produce IL-22, which protects mice from intestinal inflammation by promoting production of mucus and defensins by intestinal epithelial cells.[120,121] In all, Th17 cells enhance intestinal inflammation by promoting the recruitment of inflammatory cells, such as neutrophils, and production of inflammatory mediators, such as cytokines, chemokines, and matrix metalloproteinases (MMPs), which cause tissue damage.[122-124]

Tregs are adaptive immune cells that are crucial for the maintenance of mucosal immune homeostasis. They exert their action by producing IL-10 and TGF-β, suppressing the proliferation of naïve T helper cells and aberrant immune responses to commensal bacteria and microbial antigens.[125,126] In patients with active IBD, Treg numbers are significantly reduced in peripheral blood compared to control subjects.[127,128] In contrast, Treg activity is reduced in the intestinal mucosa of IBD patients despite increased Treg numbers.[129,130] Smad7 (inhibitory molecule) is up-regulated in the inflamed mucosa of IBD

patients and blocks Treg function by regulating TGFβ signalling.[131] Blocking Smad7 activity restores TGFβ signalling and Treg function.[132] In mice, studies have shown that, knockdown of Smad7 using a Smad7 antisense oligonucleotide (GED0301) blocked inflammatory cytokine production and reduced experimental colitis.[132] Furthermore, Phase I clinical trial studies have shown that patients with active steroid-dependent/resistant CD treated with GED0301 had reduced inflammatory cytokine-expressing $CCR9^+$ T cells in their blood.[133] Recently, Phase II clinical trials with an oral Smad7 antisense oligonucleotide (Mongersen) caused clinical remission in patients with moderate to severe CD with rates of between 55 and 65% and remission was maintained for up to 3 months,[134] suggesting that targeting Smad7 could be an important therapeutic strategy for IBD.

Taken together, IBD may develop due to defects in Treg activity resulting from increased Smad7 in the intestinal mucosa. Reduced Treg activity may lead to reduced anti-inflammatory cytokine production and uncontrolled Th1, Th2, and/or Th17 inflammatory cytokine production that promotes tissue damage and inflammation.

To summarize, the onset and pathogenesis of CD is such that, in a healthy individual, there exists a state of immune tolerance that allows commensal bacteria to live alongside the immune cells in the gut. Intraepithelial DCs sample the gut lumen for microbes and present microbial antigens to T cells, which in turn initiate a regulatory response to maintain immune tolerance. In a genetically susceptible host, the intestinal epithelial barrier may be compromised allowing luminal bacteria and antigens to enter the sterile LP where they encounter resident immune cells (DCs and macrophages). These cells sense the presence of these microbes using their PRRs, and initiate an inflammatory response with production of chemokines, such as IL-8, CCL2 (chemokine ligand 2),

CXCL8 (chemokine ligand 8), and pro-inflammatory cytokines, such as IL-6, TNFα, and IL-1β, resulting in inflammation.

1.3.6 Therapeutic options

There is currently no cure for IBD. Generally, medical management of IBD requires long-term treatment based on a combination of drugs designed to relieve patients of the symptoms, provide long-term remission, and diminish the risks of complications. Treatment takes into account the severity, location, and phenotype of disease, as well as disease complications and individual tolerance to medical intervention.[15] Futhermore, the patient's past disease course and the duration and number of relapses in a calendar year are also considered during IBD management. In addition, management of pediatric IBD takes into account the age and pubertal status of the child.[15] Treatment begins with 5-aminosalicyclic acids (5-ASAs), progresses to steroids if symptoms persist, and finally to biological therapies.[1,135]

5-ASAs, such as sulfasalazine, mesalamine, olsalazine, and balsalazide, are used for treating mild to moderate UC and can be used for mild ileocolonic and colonic CD.[1,15] For moderate disease, corticosteroids, such as prednisone, which reduce inflammation, are used. Because of the side effects of corticosteroids, long-term use is avoided. Exclusive enteral nutrition is an effective and often preferred option, which involves exclusion of normal diet for a period of time and use of liquid nutritional products.[1,136] CD Patients with complications such as perianal disease, fistulas, bacterial over-growth in the settings of strictures, may be treated with antibiotics, such as ciprofloxacin and metronidazole.[1,15] In patients with moderate to severe IBD, immunosuppressive drugs, such as azathioprine, 6-

mercaptopurine or methotrexate that suppress the immune response can be used. A disadvantage of using immune-modulatory drugs such as methotrexate is that they are non-selective and reduce the patient's ability to fight infections.[1]

Biological therapies are designed to target specific immune mediators of diseases, such as cytokines. Biological therapies have been used for IBD treatment and have produced mixed results. Some of these biological therapies 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.[137] Others, such as secukinumab (human anti-IL-17A monoclonal antibody), have produced mixed results and has been shown to reduce moderate to severe CD in patients with the *TL1A* gene variant.[115] Ustekinumab, which is a human mAb that specifically binds the p40 subunit shared by IL-12 and IL-23, has failed to induce remission in patients with moderate to severe CD in phase two clinical trials.[114] Despite that, some patients are refractory to biological therapy and for others, treatment loses its efficacy over time.[138] Loss of efficacy may be caused by low drug levels or development of antibodies to the biological therapy, but also occurs in some patients with neither of these causes.[138] This underscores the need for identification of disease subtypes and development of novel therapeutic strategies to treat disease.

1.4 The Interleukin 1 family of cytokines

The interleukin 1 (IL-1) family of cytokines is composed of eleven members including seven ligands (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ); three receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37).[139] IL-1β and IL-

18 are processed through the same mechanism, involving activation of the inflammasome and generation of active caspase-1. IL-33 is produced by many cell types including fibroblast, mast cells, macrophages, DCs, osteoblasts, epithelial cells, and endothelial cells.[140] It was initially thought to be produced by the same process as IL-1β and IL-18. However, recent studies have shown that caspase -1 cleavage is not required for production of biologically active IL-33.[141] IL-33 is increased in bronchial epithelial cells of asthma patients compared to control subjects.[142] IL-33 has been shown to promote goblet cell hyperplasia, airway hyper-responsiveness, eosinophilia, M2 macrophage polarization and accumulation of IL-4, IL-5, and IL-13 in the lungs.[143-145] IL-36 is produced by innate immune cells, lymphocytes,[146,147] and epithelial cells,[148] and activates nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs).[149]

Some members of the IL-1 family play an important role in the development and differentiation of T helper cells. IL-1β, together with IL-6, and TGFβ, induce differentiation of Th17 cells.[150] In the presence of IL-23, IL-1β induces development of RORγt$^+$ IL-17A-producing cells.[150] IL-18, directs development of IFNγ-producing Th1 cells,[151] whereas IL-33 is known to induce Th2 responses.[152] Both IL-1α and IL-33 mediate sterile inflammation since their precursors are constitutively active and are released when cells undergo necrosis.[153,154] The IL-1α precursor is present in epithelial layers of the gastrointestinal tract, the kidney, liver, and the lungs.[153,154] Furthermore, IL-36 activates release of IL-2 and enhances T-cell proliferation.[147]

The IL-1 family also includes anti-inflammatory cytokines that are critical for the regulation and control of the actions of the pro-inflammatory cytokines. These anti-inflammatory members include IL-37, IL-38, IL-1Ra, and IL-36Ra. Endogenous IL-37 is a

natural anti-inflammatory cytokine, and reduces LPS (lipopolysaccharide) and IL-1β-induced pro-inflammatory cytokine production by 2-3 folds in human blood monocytes.[155,156] IL-37 production is stimulated by TGFβ,[155] and exerts its suppressive functions by translocating to the nucleus, where it binds to Smad3 (a transcription factor of TGFβ) and inhibits transcription of pro-inflammatory genes.[157] Recombinant IL-37 binds the IL-18Rα chain and transmits its inhibitory signals.[155] IL-37 has also been shown to inhibit adaptive immune responses. DCs expressing IL-37 induce Tregs and are unable to activate effector T cell responses[155] IL-38 on the other hand, binds IL-36R and acts as a partial receptor antagonist.[158] Polymorphisms in the *IL-38* gene have been associated with increased susceptibility to inflammatory diseases, such as psoriatic arthritis and ankylosing spondylitis.[159] Furthermore, IL-38 inhibits IL-17A and IL-22 production by human memory T cells.[158]

The IL-1 receptor antagonist (IL-1Ra) is a naturally occurring cytokine that competes with IL-1β and IL-1α for IL-1R1 and prevents IL-1 signalling. The IL-1Ra gene is conserved in humans and mice with identical intron-exon organization.[160] There has been no report of interspecies differences in the IL-1Ra gene between humans and mice. Anakinra is a synthetic IL-1Ra that has been shown to reduce disease symptoms in patients with autoinflammatory diseases, such as rheumatoid arthritis.[161,162] Deficiency in IL-1Ra is associated with severe systemic and local inflammation in children.[163] In mice, deficiency in IL-1Ra leads to increased susceptibility to carcinogens, psoriatic-like skin lesions, and spontaneous arthritis.[164,165]

The IL-36 receptor antagonist (IL-36Ra) competes with IL-36 for the IL-36R and prevents IL-36 signalling. IL-36Ra also prevents IL-36-induced IL-23, IL-17, and IL-22

production as well as development of psoriasiform dermatitis.[148,166] Mutations in the *IL-36Ra* gene have been associated with psoriasis.[167]

1.4.1 Interleukin 1β (IL-1β)

The inflammatory response is critical in host defense against infectious pathogens but must be tightly regulated to prevent excessive tissue damage during injury or infection. This is particularly evident for production of the pro-inflammatory cytokine IL-1β, which has been described as the "master mediator,"[168,169] "initiator,"[168] or "gatekeeper" of inflammation.[170] Cells, such as epithelial cells, monocytes, macrophages, DCs, B lymphocytes, and NK cells can produce IL-1β to initiate and/or amplify innate immune responses.[170] IL-1β and IL-18 are produced by the same two-step process (Figure 1.3) that is tightly regulated in immune cells. In the first step, known as the priming step, microbial associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS) bind to pattern recognition receptors (e.g. TLR4) and initiate a signalling cascade, which leads to NFκB activation and translocation into the nucleus, where it drives transcription of the IL-1β precursor, pro-IL-1β,[171] and up-regulates *NLRP3* (NOD-like receptor family, pyrin domain containing 3) expression.[172,173] A second signal, typically a danger associated molecular pattern (DAMP), stimulates assembly of the inflammasome.

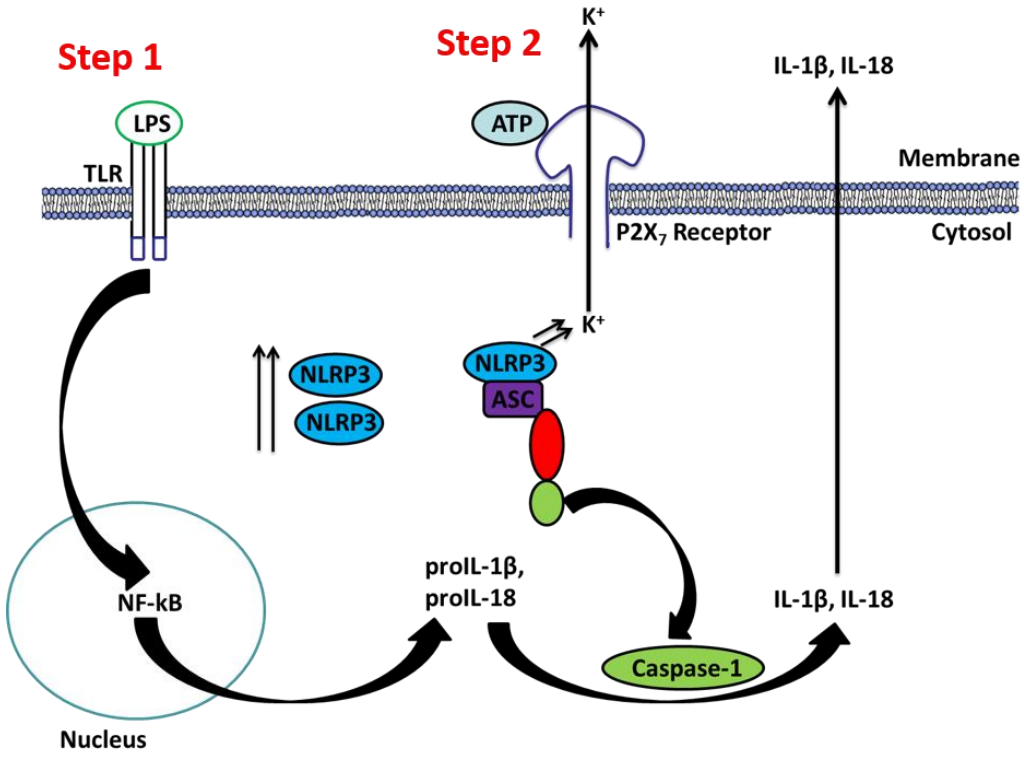

Figure 1.3 NLRP3 inflammasome activation and IL-1β production
IL-1β production is a two-step process. The first step involves activation of a PRR (such as a TLR) by a MAMP (e.g. LPS), resulting in NFκB activation, its translocation into the nucleus, and production of pro-IL-1β and pro-IL-18. Activation of PRR also results in increased *NLRP3* transcription. In the second step, ATP (a DAMP) binds to the P2X7 receptor, resulting in potassium ion (K^+) efflux. The NLRP3 inflammasome senses a drop in intracellular K^+ levels, activating caspase-1, which cleaves pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18 that are secreted out of the cell.

1.4.2 Inflammasome activation and IL-1β production

The inflammasome is a multi-protein complex composed of a nod-like receptor protein (NLRP), an adaptor protein (ASC), and an effector protein. Structurally, NLRs are composed of a C-terminus leucine rich repeat (LRR) that acts as the sensor of intracellular pathogen associated molecular patterns (PAMPs) and DAMPs,[174] a central nucleotide binding oligomerization domain (NOD or NACHT), which is important for oligomerization

and activation of the NLR,[175] and an N-terminal domain, which is usually a PYD (pyrin domain) or a CARD (caspase recruitment domain)[176] (Figure 1.4).

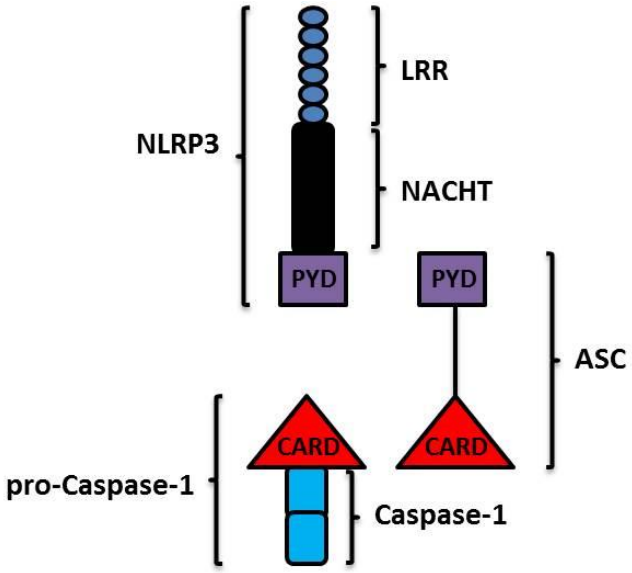

Figure 1.4 The NLRP3 inflammasome
The NLRP3 inflammasome is made up of three main components: NLRP3, which contains a LRR, NACHT, and a Pyrin domain (PYD); an adaptor protein (ASC), which contains a PYD and a caspase recruitment domain (CARD); and pro-caspase-1. In the presence of a DAMP, the NLRP3 inflammasome is assembled by interaction of the PYD of NLRP3 with the PYD of ASC, and the CARD of ASC with the CARD of pro-caspase-1.

Six inflammasomes containing a NLR sensor have been identified to date and include: NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, and NLRC4 (also known as IPAF).[85] Two other inflammasomes that contain a PYHIN (pyrin and HIN domain-containing protein) have also been described and include Absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16)[177]. These inflammasomes are usually assembled in response to stimuli, such as: Muramyl dipeptide (MDP) and *Bacillus anthracis* lethal toxin (NLRP1b); microbiota (NLRP6); cytosolic flagellin or stimuli from type III or IV secretion system-

containing bacteria, such as *Salmonella* sp serovar Typhimurium, *P. aeruginosa*, *L. pneumophilia*, and *S. flexneri* (NLRC4); cytoplasmic double-stranded deoxyribonucleic acid (DNA) viruses, *Francisella tularensis*, and *Listeria monocytogenes* (AIM2). It is still not known exactly how these inflammasomes sense the presence of DAMPs within the cell. NLRP3 is the best described of the inflammasomes and its activation is often used as a model to study the production of IL-1β by monocytes and macrophages.

The assembly and activation of the NLRP3 inflammasome occurs via many different pathways; the first is via detection of increased levels of extracellular ATP (adenosine triphosphate), which is released from dying cells and acts as a danger signal. ATP binds to the P2X7 receptor, opening the potassium channel, and induces formation of pores in the cell membrane, allowing MAMPs or DAMPs to gain access into the cell and activate the inflammasome.[178,179] Pore forming toxins, such as nigericin also create pores in the cell membrane that facilitate potassium efflux.[180] The drop in intracellular potassium concentration triggers activation of the NLRP3 inflammasome.[181] Particulate and non-particulate stimuli, such as uric acid crystals, silica, asbestos, aluminum salt, and amyloid-β also activate the NLRP3 inflammasome. These crystals and particles are thought to destabilize the phagosomes in which they are stored upon phagocytosis, causing lysosome damage and release of cathepsin B into the cytosol, which then activates NLRP3.[182-184] A wide range of pathogens including *Escherichia coli*,[185] *Neisseria gonorrhoeae*,[186] *Candida albicans*,[187] and *Vibrio cholera*[188] have also been shown to activate NLRP3. The mechanism by which this occurs is not completely known but NLRP3 activation by *C. albicans* requires release of cathepsin B and *V. cholera* acts via release of cytotoxins. Mitochondrial reactive oxygen species (ROS) was initially thought to activate the NLRP3 inflammasome, but recent

studies have now shown that mitochondrial ROS actually enhances expression of pro-IL-1β and NLRP3 via increased NFκB transcription.[172,189] It is not known if there is direct contact between the LRR and the different DAMPs that activate NLRP3 inflammasome, but considering their diversity, it seems unlikely.

Activation and assembly of the inflammasome results in cleavage of pro-caspase-1 to active caspase-1. Caspase-1 produced, cleaves pro-IL-1β/pro-IL-18 to their active forms, which are secreted out of the cell. Secreted IL-1β signals through the IL-1 receptor, driving transcription of IL-1β thus initiating positive feedback activation.[190-192] Circulating blood monocytes in humans have constitutively active caspase-1[193] and when monocytes are stimulated with PAMPs that activate PRR, they produce mature IL-1β for several hours.[170] This process can be accelerated by addition of exogenous ATP that activates the NLRP3 inflammasome.[194] Thus, the rate-limiting step in IL-1β production is the synthesis of pro-IL-1β from IL-1 mRNA.[170] Extracellular serine proteases, such as proteinase-3 released by infiltrating neutrophils,[195,196] elastase, granzyme A, and matrix metalloprotease 9,[169] can also cleave pro-IL-1β to mature IL-1β. This is relevant physiologically in that neutrophils are recruited to sites of inflammation and they release these proteases when they become apoptotic, thereby enhancing IL-1β production.

After mature IL-1β is produced, it is released out of the cell, though the mechanism of its release is not completely understood. An increase in intracellular calcium levels and the presence of phospholipase C are necessary to facilitate the process.[197] IL-1β release can occur through vesicular bodies that contain exosomes,[198] exocytosis of secretory lysosomes,[199,200] and shedding of plasma membrane micro-vesicles.[198] Even though a lot has been done to characterize the molecular mechanisms that underlie inflammasome activation,

there are still avenues for future research especially in identifying new intrinsic cellular mechanisms that regulate expression levels of NLRP3 and other inflammasome components.

To summarize, production of IL-1β is a 2 step process: The first step is activation of a TLR/cytokine receptor (e.g. IL-1R1) by a PAMP/cytokine (e.g. IL-1) resulting in downstream activation of NFκB transcription, which in turn drives IL-1β/IL-18 transcription and pro-IL-1β/pro-IL-18 production. The second step is activation of the inflammasome by DAMPs, resulting in caspase-1 activation, which permits cleavage of the pro-forms of IL-1β/IL-18 to their active forms, which are then secreted out of the cell.

1.4.3 IL-1β-mediated diseases (auto-inflammatory diseases)

Auto-inflammatory diseases are initiated by sterile inflammation caused by excessive innate immune responses to DAMPs.[168] IL-1 mediates these diseases and IL-1 blockade or treatment with a decoy IL-1 receptor (IL-1RII) or monoclonal antibody to IL-1β prevents pathology. In contrast, autoimmune diseases occur when one develops an adaptive immune response to self-antigens, usually initiated by excessive innate immune responses to DAMPs and PAMPs.[168] IFNγ and TNFα produced by Th1 cells or IL-17 produced by Th17 and γδ T cells mediate autoimmune diseases.[201] IL-1β has been shown to mediate many human diseases, which are described in the following sections.

1.4.3.1 Cryopyrin-associated periodic syndrome (CAPS)

CAPS are a group of autoinflammatory diseases including FCAS (Familial cold auto-inflammatory syndrome), MWS (Muckle-Wells syndrome), and CINCA (Chronic infantile

neurological, cutaneous, and articular syndrome), which occur when individuals with a gain of function mutation in NLRP3 are exposed to cold.[202] Patients usually have recurrent fever, increased acute phase proteins, and leukocyte infiltration.[202] CAPS are uniquely mediated by IL-1β and hence patients respond to treatment with the synthetic IL-1Ra, Anakinra,[203-205] the soluble IL-1 receptor, rilonacept,[206] or the monoclonal antibody to IL-1β, canakinumab.[207]

1.4.3.2 TNF-receptor-associated periodic syndrome (TRAPS)

This is an auto-inflammatory disease that results from a gain of function mutation in the coding region of the TNF receptor and is characterized by recurrent fever and systemic inflammation.[208] People with this mutation have defects in the translocation of the TNF receptor to the cell membrane, resulting in accumulation of misfolded proteins, endoplasmic reticulum stress (ER), and hence inflammation.[170] ER stress results in increased mitochondrial ROS production that has been associated with potassium efflux, NLRP3 activation, and IL-1β release.[209] Patients with TRAPs respond to treatment with an IL-1Ra (Anakinra) suggesting a key role for IL-1 in disease pathogenesis.[210,211]

1.4.3.3 Familial Mediterranean Fever (FMF)

FMF is a chronic inflammatory condition that results from a gain of function mutation in pyrin, a protein component of the inflammasome complex.[212] The marentosin-encoding fever gene (*MEFV*), which encodes pyrin, is associated with FMF. Pyrin is a protein found in neutrophils, monocytes, eosinophils, and fibroblasts from the skin, peritoneum, and synovium.[213,214] They regulate caspase-1 activation and IL-1β production through its

interaction with the adaptor protein, ASC.[215] A mutation in the *MEFV* gene leads to cleavage of pyrin and results in increase caspase-1 activation and IL-1β production.[215] Furthermore, caspase-1 has been shown to cleave pyrin, releasing the N-terminal fragment of this protein that is translocated to the nucleus and activates NFκB by interacting with its p65 subunit.[216] FMF is characterized by systemic and local inflammation resulting from increased activation of the inflammasome and IL-1β production.[170] Anakinra has been shown to improve clinical outcome in patients with FMF.[170]

1.4.3.4 Adult onset Still's disease (AOSD)

AOSD is a rheumatologic condition characterized by fever, sore throat, arthritis, splenomegaly, lymphadenopathy, increased neutrophil infiltration, and release of hepatic acute phase proteins.[170] Reducing IL-1β activity with Anakinra reduces systemic and local manifestations of disease.[217]

1.4.3.5 Other autoinflammatory diseases

Other diseases mediated by increased IL-1β include Systemic onset juvenile idiopathic arthritis (SOJIA),[211,218] osteoarthritis,[219] chronic granulomatous disease (CGD),[220] Type 2 diabetes,[221,222] and smoldering myeloma.[223,224] In gouty arthritis, uric acid crystals activate synovial macrophages in the presence of fatty acids to synthesize IL-1β.[225-230] In all of these diseases, there is increased release of DAMPs, which are known activators of the inflammasome. The resultant IL-1β produced feeds back to produce more IL-1β. Anakinra or a monoclonal Ab to IL-1β has been shown to reduce pathology. Blocking IL-1β signalling

reduces IL-1β production and feedback, recruitment of neutrophils, and release of proteases that cause tissue damage and DAMPs production, and consequently reduces auto-inflammation.

1.4.4 IL-1β in intestinal inflammation

Despite being a central mediator in the inflammatory response, the role of IL-1β in intestinal inflammation has been confounded by conflicting reports. In humans, reduced expression of *NLRP3* has been associated with increased susceptibility to CD.[231] Gene variants around the regulatory region of *NLRP3* (rs4353135 and rs10733113 SNPs) are associated with reduced *NLRP3* gene expression and decreased IL-1β production suggesting that NLRP3 and IL-1β may play a protective role early on during CD.[231] However, IBD patients have increased levels of IL-1 in their intestinal tissue, which correlates with the level of intestinal inflammation.[232] It has also been reported that, there is an imbalance between IL-1 and IL-1ra in the intestinal mucosa of IBD patients.[233] Moreover, elevated IL-18 levels have been reported in colonic inflammatory lesions in CD patients.[234] During the later stages of disease, inflammasome activation and IL-1β production have been shown to alter epithelial tight junctions and increase intestinal permeability in CD subjects.[235] Hence, IL-1 may play protective or pathogenic roles in IBD depending on the stage of the disease.

In mice, studies have shown that NLRP3 activation and increased IL-18 production, protects mice from chemically-induced intestinal inflammation by stimulating differentiation of epithelial stem cells and repair of damaged intestinal epithelium.[236-238] Consistent with that, deficiency in NLRP6 inflammasome[239] or IL-1β,[240] exacerbates DSS-induced colitis in mice. These findings support the hypothesis that, in the initial phase of intestinal

inflammation, IL-1β and IL-18 produced by inflammasome activation in non-hematopoietic cells play a protective role, promoting epithelial repair[237] after injury and limiting bacterial translocation into the *lamina propria*. During the later stages of disease, IL-1β promotes intestinal pathology by enhancing neutrophil recruitment and IL-17 production by innate lymphoid cells (ILCs) and CD4+ Th17 cells in the gut.[241] In IL-10 KO mice, inflammasome activation and IL-1β production promote development of spontaneous colitis.[242] Moreover, IL-1β is increased in intestinal tissues and peritoneal macrophages during DSS-induced colitis[243] and increased IL-1β relative to IL-1Ra has been shown to drive colitis in TLR5 KO mice.[244]

1.5 Autophagy

1.5.1 Description and overview

Autophagy is a multistep, catabolic self-preservation process that controls clearance and re-use of intracellular components during homeostasis and provides amino acids required for cell survival under stress conditions including starvation, accumulation of unfolded proteins, or intracellular infection.[245] During autophagy, cytoplasmic contents including damaged organelles, misfolded/unfolded proteins, or intracellular bacteria are engulfed in a double membrane vesicle called the autophagosome, and degraded by fusion with lysosomes.[246] Autophagy is usually determined by measuring expression levels of the microtubule-associated protein light chain 3 (LC3-II). LC3 is ubiquitously expressed in mammalian tissues as LC3-I in the cytosol.[247] During autophagy, cytosolic LC3-I is conjugated with phosphatidylethanolamine to form LC3-II, which is integrated into the autophagosome membrane, and usually degraded during fusion with lysosomes (Figure

1.5).[247,248] The amount of LC3-II is indicative of the number of autophagosomes present in a cell.[247]

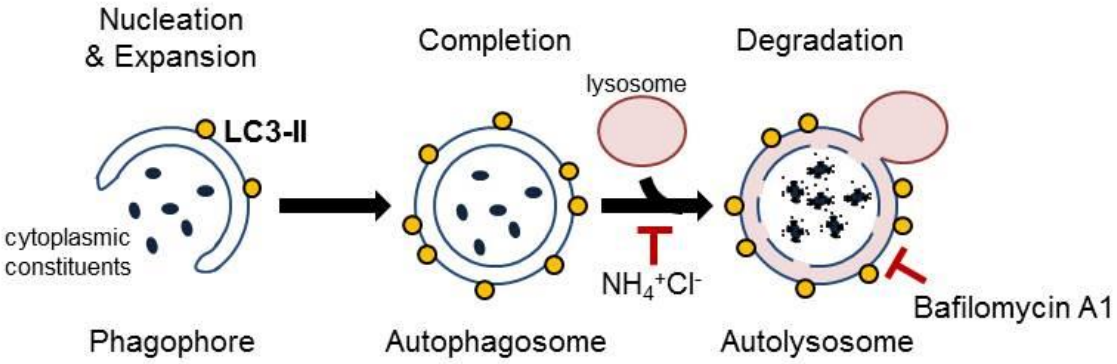

Figure 1.5 Autophagy.
Autophagy is a cell homeostatic mechanism for degradation of cytoplasmic constituents, such as intracellular bacteria, misfolded/unfolded proteins, or damaged organelles, in autophagosomes. A phagophore nucleates, expands, and engulfs cytoplasmic constituents to form an autopahgosome. Cytosolic LC3-I is conjugated with phosphatidylethanolamine to form LC3-II, which is integrated into the autophagosome membrane. The completely formed autophagosome fuses with lysosomes to form an autolysosome, where its contents are degraded by lysosomal enzymes and proteases. Ammonium chloride ($NH4^+Cl^-$) prevents autophagosome-lysosome fusion while Bafilomycin A1 prevents degradation of the autophagosome-lysosome complex (autolysosome).

Autophagy negatively regulates the inflammatory response by regulating production of pro-inflammatory IL-1β. In mice, autophagy stops inflammasome activation (signal 2) by degrading cytosolic DAMPs that activate inflammasomes, mitochondrial DNA, ROS, or by degrading inflammasomes/caspase-1.[48,249-251] Autophagy also targets and segregates pro-IL-1β in autophagosomes for degradation by lysosomes, hence, inhibition of autophagy leads to availability of more pro-IL-1β, and consequently increased IL-1β production.[251] Finally, autophagy limits IL-1β signalling by promoting degradation of p62 (multi domain protein involved in activation of NFκB) by proteasomes and other lysosomal enzymes.[252] SNPs in

genes encoding *NOD2*, *IRGM*, and *ATG16L1* have been implicated in autophagy-related defects in CD. The association of these SNPs in autophagy with CD highlights a crucial role for autophagy in the pathogenesis of CD.

1.5.2 Autophagy related 16-like 1 (ATG16L1)

ATG16L1 is crucial for autophagy in that, it sets the platform for recruitment of the ATG12-ATG5 complex to the immature phagophore membrane, which in turn permits progression, and closure of the membrane to form the autophagosome.[253] ATG16L1 is ubiquitously expressed. A non-synonymous SNP in the *ATG16L1* gene has been associated with increased risk for developing CD and specifically ileal CD.[35-37] The presence of the *ATG16L1* gene variant renders ATG16L1 susceptible to cleavage by Caspase 3/7 released during cellular/metabolic stress, death receptor ligation, or bacterial infection, resulting in a reduction in full-length, functional ATG16L1 (Figure 1.6).[254,255] The association between the *ATG16L1* gene variant and CD has been replicated in more than 50 patient cohorts and the gene variant has been associated with defects in autophagy[256] and increased inflammatory responses.[257] The *ATG16L1* CD-associated SNP, rs2241880, is common in the population, and about 33.2% of people from Western European descent are homozygous for the gene variant.[258] Individuals homozygous for the *ATG16L1* CD risk allele (GG) have an odds ratio (which is a measure of association of a disease between an exposed and a non-exposed group) of 2.38 (95% confidence interval 1.40-4.04),while heterozygotes (AG) have an odds ratio of 1.86 (95% confidence interval 1.09-3.24),[35,259] suggesting that the *ATG16L1* CD risk allele increases the risk of developing CD. The *ATG16L1* gene variant is denoted as T300A and codes for a threonine (T) to alanine (A) amino acid substitution at position 300.[36]

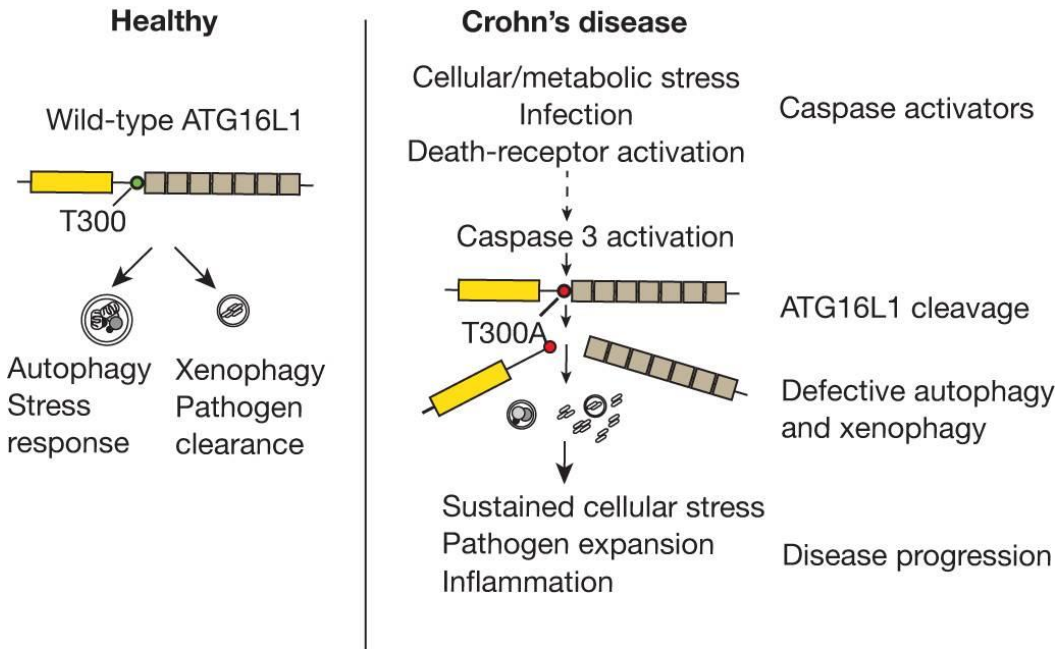

Figure 1.6 The ATG16L1 T300A-encoding gene variant predisposes people to CD
In the absence of the T300A-encoding variant, ATG16L1 is fully functional, and facilitates bacterial clearance during autophagy. The substitution of Alanine (A) for Threonine (T) at position 300 in the protein creates a cleavage site on ATG16L1 for caspase 3/7. ATG16L1 cleavage causes a defect in autophagy and xenophagy (bacterial clearance by autophagy) in the gut, and chronic inflammation. Reproduced with permission of Nature Publishing Group: Murthy A et al, Nature 2014.[254,255]

ATG16L1 deficiency in mice or homozygosity for the *ATG16L1* CD susceptibility gene variant in humans causes defects in Paneth cell granule exocytosis,[80] which may be important in susceptibility to disease in response to specific infections and/or environmental influences.[73] Moreover, CD patients, who are homozygous for the *ATG16L1* CD susceptibility SNP, are unable to adequately clear pathosymbionts and monocytes from subjects with the ATG16L1 T300A-encoding gene variant have impaired ability to clear invading pathogens.[260] In humans, defects in autophagy and the *ATG16L1* gene variant have also been associated with increased IL-1β production.[254,255,261]

1.5.3 Autophagy protein 5 (ATG5)

ATG5 is an autophagy-related protein that forms a complex with ATG12 and ATG16L1 and provides a platform for the formation of the autophagosome.[245] ATG5 plays a second role, regulating anti-viral responses. As part of the autophagy protein complex, ATG5 enhances autophagic clearance of viruses[262] and antigen presentation, [263] whereas the ATG5-ATG12 complex promotes viral replication by negatively regulating type I IFN production.[264-266] The *ATG5* SNP, rs510432, enhances ATG5 promoter activity and, together with the *ATG5* rs12201458 SNP, has been associated with childhood asthma.[267] Besides, *ATG5* mRNA expression is increased in nasal epithelial cells of asthma patients, [267] suggesting that the *ATG5* SNPs are associated with increased expression of *ATG5*. Despite its central role in autophagy, it is not known if the *ATG5* SNPs cause defects in autophagy.

1.5.4 Immunity-related GTPase family M (IRGM)

The immunity-related GTPase family M (IRGM) localizes to the mitochondria and regulates the initial phase of anti-bacterial autophagy and cellular homeostasis.[53,268-270] IRGM has been implicated in the regulation of AIEC, [271] *Salmonella* sp serova-typhimurium, and *Mycobacterium tuberculosis* infections[37,53] by autophagy. Three synonymous SNPs, rs13361189 rs4958847, and rs7714584, within the human *IRGM* locus around chromosome 5q33.1 have been shown to alter expression levels of IRGM[272] and disrupt autophagy.[53,270] The *IRGM* SNPs are associated with increased risk of developing CD, with individuals carrying the risk alleles for the rs13361189, rs4958847, and rs7714584 SNPs having an OR of 1.38, 1.36, and 1.37 respectively, suggesting that the presence of these SNPs increase the risk for developing CD.[38,272] In CD patients harboring the *IRGM*

CD-associated genetic variant, miR-196 expression is increased and this down regulates *IRGM* expression, resulting in decreased autophagy in the inflamed intestinal mucosa.[273]

1.5.5 Nucleotide-binding oligomerization domain containing 2 (NOD2)

The nucleotide-binding oligomerization domain 2, NOD2 (also known as CARD15), is an intracellular PRR, expressed in macrophages, DCs, intestinal epithelial cells,[274] and T cells.[275] NOD2 is activated by a component of bacterial peptidoglycan, muramyl dipeptide (MDP),[276,277] or by viral single stranded RNA (ssRNA),[278] which causes activation of the NFκB pathway or the transcription factor, interferon regulatory factor 3 (IRF3), respectively. Polymorphisms in *NOD2* were the first SNPs to be associated with CD.[32] These CD-associated gene variants in *NOD2* are located in the coding region of the gene and are localized in the leucine-rich-repeat (LRR) region of NOD2.[32] The odds ratio for CD in the presence of these SNPs is 2-4 for heterozygotes and 20-40 for homozygotes.[32,33,279,280] In the presence of the CD-associated *NOD2* variants, NFκB activation is reduced in response to stimulation with MDP.[281] Along these lines, TLR2-induced activation of NFκB is inhibited by the CD-associated *NOD2* variants.[282] Ileal CD patients with the *NOD2* variants have been shown to express reduced levels of α-defensins in their Paneth cells.[283] During bacterial infection, NOD2, which is localized within the cell, recruits ATG16L1 to the plasma membrane at the site of bacterial entry to initiate autophagic degradation of the bacteria.[284] In dendritic cells, the degraded bacterial antigens are processed and presented through major histocompatibility complex (MHC) class II to activated CD4+ T cells.[256] In all, the CD-associated *NOD2* SNPs result in defective autophagic processing of intracellular bacteria, which is believed to account for their contribution to intestinal inflammation.

1.5.6 Endoplasmic reticulum stress and autophagy

Accumulation of unfolded or misfolded proteins within the ER causes ER stress.[285,286] In response to ER stress, cells initiate the unfolded protein response (UPR), a process that facilitates the folding, processing, export, and degradation of proteins emanating from the ER during stress conditions.[287,288] Autophagy is also induced downstream of ER stress as an alternative pathway for degradation of misfolded proteins.[289-291] Hence, it is evident that autophagy and ER stress may interact at various levels to regulate the cellular stress response. Two CD susceptibility SNPs in genes encoding proteins required for the UPR have been identified, X-box binding protein 1 (XBP1) and orosomucoid 1-like 3 (ORMDL3*)*.[49] It is not yet known if they cause defects in autophagy.

1.5.6.1 X-box binding protein 1 (XBP1)

The X-box binding protein 1 (XBP1) is a transcription factor that regulates expression of genes crucial for the functioning of the ER, the immune system, and the UPR.[292,293] TLR 2, 4, and 5 agonists activate XBP1, enhancing sustained production of inflammatory mediators IL-6 and TNFα.[294] *XBP1* deficiency in mice leads to spontaneous intestinal inflammation with impaired Paneth cell function, reduced goblet cell number, and increased ER stress, and increased pro-inflammatory responses to microbial pathogens.[288] Non-synonymous SNPs within the *XBP1* gene locus around chromosome 22q12.1 have been associated with increased risk for IBD.[295-297] Carriers of the minor C allele of *XBP1* rs35873774 SNP, which is strongly associated with both CD and UC, have an odds ratio of 0.74,[298] suggesting that this SNP could play a protective role in IBD.

1.5.6.2 Orosomucoid 1-like 3 (ORMDL3)

Orosomucoid 1-like 3 (ORMDL3) is expressed ubiquitously and is localized on the ER membrane where it mediates Ca^{2+} signalling and homeostasis, and regulates protein folding and the UPR.[42,299] The *ORMDL3* rs2872507 SNP located on chromosome 17q21 have been associated with increased susceptibility to asthma and CD.[39,42] Individuals carrying the *ORMDL3* CD-associated risk allele have an OR of 1.14 of developing CD.[39,42] The *ORMDL3* SNP disrupts Ca^{2+} signalling, which causes the accumulation of misfolded proteins, and subsequently ER stress that initiates the UPR.[300,301] In children with the *ORMDL3* SNP, IL-17 secretion is increased in peripheral blood mononuclear cells (PBMCs).[302] All of these studies demonstrate that ORMDL3 is involved in many pathways in the ER that regulate inflammation.

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

1.6.1 Description and function

The src homology 2 domain-containing inositol polyphosphate 5´- phosphatase (SHIP) is a hematopoietic-specific lipid phosphatase that negatively regulates class I phosphatidylinositol 3-kinase (PI3K) activity. SHIP is also expressed in osteoblasts and mesenchymal stem cells.[303,304] The human gene encoding the 145kDa SHIP protein (*INPP5D*) is located at chromosome 2q37.1.[305] Two other SHIP isoforms exist, the 150kDa SHIP2 that is similar in structure and biochemical function to SHIP,[306-308] and the 104kDa sSHIP, which lacks the SH2 domain. SHIP2 is ubiquitously expressed and is seen in high levels in human skeletal muscles, placenta and heart.[309] sSHIP is restricted to murine hematopoietic and embryonic stem cells.[306,310]

PI3Ks are a family of enzymes that are critical in cellular processes including cell growth, differentiation, proliferation, and inflammation.[311,312] These enzymes phosphorylate the 3´position of the inositol ring of phosphatidylinositol-4,5-bisphosphate PI(4,5)P_2 to generate PI(3,4,5)P_3, a critical second messenger. PI3Ks can be grouped into three main classes, class I, II, and III, based on their substrates, molecular structures, and regulation within the cell.[311,313] Class I PI3Ks are heterodimeric enzymes: Class IA is composed of 1 of 5 regulatory subunits, p50α, p55α or p55γ, p85α, p85β, and 1 of 3 catalytic subunits, p110α, p110β, or p110δ; and Class IB is composed of 1 of 2 regulatory subunits, p87 or p101, and the catalytic subunit, p110γ. p110α and p110β are ubiquitously expressed whereas p110γ and p110δ are mainly restricted to hematopoietic cells.[312] PI3Kp110 catalytic subunits have overlapping as well as unique functions downstream of specific receptor tyrosine kinases, growth factor, cytokine, and TLRs.[314] Class II PI3K is membrane bound, usually activated by tyrosine kinases and integrins,[315,316] and is involved in cell migration.[317] Class III PI3K consists of a single catalytic subunit Vps34 and a regulatory subunit Vps15, and has been implicated in autophagy.[318] Class II PI3K catalyzes the phosphorylation of PI and PIP to PI(3)P and PI(3,4)P 2,[318] whereas class III PI3K only catalyzes the production of PI(3)P from PI.[313]

1.6.2 SHIP enzymatic activity

To exert its action, SHIP is translocated from the cytoplasm, where it resides, to the site of synthesis of PI(3,4,5)P_3 at the inner leaflet of the cell membrane through association with adaptor and scaffold proteins and/or direct binding of its SH2 domain. SHIP antagonizes class I PI3K signalling by dephosphorylating the 5' position of the inositol ring

to form PI(3,4)P_2 (Figure 1.5).[305] PI(3,4,5)P_3 recruits serine-threonine kinases, such as the serine/threonine protein kinase, Akt, and PDK1 to the plasma membrane,[311] driving cellular processes, such as growth, proliferation, differentiation, and immune activation.[319] SHIP therefore blocks recruitment of Akt and PDK1; and inhibits downstream cellular processes, such as cytokine production and inflammation (Figure 1.7).[320]

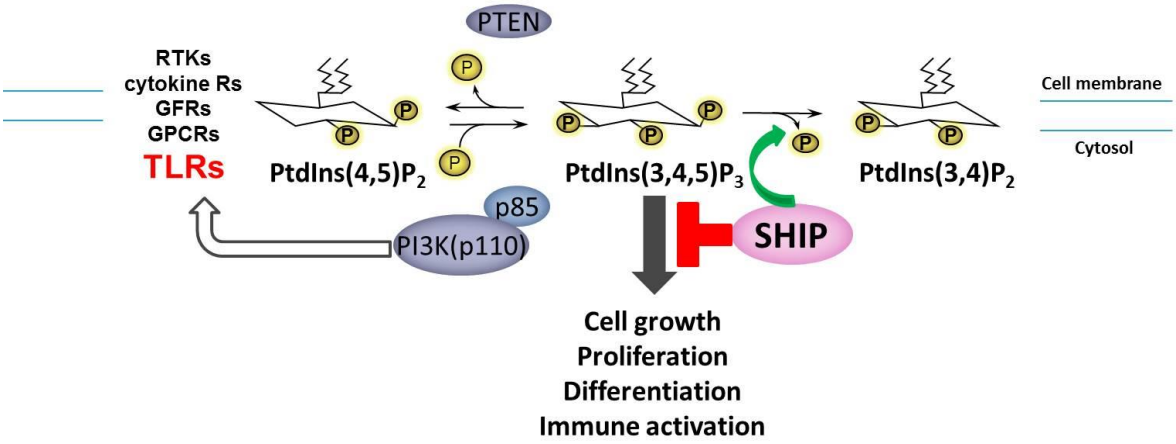

Figure 1.7 SHIP negatively regulates the immune response
Ligation of receptor tyrosine kinases (RTKs), cytokine receptors (cytokine Rs), growth factor receptors (GFRs), G protein-coupled receptors (GPCRs), and TLRs activate Class I PI3K, which is comprised of a p110 catalytic and a p85 regulatory subunit. 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)P2, and blocks cellular processes, such as growth, proliferation, differentiation, and immune activation. PTEN reverses the action of class I PI3K dephosphorylating PI(3,4,5)P3 to PI(4,5)P2.

In addition, SHIP has also been shown to promote activation of Akt via production of PI(3,4)P_2,[321,322] suggesting that SHIP can have either an inhibitory or enhancing role in cell signalling and inflammation. SHIP also regulates cell signalling events independent of its enzymatic activity, by binding to DNAX-activating protein of 12kD (DAP12), blocking

TREM2- (Triggering receptor expressed on myeloid cells 2) and DAP12-induced signalling in macrophages, and osteoclasts.[323]

SHIP can be regulated either at the level of transcription or post-transcriptionally.[324] Transcriptionally, Activin and TGFβ up regulate SHIP mRNA expression in both human and mouse cells,[325] while microRNA-155 (miR-155) negatively regulates SHIP expression during physiological conditions through direct 3′ UTR interaction.[326] SHIP protein is up regulated by LPS/CpG-induced TGFβ via SMAD4,[327] while SMAD7, which blocks TGFβ activity by competing locally with SMAD2/3 for TGFβ receptor,[328] may reduce SHIP expression. On the other hand, miR-155-induced reduction in SHIP protein levels result in increased pro-inflammatory cytokine production.[329] Post transcriptionally, IL-4 induces SHIP protein degradation in macrophages.[330] Studies have also shown that, tyrosine phosphorylation of SHIP, reduces SHIP protein levels through polyubiquitination and proteasomal degradation.[330]

1.6.3 The SHIP deficient mouse

The SHIP deficient (SHIP-/-) mouse was developed in 1998 by deleting the first exon of SHIP.[331] These mice are relatively smaller in size than their wild type counterparts because they fail to thrive, they have asthmatic-like lungs, and reduced lifespan, and suffer from splenomegaly and myeloproliferative disorder (characterized by abnormal growth of blood cells; red blood cells, white blood cells, and platelets).[331] Moreover, SHIP-/- mice have increased numbers of Gr-1^{+}Mac-1^{+} myeloid suppressor cells[332] and reduced B cell numbers[333] compared to SHIP+/+ mice.

SHIP-/- macrophage responses to microbial pathogens vary, and depend on whether they are derived *in vivo* or *in vitro*. *In vivo* derived SHIP-/- peritoneal and alveolar macrophages are profoundly M2 skewed,[334] and are hyper-responsive to cytokine, growth factor, and chemokine stimulation compared to SHIP+/+ macrophages.[331,335] These macrophages constitutively express high levels of the M2 markers, Arginase I (Arg I) and Ym1.[334] When stimulated with LPS, SHIP-/- peritoneal and alveolar macrophages secrete low levels of IL-6, TNFα, IL-12, and nitric oxide (NO), and high levels of the anti-inflammatory cytokines IL-10 and TGF-β,[334] confirming their M2 properties. In contrast, *in vitro* derived SHIP-/- bone marrow macrophages (BMMs) produce high levels of TNFα, IL-6, IL-1β, and NO when stimulated with LPS.[327] Studies have shown that, SHIP-/- BMMs derived *in vitro* in standard culture medium (MCSF), develop an M1 phenotype whereas, when these macrophages are derived in standard culture medium in the presence of mouse or human serum, IL-10, or TGFβ, or when derived in GM-CSF or IL-3, they develop an M2 phenotype.[334] GM-CSF and IL-3 skew SHIP-/- macrophages to an M2 phenotype because they act on basophils, which use STAT5 to drive transcription of IL-4.[336] IL-4 in turn acts through type I/II receptors on macrophages, leading to phosphorylation, dimerization, and translocation of STAT6 to the nucleus, where it drives transcription of STAT6 responsive genes, and skews macrophages to an M2 phenotype.[336]

1.6.4 The SHIP-/- mouse model of Crohn's disease-like intestinal inflammation

SHIP-/- mice develop spontaneous CD-like ileal inflammation beginning at the age of 4 weeks.[337] Inflammation, which is present in all SHIP-/- mice from the age of 6 weeks onwards is restricted to the distal part of the ileum and is characterized by increased

Th2/Th17 cytokines, massive granulocyte infiltration, increased collagen deposition, and fibrosis, thickened muscularis, goblet cell hyperplasia, and the presence of immune cell aggregates that resemble poorly formed granulomas.[337,338] There is a paucity of T cells in the inflamed mucosa of SHIP-/- mice, suggesting that T cells might not play an important role in onset of inflammation.[338] Furthermore, both arginase-1 expression and activity are increased in the SHIP-/- mouse ilea compared to their wild type littermates.[337]

1.6.5 The role of SHIP in inflammation

The role of SHIP in inflammation has been studied extensively in mice, with very limited studies done in humans. SHIP has been shown to play a critical role in regulating immune homeostasis. It acts in concert with other negative regulators of inflammation, such as the interleukin 1 receptor-associated kinase M (IRAK-M), Suppressor of cytokine signalling proteins 1 and 3 (SOCS1 and SOCS3), and the spliced variant of myeloid differentiation primary response gene (MyD88s), to promote endotoxin tolerance.[327] SHIP has been shown to play a critical role in mast cell biology in that, it sets the threshold,[339] and reduces adhesion, degranulation, and cytokine production by mast cells.[340] Moreover, SHIP enhances neutrophil apoptosis,[341] decreases B cell proliferation, chemotaxis, and activation.[342-344] Furthermore, SHIP-/- B cells have reduced FcRγIIB inhibitory signals.[342] SHIP has also been shown to be involved in NK cell physiology. Studies have shown that, SHIP inhibits FCγRIII signalling[345] and promotes NK cell effector function by enhancing IFNγ production.[346] In addition, SHIP promotes T cell survival and maintains innate immune balance at mucosal surfaces.[347] SHIP deficient mice have reduced CD4+ and CD8+ T cell numbers in the small intestine.[338] Moreover, myeloid cells from SHIP deficient mice are

hyper-proliferative,[331] and when recruited to the intestinal mucosa secret numerous inflammatory mediators and proteolytic enzymes that promote tissue destruction and fibrosis.[348] Regulation of SHIP expression levels and activity could therefore be an important strategy to limit chronic inflammation.

In humans, SHIP mRNA expression has been shown to positively correlate with both *FOXP3* and *IL-10* mRNA expression, suggesting that, SHIP may be an important regulator of Treg function.[349]

1.7 Thesis hypothesis and objectives

1.7.1 Summary of rationale

SHIP negatively regulates NFκB transcription and pro-inflammatory cytokine production. As such, SHIP-/- macrophages are hyper-responsive to immune stimuli including those found on commensal microorganisms in the gastrointestinal tract, such as LPS, and secrete high levels of IL-6 and TNFα.[314,350] Moreover, SHIP-/- mice develop spontaneous CD-like intestinal inflammation that is restricted to the distal ileum.[323,337] Il-1β is a critical mediator of pro-inflammatory cytokine and chemokine production since it acts on cells in an autocrine fashion to amplify its own production[351,352] and drives auto-inflammation.[170] The goal of Chapter 2 was to investigate the role of SHIP in macrophage IL-1β production. I also determined whether depleting intestinal macrophages was sufficient to prevent intestinal inflammation in the SHIP-/- mouse. Furthermore, I used an IL-1 receptor antagonist, Anakinra, to block IL-1 signalling to determine its role in intestinal pathology in SHIP deficient mice.

In humans SHIP mRNA expression is increased in inflamed colonic biopsies from people with ulcerative colitis and colonic CD, but strangely, not in ileal biopsies from subjects with ileal CD.[349] The human gene encoding SHIP protein (*INPP5D*) is located at chromosome 2q37.1 and is upstream of the gene encoding ATG16L1.[338] The goal of Chapter 3 was to determine whether SHIP mRNA expression and activity were altered in CD subjects compared to control subjects, who did not have IBD. I also determined whether SHIP mRNA expression and activity were regulated by ATG16L1, and if SHIP may contribute to increased IL-1β production in subjects with the *ATG16L1* risk variant.

Humans carrying the *ATG16L1, NOD2, IRGM, or XBP1* CD gene variant have increased susceptibility to CD, and macrophages from subjects homozygous for the *ATG16L1* T300A-encoding gene variant have increased ability to produce IL-1β.[261] To clearly understand the role of autophagy in regulating SHIP expression and IL-1β production, I genotyped healthy control subjects for five autophagy and CD-related genetic variants, and determined their effects on IL-1β and other pro-inflammatory cytokine production in PBMCs in response to different PRR ligands that are present on commensal bacteria in the gut.

1.7.2 Hypothesis and objectives

I hypothesize that SHIP deficiency leads to increased macrophage-derived IL-1β production that may cause, or contribute to, intestinal inflammation in people with CD.
Aim 1 (Chapter 2): To determine the cause of intestinal inflammation in SHIP-/- mice and to examine the role of macrophage-derived IL-1β in intestinal pathology.

Aim 2 (Chapter 3): To determine whether SHIP mRNA expression and activity are regulated by ATG16L1 and autophagy, and how this affects IL-1β production in human cells.

Aim 3 (Chapter 4): To determine the effects of autophagy related CD-associated gene variants on IL-1β and other pro-inflammatory cytokine production in human cells.

1.7.3 Significance

These studies contribute to our understanding of the mechanisms by which ATG16L1/autophagy regulate IL-1β production and intestinal auto-inflammation, by suggesting that this may occur via SHIP up-regulation. This work also helps to identify a sub-group of CD patients with a particular genotype (homozygosity for *ATG16L1* T300A-encoding gene variant, or the presence of the *XBP-1* gene variant), who may be amenable to treatment with Anakinra (IL-1Ra). Moreover, these studies suggest that Anakinra could be used as a prophylactic treatment to prevent relapse in CD subjects, who are homozygous for the T300A-encoding gene variant.

Chapter 2: SHIP deficiency leads to increased IL-1β transcription in macrophages and intestinal autoinflammation in mice

2.1 Introduction and rationale

Crohn's disease (CD) is a subtype of inflammatory bowel disease (IBD) characterized by chronic, relapsing and remitting, or progressive inflammation along the gastrointestinal tract.[353] IBD affects up to 1 in 150 people in Canada and incidence of disease is increasing in developed countries.[10,353] Although the etiology of disease remains unknown, current thinking is that CD and IBD occur in genetically susceptible individuals due to an inappropriate initiation or perpetuation of an immune response to intestinal flora.[106,354] Biological therapy, monoclonal antibodies directed against the pro-inflammatory cytokine TNFα, is effective at inducing and maintaining remission in patients and has revolutionized the treatment for CD.[355] Despite that, some patients are refractory to biological therapy and for others; biological therapy becomes ineffective because they develop antibodies to the drug.[138,356]

The pro-inflammatory cytokine, IL-1β, acts as an alarm cytokine, initiating the inflammatory response, thus its production is tightly regulated by a two-step process. In the first step, toll-like receptor (TLR) or endogenous ligands induce transcription of *IL1B*, which is translated to pro-IL-1β, an inactive precursor that resides in the cell cytosol. In the second step, numerous stimuli, typically danger associated molecular patterns, lead to assembly of the inflammasome, a hetero-oligomeric protein complex. This complex includes one of several NOD-like receptors (NLRs), the apoptosis-associated speck-like protein (ASC) adaptor, and the zymogen, pro-caspase-1, which is cleaved, activated, and catalyzes the

processing of pro-IL-1β, leading to its activation and secretion.[190-192] Monogenic gain of function mutations leading to increased IL-1β production cause a group of autoinflammatory diseases known as periodic fever syndromes, which can be treated with anakinra, an IL-1 receptor antagonist.[190,353] Intestinal inflammation is a common complication of canonical monogenic autoinflammatory diseases as well as primary immune deficiencies characterized by increased IL-1β production.[353,357-359] IL-1β antagonism has been used effectively to treat some genetically defined forms of very early onset IBD[360] and may be more broadly applicable for the treatment of sub-groups of IBD.

Based on previous findings from our group and others showing that SHIP-/- mice developed spontaneous CD-like intestinal inflammation, I investigated the cause of intestinal inflammation in SHIP$^{-/-}$ mice and determined the role and contribution of SHIP deficient macrophages to pathology.

2.2 Materials and methods

Mice. Mice heterozygous for SHIP expression (*Inpp5d*$^{+/-}$) and derived from an F2 generation of C57BL/6 X 129Sv mice were used to generate SHIP+/+ and SHIP-/- littermates for experiments. The mice were maintained in sterilized filter-top cages and were housed in the Animal Research Centre at the Child & Family Research Institute, which is specific pathogen- and *Helicobacter*-free. Mice used for experiments were between 4 and 8 weeks of age and were fed with autoclaved food and water under specific pathogen-free conditions. All experiments were performed in accordance with institutional and Canadian Council on Animal Care guidelines.

Subjects with Crohn's disease and control subjects. Experiments were performed in accordance with ethical guidelines and with approval by the University of British Columbia Research Ethics Boards (protocol number H09-01826). Subjects seen in the Division of Gastroenterology at BC Children's Hospital were recruited into the study. No subjects had been previously diagnosed with, or treated for, IBD or other inflammatory pathology. Four ileal and four colonic biopsies were taken from sites of inflammation that were adjacent to tissues harvested for pathological assessment in subjects with CD. Biopsies were taken from comparable, uninflamed sites in subjects, who were not subsequently diagnosed with IBD. Peripheral blood was taken from the site of intravenous insertion during colonoscopy. Diagnosis of CD with ileal inflammation or no disease was based on pathological assessment and colonoscopy. Eight subjects diagnosed with ileal CD (no colonic involvement) and 14 subjects, who did not have IBD, were included in analyses. Biopsies were fixed for H&E staining or used immediately for analyses. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque^{TM} PLUS (GE Healthcare, Piscataway, NJ). PBMCs were washed and resuspended at 0.5×10^6 cells/mL in IMDM/10% FBS for assays.

Macrophage derivation and isolation. Bone marrow macrophages (BMMϕs) were generated by flushing out bone marrow aspirates from femura and tibiae of SHIP+/+ and SHIP-/- mice. Aspirates were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum (FBS) in 75 cm^2 Falcon flasks (BD Biosciences) for 1 hr. Following adherence depletion, bone marrow aspirates were then resuspended in IMDM, 10% FBS, and penicillin/streptomycin at a concentration of 0.5×10^6 cells/mL for 10 days in the presence of 10 ng/mL monocyte colony-stimulating factor (MCSF), granulocyte-

monocyte colony-stimulating factor (GM-CSF), or interleukin-3 (IL-3) (StemCell Technologies, Vancouver, BC), with complete media changes at day 4 and 7. BMMϕ cultures were >95% Mac-1^+ and F4/80^+ after 10 days in culture. Peritoneal macrophages were isolated by lavaging the peritoneal cavity with 3×5 mL of complete medium containing 10 ng/mL MCSF. To isolate ileal macrophages, *lamina propria* cells were prepared from whole mouse ilea, as follows: longitudinal sections of SHIP+/+ and SHIP-/- ilea were cut and rinsed with a solution of phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS), to wash off luminal contents. Ileal sections were further cut into smaller sections and washed for 3 x 15 min each, after which, tissue were shaken at 37^{o}C for 1.5 h in a solution of PBS containing 5% FCS and 2mM ethylenediaminetetraacetic acid (EDTA). The solution was replaced every 20 min. To remove EDTA, tissue sections were washed 2 x 5 min with wash solution. Next, tissue was incubated in collagenase digestion media comprised of 20 ml RPMI (Roswell Park Memorial Institute medium), 5% FCS and 1 mg/ml collagenase (Worthington, New Jersey) at 250 rpm for 40 min at 37^{o}C. Digested fractions were pooled and cells were selected using the mouse monocyte enrichment kit (StemCell Technologies). Macrophage populations were ≥ 95% F4/80^+ and Mac-1^+.

Cell stimulations. Cells were plated at a density of 0.5×10^6 cells/mL and stimulated with 10 ng/mL of LPS (*E. coli* serotype 127:B8, Sigma Aldrich, St. Louis, MO) for 5 h, 5 mM ATP for 1 h, or LPS for 5 h with addition of ATP for the final 1 h. After incubation, cell supernatants were harvested and clarified by centrifugation for analysis. Inhibitors were added to cultures 30 min prior to addition of LPS or 30 min prior to addition of ATP, where indicated. Commercially available inhibitors, controls, and final concentrations of each

were: glybenclamide (100 μM, Sigma Aldrich), Z-YVAD-fmk (40 μM, Sigma Aldrich), LY303511 (14 μM, Calbiochem, San Diego CA), LY294002 (14 μM, Calbiochem), DMSO (dimethyl sulphoxide) (0.1%), and wortmannin (50 nM, Calbiochem). Isoform-specific class I PI3K inhibitors were synthesized by our collaborator as described previously and used at a concentration of 10 μM.[361,362]

Cytokine measurements. Cytokine measurements were performed on clarified full thickness tissue homogenates from mice or cell-free tissue culture supernatants using the Mouse Cytokine Array Panel A kit (R&D Systems; Minneapolis, MN) or by enzyme-linked immunosorbent assay (ELISA). During an ELISA, cell-free supernatants are incubated with a primary antibody (capture antibody) in an ELISA plate to immobilize the antigen (Ag). Next, the Ag-Ab complex is incubated with a secondary Ab (detection Ab) that links to an enzyme. The conjugated enzyme activity is detected by incubation with a substrate solution that produces a signal, which is read as absorbance in an ELISA plate reader. The concentration of the targeted Ag is then determined from the absorbance. For Mouse Cytokine Array, ImageJ version 1.43 and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) were used to quantify spot densities, which were further corrected for individual background to reduce variations. ELISA kits for mouse IL-1β, IL-6, and human IL-β were from BD Biosciences (Mississauga, ON). The mouse IL-18 ELISA kit was from MBL International (Woburn, MA).

Gene Expression Analysis. RNA was prepared from mouse tissue or cells using the NucleoSpin RNA II Total RNA Isolation Kit (Macherey-Nagel, Bethlehem, PA) and reverse

transcribed using Superscript II (Invitrogen, Burlington, ON). Gene expression was measured by quantitative polymerase chain reaction (qPCR) using the AB Applied Biosystems Taqman Universal Master Mix II (Invitrogen). IL-1β (*IL1B*) gene expression was normalized to gene expression for ribosomal protein RPLP0 (*Rplp0*). Primer and probe sequences were:

IL1B forward 5'-ACGGACCCCAAAAGATGAAG-3'

IL1B reverse 5'-TTCTCCACAGCCACAATGAG-3'

IL1B probe 5'-/56-FAM/AGAGCATCC/ZEN/AGCTTCAAATCTCGCA/3IABkFQ/-3'

Rplp0 forward 5'-TGACATCGTCTTTAAACCCCG-3'

Rplp0 reverse 5'-TGTCTGCTCCCACAATGAAG-3'

Rplp0 probe 5'-/56-FAM/TGTCTTCCC/ZEN/TGGGCATCACGTC/3IABkFQ/-3'

Macrophage Depletion. Macrophages were depleted from mouse ilea using clodronate-containing liposomes (clod-lip). 200 μl of Clod-lip or PBS injection controls were administered to SHIP-/- mice intraperitoneally every two days between 4 and 6 weeks of age. Mice were euthanized at 6 weeks of age, ilea were photographed, sections were fixed for histological analyses, and full thickness tissue homogenates were prepared for cytokine analyses.

Anakinra treatment. Anakinra was injected intraperitoneally into mice daily at a dose of 150 mg/kg between 4 and 6 weeks of age. An equal volume of PBS was injected into mice as an injection control. Mice were euthanized at 6 weeks of age, ilea were photographed, sections were fixed for histological analyses, and full thickness tissue homogenates were

prepared for cytokine analyses.

Histological analyses. Ileal and colonic biopsies from human subjects and ileal sections from SHIP+/+ and SHIP-/- mice were fixed in PBS-buffered 10% formalin at 4°C for 24 h. Tissue sections were embedded in paraffin, and 5 μm cross-sections were cut and stained with hematoxylin and eosin (H&E). Images were acquired using a Zeiss Axiovert 200 microscope, a Zeiss AxiocamHR camera, and the Zeiss Axiovision 4.0 software imaging system. Immune cell infiltrates were counted at 20× magnification in six H&E stained sections separated by ≥50 μm, by two individuals blinded to experimental condition. Crypt/villus length (mouse) was determined by counting epithelial cell nuclei from the base of the crypt to the villus tip on uniform horizontal ileal cross-sections. Representative crypt/villi (10 per section) were counted in six H&E stained ileal sections for each mouse.

For macrophage staining, slides were mounted and stained with F4/80. Samples were incubated with 20 μg/mL proteinase K in PBS for 15 min at room temperature and rinsed in Tris-buffered saline with 0.1% Tween 20. Endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide (H_2O_2) in PBS for 10 min and endogenous avidin and biotin were blocked with an avidin-biotin blocking kit, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Rat anti-F4/80 IgG (Immunoglobulin G) (AbD serotec, Oxford, UK) was the primary antibody used. Blocking buffers, biotinylated anti-rat IgG secondary and avidin-biotin-horseradish peroxidase detection complex were prepared and used from immunohistochemistry detection kits according to the manufacturer's instructions (Vector laboratories). Signal was detected with a diaminobenzidine chromogen system (Dako, Carpinteria, CA) and developed sections were counter stained with Harris'

hematoxylin (Sigma, St. Louis, MO). Macrophages were counted at 20× magnification at six points in six H&E stained sections separated by ≥50 μm by two individuals blinded to experimental condition.

For detection of active caspase-1, F4/80 stained slides were co-stained with YVAD-FLICA (a fluorescent labeled irreversible inhibitor of caspase-1) immediately prior to counterstaining with Harris' hematoxylin (ImmunoChemistry Technologies, Bloominton, MN). YVAD-FLICA was resuspended in blocking buffer and tissue sections were stained for 1 h at room temperature in the dark. Tissue sections were thoroughly rinsed and then stained with DAPI (4′ 6-diamino-2-phenylindole) (Invitrogen).

Statistical Analyses. Unpaired two-tailed Student's *t* tests were performed when indicated using GraphPad Prism version 5 software. For multiple comparisons, the Bonferroni correction was applied. Differences were considered significant at $P < 0.05$.

2.3 Results

2.3.1 Ileal macrophages from SHIP-/- mice produce high levels of IL-1β and IL-18

Our laboratory recently reported that SHIP-/- mice develop spontaneous CD-like intestinal inflammation.[337] We found that full thickness tissue homogenates from SHIP-/- mice did not have elevated levels of pro-inflammatory cytokines expected, including IFNγ, IL-12p70, IL-23, and TNFα.[337] However, we noted that protein levels of IL-1 family cytokines, IL-1α, IL-1β, and IL-1ra, were higher in SHIP-/- mice relative to their wild type littermates (Figure 2.1A). Based on this, we measured absolute values of IL-1β and IL-18 in

full thickness ileal tissue homogenates from 8-week-old SHIP+/+ and SHIP-/- by ELISA and found that they were significantly higher in SHIP-/- mice (Figure 2.1B). To investigate the cellular source of IL-1β and IL-18 in ileal tissues, fixed tissue cross sections were co-stained with YVAD-FLICA, which stains active caspase-1, and F4/80, which is a macrophage marker. YVAD-FLICA^{+} cells were found in the sub-epithelial region of the distal tips of villi in SHIP$^{-/-}$ mice and co-stained with F4/80 (Figure 2.1C).

To determine the concentration of LPS and the time point for ATP stimulation, GM-CSF-derived bone marrow macrophages were either stimulated with different concentrations of LPS for 4 hours followed by addition of ATP for 1 hour (left) or were stimulated with LPS for 4 hours followed by addition of ATP at different time points. We found that SHIP-/- macrophages produced significantly more IL-1β compared to wild type macrophages at a high concentration of LPS (10 ng/ml) and when ATP was added after 4 hours (Figure 2.2A).

To determine whether this was a cell-intrinsic effect of SHIP deficiency in macrophages, we isolated ileal macrophages from SHIP+/+ and SHIP-/- mice and stimulated them with LPS (5 h), to induce IL-1β and IL-18 transcription, ATP (1 h), to induced inflammasome activation, or LPS (5h) + ATP (for the last hour), to induce secretion of mature IL-1β and IL-18, and we measured IL-1β and IL-18 in cell culture supernatants. Unlike their wild type counterparts, SHIP-/- ileal macrophages secreted IL-1β and IL-18 in response to LPS alone, consistent with staining for active caspase-1 observed, and SHIP-/- ileal macrophages secreted significantly more IL-1β and IL-18 in response to stimulation with LPS+ATP (Figure 2.2B).

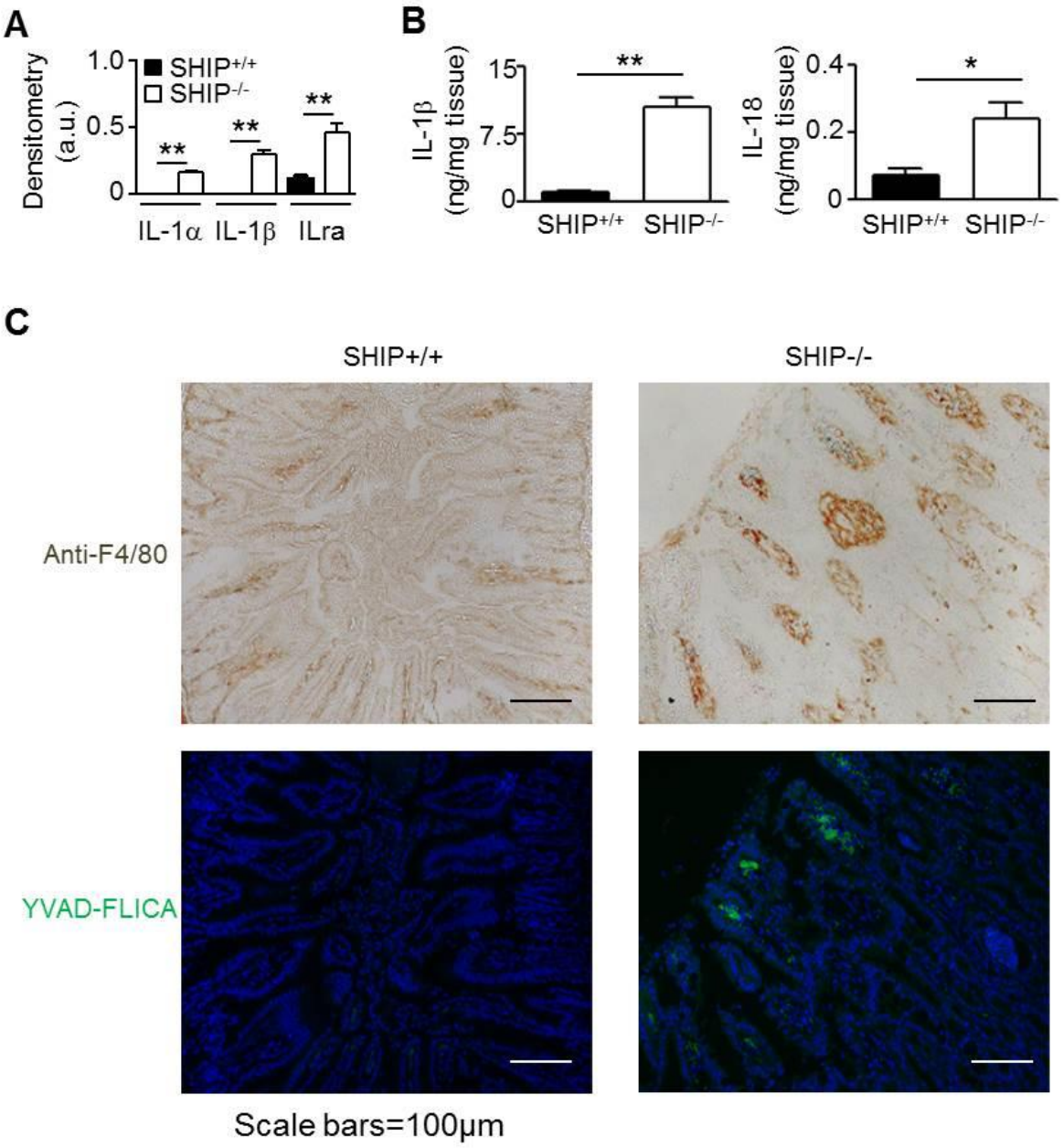

Figure 2.1 IL-1β production is increased in ileal sections from SHIP-/- mice compared to SHIP+/+ mice.

Full thickness ileal tissue homogenates from 8-week-old SHIP+/+ and SHIP-/- mice were assayed for IL-1 family members by cytokine array (A) and by ELISA (B). (C) Fixed ileal sections were co-stained with anti-F4/80 for macrophages and with YVAD-FLICA for active caspase-1. Data are representative of sections from 6 mice/group. Data are means ±SD for n=4 mice/group in (A) and n=6 mice/group in (B). *P<0.05, **P<0.01 (unpaired Student's *t*-test).

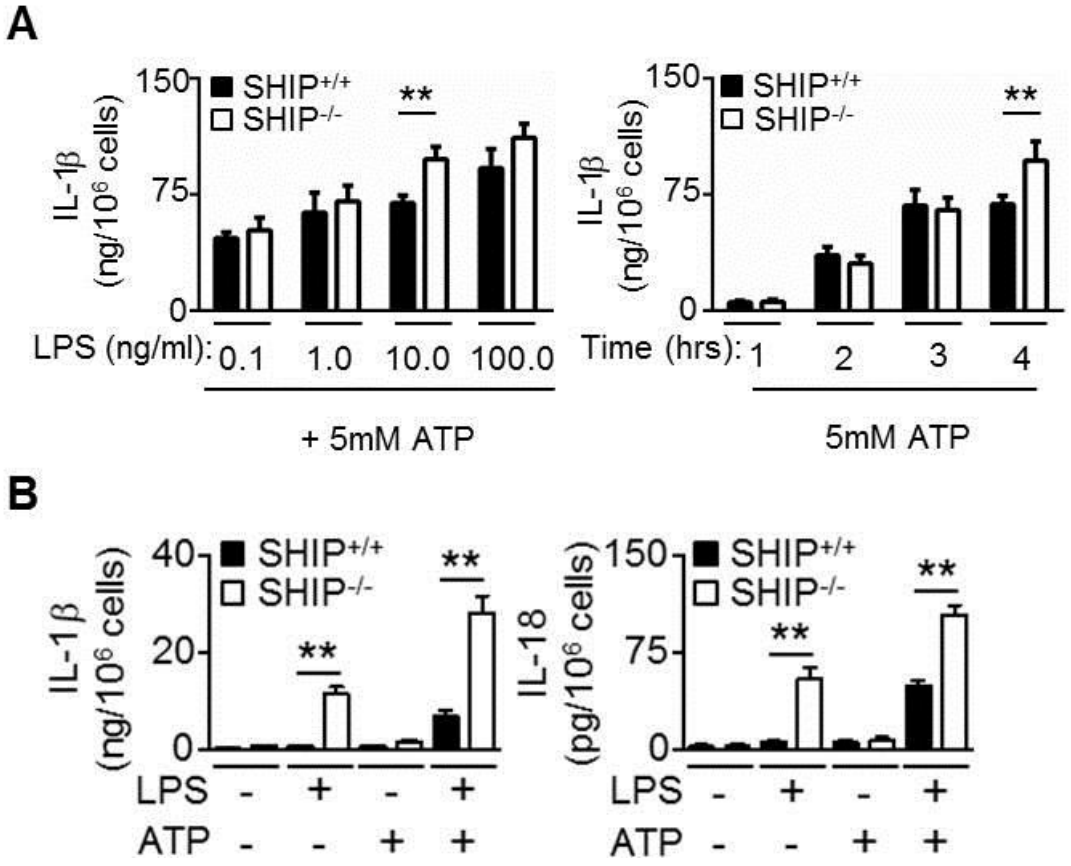

Figure 2.2 SHIP-/- ileal macrophages produce more IL-1β and IL-18 than wild type ileal macrophages.
(A) GM-CSF-differentiated bone marrow macrophages were stimulated with different concentrations of LPS for 4 hours followed by addition of ATP (5mM) for 1 hour (left) or stimulated with 10ng/ml LPS for 4 hours followed by addition of ATP (5mM) at different time points (right). (B) Ileal mononuclear phagocytes were isolated and stimulated with LPS, ATP, or LPS+ATP. Cell supernatants were assayed for IL-1β or IL-18 by ELISA. Data are means ±SD for n=6 mice/group in (A) and (B). **P<0.01 (unpaired Student's *t*-test).

2.3.2 *In vivo* differentiated SHIP-/- macrophages produce high levels of IL-1β and IL-18

We next sought to establish an *in vitro* culture model to investigate the mechanism(s) for increased IL-1β and IL-18 secretion by SHIP-/- macrophages. SHIP+/+ and SHIP-/- bone marrow progenitors were differentiated into macrophages in the presence of macrophage

growth factors, MCSF (macrophage colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), or IL-3. Macrophages were stimulated with LPS, ATP, or LPS+ATP and IL-1β was measured in culture supernatants. Surprisingly, SHIP-/- bone marrow macrophages differentiated in the presence of MCSF or IL-3 did not produce more IL-1β and IL-18 than SHIP+/+ macrophages in response to stimulation. Moreover, although GM-CSF-derived SHIP-/- macrophages produced significantly more IL-1β and IL-18 than those from SHIP+/+ mice, the effect on IL-1β production was modest compared to the differences observed for ileal macrophages (Figure 2.3A). Given that SHIP-/- macrophages may be influenced by the complex inflammatory environment in the SHIP-/- ileum, we treated GM-CSF-derived macrophages from SHIP+/+ and SHIP-/- mice for 3 days with IFNγ, to induce a classically activated phenotype, IL-4, (elevated in SHIP-/- ilea)[337] to induce an alternatively activated phenotype, or IFNγ+IL-4. Resulting macrophages were then stimulated with LPS, ATP, or LPS+ATP. IFNγ and (IFNγ+IL-4)-treated, GM-CSF-derived SHIP-/- macrophages produced significantly more IL-1β than SHIP+/+ macrophages in response to LPS+ATP (Figure 2.3B). However, these *in vitro* culture systems did not replicate the dramatic differences in IL-1β secretion observed comparing SHIP+/+ and SHIP-/- ileal macrophages. Reasoning that differences observed may be due to *in vivo* differentiation in the complex inflammatory environment in the SHIP-/- mouse, peritoneal macrophages were isolated from SHIP+/+ and SHIP-/- mice and IL-1β and IL-18 secretion were assayed in response to stimulation. IL-1β and IL-18 secretion were significantly higher in SHIP-/- peritoneal macrophages compared to SHIP+/+ peritoneal macrophages (Figure 2.4A and B, left). Both peritoneal and bone marrow-derived macrophages from SHIP+/+

and SHIP-/- mice were also stimulated with LPS+ATP in the presence of glybenclamide, to inhibit ATP-induced potassium efflux required for inflammasome activation, or YVAD, to inhibit caspase-1. Glybenclamide and YVAD blocked (LPS+ATP)-induced IL-1β and IL-18 secretion by $SHIP^{-/-}$ macrophages as effectively as in wild type macrophages (Figure 2.4A and B, middle and right panels).

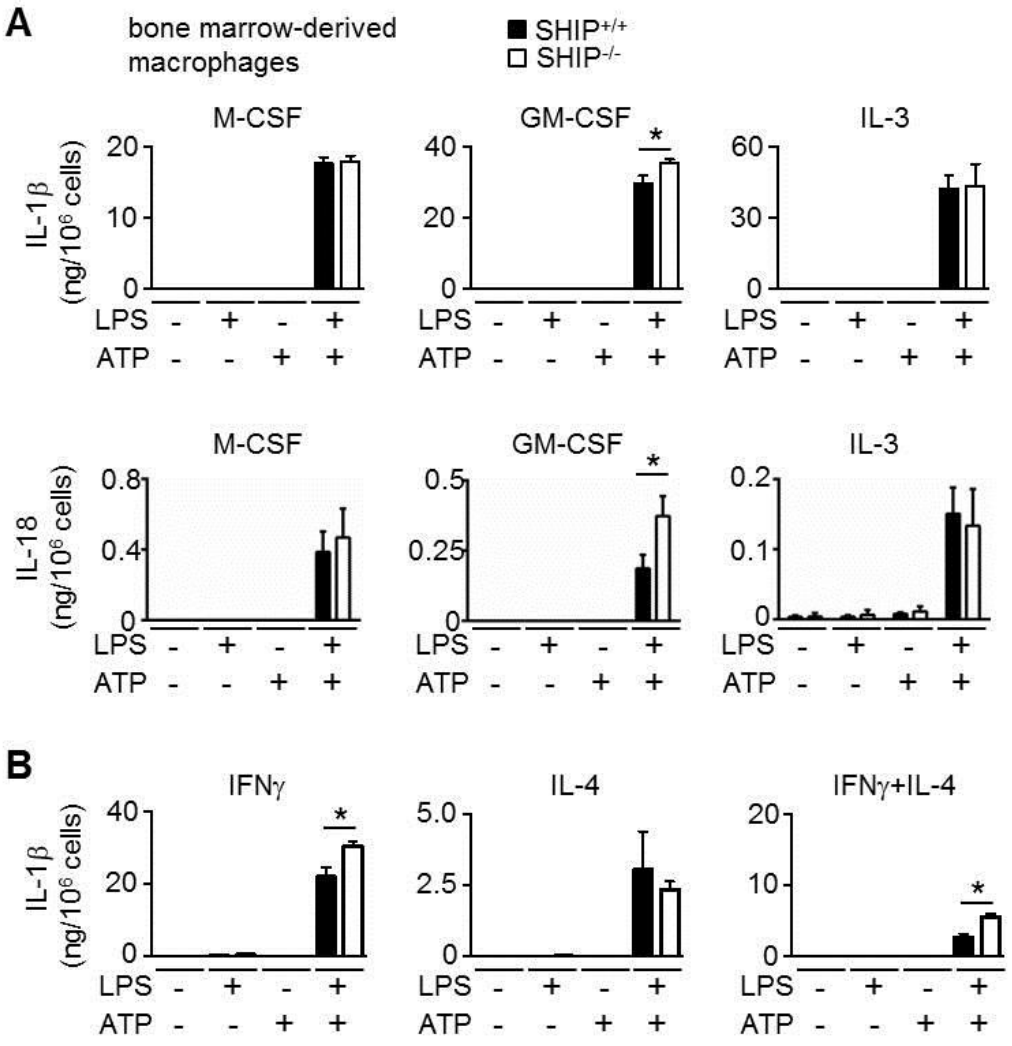

Figure 2.3 ***In vivo*** **differentiated SHIP-/- macrophages produce high levels of IL-1β and IL-18.**

(A) Macrophages were differentiated from SHIP+/+ and SHIP-/- bone marrow for 10 days in the presence of 10 ng/mL of macrophage growth factors, MCSF, GM-CSF, or IL-3. Macrophages were unstimulated or stimulated with LPS, ATP, or LPS+ATP and supernatants were assayed for IL-1β and IL-18 by ELISA. (B) 10-day-old GM-CSF-derived bone marrow macrophages were treated for 3 days with 10 ng/mL of IFNγ, IL-4, or IFNγ+IL-4, washed, and stimulated with LPS, ATP, or LPS+ATP. Supernatants were assayed for IL-1β by ELISA. Data are means ±SD for n=4 in (A) and (B). *P<0.05 (unpaired Student's *t*-test).

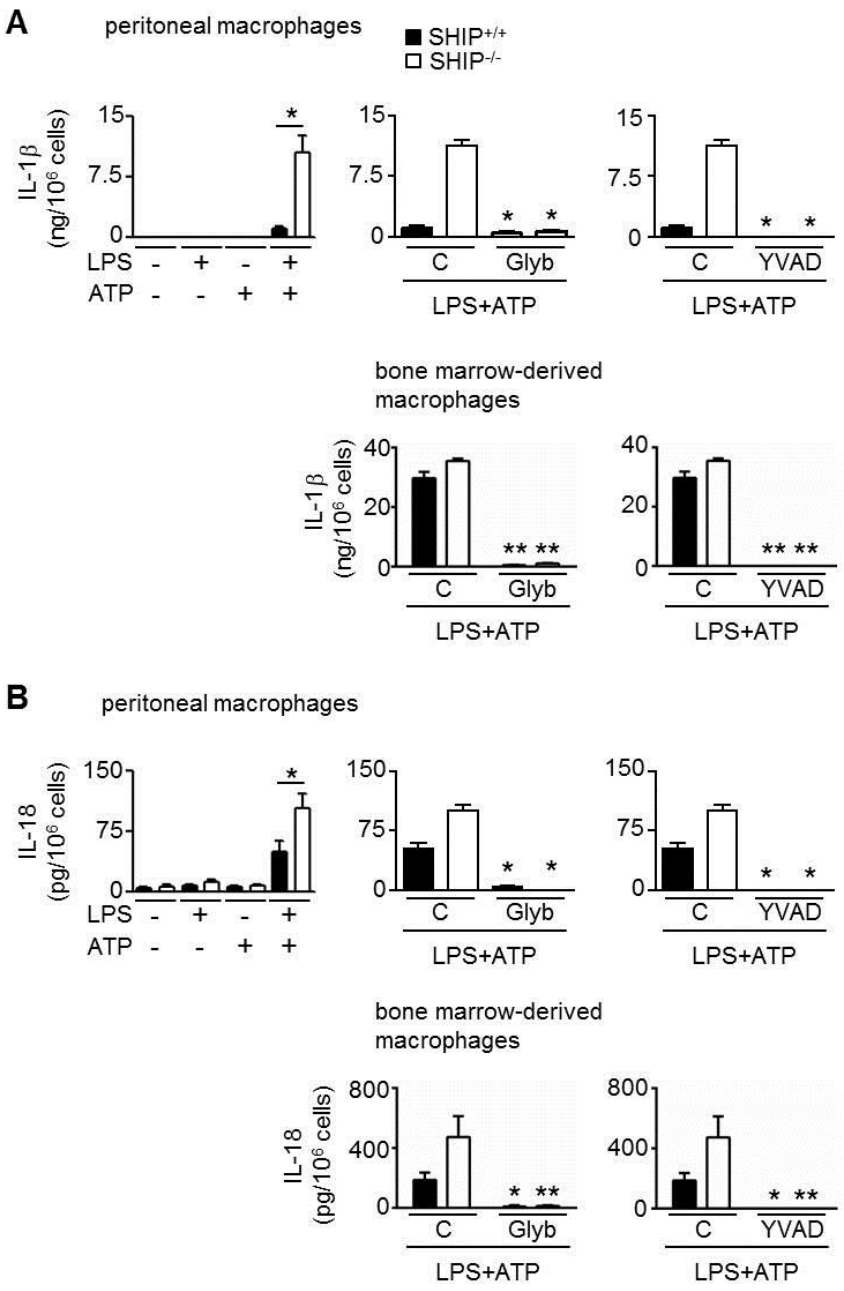

Figure 2.4 IL-1β and IL-18 production are dependent on the NLRP3 inflammasome and caspase-1 activity.

Peritoneal macrophages were harvested from 8-week-old SHIP+/+ and SHIP-/- mice. Macrophages were differentiated from $SHIP^{+/+}$ and $SHIP^{-/-}$ bone marrow for 10 days in the presence of 10 ng/mL of GM-CSF. Cells were stimulated with LPS, ATP, or LPS+ATP (left panels). Cells were also stimulated with LPS+ATP ± glybenclamide (NALP3 inhibitor; middle panels) or YVAD (caspase-1 inhibitor; right panels). Cell supernatants were assayed for IL-1β (A) or IL-18 (B). Data are means ±SD for n=6. *P<0.05, **P<0.01 (unpaired Student's *t*-test).

2.3.3 Class I PI3Kp110α drives transcription of *IL1B*

SHIP is a critical negative regulator of class I PI3Ks. Thus, to investigate the mechanism by which SHIP deficiency increases (LPS+ATP)-induced IL-1β and IL-18 secretion, SHIP-/- peritoneal macrophages were stimulated with LPS+ATP in the presence of pan PI3K inhibitors, LY294002 (LY29) or wortmannin (Wm), or their respective controls, LY303511 (an inactive structural analogue) or DMSO (vehicle control). Inhibitors were added to cultures 30 min prior to stimulation with LPS or 30 min prior to addition of ATP, which is added for the last hour of stimulation to activate the inflammasome. LY29 and Wm blocked production of IL-1β when added to cultures prior to stimulation with LPS but did not block IL-1β production when added to culture prior to addition of ATP suggesting that class I PI3K is required for LPS-induced transcription of *IL1B*, but not for inflammasome activation by ATP (Figure 2.5A). To determine which class I PI3K(s) was required for (LPS+ATP)-induced IL-1β secretion, inhibitors that target specific catalytic isoforms of PI3K, p110α, β, γ, and δ, were added to peritoneal macrophages prior to stimulation with LPS. Class I PI3K p110α inhibitors, PIK-90 and PI-103, reduced (LPS+ATP)-induced IL-1β secretion, whereas inhibitors for other isoforms had no effect (Figure 2.5B). To determine whether SHIP-/- peritoneal macrophages have increased LPS-induced transcription of *IL1B*, SHIP+/+ and SHIP-/- peritoneal macrophages were stimulated with LPS, ATP, or LPS+ATP, and total RNA was harvested for analysis of *IL1B* gene expression. Peritoneal macrophages from SHIP-/- mice had higher gene expression for *IL1B* relative to SHIP+/+ in response to LPS or LPS+ATP (Figure 2.6A, left). In addition, *IL1B* gene expression was elevated in ileal tissues from $SHIP^{-/-}$ mice compared to wild type littermates, where it was not detected (Figure 2.6A,

right). Finally, to determine whether PI3K and specifically PI3Kp110α is required for LPS-induced *IL1B* gene expression, *IL1B* gene expression was measured in SHIP+/+ and SHIP-/- peritoneal macrophages stimulated with LPS in the presence of pan-PI3K inhibitors, LY29 and Wm, PI3Kp110α inhibitors, PIK-90 and PI-103, or controls. As expected, pan- and p110α-specific PI3K inhibitors reduced *IL1B* transcription in peritoneal macrophages from both SHIP+/+ and SHIP-/- mice (Figure 2.6B).

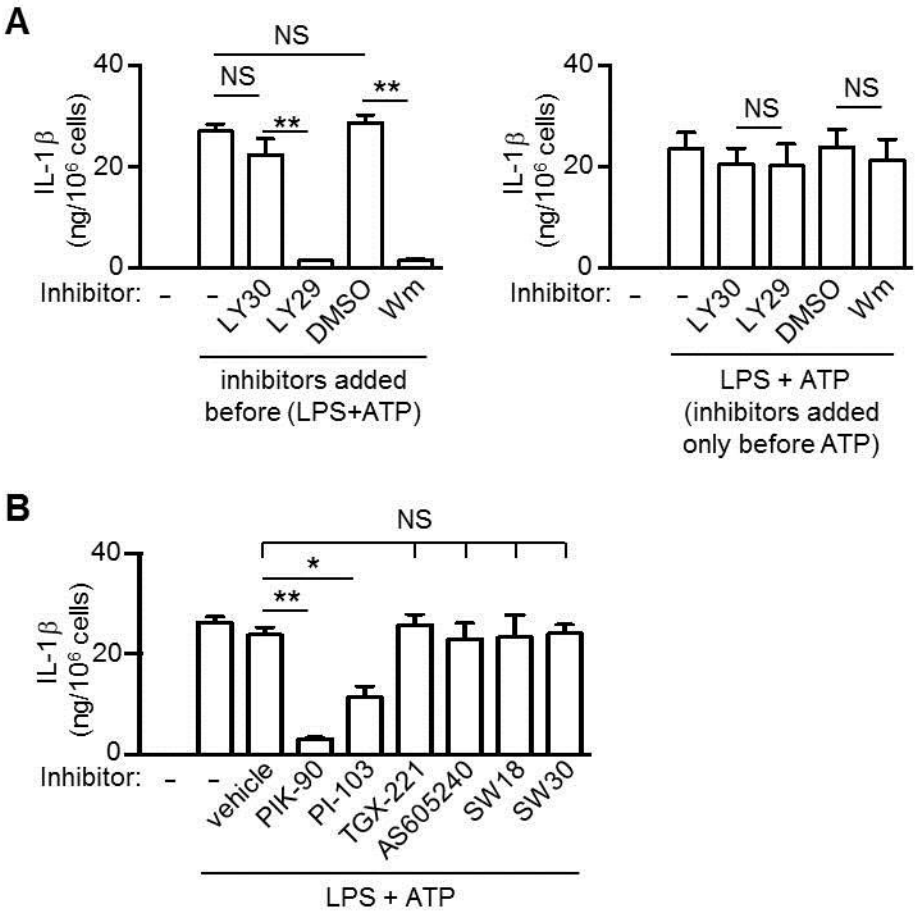

Figure 2.5 IL-1β production is dependent on class I PI3K p110α activity
(A) SHIP-/- peritoneal macrophages were stimulated with LPS for 4 hours followed by addition of ATP for 1 h ± pan-PI3K inhibitor, LY294002 (LY29), its inactive analogue LY305311 (LY30), pan-PI3K inhibitor, wortmannin (Wm), or DMSO, as a vehicle control. PI3K inhibitors or controls were added to cultures prior to LPS (left) or 30 min prior to ATP (right) and cell supernatants were assayed for IL-1β by ELISA. (B) SHIP-/- peritoneal macrophages were stimulated with LPS+ATP ± isoform-specific inhibitors for class I PI3Ks or DMSO (vehicle). IL-1β was assayed in cell supernatants by ELISA. Data are means ±SD for n=6 in (A) and n=4 in (B)-(D). NS=not significantly different, *P<0.01, **P<0.0001 (unpaired Student's *t*-test).

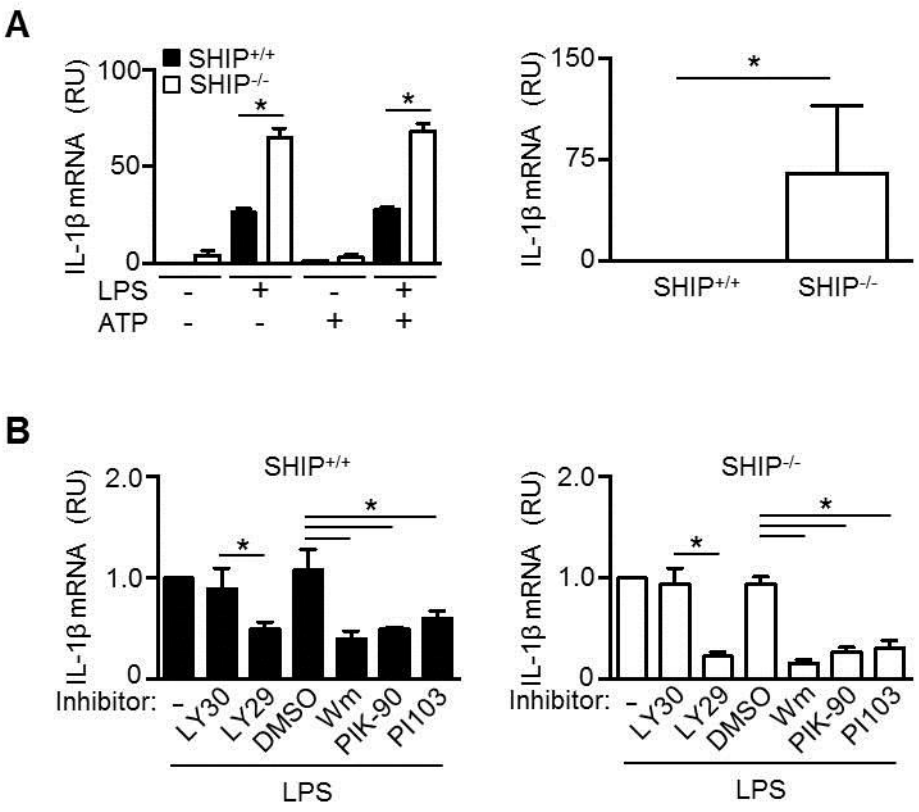

Figure 2.6 SHIP deficiency increases transcription of IL-1β
(A) SHIP+/+ and SHIP-/- peritoneal macrophages were stimulated with LPS, ATP, or LPS+ATP. RNA was isolated from cells and IL-1β transcription was assayed by Q-PCR (left). RNA was isolated from ileal tissues of 6-week-old SHIP+/+ and SHIP-/- mice and IL-1β transcription was assayed by Q-PCR (right). (B) SHIP+/+ (left) and SHIP-/- (right) peritoneal macrophages were stimulated with LPS+ATP ± pan-PI3K inhibitors, LY29 or Wm, or PI3Kp110α isoform-specific inhibitors, PIK-90 or PI-103, or controls, LY30 or DMSO. RNA was isolated from cells and IL-1β transcription was assayed by Q-PCR. Data are means ±SD for n=4. *P<0.01 (unpaired Student's *t*-test).

2.3.4 Macrophage depletion reduces intestinal inflammation in SHIP-/- mice

To understand the contribution of macrophages to intestinal inflammation in SHIP-/- mice, we treated SHIP-/- mice with clodronate-containing liposomes (Clod-lip) to deplete macrophages, or injection controls, for two weeks during the development of disease and assessed pathology in mice after treatment. SHIP-/- mice treated with Clod-lip had reduced

gross and histological pathology (Figure 2.7A). Ileal tissue sections were co-stained with F4/80, for macrophages, and YVAD-FLICA, for active caspase-1, and macrophages were counted in ileal tissue sections. Clod-lip depleted 55±5% of macrophages in the ilea of SHIP-/- mice and eliminated staining for active capase-1 (Figure 2.7B). Macrophage depletion also reduced histological evidence of inflammation including crypt-villus hyperplasia (Figure 2.7C, left) and the number of immune cells in ileal sections (Figure 2.7C, right). Furthermore, macrophage depletion effectively reduced the levels of IL-1β in ileal tissue homogenates (Figure 2.8, left). The level of IL-6, an IL-1β-induced pro-inflammatory cytokine that is up-regulated during intestinal inflammation, was also reduced (Figure 2.8, right).

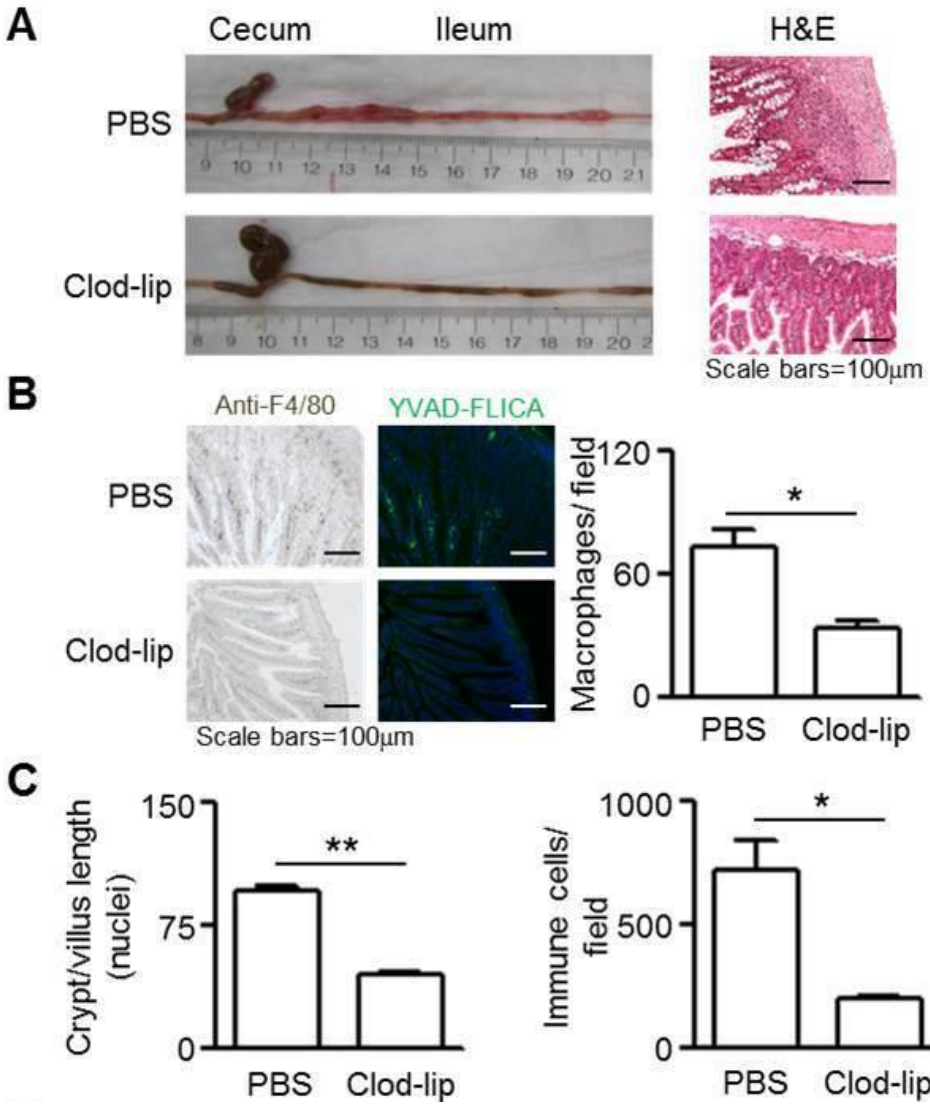

Figure 2.7 Macrophage depletion reduces development of intestinal inflammation in SHIP-/- mice.

SHIP-/- mice were injected intravenously with clodronate-containing liposomes (clod-lip) or PBS, as an injection control, from 4-6 weeks of age. (A) Gross pathology of distal ilea (left) and H&E stained ileal cross-sections (right) of 6-week-old SHIP$^{-/-}$ mice. (B) Ileal cross-sections co-stained with anti-F4/80 (macrophages) and YVAD-FLICA (active caspase-1) (left) and F4/80^{+} macrophages in ileal cross-sections quantitated by microscopy. (C) Crypt-villus length (left) and immune cell infiltration (right) quantitated by microscopy. Photographs in (A) and (B) are representative images from 6 mice/group. In (B)-(C), counting was performed on 6 mice/group counting 6 fields in 6 sections separated by ≥50 μm and by two individuals blinded to experimental condition. Data are means ±SD for n=6. *P<0.01, **P<0.001(unpaired Student's *t*-test).

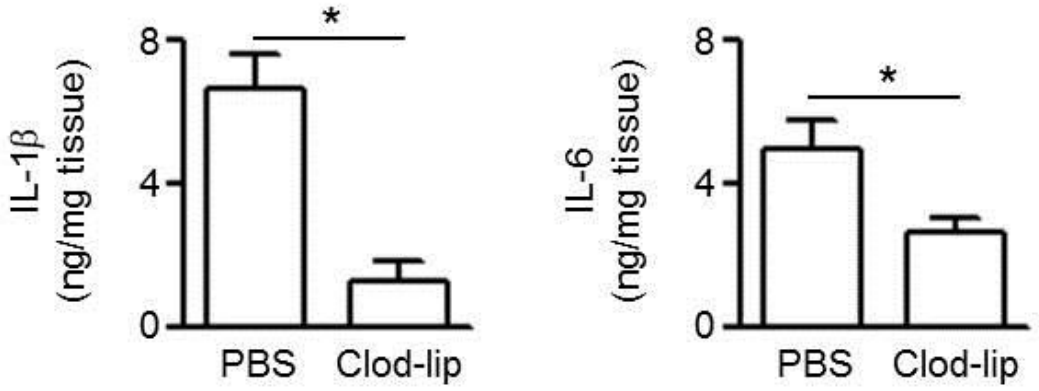

Figure 2.8 Macrophage depletion reduces IL-1β and IL-6 levels in the ileum of SHIP-/- mice.
SHIP-/- mice were injected intravenously with clodronate-containing liposomes (clod-lip) or PBS, as an injection control, from 4-6 weeks of age. IL-1β (left) and IL-6 (right) were assayed in full thickness ileal tissue homogenates by ELISA. Data are means ±SD for n=6. *P<0.01, (unpaired Student's *t*-test).

2.3.5 Anakinra treatment reduces intestinal inflammation in SHIP-/- mice

Anakinra is an IL-1 receptor antagonist that is used to treat autoinflammatory diseases. To determine whether blocking IL-1 signalling could block intestinal inflammation in SHIP deficient mice, we treated SHIP-/- mice with anakinra or PBS, as an injection control. SHIP-/- mice treated with anakinra had reduced gross and histological pathology (Figure 2.9A). Anakinra did not reduce the number of macrophages in the ilea of SHIP-/- mice; however, it eliminated staining for active caspase-1 (Figure 2.9B) and dramatically reduced *IL1B* gene expression in ileal tissues (Figure 2.10A). Anakinra reduced crypt-villus hyperplasia and the number of immune cells in ileal sections (Figure 2.9C). Anakinra also reduced the levels of IL-1β and IL-6 in ileal tissue homogenates (Figure 2.10B).

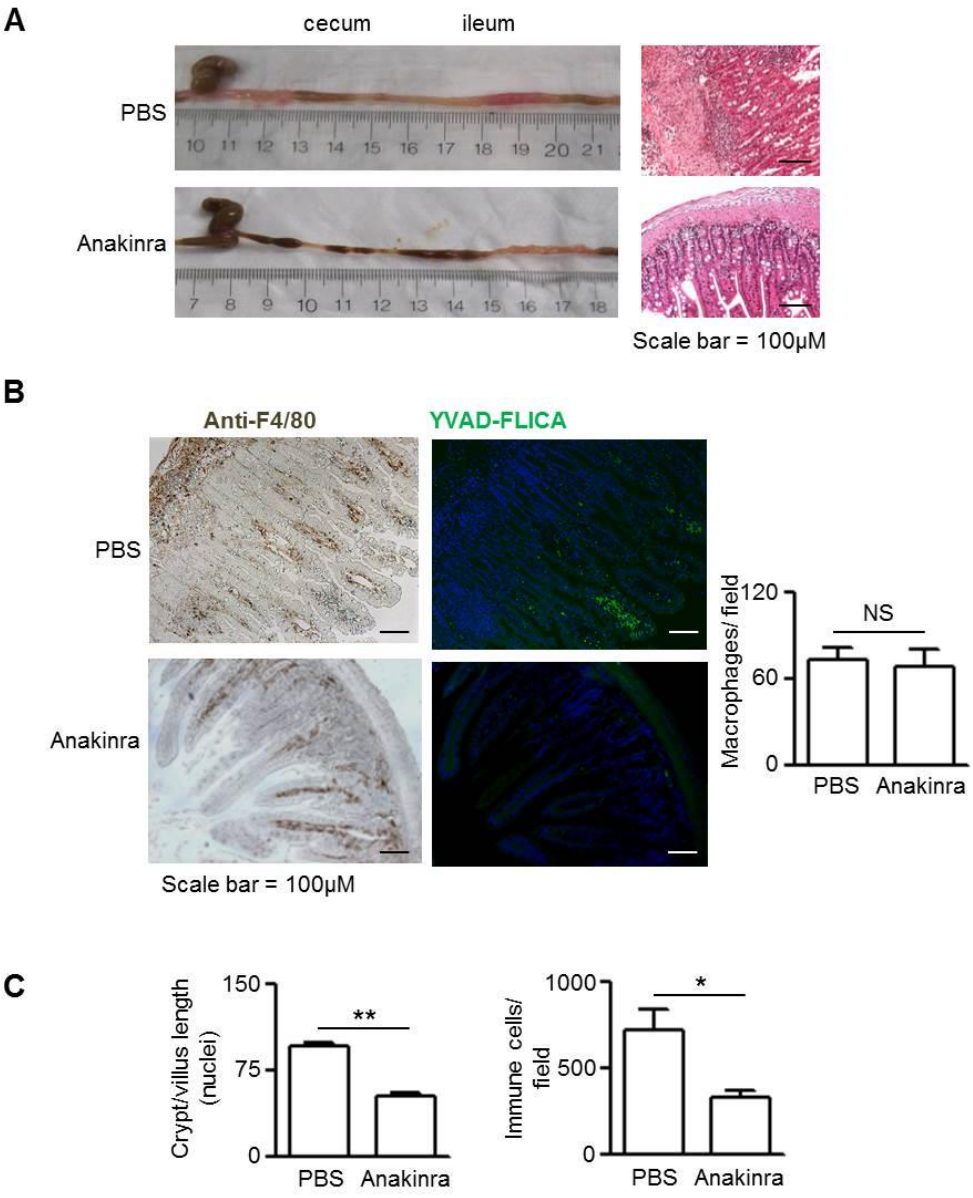

Figure 2.9 Anakinra reduces development of intestinal inflammation in SHIP-/- mice. SHIP$^{-/-}$ mice were injected intravenously with the IL-1 receptor antagonist, anakinra, or PBS, as an injection control, from 4-6 weeks of age. (A) Gross pathology of distal ilea (left) and H&E stained ileal cross-sections (right) of 6-week-old SHIP-/- mice. (B) Staining for macrophages (F4/80) and active caspase-1 (FLICA) in fixed ileal tissue sections (left). Quantitation of F4/80^{+} macrophages in ileal cross-sections by microscopy (right). (C) Crypt-villus length (left) and immune cell infiltration (right) quantitated by microscopy. Photographs in (A) and (B) are representative images from 6 mice/group. In (B)-(C), counting was performed on 6 mice/group counting 6 fields in 6 sections separated by ≥50 μm and by two individuals blinded to experimental condition. Data are means ±SD for n=6. *P<0.05, **P<0.0001(unpaired Student's *t*-test).

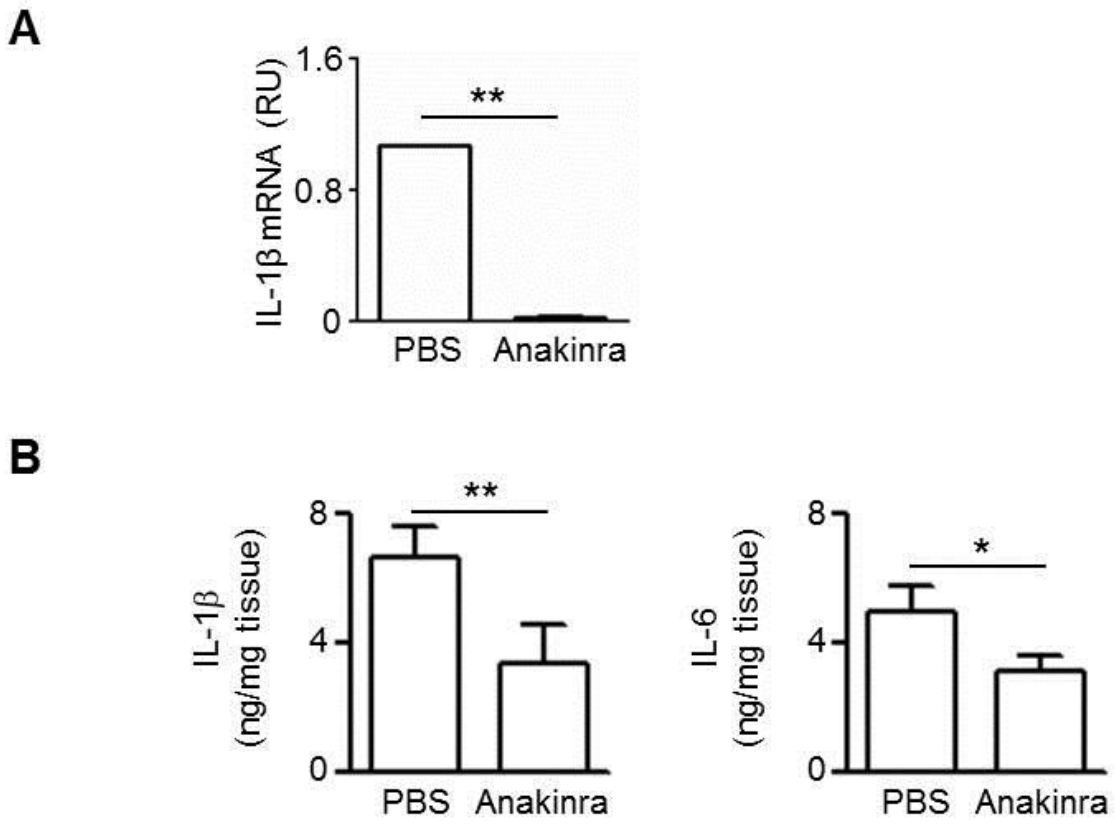

Figure 2.10 Anakinra reduces IL-1β levels in the ileum of SHIP-/- mice.
SHIP$^{-/-}$ mice were injected intraperitoneally with the IL-1 receptor antagonist, anakinra, or PBS, as an injection control, from 4-6 weeks of age. (A) RNA was isolated from ileal tissues and IL-1 β transcription was assayed by Q-PCR. (B) IL-1β (left) and IL-6 (right) assayed in full thickness ileal tissue homogenates by ELISA. Data are means ±SD for n=6. *P<0.05,**P<0.001 (unpaired Student's *t*-test).

2.3.6 SHIP activity is lower in subjects with Crohn's disease.

To determine whether SHIP may play a role in intestinal inflammation in people with CD, we compared SHIP activity in subjects with CD (no colonic involvement) to that in control subjects, who did not have IBD. Ileal and colonic biopsies and peripheral blood were collected from treatment-naïve subjects, who were undergoing colonoscopy as part of their diagnosis. SHIP activity was assayed in fresh ileal and colonic biopsies and tissues were fixed for H&E staining. SHIP activity was significantly lower in ileal biopsies from subjects,

who were subsequently diagnosed with CD compared to control subjects (Figure 2.11A). SHIP is hematopoietic-specific so it is interesting to note that SHIP activity is lower in ileal tissues from subjects with CD despite dramatic immune cell infiltration visible in biopsies (Figure 2.11B). Immune cells were quantitated in biopsy sections and confirmed a 2.2-fold increase in immune cells in CD subjects (Figure 2.11C). PBMCs were prepared from subjects with CD and control subjects to measure SHIP activity and (LPS+ATP)-induced IL-1β production. As in ileal biopsies, SHIP activity was lower in PBMCs from subjects with CD compared to control subjects (Figure 2.12A) and SHIP activity in PBMCs inversely correlated with (LPS+ATP)-induced IL-1β production by these cells (Figure 2.12B).

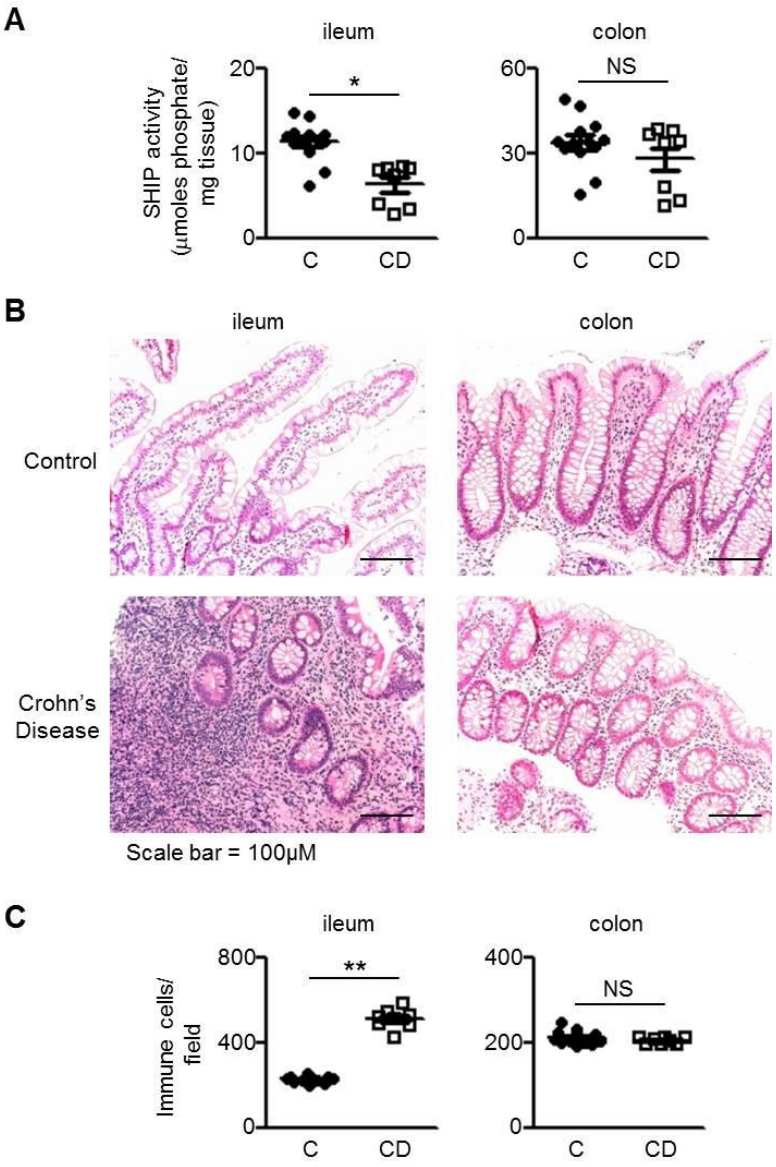

Figure 2.11 SHIP activity is lower in ileal biopsies from subjects with CD compared to control subjects.

Ileal and colonic biopsies and PBMCs were collected from treatment-naïve subjects undergoing colonoscopy as part of diagnosis of IBD. N=8 subjects were subsequently diagnosed with CD (no colonic involvement) and n=11 subjects were not diagnosed with IBD. (A) SHIP activity in ileal and colonic biopsies from control subjects (C) and subjects with CD (CD). (B) H&E stained ileal and colonic biopsy sections. (C) Quantitation of immune cells in tissue sections by microscopy. Horizontal lines indicate means ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, NS = not significantly different.

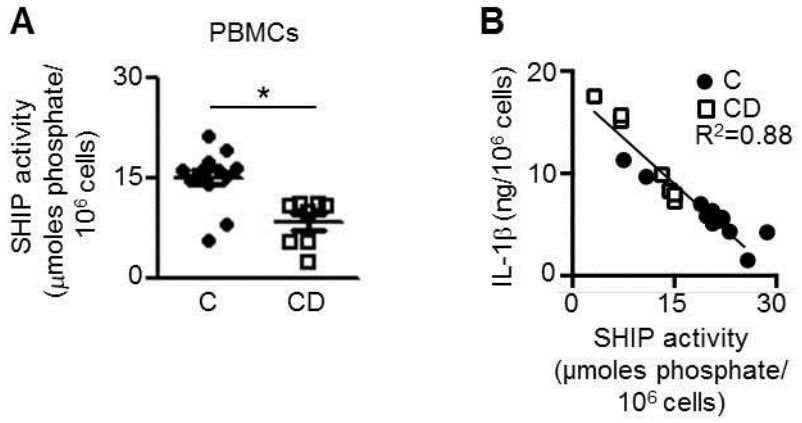

Figure 2.12 SHIP activity is lower in peripheral blood mononuclear cells (PBMCs) from subjects with CD compared to control subjects and is inversely proportional to IL-1β production.
PBMCs were collected from treatment-naïve subjects undergoing colonoscopy as part of diagnosis of IBD. N=8 subjects were subsequently diagnosed with CD (no colonic involvement) and n=11 subjects were not diagnosed with IBD. (D) SHIP activity in peripheral blood mononuclear cells (PBMCs) from control subjects (C) and subjects with CD (CD; left). (B) (LPS+ATP)-induced IL-1β production by PBMCs assayed by ELISA versus SHIP activity in control subjects (black circles) and subjects with CD (open squares; right). Horizontal lines indicate means ± SEM. $*P \leq 0.05$.

2.4 Discussion

We have demonstrated that chronic ileitis in SHIP-/- mice are associated with elevated levels of macrophage-derived IL-1β. We also found that increased IL-1β production occurs in *in vivo* differentiated SHIP-/- macrophages, which have increased Class IA PI3Kp110α-driven transcription of *ILIB* and that SHIP deficiency does not affect inflammasome activation directly. Development of ileitis was ameliorated by macrophage depletion or treatment with the IL-1 receptor antagonist, anakinra. Finally, we demonstrated that SHIP activity is lower in the inflamed ileum and PBMCs from treatment-naïve subjects with ileal CD compared to control subjects and SHIP activity inversely correlated with the ability of PBMCs to produce IL-1β.

SHIP plays pleotropic roles in macrophage activation by limiting PI3K activity downstream of receptor stimulation. Thus, SHIP deficient macrophages are hyper-responsive to immune stimuli. Herein, we demonstrate a critical role for SHIP in transcription of *ILIB* in macrophages where SHIP limits PI3Kp110α activity downstream of LPS/TLR4 signalling and contributes to intestinal inflammation. PI3K and its downstream targets, Akt and mTOR (mammalian target of rapamycin), are activated by TLR4 signalling and both contribute to increased *ILIB* transcription in SHIP$^{-/-}$ macrophages (data not shown). Additional evidence in the literature implicates the PI3K/SHIP axis in IL-1β production and intestinal inflammation. The micro RNA, miR-155, targets SHIP protein.[363] Triptolide (a potent anti-inflammatory/ immunosuppressive plant component) ameliorates inflammation post ileocolonic anastomosis in IL-10-/- mice by decreasing miR-155 levels.[364] miR-155 levels are also increased during DSS-induced colitis[365] and spontaneous colitis in IL-10-/- mice[366], both of which have been associated with increased IL-1β production, though a direct correlation with SHIP activity has not been investigated. Finally, targeting mTOR, downstream of PI3K, effectively reduces inflammation during DSS-induced colitis in mice and has been used effectively to treat refractory CD.[367]

Our data demonstrate that SHIP$^{-/-}$ bone marrow macrophages derived in MCSF or IL-3 did not produce more IL-1β in response to stimulation than those derived from their wild type littermates. GM-CSF-derived SHIP-/- bone marrow macrophages did produce significantly more IL-1β than their wild type counterparts, but the effect of SHIP deficiency on IL-1β production was modest, particularly compared with differences observed for *in vivo* differentiated SHIP-/- ileal and peritoneal macrophages. Furthermore, the dramatic

differences in IL-1β production for *in vivo* differentiated SHIP-/- macrophages could not be recapitulated *in vitro* by addition of pro-inflammatory IFNγ, or IL-4, which we have reported is increased in SHIP$^{-/-}$ ileum[337], or both IFNγ+IL-4. Though unexpected, this data is consistent with a recent report, which showed that LysM-cre×SHIP$^{fl/fl}$ mice, that is, mice deficient in SHIP in myeloid cells including macrophages, did not develop the ileal inflammation characteristic of the germ line SHIP$^{-/-}$ mouse.[348] Taken together, these data suggest that high IL-1β production by *in vivo* differentiated SHIP$^{-/-}$ macrophages requires cell-extrinsic factors produced by other SHIP-/- cells that contribute to the complex inflammatory environment in the germ line SHIP-/- mouse. Though the cell type that initiates the inflammatory response has not been determined, data from Kerr *et al.* (2011)[338] suggests that bone marrow-derived hematopoietic cells drive ileitis in germ line SHIP-/- mice because disease can be transferred to wild type mice by bone marrow transplantation, but not by adoptive transfer of T cells or NK cells, and because disease can be cured in SHIP-/- mice by transplantation of wild type bone marrow.[338]

In our study, SHIP activity inversely correlated with IL-1β production in three model systems: in *ex vivo* isolated macrophages from mice, in ileal tissues from mice, and in PBMCs from human subjects. Intriguingly, increased IL-1β production by SHIP-/- macrophages is consistent with some previously described, but unexplained features of the SHIP$^{-/-}$ mouse. IL-1 production is associated with neutrophil influx[368] and our laboratory previously reported that ileal inflammation in the SHIP-/- mice is associated with dramatic neutrophilia.[337] IL-1 is also a potent inducer of fever and, while monitoring temperature in SHIP$^{-/-}$ mice, it was previously reported that SHIP-/- mice have a chronic, low-grade fever.[369]

These unexplained phenotypes may be accounted for by hyper-responsiveness to *IL1B* transcription in SHIP-/- mouse macrophages.

The role of IL-1β production in intestinal inflammation is considered controversial. In a model of acute intestinal inflammation in mice, DSS-induced colitis, concurrent studies reported that loss of the NLRP3 inflammasome led to reduced[370] or exacerbated[236] intestinal inflammation and disease severity. Consistent with the former report, caspase-1 inhibition[370] or caspase-1 deficiency,[371] which would lead to an inability to process and secrete mature IL-1β, protected mice from DSS-induced colitis. Consistent with the latter report, NLRP6[239] or IL-1β[240] deficiency exacerbated DSS-induced colitis in mice. These last two studies may provide insight into the discrepancies reported in the literature highlighting key roles for dysbiosis in development of intestinal inflammation[239] and the central role of macrophage-derived IL-1β in colon repair post DSS-induced epithelial cell injury.[240] In contrast, the role of IL-1β during chronic intestinal inflammation is not controversial and IL-1β has consistently been reported to contribute to disease. Caspase-1 deficiency protected mice during chronic inflammation induced by DSS[371]; $TLR5^{-/-}$ mice treated with a neutralizing antibody to the IL-10 receptor developed spontaneous colitis, which was driven by IL-1β and dependent on the IL-1 receptor[244]; and inflammasome activation and IL-1β production contributed to the development of spontaneous intestinal inflammation in $IL\text{-}10^{-/-}$ mice, which was inhibited by antagonizing the IL-1 receptor or caspase-1 activity.[242] Our data provide additional evidence supporting the thesis that IL-1β contributes to chronic intestinal inflammation in mice because reducing IL-1β levels by either macrophage depletion or anakinra treatment ameliorated spontaneous intestinal inflammation in SHIP-/- mice.

Moreover, the efficacy of anakinra treatment suggests that SHIP$^{-/-}$ mice suffer from autoinflammation. Anakinra is an IL-1 receptor antagonist. As such, it does not interfere with IL-1β production directly, but rather blocks IL-1 signalling through the IL-1 receptor, which can drive transcription of more *IL1B*. Anakinra treatment in SHIP-/- mice led to a dramatic decrease in IL-1β mRNA, which is consistent with it blocking the auto amplification of IL-1β.

Finally, we report that SHIP activity was reduced in ileal biopsies and PBMCs from treatment-naïve subjects with ileal CD compared to control subjects, who did not have IBD. This is particularly compelling given that SHIP expression is restricted to hematopoietic cells and there is a dramatic influx of hematopoietic cells into intestinal tissue in patients with IBD. Arijs *et al.* (2011)[349] previously reported that SHIP mRNA levels are increased in colonic biopsies from IBD subjects with colonic inflammation, both ulcerative colitis and Crohn's colitis, but remain unaffected in ileal biopsies from subjects with ileal CD.[349] Our data extend these observations suggesting that SHIP protein levels are regulated post-transcriptionally thus reducing net SHIP activity in subjects with ileal inflammation. It has previously been reported that SHIP is up-regulated in response to LPS and MyD88-dependent TLR signalling,[327,369] which provides a mechanism for maintenance of SHIP mRNA levels and activity in the colon where commensal microorganisms are abundant. Our laboratory has also reported that SHIP protein expression and activity are reduced post-transcriptionally after activation and phosphorylation by Src family tyrosine kinases, which trigger SHIP's polyubiquitination and degradation by the proteasome.[330,361] This suggests a model in which SHIP is activated to dampen down PI3K signalling during intestinal inflammation, but is ultimately degraded in the ileum leading to increased inflammation.

Chapter 3: The Crohn`s disease-associated polymorphism in *ATG16L1* reduces SHIP gene expression and activity in human subjects

3.1 Introduction and rationale

Crohn's disease (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] The interactions between genetic and environmental factors,[16] intestinal microbes and the epithelial lining, as well as the immune system are believed to be crucial for the development of chronic inflammation during CD.[17]

Genome-wide association studies have identified 163 susceptibility loci associated with inflammatory bowel disease (IBD), 140 of which are associated with CD.[16] A non-synonymous single nucleotide polymorphism (SNP) in the gene encoding the autophagy-related 16-like protein (ATG16L1) has been associated with increased risk for developing CD in multiple populations[35,372-374] and specifically with the development of ileal CD.[36] The *ATG16L1* CD-associated gene variant is common in the population with 33.2% of people from Western European descent being homozygous for the risk variant.[258] People, who have one copy of the *ATG16L1* CD-associated gene variant, have a 1.86-fold increased risk of developing CD (95% confidence interval 1.09-3.24) and people, who have 2 copies of the *ATG16L1* risk allele, have a 2.38-fold increased risk of developing CD (95% confidence interval 1.40-4.04) thus the effect of this risk allele is gene dose dependent.[259] The *ATG16L1* risk allele codes for a threonine to alanine amino acid substitution at position 300 in the ATG16L1 protein (T300A).[36] ATG16L1 is crucial for autophagy, a multi-step, catabolic self-

preservation process that controls clearance and re-use of intracellular components during homeostasis and provides amino acids required for cell survival under stress conditions including starvation, accumulation of unfolded proteins, or intracellular infection.[245] ATG16L1 sets the platform for recruitment of the ATG12-ATG5 complex to the immature phagophore membrane, which in turn permits progression and closure of the membrane to form the autophagosome.[253] ATG16L1 deficiency in mice or homozygosity for the *ATG16L1* CD-associated gene variant in humans causes defects in Paneth cell granule exocytosis,[80] which may be important in susceptibility to disease in response to specific infections and/or environmental influences.[73] Moreover, CD patients, who are homozygous for the *ATG16L1* CD risk allele, are unable to adequately clear pathosymbionts and monocytes from subjects with the ATG16L1 T300A risk protein have impaired ability to clear invading pathogens.[260]

In humans, defects in autophagy and the *ATG16L1* CD risk variant have been associated with increased IL-1β production.[254,255,261] IL-1β is a critical cytokine, which can be produced by monocytes and macrophages to initiate and amplify the innate immune responses.[170] Thus, its production is tightly regulated by a two-step process. First, innate immune stimuli induce transcription of *IL1B*, which is translated to pro-IL-1β, an inactive precursor. Second, danger associated molecular patterns cause assembly of the inflammasome activating caspase-1, which cleaves pro-IL-1β, leading to activation and secretion of mature IL-1β.[190-192] The ATG16L1 T300A protein has recently been shown to be cleaved by caspase 3/7, resulting in defective autophagic control of intracellular bacteria and increased IL-1β secretion.[254,255] Consistent with these results, PBMCs from patients, who are homozygous for *ATG16L1* CD risk variant, produce more IL-1β in response to muramyl dipeptide (MDP) than those from subjects, who are homozygous for the variant that

is not associated with CD.[261] Moreover, differences observed were attributed to increased *ILIB* transcription.[261] In mice, increased IL-1β production associated with autophagy defects has been attributed to increased inflammasome activity but the molecular mechanism(s) by which autophagy and/or ATG16L1 regulate *ILIB* transcription in humans remains unknown.

The SH2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP) is a hematopoietic-specific lipid phosphatase that negatively regulates class I phosphatidylinositol 3-kinase (PI3K) activity. SHIP antagonizes class I PI3K signalling by dephosphorylating the 5' position of class I PI3K-generated phosphatidylinositol 3,4,5 trisphosphate ($PI(3,4,5)P_3$). Class I PI3K is critical in many cellular processes including immune activation, thus, SHIP deficient mice are hyper-responsive to immune stimuli, [327,337,369,375] including increased IL-1β production in response to innate immune activation.[327] We, and others, have reported that SHIP deficient mice develop spontaneous CD-like intestinal inflammation that is primarily restricted to the distal ileum.[337,338] In humans, SHIP mRNA expression is increased in inflamed colonic biopsies from people with ulcerative colitis and colonic CD, which is consistent with dramatic immune cell infiltration into tissues during inflammation and SHIP expression being restricted to hematopoietic cells.[349] More surprisingly, SHIP mRNA expression was not elevated in inflamed biopsies from subjects with ileal CD. [349] The human gene encoding SHIP protein (*INPP5D*) is located at chromosome 2q37.1 and is 40 kilobases upstream of the gene encoding ATG16L1.[305] *INPP5D* and *ATG16L1* are located on the same DNA strand and are transcribed in the same direction. Though there are SNPs in *ATG16L1*, which are in linkage disequilibrium with rs2241880,[376] there are no SNPs in *INPP5D*, which co-segregate with rs2241880.

Humans carrying the *ATG16L1* risk variant and mice deficient in SHIP have increased susceptibility to CD or ileal inflammation, respectively, and their macrophages have increased ability to produce IL-1β as shown in chapter 2. Thus, we asked whether SHIP mRNA expression and activity are regulated by ATG16L1 and whether SHIP may contribute to increased IL-1β production in subjects with the *ATG16L1* risk variant. Herein, we report that SHIP mRNA expression was reduced in subjects with ileal CD. In our patient cohort and a second cohort of healthy control subjects, both SHIP mRNA expression and activity levels were extremely low in subjects, who were homozygous for the ATG16L1 T300A-encoding risk allele. Furthermore, we show that autophagy up-regulated SHIP protein and that SHIP up-regulation was dependent on ATG16L1 protein levels and/or autophagy, and the *ATG16L1* gene variant. Finally, we demonstrate that the *ATG16L1* genotype and low SHIP mRNA expression correlated with increased IL-1β production by (LPS+ATP)-stimulated PBMCs, and was dependent, at least in part, on *IL1B* transcription. Taken together, these results identify SHIP as a critical link between the *ATG16L1* CD-associated gene variant and increased *IL1B* transcription, which may contribute to increased risk of developing intestinal inflammation.

3.2 Materials and methods

Subjects with ileal Crohn's disease and control subjects. This study was performed in accordance with ethical guidelines approved by the University of British Columbia Research Ethics Boards (H09-01826). Written informed consent was obtained from all subjects. Patients seen in the Division of Gastroenterology at BC Children's Hospital and scheduled

for colonoscopy were recruited into the study. None of the patients had been previously diagnosed or treated for IBD or other inflammatory disease. Four biopsies were taken from the ileum and colon at sites of inflammation, which were adjacent to tissues harvested for pathological assessment in patients diagnosed with CD. Similar sites were biopsied in control subjects. During the procedure, peripheral blood was drawn from the site of intravenous insertion. The diagnosis of CD with inflammation in the ileum, or absence of IBD, was based on colonoscopy and pathological assessment. Twenty-one subjects were included in the analyses, 8 patients diagnosed with CD with ileal inflammation and 13 subjects with no IBD. Biopsy samples and PBMCs isolated from peripheral blood were either stored in RNA*later* (Invitrogen; Burlington, ON) for gene expression analyses, or used immediately for biochemical and immunological assays. All analyses were performed on all participants prior to diagnosis.

Healthy control subjects. Experiments were performed in accordance with ethical guidelines and with approval by the University of British Columbia Research Ethics Boards (H04-0534). Written informed consent was obtained from all subjects. Isolated PBMCs were used for analyses. Eighteen subjects, 6 from each ATG16L1 genotype, were used for analyses. PBMCs were isolated from peripheral blood and DNA was stored for genotyping, or fresh PBMCs were used for biochemical and immunological assays. All analyses were performed on all participants prior to stratification by genotype.

Cell isolation and derivation conditions. PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll-Paque^{TM} PLUS (GE Healthcare, Piscataway, NJ). Cells were washed and resuspended at a density of 0.5×10^6 cells/mL in Iscove's Modified

Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) for assays. DNA was prepared for genotyping using the QiaAMP DNA blood mini kit, as per the manufacturer's instructions (Qiagen, Toronto, Ontario, Canada), and fresh PBMCs were used for analyses of gene expression, SHIP activity, autophagy, and cytokine production. THP-1 cells from the ATCC and free of mycoplasma contamination, were maintained in RPMI 1640, 10% FCS, and penicillin/streptomycin. Cells (0.5×10^6 cells/mL) were differentiated for 16 h in 20 ng/mL phorbol 12-myristate 13-acetate (PMA) for use in autophagy assays. Bone marrow macrophages (BMMϕs) were generated from bone marrow aspirates from femura and tibiae of SHIP+/+ and SHIP-/- mice. Aspirates were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum (FBS) in 75 cm^2 Falcon flasks (BD Biosciences) for 1 hr. Following adherence depletion, bone marrow aspirates were then resuspended in IMDM, 10% FBS, and penicillin/streptomycin at a concentration of 0.5×10^6 cells/mL for 10 days in the presence of 5 ng/mL MCSF (StemCell Technologies, Vancouver, BC), with complete media changes at day 4 and 7. At day 10, macrophages were $\geq$ 95% $F4/80^+$ and $Mac\text{-}1^+$. In THP-1 cells, ATG16L1 was knocked down using two Stealth RNAi designed against non-overlapping sequences (HSS147873 and HSS182825) or a non-silencing control (Life Technologies). Stealth RNAi or control RNAi was transfected into THP-1 cells using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies).

Genotyping the *ATG16L1* Crohn's disease susceptibility SNP, rs2241880. Stored DNA samples were used to genotype the ATG16L1 SNP, rs2241880, using a commercially available Taqman assay (C_9095577_20; Applied Biosystems, Burlington, ON, Canada).

SNPs were deemed acceptable for analysis if they had call rates > 95% and frequencies did not deviate from Hardy-Weinberg equilibrium ($P > 0.05$).

Gene Expression Analyses. Human tissue sections or PBMCs were stored in RNA*later* at -20°C. RNA was prepared using the either Qiagen RNeasy Mini Kit (Qiagen, Toronto, ON) or NucleoSpin RNA II Total RNA Isolation Kit according the manufacturer's protocol (Macherey-Nagel, Bethlehem, PA). RNA concentration was measured using the NanoDrop 1000 Spectrophotometer. RNA was reverse transcribed using Superscript II (Invitrogen, Burlington, ON).

For analysis of murine SHIP (*Inpp5d*) and β-actin (*ActB*) gene expression, qPCR was performed using 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the CFX96 C1000 Thermal Cycler (BioRad Laboratories, Mississauga, ON). Primers were from BioRad Laboratories, catalogue number 100-25636, with unique identifiers qMmuCED0045767 (*Inpp5D*), and qMmuCED0027505 (*ActB*).

For analysis of human SHIP (*INPP5D*), ATG16L1 (*ATG16L1*), CD45 (*PTPRC*), and β-actin (*ACTB*) gene expression, qPCR was performed using 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the CFX96 C1000 Thermal Cycler (BioRad Laboratories, Mississauga, ON). Primers sequences used were:

INPP5D (SHIP) forward	5'-GGGAGATGTAGTGTCACTGG-3'
INPP5D (SHIP) reverse	5'-TACAGGAATGAGCCCCAAAG-3'
PTPRC (CD45) forward	5'-CTGACATCATCACCTAGCAG-3'
PTPRC (CD45) reverse	5'-TGCTGTAGTCAATCCAGTGG-3'
ACTB (β-actin) forward	5'-AAATCTAGCTGCCCGTCATT-3'

ACTB (β-actin) reverse 5'-GCATCGAGTCCCTGATTTCT-3'

Primers for *ATG16L1* were from BioRad Laboratories, catalogue number 100-25636, and sequences are proprietary.

For analysis of IL-1β gene expression (*IL1B*), qPCR was performed using the AB Applied Biosystems Taqman Universal Master Mix II (Invitrogen). Primers sequences used were:

ILIB (IL-1β) forward 5'-ATGCACCTGTACGATCACTG-3'

ILIB (IL-1β) reverse 5'-ACAAAGGACATGGAGAACACC-3'

ILIB (IL-1β) probe 5'-/56 FAM/CCAGGGACA/ZENGGATATGGAGCAACA/3IABkFQ/-3'

ACTB forward 5'-ACCTTCTACAATGAGCTGCG-3'

ACTB reverse 5'-CCTGGATAGCAACGTACATGG-3'

ACTB probe 5'-/56-FAM/ATCTGGGTC/ZEN/ATCTTCTCGCGGTTG/3IABkFQ/-3'

SHIP activity assays. To quantify SHIP activity, SHIP was immunoprecipitated with anti-human SHIP1 antibody (N-1, sc6244; Santa Cruz Biotechnology, CA). Intestinal biopsy sections were weighed or PBMCs were counted and immunoprecipitates prepared. Substrate, 100 μM IP_4 (Echelon Biosciences, Salt Lake City, UT, USA) was incubated with immunoprecipitates for 20 min and the reaction was stopped by heating samples to 80°C for 5 min. Inorganic phosphate released was detected using Malachite Green (Echelon Biosciences) and absorbance was read at 650 nm and compared to a standard curve.

Autophagy induction and Western blot analyses. Autophagy was induced in murine macrophages, THP-1 cells, or PBMCs by starvation. Media was removed and cells were washed twice and resuspended in Hanks Balanced Salt Solution (HBSS) for 4 h. Cells, 2.5×10^6 murine bone marrow derived macrophages or 0.5×10^6 THP-1 cells or PBMCs, were harvested for Western immunoblot analysis. In THP-1 cells, ATG16L1 was knocked down using two Stealth RNA interference (RNAi) designed against non-overlapping sequences (HSS147873 and HSS182825) or a non-silencing control RNAi (Life Technologies). Stealth RNAi or non-silencing control RNAi was transfected into THP-1 cells using Lipofectamine 2000, according to the manufacturer's protocol (Life Technologies).

Whole cell lysates were subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and transferred onto polyvinylidene fluoride (PVDF) for immunodetection, as described previously.[377] The following antibodies were used for analyses: anti-mouse SHIP (P1C1, Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse and -human LC3 (NB100-2220, Novus Biologicals, Oakville, ON), anti-mouse GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Fitzgerald Industries International, Acton, MA), anti-human ATG16L1 (Abcam, Toronto, ON), anti-human SHIP (N1, Santa Cruz), and anti-human GAPDH (eBiosciences, San Diego, CA). Densitometry was performed using ImageJ software (National Institute of Health, USA).

Cell stimulations for cytokine production and analyses. Murine bone marrow macrophages or PBMCs were plated at a density of 0.5×10^6 cells/mL of complete medium. For *ILIB* gene expression analysis, PBMCs were unstimulated or stimulated with 10 ng/mL of LPS (*E. coli* serotype 127:B8, Sigma Aldrich, St. Louis, MO) for 3 h, and RNA was

isolated using the Qiagen RNeasy Mini Kit (Qiagen, Toronto, ON) and gene expression measured.

For IL-1β cytokine production, cells were unstimulated or stimulated with 10 ng/mL LPS for 5 h, 5 mM ATP for 1 h, or LPS for 4 h with the addition of ATP for a final 1 h. After incubation (5 h in total), clarified cell supernatants were analyzed by ELISA. For TNFα and IL-6 production, cells were unstimulated or stimulated with 10 ng/mL LPS for 24 h. Cytokine measurements were performed on clarified, cell-free tissue culture supernatants by ELISA, according to the manufacturers' instructions. ELISA kits for human IL-β, TNFα, and IL-6 were from BD Biosciences (Mississauga, ON).

Statistical Analyses. Unpaired two-tailed Student's *t* tests, one-way analysis of variance (ANOVA), and linear regression analyses were performed using GraphPad Prism version 5 software. For multiple comparisons, the Bonferroni correction was applied. Differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 SHIP mRNA expression is reduced in subjects with ileal Crohn's disease

Previous studies from our laboratory, and others, have shown that SHIP deficient mice develop spontaneous intestinal inflammation with similar features to human CD.[337,338] Subsequent studies demonstrated that SHIP mRNA is not up-regulated in ileal biopsies from people with ileal CD,[349] despite the fact that SHIP is hematopoietic-restricted and there is dramatic immune cell infiltration into the ileum during ileal CD. Based on that, we asked whether SHIP mRNA expression was reduced in immune cells from subjects with ileal CD

compared to control subjects (C), who did not have IBD. Ileal and colonic biopsies and peripheral blood were collected from 21 treatment-naïve subjects, who were undergoing colonoscopy as part of their diagnosis. Subjects were subsequently diagnosed with ileal CD (CD = 8) or were control subjects, who were not diagnosed with IBD (C = 13). Relative gene expression for genes encoding SHIP (*INPP5D*), CD45 (*PTPRC*, a hematopoietic cell marker), and β-actin (*ACTB*) were measured. SHIP mRNA expression was reduced in the ileum and colon, relative to *PTPRC* expression, and in PBMCs, relative to *ACTB*, from subjects diagnosed with ileal CD compared to control subjects (Figure 3.1A). SHIP mRNA expression in the ileum, colon, and PBMCs was associated with SHIP activity in both control and CD groups with $R^2 = 0.85$, 0.83, and 0.92 ($P < 0.0001$), respectively (Figure 3.1B).

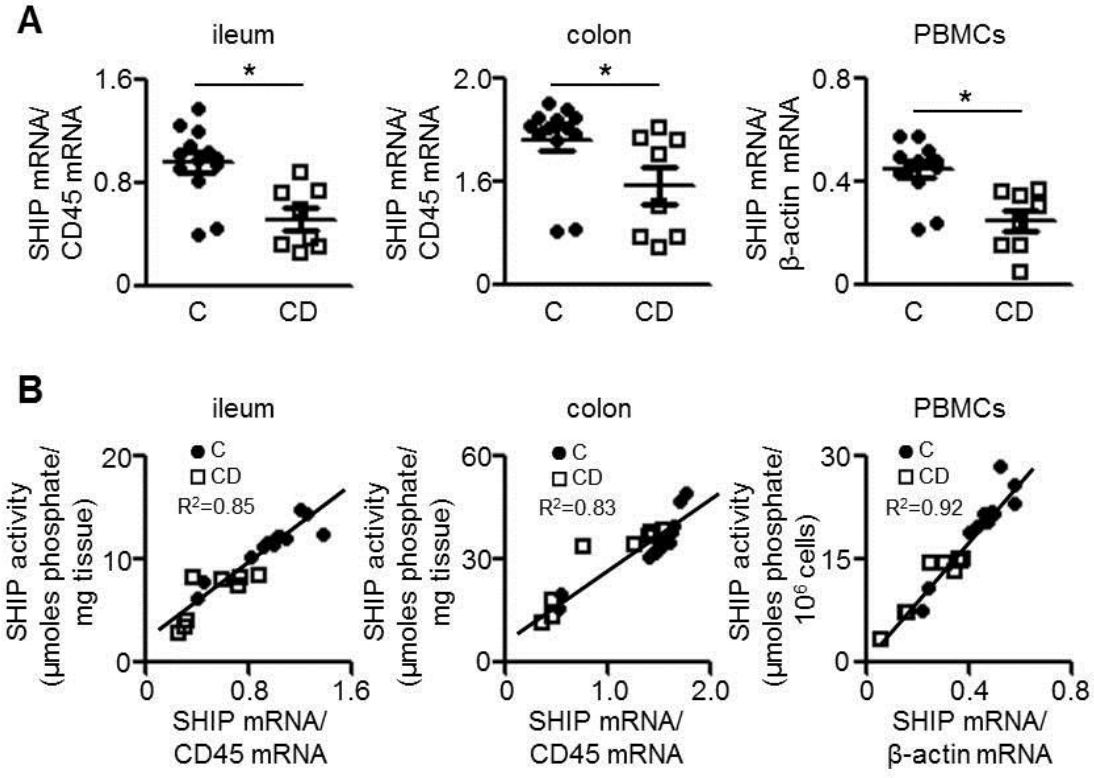

Figure 3.1 Subjects with ileal CD have lower SHIP mRNA expression compared to control subjects.
(A) SHIP mRNA expression in biopsies from the ileum and colon, relative to CD45 gene expression (a hematopoietic cell marker), and in PBMCs, relative to β-actin gene expression, from control subjects (C) and subjects with ileal CD (CD) were determined by qRT-PCR. (B) Linear regression analyses were used to compare SHIP mRNA expression levels with SHIP activity, from control subjects (black circles) and subjects with ileal CD (open squares). For (A) and (B), each symbol represents an individual subject (n = 13 control subjects, n = 8 subjects with ileal CD). In (A), horizontal lines indicate means ± SEM, $*P \leq 0.05$ (unpaired Student's *t*-test); in (B), $R^2 = 0.85$, 0.91, and 0.92 with $P < 0.0001$.

3.3.2 Homozygosity for the ATG16L1 T300A-encoding gene variant is associated with low SHIP mRNA expression and activity

SHIP mRNA expression varied more than 10-fold in PBMCs from different subjects. Intriguingly, some subjects in both control and CD groups had very low SHIP mRNA

expression and activity in all tissues samples, suggesting that there may be a genetic cause, which predisposes individuals to express low levels of SHIP mRNA. We next asked whether low SHIP mRNA and activity were associated with the *ATG16L1* genotype. As shown in Figure 3.2, both SHIP mRNA expression (Figure 3.2A) and SHIP activity (Figure 3.2B) were significantly lower in biopsies from the ileum and colon and in PBMCs from all subjects (controls and CD), who were homozygous (GG, n=5) for the *ATG16L1* CD susceptibility allele, compared to subjects, who were heterozygous (AG, n=10) or did not have the susceptibility allele (AA, n=6). There was no difference in SHIP mRNA expression or activity between subjects with the AA or AG genotypes.

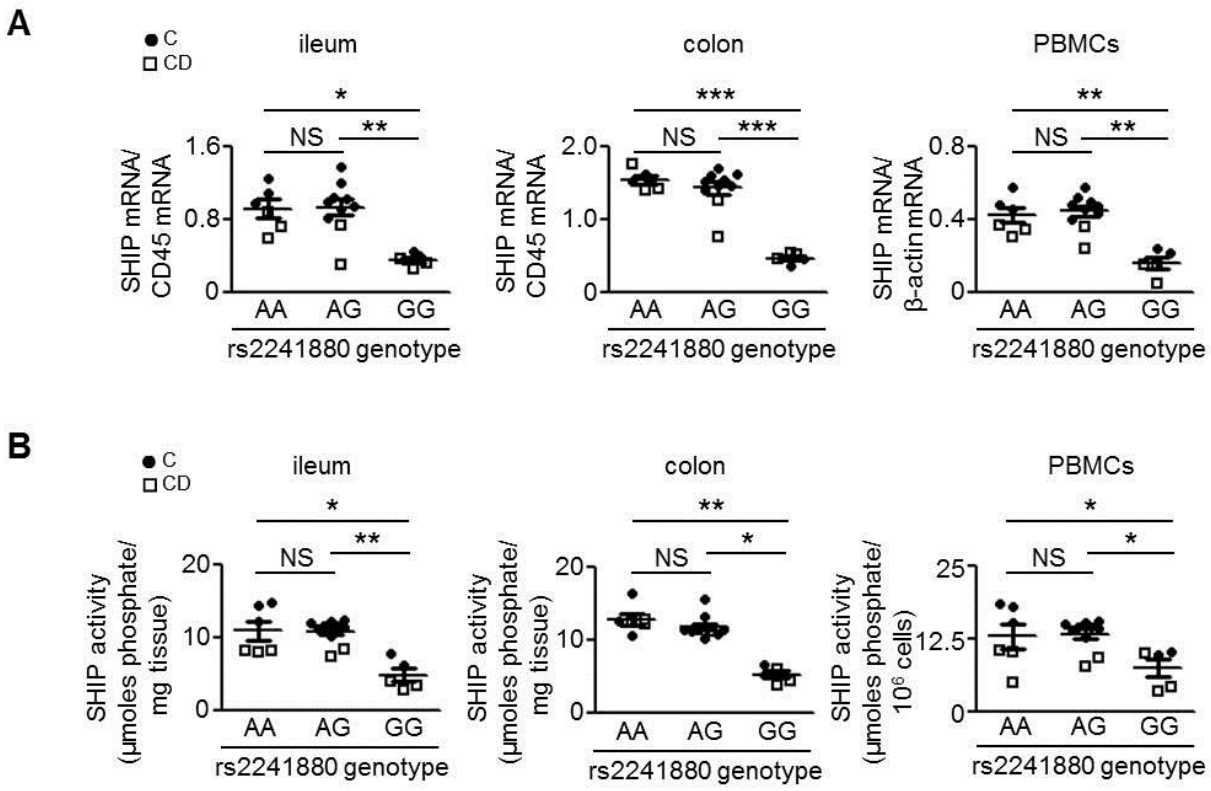

Figure 3.2 Homozygosity for the ATG16L1 T300A-encoding gene variant is associated with low SHIP mRNA expression and activity.
(A) SHIP mRNA expression and (B) SHIP activity in biopsies from the ileum and colon and in PBMCs from control subjects (black circles) and subjects with ileal CD (open squares), who did not have (AA), were heterozygous (AG), or were homozygous (GG) for the *ATG16L1* CD susceptibility SNP (rs2241880). For (A) and (B), each symbol represents an individual subject (n = 13 control subjects, n = 8 subjects with ileal CD). Horizontal lines indicate means ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, NS = not significantly different (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

3.3.3 SHIP mRNA expression and activity are lower in PBMCs from healthy control subjects, who are homozygous for the ATG16L1 T300A-encoding gene variant

To determine whether low SHIP mRNA expression and activity were independent of inflammation in CD, we recruited a second cohort of 18 healthy control subjects, who were genotyped for the *ATG16L1* SNP (rs2241880) (n = 6 subjects per genotype). We measured

SHIP mRNA expression and activity in their PBMCs. Healthy control subjects, who were homozygous for the *ATG16L1* CD susceptibility allele, had significantly lower SHIP mRNA expression (Figure 3.3A) and lower SHIP activity (Figure 3.3B) compared to subjects, who did not have the susceptibility allele. SHIP mRNA expression and activity in subjects, who were heterozygous for the *ATG16L1* genotype (AG), were not significantly different from the AA and GG groups (Figures 3.3A and 3.3B).

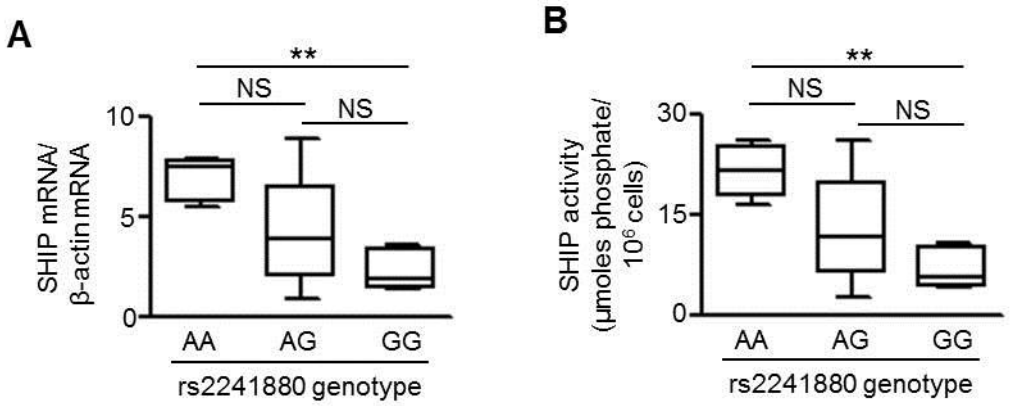

Figure 3.3 SHIP mRNA expression and activity are lower in PBMCs from healthy control subjects, who are homozygous for the ATG16L1 T300A-encoding gene variant. (A) SHIP mRNA expression and (B) SHIP activity in healthy control subjects stratified for *ATG16L1* SNP (rs2241880) genotype. For (A) and (B), horizontal lines indicate means ± SEM for n = 6 subjects per genotype. $*P \leq 0.01$, NS = not significantly different (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

3.3.4 SHIP and ATG16L1 are not transcriptionally co-regulated

Low SHIP mRNA expression in subjects, who were homozygous for the *ATG16L1* risk variant, suggested that SHIP (*INPP5D*) gene transcription may be co-regulated with *ATG16L1* and expressed at lower levels in subjects, who were homozygous for the *ATG16L1*

risk allele. To investigate this possibility, we measured ATG16L1 gene expression in our subject cohorts. In our patient group, both control (C) and CD subjects had similar mRNA expression levels for ATG16L1 in intestinal tissues (Figure 3.4A, left), which did not vary linearly with SHIP mRNA expression ($R^2 = 0.065$; $P = 0.356$) (Figure 3.4A, right). Similarly, in our healthy control subject cohort, there was no difference in ATG16L1 mRNA expression in PBMCs from subjects with different *ATG16L1* SNP genotypes (Figure 3.4B, left) and no relationship between ATG16L1 and SHIP mRNA expression ($R^2 = 0.005$; $P = 0.812$) (Figure 3.4B, right).

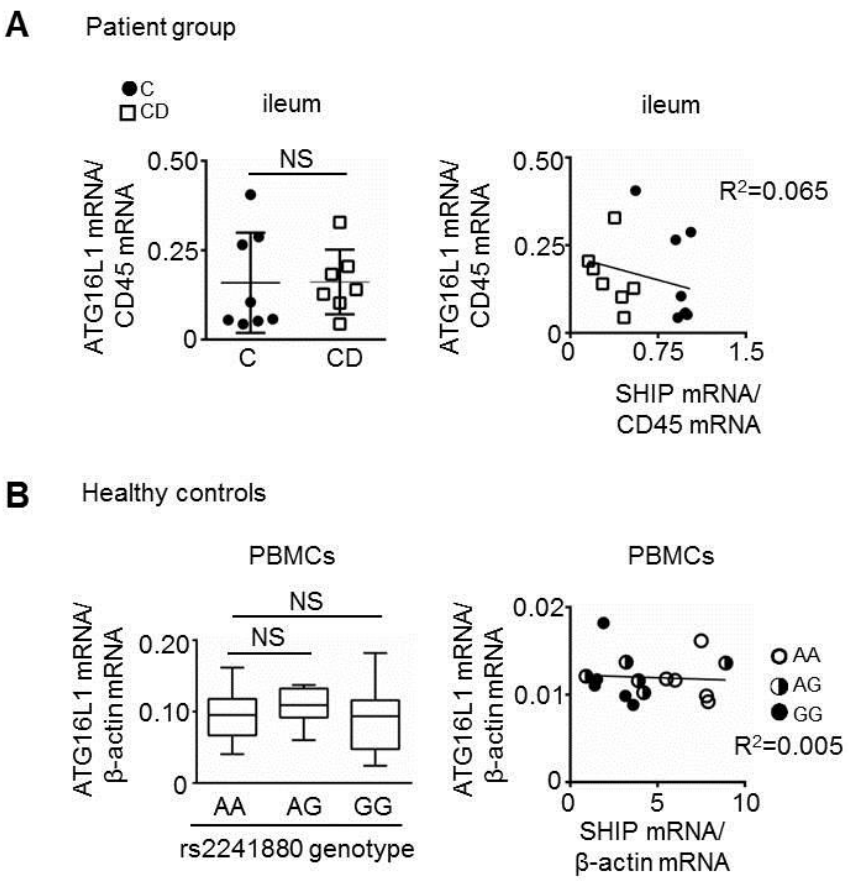

Figure 3.4 ATG16L1 mRNA expression does not vary linearly with SHIP mRNA expression.

(A) ATG16L1 and SHIP mRNA expression in biopsies from the ileum of control subjects (C, black circles) and subjects with ileal CD (CD, open squares) were determined by qRT-PCR. Linear regression analyses were used to compare ATG16L1 mRNA expression with SHIP mRNA expression. Each symbol represents an individual subject (n = 8 control subjects, n = 7 subjects with ileal CD). Horizontal lines indicate means ± SEM. NS = not significantly different (unpaired Student's *t*-test); $R^2 = 0.065$ with $P = 0.356$. (B) ATG16L1 mRNA expression in PBMCs from healthy control subjects that did not have (AA), were heterozygous (AG), or were homozygous (GG) for the *ATG16L1* CD susceptibility SNP (rs2241880) were measured by qRT-PCR. Horizontal lines indicate means ± SEM for n = 6 subjects per genotype. NS = not significantly different (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes). Linear regression analyses were used to compare ATG16L1 mRNA expression and SHIP mRNA expression in all genotypes. Each symbol represents an individual subject (n = 5 subjects with each genotype; AA open circles, AG half-filled circles, GG black circles). $R^2 = 0.005$ with $P = 0.812$.

3.3.5 Starvation-induced autophagy up-regulates SHIP protein, which is dependent on ATG16L1 or autophagy, and the risk allele genotype

ATG16L1 is required for autophagy so we next asked whether autophagy affected SHIP protein levels. First, we compared SHIP protein levels during starvation-induced autophagy in murine MCSF-derived bone marrow macrophages from wild type (SHIP+/+) and SHIP deficient mice (SHIP-/-). Intriguingly, SHIP protein levels were 4.3-fold higher during starvation-induced autophagy in macrophages from wild type mice (Figure 3.5A, left, top panel). Also, in contrast to wild type macrophages, SHIP deficient macrophages did not express LC3-II protein upon starvation, a marker of autophagosome formation (Figure 3.5A, left, middle panel, lanes 2 and 5). In the presence of bafilomycin A1, which blocks autophagosome-lysosome fusion and flux through the autophagy pathway, LC3-II was detected in SHIP deficient macrophages, albeit at lower levels than in comparably treated wild type macrophages (Figure 3.5A, left, middle panel, lanes 3 and 6). To determine whether SHIP was transcriptionally up-regulated during starvation-induced autophagy, SHIP mRNA expression was measured in wild type (SHIP+/+) murine bone marrow macrophages during starvation. SHIP mRNA expression was increased after 4 h in starvation medium (Figure 3.5A, right).

To determine whether SHIP was up-regulated during autophagy in human cells, we compared SHIP protein levels during autophagy in PMA-differentiated THP-1 cells, a human myelomonocytic cell line. SHIP protein levels were induced 4.8-fold during starvation-induced autophagy in THP-1 cells (Figure 3.5B, left, 2nd panel). To determine whether ATG16L1 was required for autophagy-induced up-regulation of SHIP protein, autophagy was induced by starvation in differentiated THP-1 cells in the presence of non-silencing

RNA (nsRNA) or a small interfering RNA (siRNA) construct targeting ATG16L1. The siRNA construct reduced ATG16L1 protein levels by 83% in untreated THP-1 cells (Figure 5b, left, top panel). Knockdown of ATG16L1 by siRNA was sufficient to block autophagy, indicated by reduced LC3-II expression, and blocked autophagy-induced up-regulation of SHIP protein, whereas the control nsRNA had no effect (Figure 3.5B, left). As in bone marrow derived macrophages, SHIP protein induction by starvation correlated with increased SHIP mRNA expression in THP-1 cells. Moreover, siRNA targeting ATG16L1, which blocked SHIP protein induction, prevented SHIP mRNA induction during autophagy compared to the nsRNA control (Figure 3.5B, right).

Finally, we measured SHIP induction in response to starvation in PBMCs from six healthy control subjects with each *ATG16L1* SNP genotype. Figure 3.6, left, shows a representative Western blot analysis of SHIP and GAPDH protein levels in one subject for each *ATG16L1* genotype; AA, AG, and GG. Average densitometry from six subjects in each group demonstrated that SHIP protein levels were 50% lower in subjects, who were homozygous for the *ATG16L1* CD risk allele, compared to subjects, who did not have the risk allele (Figure 3.6, right). Together, these data demonstrate that SHIP mRNA and protein are up-regulated during autophagy in three different cell models; murine bone marrow macrophages, a human myelomonocytic cell line, and in primary PBMCs from human subjects. Though SHIP induction is compromised during siRNA knockdown of ATG16L1 in THP-1 cells, it is not possible to distinguish whether this is cause by reduced ATG16L1 protein levels directly, or by diminished autophagy. Notably, SHIP protein levels are lower in PBMCs from subjects, who are homozygous for the *ATG16L1* CD susceptibility genotype at baseline and upon induction of autophagy by starvation.

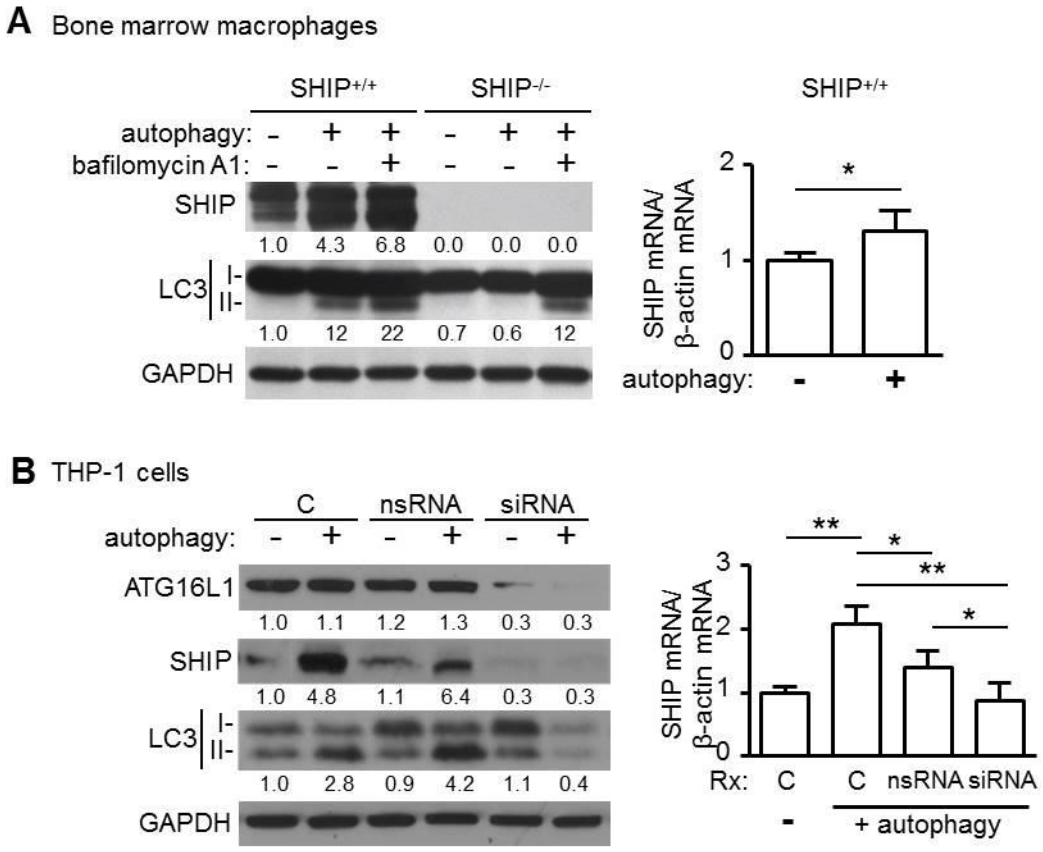

Figure 3.5 Autophagy up-regulates SHIP protein and SHIP up-regulation is dependent on ATG16L1 expression and/or autophagy.
(A) Left, immunoblot analysis of SHIP and LC3 in wild type (SHIP+/+) and SHIP deficient (SHIP-/-) bone marrow macrophages that were untreated or incubated in starvation medium for 4 h in the absence or presence of bafilomycin A1. Densitometry for SHIP and LC3-II, normalized to GAPDH, averaged from four independent experiments are shown below each band. Right, relative SHIP mRNA expression in SHIP+/+ bone marrow macrophages that were untreated or incubated in starvation medium for 4 h. Data are mean $\pm$ SEM for n = 9 independent experiments. (B) Left, immunoblot analysis of SHIP and LC3 in THP-1 cells that was untreated or incubated in starvation medium in the absence or presence of non-silencing control RNA (nsRNA) or siRNA to ATG16L1. Densitometry for ATG16L1, SHIP, and LC-II; normalized to GAPDH, averaged from four independent experiments are shown below each band. Right, relative SHIP mRNA expression in THP-1 cells incubated in starvation medium in the absence or presence of nsRNA or siRNA targeting ATG16L1. Data are means $\pm$ SEM for n = 3 independent experiments, $*P \leq 0.05$, $**P < 0.01$ (unpaired Student's *t*-test).

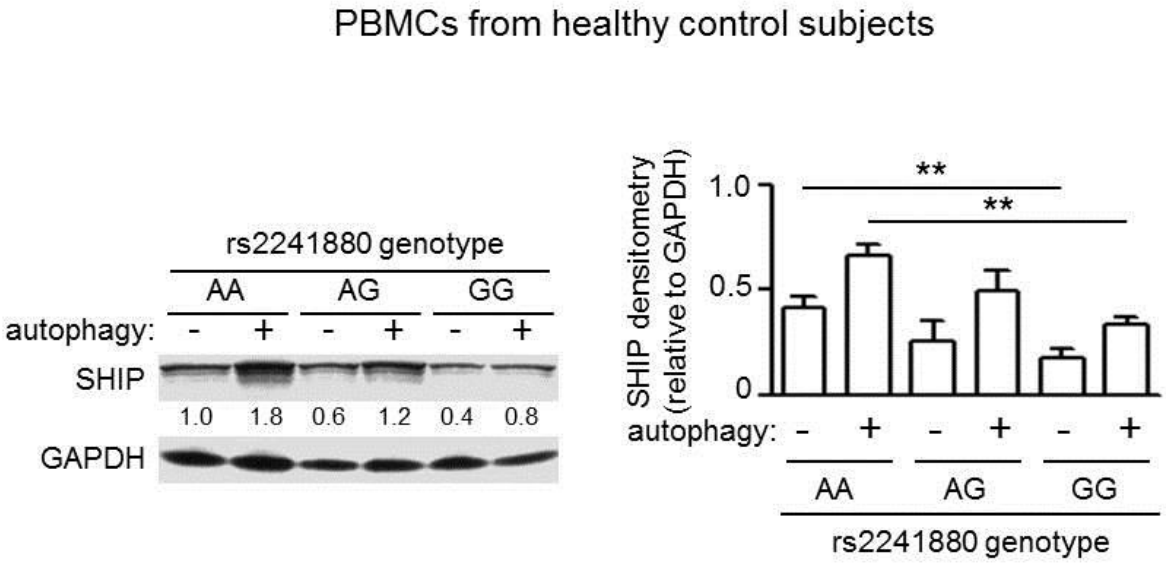

Figure 3.6 SHIP up-regulation is dependent on the *ATG16L1* genotype.
Left, representative immunoblot analysis of SHIP in PBMCs from one healthy control subject with each ATG16L1 genotype (AA, AG, or GG) that were untreated or incubated in starvation medium. Densitometry for ATG16L1 and SHIP, normalized to GAPDH, averaged from six independent experiments are shown below each band. Right, densitometry for SHIP protein levels, normalized to GAPDH, in PBMCs from healthy control subjects from each *ATG16L1* genotype (AA, AG, or GG) that were untreated or incubated in starvation medium. N = 6 subjects for each genotype; AA, AG, or GG; $^{**}P \leq 0.001$ (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

3.3.6 Homozygosity for the Crohn's disease-associated *ATG16L1* gene variant is associated with high IL-1β production and *IL1B* transcription

The CD-associated *ATG16L1* gene variant has been associated with defective autophagy[49] and high IL-1β production in response to NOD2 activation.[261] In a previous study, stimulation with MDP increased IL-1β and IL-6 in PBMCs from subjects, who carry the *ATG16L1* risk variant.[261] Along with IL-1β and IL-6, TNFα is a critical cytokine that is up-regulated and contributes to immune mediated inflammation in Crohn's disease.[378] Thus, we next measured production of these pro-inflammatory cytokines in (LPS+ATP)-stimulated

PBMCs from control (C) and CD subjects as well as in our cohort of healthy control subjects, who were homozygous (GG), heterozygous (AG), or did not have the ATG16L1 T300A-encoding gene variant (AA). In our patient cohort, PBMCs from both control and CD subjects, who were homozygous for the *ATG16L1* CD susceptibility gene variant, produced more IL-1β compared to subjects, who were heterozygous or did not carry the susceptibility allele (Figure 3.7A). In contrast, there was no difference for TNFα or IL-6 production between the genotypes (Figure 3.7A).

In our second cohort of healthy control subjects, subjects, who were homozygous (GG) for the ATG16L1 T300A-encoding gene variant, produced high levels of IL-1β compared to subjects, who were heterozygous (AG) or did not carry the risk variant (AA) (Figure 3.7B). TNFα and IL-6 production were not affected by the *ATG16L1* CD-associated gene variant in healthy control subjects (Figure 3.7B).

To determine whether increased transcription of *ILIB* contributes to increased IL-1β production by human PBMCs, *IL1B* transcription was measured in LPS-stimulated PBMCs from healthy control subjects with each genotype. Healthy control subjects, who were homozygous (GG) or heterozygous (AG) for the ATG16L1 risk variant, had significantly higher IL-1β mRNA expression compared to subjects without the risk variant (AA) (Figure 3.7C). There was no difference in IL-1β mRNA expression levels between subjects with AG and GG genotypes (Figure 3.7C).

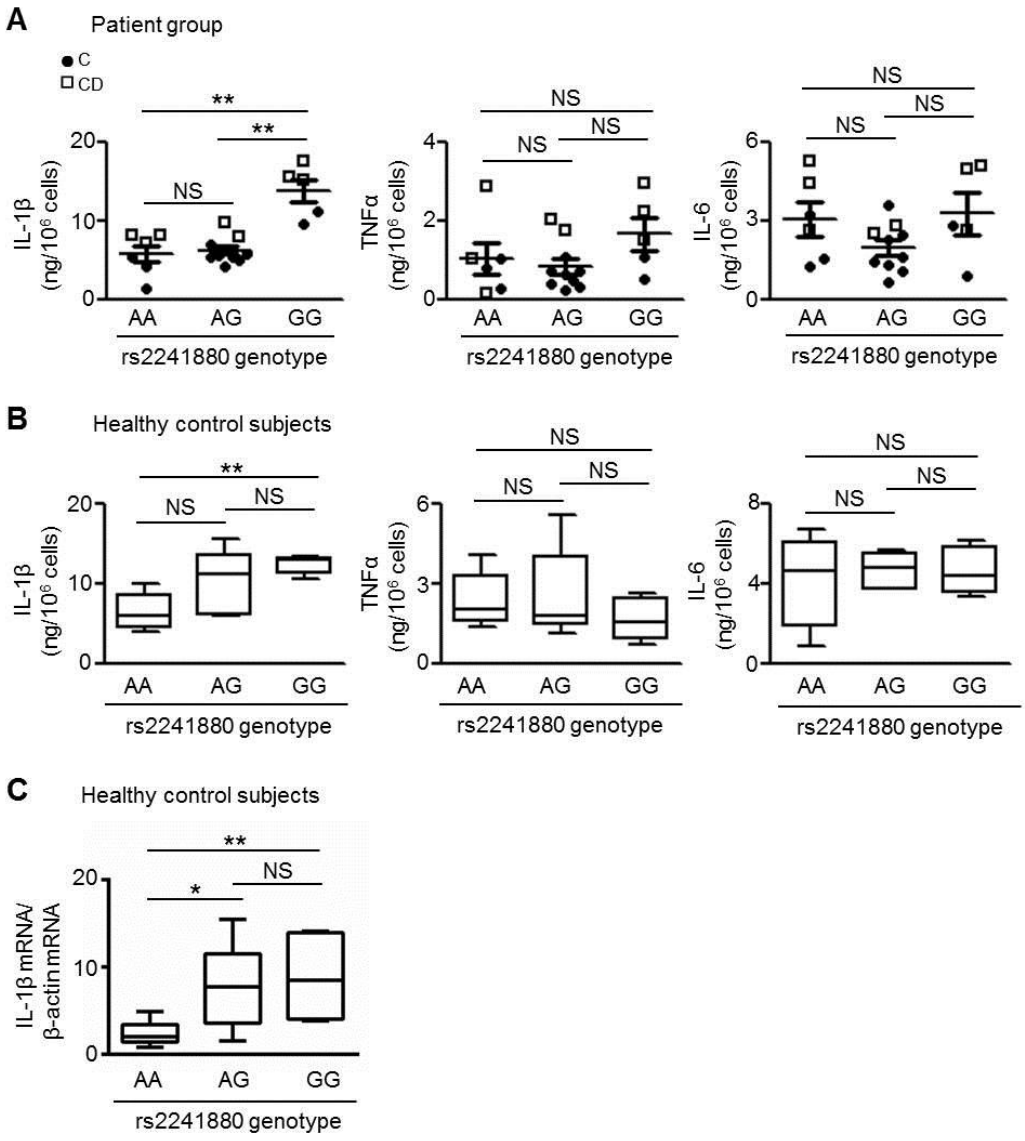

Figure 3.7 Homozygosity for the ATG16L1 T300A-encoding gene variant is associated with increased (LPS+ATP)-induced IL-1β production.
(A and B) IL-1β (left), IL-6 (middle), and TNFα (right) assayed by ELISA in clarified tissue culture supernatants from (LPS+ATP)-stimulated PBMCs, stratified for the *ATG16L1* CD susceptibility SNP (rs2241880) genotype from (A) control subjects (black circles) and subjects with ileal CD (open squares) and (B) a second cohort of healthy control subjects. (C) IL-1β mRNA expression, normalized to β-actin gene expression, in LPS-stimulated PBMCs from healthy control subjects stratified for the *ATG16L1* CD susceptibility SNP (rs2241880) genotype was determined by qRT-PCR. In (A), each symbol represents an individual subject (n = 13 control subjects, n = 8 subjects with ileal CD). In (A) - (C), horizontal lines indicate means ± SEM. In (B) and (C), n = 6 subjects per genotype. $*P \leq 0.05$, $**P \leq 0.01$, NS = not significantly different (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

3.3.7 Reduced SHIP mRNA expression is associated with high IL-1β production and *IL1B* transcription

Next, we determined whether increased IL-1β production and *IL1B* transcription was associated with SHIP mRNA expression. Indeed, IL-1β production inversely related to SHIP mRNA expression in (LPS+ATP)-stimulated PBMCs from control subjects (black circles) and subjects with CD (open squares; $R^2 = 0.3723$, $P = 0.0033$) (Figure 3.8A). Similarly, in our second cohort of healthy control subjects, IL-1β production by (LPS+ATP)-stimulated PBMCs was inversely related to SHIP mRNA expression ($R^2 = 0.4438$, $p = 0.0129$) (Figure 3.8B) and *IL1B* transcription also inversely correlated with SHIP mRNA expression ($R^2 = 0.4896$, $p = 0.0078$) (Figure 3.8C). Taken together, these data suggest that low SHIP expression contributes to increased *IL1B* transcription and thereby regulates the level of IL-1β secreted by human PBMCs.

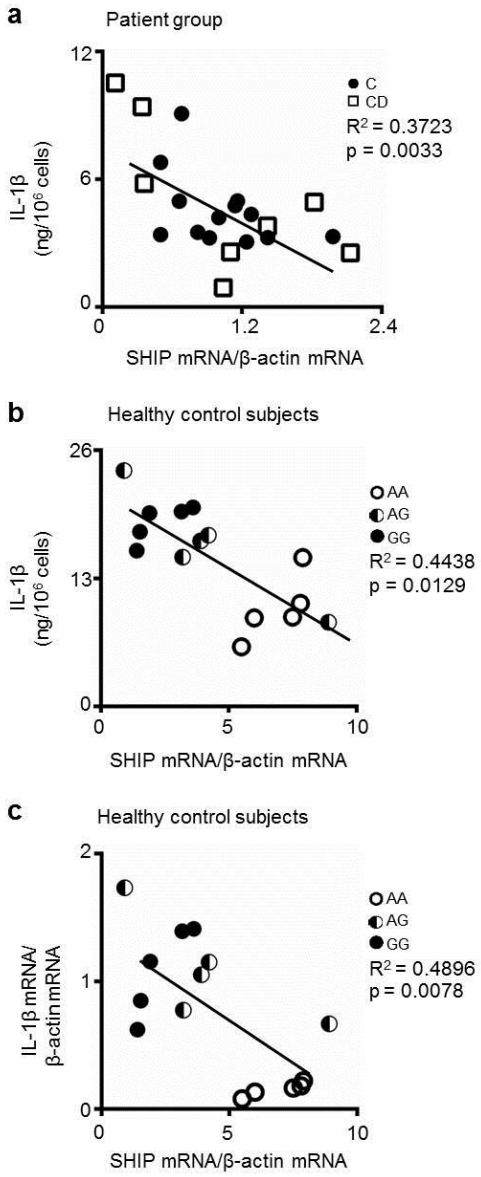

Figure 3.8 Reduced SHIP mRNA expression is associated with increased LPS-induced IL-1β production.

(A and B) IL-1β assayed by ELISA in clarified tissue culture supernatants from (LPS+ATP)-stimulated PBMCs versus SHIP mRNA expression, normalized to *ACTB* expression, determined by qRT-PCR in (A) control subjects (black circles) and subjects with ileal CD (open squares) and (B) a second cohort of healthy control subjects. (C) IL-1β mRNA expression, normalized to *ACTB* expression, in LPS-stimulated PBMCs from healthy control subjects versus SHIP mRNA expression, both determined by qRT-PCR. In (A) – (C), each symbol represents an individual subject. In (A), n = 13 control subjects (black circles) and n = 8 subjects with ileal CD (open squares). In (B) and (C), n = 5 subjects per genotype. In (A) – (C), R^2 = 0.3723, 0.4438, and 0.4896; with P = 0.0033, 0.0129, and 0.0078; respectively.

3.4 Discussion

Herein, I demonstrate that SHIP mRNA expression and activity are reduced in subjects with ileal CD and are associated with homozygosity for the *ATG16L1* CD-associated gene variant. Furthermore, I also show that SHIP is up-regulated during autophagy and that SHIP protein levels and up-regulation are compromised in subjects, who are homozygous for the *ATG16L1* CD-associated gene variant. Finally, I report that IL-1β production and *IL1B* transcription are higher in PBMCs from subjects, who are homozygous for the *ATG16L1* CD-associated gene variant and is associated with reduced SHIP mRNA expression in PBMCs from our subject cohorts.

The fact that we see reduced SHIP mRNA expression in subjects with ileal CD, confirms previous report by Arijs *et al,* 2012, in which they showed that SHIP mRNA is not up-regulated in the ileum during ileal CD.[349] We extend that finding, demonstrating that SHIP mRNA expression is actually decreased during ileal CD, when normalized to infiltrating immune cells, and that reduced SHIP mRNA expression correlates with reduced SHIP activity. SHIP mRNA expression may be up-regulated maintaining SHIP protein levels and activity in the colon, but not the ileum, due to higher abundance of commensal microorganisms in the colon[379] as SHIP mRNA and protein are induced by MyD88-dependent Toll-like receptor signalling.[327,369]

In two independent cohorts, I demonstrate that homozygosity for the *ATG16L1* CD-associated gene variant was associated with low SHIP mRNA expression and activity. Moreover, the relationship between homozygosity for the *ATG16L1* CD-associated gene variant and low SHIP mRNA and activity is independent of inflammation. In healthy control subjects who were heterozygous for the SNP (AG genotype), SHIP mRNA expression and

activity varied widely, ranging from low levels, comparable to that for people with the GG genotype, to high levels, comparable to that for people with the AA genotype. This suggests that additional environmental and/or genetic factors may contribute to low SHIP mRNA expression and activity in some individuals. SHIP protein levels are reduced by the type II inflammatory cytokines, IL-4 and IL-13.[361] In addition, 140 CD-associated risk variants have been identified,[16] including some that code for non-synonymous SNPs in proteins that cause autophagy defects (NOD2)[49] or that are predicted to affect autophagy (IRGM, ATG5, ORMDL3, and XBP-1).[16]

There was no association between SHIP mRNA expression and ATG16L1 mRNA expression in the two cohorts studied. These data are consistent with a previous report demonstrating that ATG16L1 mRNA expression is not influenced by the rs2241880 genotype.[35] This does not, however, preclude the possibility that SHIP gene expression may be reduced epigenetically by the presence of the *ATG16L1* CD risk allele.

Intriguingly, SHIP deficient macrophages had a defect in starvation-induced autophagy. Furthermore, SHIP protein expression was up-regulated during starvation conditions in both PBMCs from our healthy cohort and THP-1 cells, but was compromised in the presence of the T300A CD risk variant and siRNA constructs targeting ATG16L1 respectively. These results suggest that SHIP may be required for starvation-induced autophagy and that fully functional ATG16L1 protein was required for autophagy-induced up-regulation of SHIP protein expression. Class I PI3K is a positive regulator of autophagy[380] and rapamycin-mediated inhibition of mTOR, which is activated downstream of the PI3K/SHIP axis, is frequently used to induce autophagy.[381] Thus, SHIP may regulate autophagy in hematopoietic cells and increased SHIP expression may be required for the

induction and/or maintenance of autophagy. In addition, a defect in autophagy could cause and/or contribute to the intestinal inflammation observed in SHIP deficient mice.

IL-1β production and *IL1B* transcription were higher in PBMCs from subjects, who are homozygous for the *ATG16L1* CD-associated gene variant and was associated with reduced SHIP mRNA expression. In a previous study by Plantinga et al, stimulation with MDP increased IL-1β and IL-6 in PBMCs from subjects, who carry the *ATG16L1* risk variant.[261] I did not see any difference in IL-6 between the genotypes in our cohorts. Higher IL-6 production by PBMCs may be specific to MDP stimulation and may be induced downstream of IL-1β production.[382,383] TNFα production was also not elevated in PBMCs from subjects, who carry the *ATG16L1* risk variant, which may reflect differences in the signalling pathways required for TNFα versus IL-1β transcription.[384,385] Production of both IL-6 and TNFα may be increased by PBMCs at later time points, downstream of IL-1β production.

IL-1β is an important cytokine in IBD. It is secreted from intestinal tissues and macrophages isolated from patients with IBD and IL-1β levels correlate with disease severity.[232,386-388] IL-1β antagonism has been used effectively to treat some genetically defined forms of very early onset IBD[389] and may be more broadly applicable for the treatment of sub-groups of IBD. To our knowledge, this is the first mechanistic insight into the regulation of *IL1B* transcription by autophagy and ATG16L1. Evidence suggests that autophagy modulates the inflammatory response by regulating pro-inflammatory IL-1β production in both mice and in humans.[85] In mice, autophagy blocks inflammasome activation by degrading cytosolic danger associated molecular patterns (DAMPs) that activate inflammasomes, mitochondrial DNA, or reactive oxygen species,[249] or by degrading

inflammasomes/caspase-1[250] or pro-IL-1β directly.[251] Thus, in mice, inhibition or defects in autophagy may lead to uncontrolled IL-1β production by increasing inflammasome activation, rather than by affecting *IL1B* transcription.[249-251,257,390] In contrast, in humans, increased IL-1β production in people with the *ATG16L1* risk variant has been attributed to increased *ILIB* transcription.[261] Our human data are consistent with a model in which autophagy up-regulates SHIP, and SHIP limits the transcription of *IL1B*.

In summary, we have demonstrated that SHIP mRNA expression and activity are reduced in subjects with ileal CD and are also lower in subjects, who are homozygous for the *ATG16L1* CD-associated gene variant, in two independent human cohorts. SHIP is up-regulated during autophagy and SHIP protein levels and induction are compromised in subjects, who are homozygous for the *ATG16L1* CD-associated gene variant. IL-1β production and *IL1B* transcription are higher in PBMCs from subjects, who are homozygous for the *ATG16L1* CD-associated gene variant. Importantly, SHIP mRNA expression inversely correlates with IL-1β production and *IL1B* transcription induced in PBMCs from our subject cohorts. These results identify SHIP as a critical link between the *ATG16L1* CD-associated gene variant and increased *IL1B* transcription, which may contribute to the increased risk of developing intestinal inflammation in subjects, who carry the *ATG16L1* CD-associated gene variant.

Chapter 4: Autophagy-related CD genetic variants modulate IL-1β production upon selective action of PRRs

4.1 Introduction and rationale

CD is a chronic polygenic disease of the gastrointestinal tract characterized by painful intestinal inflammation. Current evidence indicates that CD arises from a disproportionate immune response to intestinal microflora in genetically susceptible individuals. GWAS have identified several genetic variants encoding proteins linked to the cell homeostatic process, autophagy, as associated with increased susceptibility to CD. Despite that, very little is known of how these genetic variants affect commensal-driven inflammation.

SNPs in genes encoding ATG16L1,[35,37,391] NOD2,[48] and IRGM[38,392] that are associated with CD, result in defective autophagy. Defective autophagy results in enhanced bacterial persistence and intestinal inflammation, [256] and has been associated with increased IL-1β production in both mouse and human cells. In humans, homozygosity for the ATG16L1 T300A-encoding gene variant is associated with increased IL-1β and IL-6 production in response to muramyl dipeptide.[261] In mice, ATG16L1 deletion leads to increased LPS-induced IL-1β production.[253] Also, the CD-associated *NOD2* variants are associated with reduced NFκB activation in response to MDP.[281] Furthermore, TLR2/4-induced NFκB activation is reduced in subjects homozygous for the CD-associated *NOD2* variants, R702W, G908R, and L1007fsinsC.[282 393]

Accumulation of misfolded or unfolded proteins leads to endoplasmic reticulum (ER) stress that initiates the unfolded protein response (UPR) to restore homeostasis.[50,51] UPR

induces autophagy genes that are important for cell survival and selectively sequester ER membranes into autophagosome-like structures.[50] SNPs in *XBP1* and *ORMDL3*, two CD-associated genes required for the UPR have also been implicated in autophagy-related defects in CD,[49] but their effects on autophagy and the regulation of IL-1β production in response to PRRs are unknown.

IL-1β has been shown to mediate intestinal inflammation in many mouse models as well as in human CD. In IL-10 KO mice, IL-1β is increased in the small intestine and the colon compared to similar tissue sections from WT mice.[242] IL-1β also drives intestinal pathology in both *Helicobacter hepaticus*-induced and the T cell transfer model of colitis.[241] Moreover, colitis in TLR5 KO mice is associated with increased levels of IL-1β in the inflamed tissues.[244]

We have previously demonstrated that homozygosity for the ATG16L1 T300A-encoding gene variant is associated with reduced mRNA expression and activity of the lipid phosphatase, SHIP, and that, in turn, results in increased *IL1B* transcription. Based on these findings, we hypothesized that autophagy-related CD genetic variants may be associated with increased IL-1β production. Herein, we measured the effects of five autophagy and CD-related gene variants on cytokine production by human peripheral blood mononuclear cells (PBMCs) in response to TLR and NOD2 ligands. DNA samples were obtained from 45 healthy control subjects, which we genotyped for gene variants in five CD-related genes (*ATG16L1*, *ATG5*, *NOD2*, *IRGM*, and *XBP1*), encompassing six SNPs that may be associated with defects in autophagy. Since PBMCs circulate in blood and infiltrate intestinal tissues during inflammation, we measured cytokine production by PBMCs in response to eight TLR ligands and NOD2 ligand comparing responses of the different genotypes.

4.2 Materials and methods

Subject recruitment and blood collection

The study was performed according to the ethical guidelines approved by the University of British Columbia Research Ethics Boards (H14-00622). All subjects provided written informed consent for the study. Peripheral blood was obtained from 45 healthy control subjects and DNA was prepared for genotyping using the QiaAMP DNA blood mini kit (Qiagen, Toronto, Ontario, Canada). PBMCs were isolated by density gradient centrifugation using Ficoll-Paque^{TM} PLUS (GE Healthcare, Piscataway, NJ). Cells were washed and resuspended at a density of 0.5×10^6 cells/mL in RPMI supplemented with 10% FBS.

Genotyping

Stored DNA samples were used to genotype CD-associated SNPs (Table 4.1), using commercially available Taqman assay (Applied Biosystems, Burlington, ON, Canada). Table 4.1 also shows the allelic frequency (as percentage) in subjects of European descent for each of the SNPs examined. SNPs were accepted at call rates >95% and frequencies did not deviate from Hardy-Weinberg equilibrium ($P>0.05$). Subject numbers (n) per genotype are shown in Table 4.2. Total number of subjects per SNP was less than 45 (total number of subjects whose DNA were collected) because the genotyping for some of the subjects for specific SNPs did not work.

Table 4.1 Crohn's disease-associated single nucleotide polymorphisms

Gene	SNP identifier	Frequency (%)			Applied Biosystems assay cat #
		Non-disease homozygous	heterozygous	CD-associated homozygous	
ATG16L1	rs2241880	21.6 (AA)	45.2 (AG)	33.2 (GG)	c_9095577_20
ATG5	rs510432	31.9 (AA)	44.2 (AG)	23.9 (GG)	c_910351_10
	rs12201458	81.7 (CC)	16.7 (CA)	01.7 (AA)	c_30905134_10
NOD2	rs2066844	94.3 (CC)	05.6 (CT)	00.1 (TT)	c_11717468_20
IRGM	rs7714584	91.2 (AA)	08.8 (AG)	00.0 (GG)	c_29565819_10
XBP1	rs35873774	95.1 (TT)	04.9 (TC)	00.0 (CC)	c_25625445_10

Table 4.2 Subject numbers (n) per genotype

Gene	SNP	Non-disease homozygous (n)	Heterozygous (n)	CD-associated homozygous (n)
ATG16L1	rs2241880	AA (n=9)	AG (n=17)	GG (n=11)
ATG5	rs510432	AA (n-10)	AG (n=20)	GG (n=8)
ATG5	rs12201458	CC (n=26)	CA (n=9)	AA (n=0)
NOD2	rs2066844	CC (n=26)	CT (n=5)	TT (n=0)
IRGM	Rs7714584	AA (n=26)	AG (n=11)	GG (n=4)
XBP1	rs35873774	TT (n=33)	TC (n=5)	CC (n=0)

Cell stimulations

For IL-1β production, PBMCs were unstimulated or stimulated with TLR/NOD2 ligands for 5 h, 5 mM ATP for 1 h, or ligands for 4 h with the addition of ATP for the final 1 h. For IL-6, TNFα, and IL-10 production, cells were unstimulated or stimulated with TLR/NOD2 ligands for 24 h. TLRs and NOD2 ligands (InvivoGen, San Diego, CA) used and the concentrations at which they were used are shown in Table 4.3. After incubation (5 h in total for IL-1β and 24 h in total for IL-6, TNFα, and IL-10), supernatants were collected, centrifuged at 10 000 rpm for 5 min to remove floating cells, and were stored at -80°C until they were analyzed by ELISA.

Cytokine measurements

Cytokine measurements were performed on clarified, cell-free tissue culture supernatants by ELISA according to the manufacturers´ instructions. ELISA kits for human IL-β, TNFα, IL-6, and IL-10 were from BD Biosciences (Mississauga, ON).

Statistical Analyses

Unpaired two-tailed Student's *t* tests and one-way ANOVA were performed using GraphPad Prism version 5 software. For multiple comparisons, the Bonferroni correction was applied. Differences were considered significant at $P < 0.05$.

Table 4.3 PRR ligands and concentrations used for stimulations

PRR	Ligand	Concentration
TLR1/2	Pam3CysSerLys4 (Pam3CSK4)	50 ng/mL
TLR2	Heat-killed *Listeria monocytogenes* (HKLM)	10^8 cells/mL
TLR3	Poly (I:C) low molecular weight (LMW)	10 μg/mL
TLR4	*E. coli* K12 lipopolysaccharide (LPS)	10 ng/mL
TLR5	*S. typhimurium* flagellin	100 ng/mL
TLR6	Pam2CGDPKHPKSF (FSL)	100 ng/mL
TLR7	Imiquimod (R837)	5 μg/mL
TLR8	Single stranded RNA40 (ssRNA40)	5 μg/mL
TLR9	CpG ODN 2006 (type B)	5 μM
NOD2	Muramyl dipeptide (MDP)	10 μg/mL

4.3 Results

4.3.1 Determination of PRR ligand concentrations to be used for stimulations

To determine the concentrations of ligands used for PBMC stimulations, cells were stimulated with different concentrations of ligands for 4 h followed by addition of ATP (5 mM) for 1 h and IL-1β production measured. We found that, all ligands except TLR9 ligand (CpG ODN 2006 type B) activated PBMCs to produce IL-1β (Figure 4.1). TLR9 ligand did not stimulate IL-1β production at low or high doses. As such, TLR9 ligand was not used in the rest of the study. Concentrations used for the different TLR/NOD2 ligands indicated in Table 4.3 are highlighted in red (Figure 4.1).

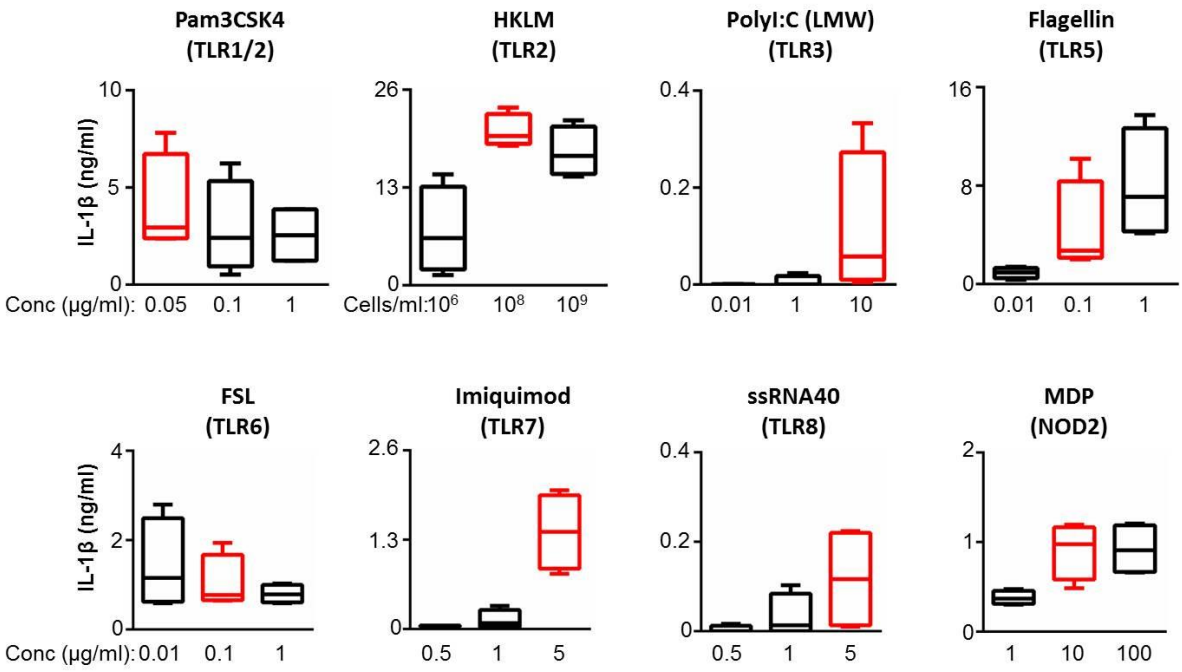

Figure 4.1 TLRs/NOD2 ligand dose response assays
IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs of 4 healthy control subjects stimulated with PRR ligands for 4 h followed by addition of ATP (5 mM) for 1 h. Horizontal lines indicate means ± SEM for PBMCs from 4 subjects.

4.3.2 Homozygosity for the ATG16L1 T300A-encoding gene variant is associated with increased IL-1β production in response to TLR4 and TLR5 ligands

The ATG16L1 T300A-encoding gene variant is associated with increased susceptibility to CD.[35-37] Subjects homozygous for the risk allele (GG) have an odds ratio (OR) of 2.38 while heterozygotes have an OR of 1.86,[35,259] suggesting that subjects with the *ATG16L1* CD-associated gene variant are more likely to develop CD than subjects, who do not have the gene variant. The T300A gene variant significantly increases ATG16L1 susceptible to cleavage by Caspase 3/7 released during cellular/metabolic stress, death receptor ligation, or bacterial infection, resulting in a reduction in full-length, functional ATG16L1, and defective autophagy.[254,255] Autophagy acts as a self-regulatory mechanism to

limit secretion of inflammatory cytokines, such as IL-1β and IL-23,[257] as such, the presence of ATG16L1 T300A-encoding gene variant enhances proinflammatory cytokine secretion.[261] To determine the effect of the *ATG16L1* rs2241880 risk allele (G) on cytokine production in response to PRR ligands, PBMCs obtained from healthy control subjects with different *ATG16L1* genotypes were stimulated with the following ligands (receptors): Pam3CSK4 (TLR1/2), HKLM (TLR2), polyI:C LMW (TLR3), LPS (TLR4), flagellin (TLR5), FSL (TLR6/2), Imiqimod (TLR7), ssRNA40 (TLR8), and MDP (NOD2) in the presence or absence of ATP, and cytokine production was measured in cell-free supernatants (Figure 4.2). Data showing significant differences in cytokine production between genotypes are shown in a red box. Significantly higher IL-1β production was observed in subjects homozygous (GG) for the gene variant associated with CD in response to LPS (TLR4) and flagellin (TLR5) (Figure 4.2A). The rs2241880 genotype did not affect IL-1β production in response to TLR1/2, TLR2, TLR3, TLR6/2, TLR7, TLR8, or NOD2 ligands (Figure 4.2A). Both IL-6 (Figure 4.2B) and TNFα (Figure 4.2C) production were not affected by the *ATG16L1* rs2241880 genotype. Interestingly, homozygosity (GG) for the *ATG16L1* gene variant was associated with increased IL-10 production in response to LPS (Figure 4.2D). There was no TNFα (Figure 4.2C) or IL-10 (Figure 4.2D) production in response to TLR3, TLR6/2, and NOD2 ligands, and the TLR7 ligand did not stimulate TNFα production (Figure 4.2C).

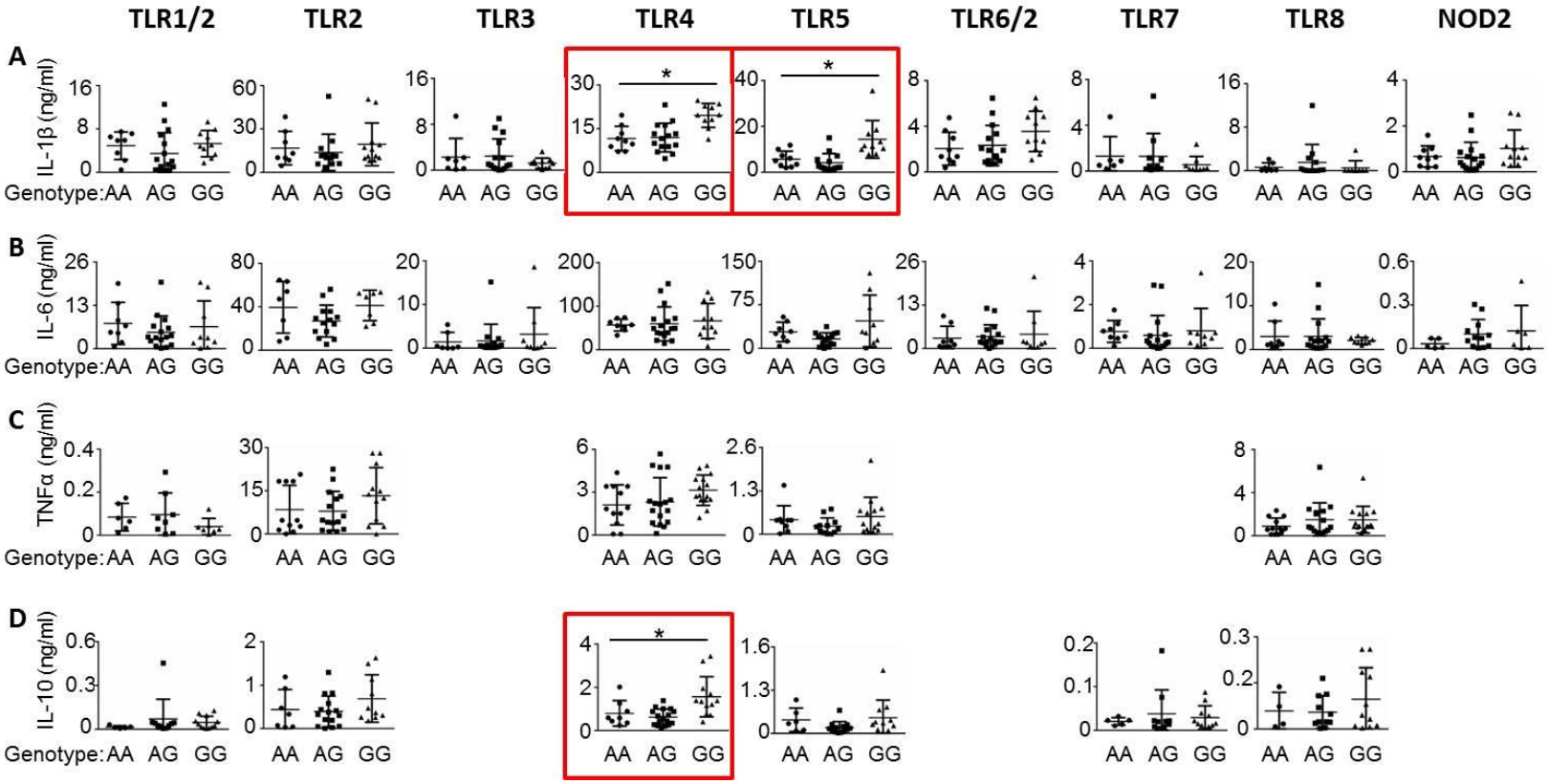

Figure 4.2 Homozygosity for the ATG16L1 T300A-encoding gene variant is associated with increased IL-1β production in response to TLR4/5 ligands+ATP.

(A) IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands+ATP, stratified for the *ATG16L1* rs2241880 genotype. Each symbol represents an individual subject. (B) IL-6, (C) TNFα, and (D) IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands, stratified for the *ATG16L1* rs2241880 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: AA (n=9), AG (n=17), and GG (n=11). In (A) - (D), horizontal lines indicate means ± SD. *$P \leq 0.05$ (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

4.3.3 *ATG5* rs12201458 gene variant is associated with an anti-inflammatory response to TLR4 ligand

ATG5 forms a complex with ATG12 and is important for the conjugation of LC3-I with phosphatidylethanolamine to form LC3-II, and formation of the autophagosome. ATG5 is a key component of the autophagy machinery, and enhances autophagic clearance of viruses[262] and antigen presentation.[263] In mice, *Atg5* deletion leads to impaired autophagy and defective lysozyme secretion by Paneth cells.[80] The *ATG5* rs510432 gene variant is associated with increased *ATG5* mRNA expression in human nasal epithelial cells from asthma patients, [267] suggesting that, the *ATG5* rs510432 gene variant may increase autophagy, which in turn, may lead to reduced inflammatory cytokine production. We next determined whether the presence of the *ATG5* rs510432 or rs12201458 gene variants affected cytokine production in response to PRR ligands. The rs510432 gene variant did not affect cytokine production in response to PRR ligands (Figure 4.3). Intriguingly, the *ATG5* rs12201458 gene variant (CA genotype), which is associated with CD, was associated with increased IL-10 production (Figure 4.4D) in response to a TLR4 ligand. The rs12201458 gene variant did not affect IL-1β production (Figure 4.4A), IL-6 production (Figure 4.4B), or TNFα production (Figure 4.4C) in response to PRR ligands. All other ligands tested did not show any difference in IL-10 production (Figure 4.4D) between genotypes.

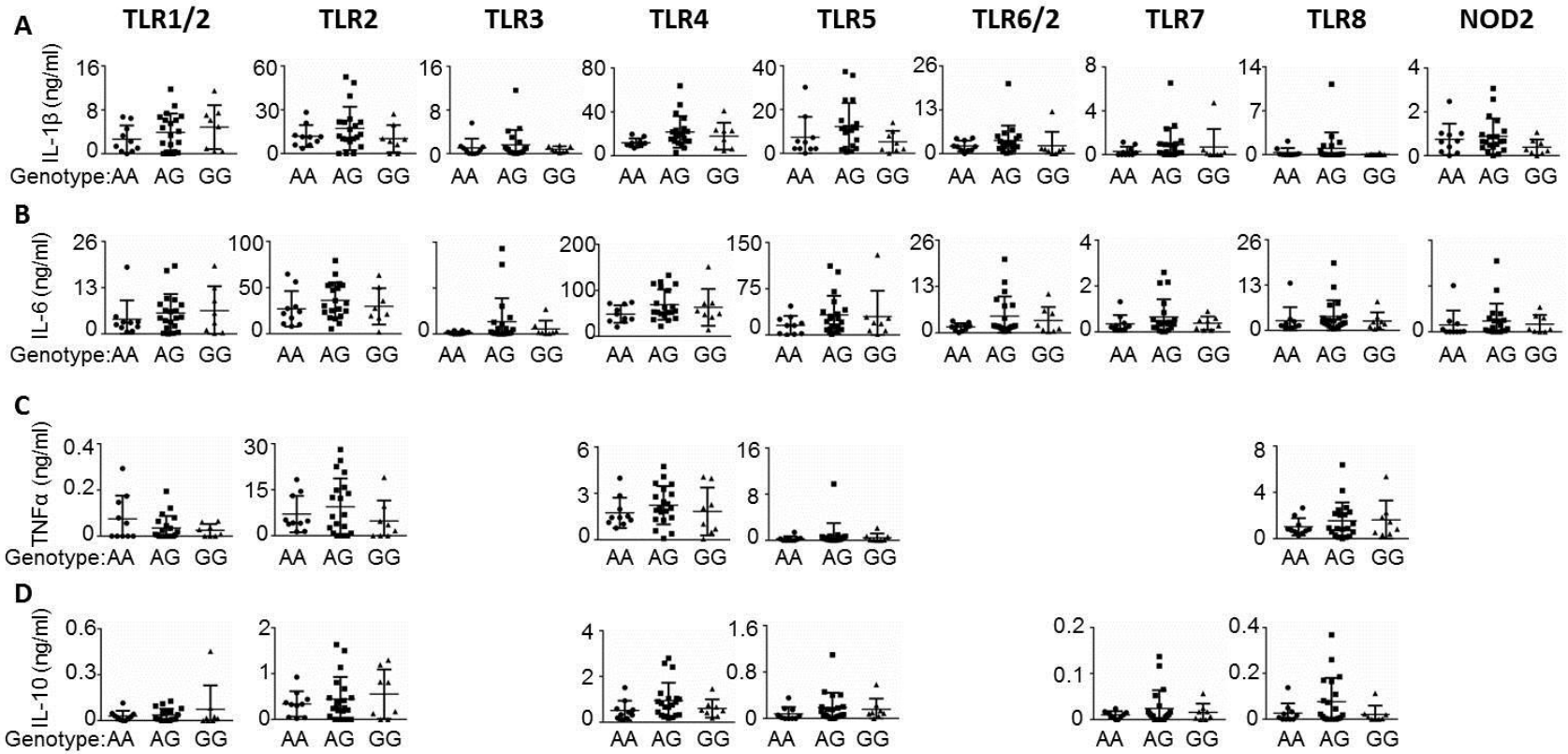

Figure 4.3 The *ATG5* rs510432 gene variant does not affect IL-1β production in response to TLRs and NOD2 ligands. (A) IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands+ATP, stratified for the *ATG5* rs510432 genotype. (B) IL-6, (C) TNFα, and (D) IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands, stratified for the *ATG5* rs510432 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: AA (n=10), AG (n=20), and GG (n=8). In (A) - (D), horizontal lines indicate means ± SD. **P* ≤ 0.05 (one-way ANOVA with Bonferroni correction for multiple pairwise comparisons between genotypes).

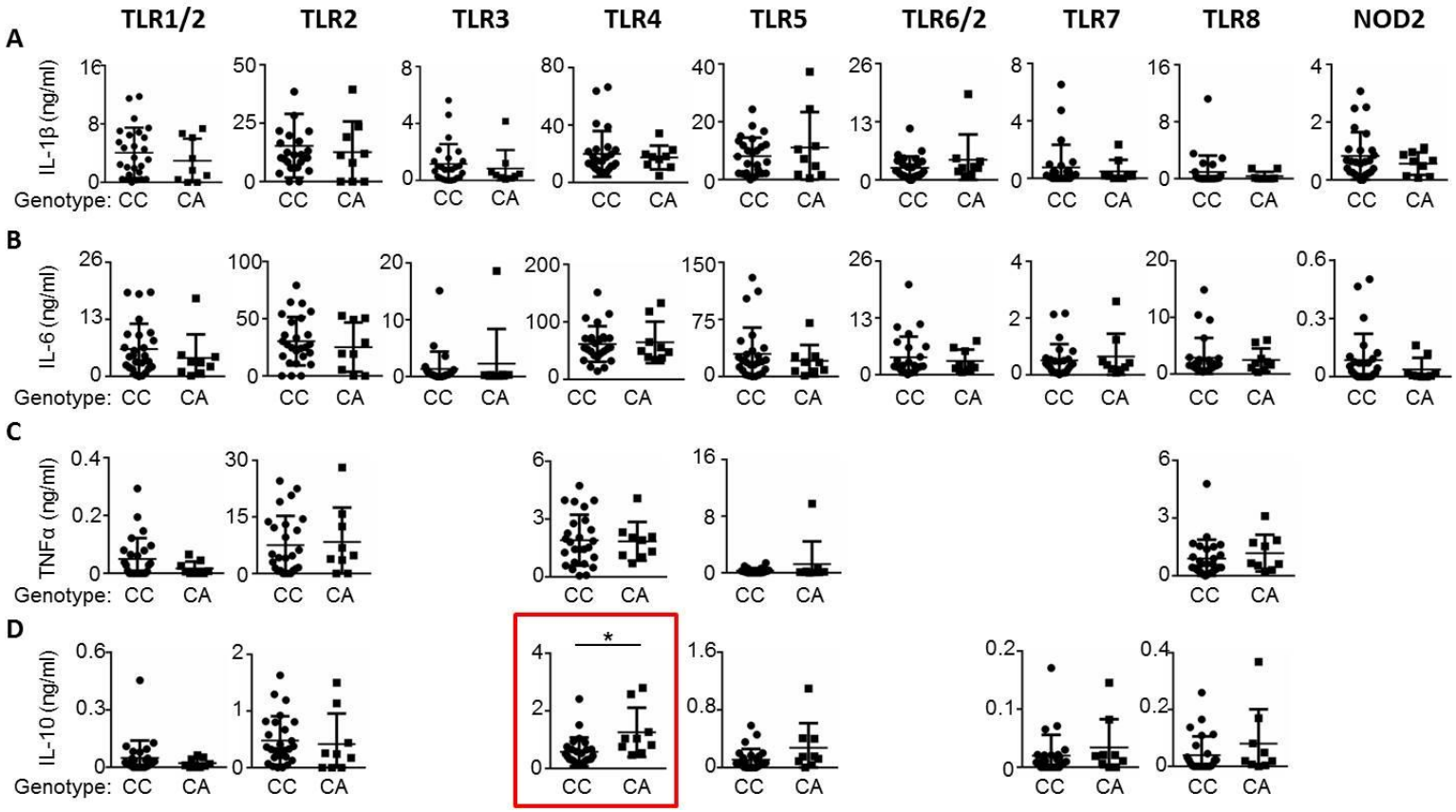

Figure 4.4 The *ATG5* rs12201458 gene variant is associated with an increase in IL-10 production in response to TLR4 ligand.

(A) IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands+ATP, stratified for the *ATG5* rs12201458 genotype. **(B)** IL-6, **(C)** TNFα, and **(D)** IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands, stratified for the *ATG5* rs12201458 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: CC (n=26), CA (n=9), and AA (n=0). In **(A) - (D)**, horizontal lines indicate means ± SD. *$P \leq 0.05$ (unpaired Student's *t*-test.

4.3.4 The *NOD2* rs2066844 gene variant is associated with increased IL-1β production in response to TLR4 ligand

The *NOD2* rs2066844 SNP is strongly associated with increased susceptibility to CD.[32,33] The OR for individuals homozygous for the SNP (TT) ranges from 20-40, while the OR for heterozygotes (CT) ranges from 2-4,[32,33,279,280] suggesting that individuals with the *NOD2* rs2066844 risk allele are more likely to develop CD. In the presence of the CD-associated *NOD2* gene variants, NOD2 fails to recruit ATG16L1 to the cell membrane at the site of bacterial entry, resulting in defective autophagy, and clearance of intracellular bacteria.[284] To determine whether the *NOD2* CD-associated risk allele (T) affected cytokine production in response to PRR ligands, we measured cytokine production in PBMCs stimulated with various PAMPs. The *NOD2* rs2066844 gene variant was associated with increased IL-1β production in response to LPS+ATP (Figure 4.5A). Activation of TLR1/2 and NOD2 resulted in reduced IL-1β production (Figure 4.5A). No significant differences were observed for IL-6 production (Figure 4.5B), TNFα production (Figure 4.5C), or IL-10 production (Figure 4.5D) between the genotypes.

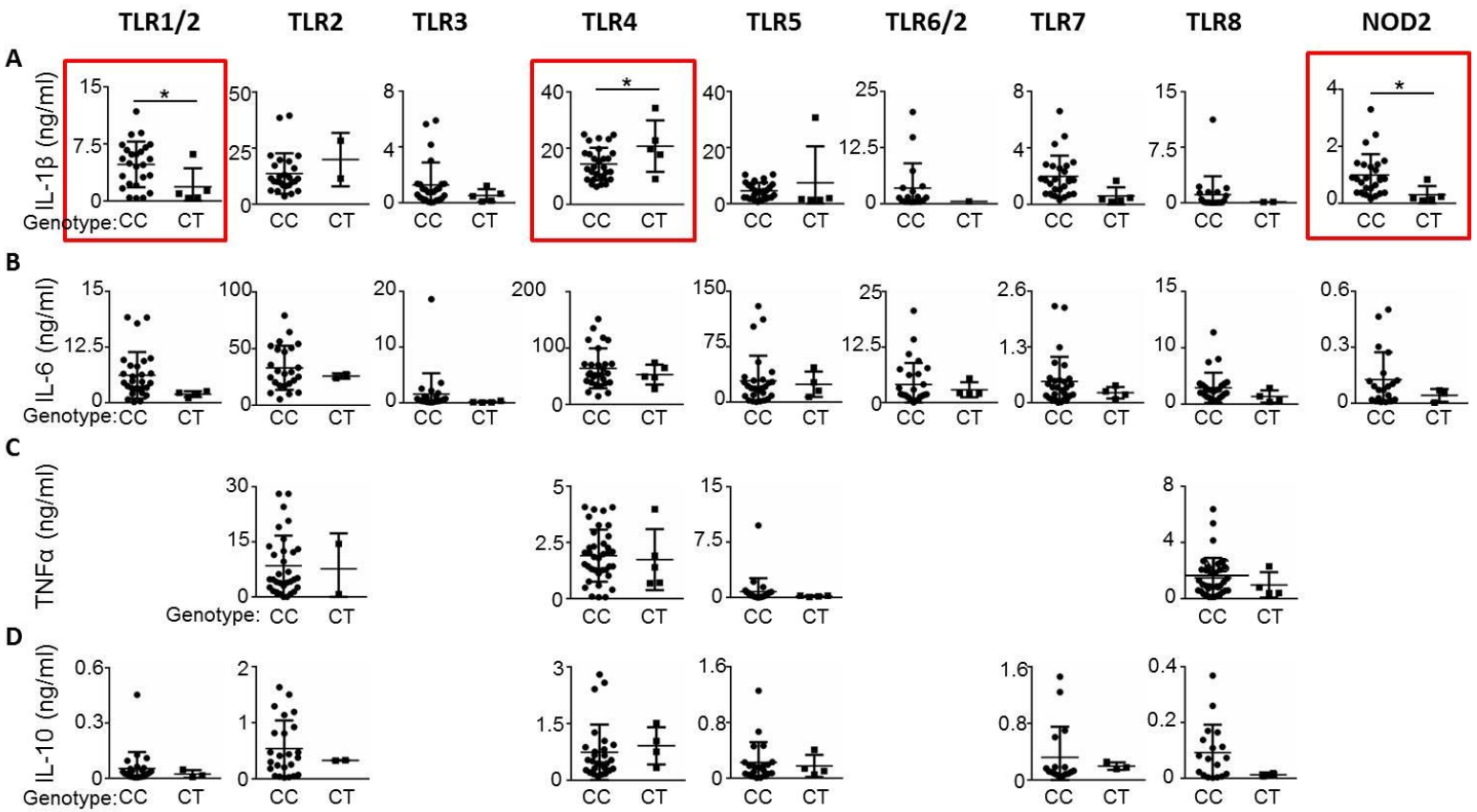

Figure 4.5 The *NOD2* rs2066844 gene variant is associated with increased IL-1β production in response to TLR4 ligand.

(A) IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands+ATP, stratified for the *NOD2* rs2066844 genotype. (B) IL-6, (C) TNFα, and (D) IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands, stratified for the *NOD2* rs2066844 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: CC (n=30), CT (n=5), and TT (n=0). In (A) - (D), horizontal lines indicate means ± SD. *$P \leq 0.05$ (unpaired Student's *t*-test)

4.3.5 The *IRGM* rs7714584 gene variant does not affect IL-1β production in response to TLRs and NOD2 ligands

IRGM is a mitochondrial protein that regulates the initial phase of anti-bacterial autophagy and cellular homeostasis.[53,268-270] The *IRGM* rs7714584 SNP has been strongly associated with increased susceptibility for developing CD.[38,272,273] Individuals carrying the *IRGM* rs7714584 CD-associated gene variant have an OR of 1.37,[39] suggesting that the presence of this SNP increases the risk of developing CD. The *IRGM* SNP has been reported to reduce expression levels of IRGM, there by affecting conversion of LC3-I to LC3-II and results in defective autophagy.[272] CD patients harboring the *IRGM* CD-associated gene variant have reduced autophagy in the inflamed intestinal mucosa,[273] as such, the presence of the *IRGM* rs7714584 gene variant may be associated with increased pro-inflammatory cytokine production. We next determine whether the CD-associated *IRGM* rs7714584 gene variant altered cytokine production in PBMCs. IL-1β (Figure 4.6A), IL-6 (Figure 4.6B), TNFα (Figure 4.6C), and IL-10 (Figure 4.6D) production were not affected by the presence of rs7714584 gene variant.

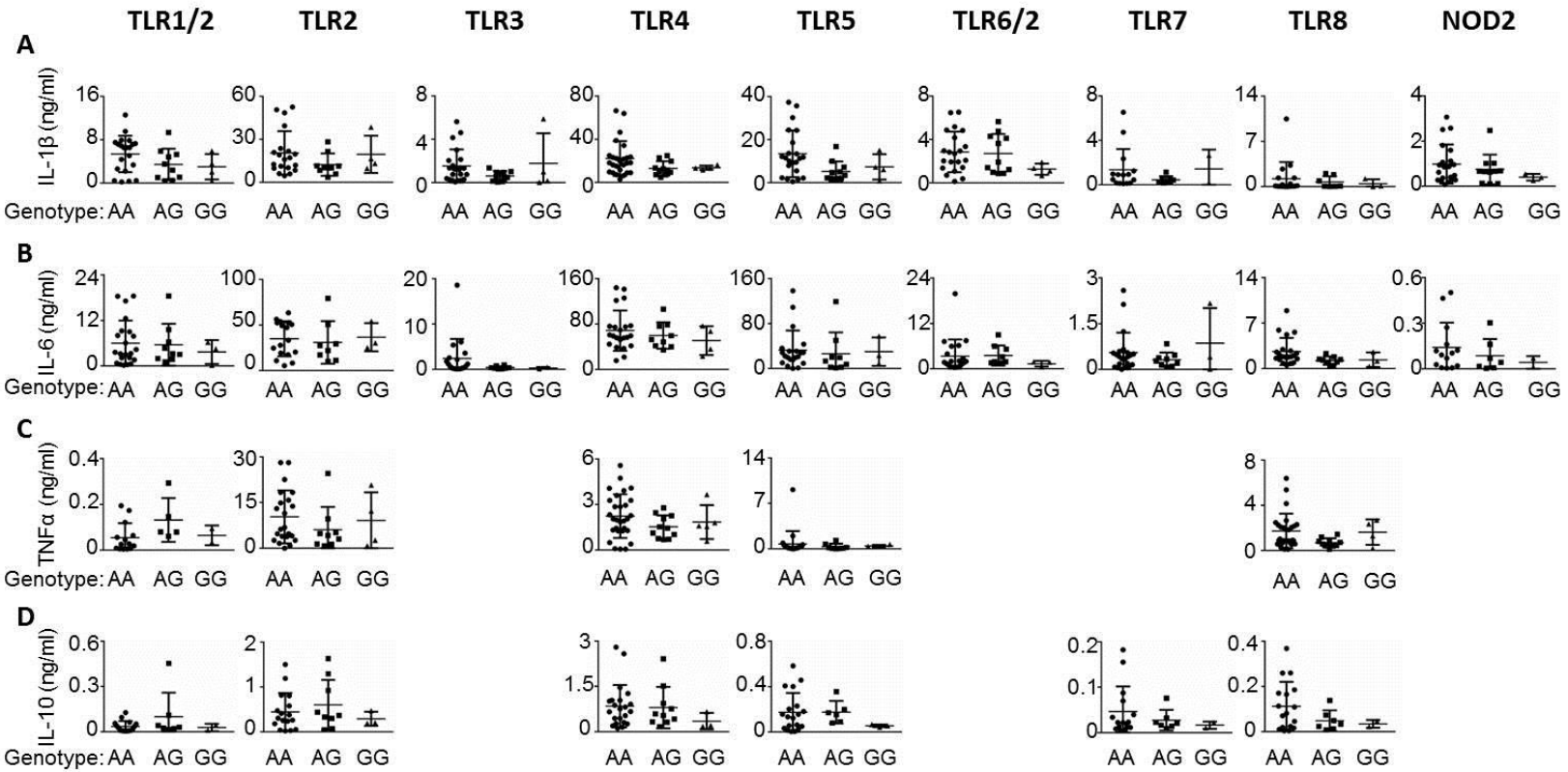

Figure 4.6 The *IRGM* rs7714584 gene variant does not affect IL-1β production in response to TLRs and NOD2 ligands. (A) IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands+ATP, stratified for the *IRGM* rs7714584 genotype. Each symbol represents an individual subject. (B) IL-6, (C) TNFα, and (D) IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands, stratified for *IRGM* rs7714584 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: AA (n=26), AG (n=11), and GG (n=4). In (A) - (D), horizontal lines indicate means ± SD.

4.3.6 The *XBP-1* rs35873774 gene variant is associated with increased IL-1β production in response to PRR ligands

XBP-1 is a transcription factor that is activated downstream of the UPR during ER stress.[292,293] Accumulation of misfolded proteins within the ER causes ER stress that initiates the UPR.[285,286] UPR enhances production of the spliced variant of XBP1 (*XBP1s*), which is translocated to the nucleus, and up regulates transcription of *XBP1* and other UPR target genes to restore protein folding.[292-294] TLR2 or TLR4 have also been shown to activate XBP1 in macrophages.[294] XBP1 in turn increases transcription of pro-inflammatory genes, such as *IL6* and *TNFα*.[294] The *XBP1* rs35873774 SNP has been strongly associated with both CD and UC.[295-297] The OR for individuals carrying the *XBP1* rs35873774 risk variant, associated with IBD is 0.74 (95% confidence interval 0.66-0.84), suggesting that, the risk allele may confer protection from IBD.[298] We next determined whether the *XBP-1* rs35873774 gene variant associated with IBD affects cytokine production in PBMCs from healthy control subjects. Increased IL-1β production was associated with the *XBP-1* gene variant in response to TLR1/2, TLR3, TLR4, TLR7, TLR8, and NOD2 ligands (Figure 4.7A). IL-1β production in response to TLR5 and TLR6/2 agonists were not significantly different (Figure 4.7A). The *XBP1* gene variant was also associated with increased IL-6 production (Figure 4.7B) in response to TLR1/2 and TLR8 ligands, and increased TNFα production (Figure 4.7C) in response to TLR5 and TLR8 ligands. There was no effect of the *XBP1* rs35873774 genotype on IL-6 production in response to TLR2, TLR3, TLR4, TLR5, TLR6/2, TLR7, and NOD2 ligands (Figure 4.7B); on TNFα production in response to TLR1/2, TLR2, and TLR4 ligands (Figure 4.7C); or on IL-10 production in response to ligands for TLR1/2, TLR2, TLR4,

TLR5, and TLR8. TNFα (Figure 4.7C), and IL-10 (Figure 4.7D) were not produced in response to TLR3, TLR6/2, TLR7, or NOD2 ligands.

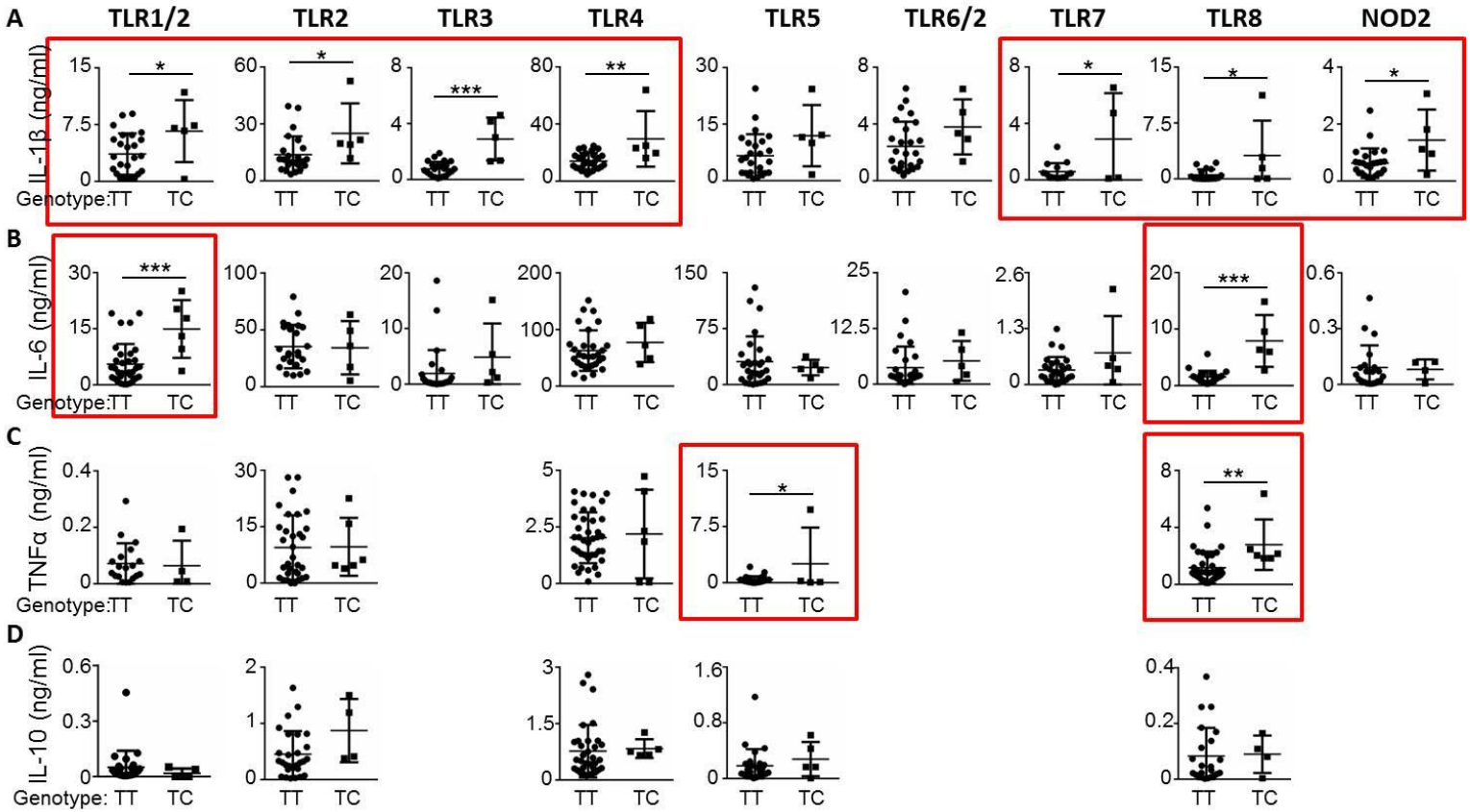

Figure 4.7 The *XBP1* rs35873774 gene variant is associated with increased IL-1β production in response to PRR ligands. **(A)** IL-1β measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects and stimulated with TLR/NOD2 ligands+ATP, stratified for the *XBP1* rs35873774 genotype. Each symbol represents an individual subject. **(B)** IL-6, (C) TNFα, and **(D)** IL-10 measured by ELISA in clarified tissue culture supernatants from PBMCs from healthy control subjects stimulated with TLR/NOD2 ligands, stratified for *XBP1* rs35873774 genotype. Each symbol represents an individual subject. Number of subjects (n) per genotype was: TT (n=33), TC (n=5), and CC (n=0). In **(A) - (D)**, horizontal lines indicate means ± SD. *$P \leq 0.05$ (unpaired Student's *t*-test).

4.4 Discussion

The effects of CD-associated genetic variants in ATG16L1,[253,261] and NOD2[394,395] on IL-1β production have been shown in mouse and human studies. These studies have assessed IL-1β production in response to LPS (TLR4), Pam3CSK4 (TLR2), or MDP (NOD2). No studies have reported the effects of these polymorphisms on IL-1β production in response to other TLR ligands, which are also represented in intestinal flora. Herein, I extend these observations by showing that increased IL-1β production is associated with the ATG16L1 T300A-encoding gene variant in response to flagellin, and the *XBP1* rs35873774 CD variant in response to Pam3CSK4 (TLR1/2), HKLM (TLR2), polyI:C (LMW) (TLR3), LPS (TLR4), Imiquimod (TLR7), ssRNA (TLR8), and MDP (NOD2). Furthermore, the *XBP1* rs35873774 variant was also associated with increased IL-6 and TNFα production in response to ssRNA (TLR8). Increased IL-10 production by PBMCs was associated with both the *ATG16L1* rs2241880 and the *ATG5* rs12201458 gene variants in response to LPS.

In the intestine, autophagy is an important component of the innate immune response to invading pathogens.[396] As such, defects in autophagy due to the presence of the ATG16L1 T300A-encoding gene variant, [35,37,254,255,391] have been associated with defective bacterial clearance, [80,245,260] increased IL-1β production, [253] and CD.[16,42,254] We, and others,[261] have demonstrated that homozygosity for the *ATG16L1* rs2241880 gene variant is associated with increased IL-1β in response to LPS+ATP and MDP, respectively. The present data is consistent with our previous finding and demonstrates that IL-1β is increased in subjects homozygous for the T300A expressing gene variant in response to LPS+ATP in a larger cohort. In addition, our results indicate that homozygosity for the ATG16L1 T300A-encoding gene variant is associated with increased IL-1β production in response to

flagellin+ATP. These results suggest that the effect of the rs2241880 gene variant is TLR-specific and affects IL-1β production only when certain PRRs (TLR4, TLR5, and NOD2) are involved. Increased IL-10 production in subjects homozygous for the ATG16L1 T300A-encoding gene variant in response to TLR4 ligand could be associated with a compensatory response aimed at dampening down increased IL-1β and/or a robust immune response leading to the resolution of inflammation.

The *ATG5* SNPs (rs510432 and rs12201458) have been associated with increased susceptibility to CD.[16,52] However, the effect of these CD-associated SNPs in ATG5 on autophagy and IL-1β remains to be determined. In this study, we show an association between the *ATG5* rs12201458 gene variant and increased IL-10 production. We also saw a trend towards increased IL-10 production in response to a TLR5 ligand. These results suggest that the CD-associated *ATG5* rs12201458 SNP is associated with an anti-inflammatory response and hence its potential involvement in CD susceptibility needs to be clarified. Based on our observations, we hypothesize that the *ATG5* rs12201458 SNP may increase autophagy. This, in turn, may up-regulate the lipid phosphatase, SHIP, and result in reduced IL-1β production in response to stimulation. The effects of the *ATG5* rs12201458 SNP on increased susceptibility to CD may be independent of its role in autophagy. The fact that we did not see a difference in cytokine production in the presence of the *ATG5* rs510432 SNP, suggests that the effects of this SNP may be cell specific.

The *NOD2* gene variants result in defective autophagosome formation and autophagic clearance of intracellular bacteria.[256,284] Our data for the *NOD2* gene variant are consistent with previous findings. *NOD2* variants are associated with increased pro-inflammatory cytokine production in mice due to a gain of function phenotype, which leads to higher IL-1β

production after MDP stimulation.[397] Previous studies in mice have shown that homozygosity for the *Nod2*^{2939ic} mutation (the mouse homolog of the CD susceptibility allele, *3020insC*) does not affect responses to TLR ligands.[397] In contrast, in humans, reduced *IL1β* transcription has been associated with reduced LPS-induced NFκB activation resulting from a loss of function phenotype.[398] Consistent with that, we show that IL-1β production is increased in subjects with the CD-associated *NOD2* SNP in response to TLR4 ligand, but reduced in response to TLR1/2 and NOD2 ligands. In addition, the limited number of subjects with the *NOD2* rs2066844 gene variant within our subject cohort makes it extremely difficult to draw conclusions about its effects in our study.

The *IRGM* rs7714584 gene variant did not affect cytokine production in our cohort. The CD-associated *IRGM* rs7714584 gene variant has been shown to alter IRGM expression levels leading to defective autophagy.[272] In mice, autophagy negatively regulates IL-1β production by degrading cytosolic DAMPs, ROS, and mitochondrial DNA that are known activators of the NLRP3 inflammasome, or by directly degrading pro-IL-1β or active caspase-1.[49] Furthermore, we have previously reported that starvation-induced autophagy up regulates SHIP mRNA expression and protein levels in both mouse and human cells, leading to reduced IL-1β production (Chapter 3). Our results suggest that autophagy may not be affected in PBMCs from subjects with the *IRGM* gene variant, which may result in no change in SHIP mRNA expression and protein levels, and consequently no difference in IL-1β production.

The UPR is triggered upon accumulation of misfolded proteins in the ER and induces autophagy to restore homeostasis.[49,50] Accumulation of misfolded proteins in the ER results in unconventional XBP1 mRNA splicing by endonuclease inositol-requiring enzyme 1

(IRE1) in the cytoplasm.[399] The spliced form of XBP1 (*XBP1s*) that is produced, up regulates the transcription of proinflammatory cytokine genes, such as IL-6 and TNFα in the nucleus.[294] SNPs in genes encoding XBP1 and ORMDL3, key proteins involved in the UPR, have been associated with increased susceptibility to CD,[49] but their role in autophagy and IL-1β-mediated intestinal inflammation have not been assessed. Interestingly, our results show that, the *XBP1* rs35873774 CD-associated gene variant is associated with increased IL-1β production in response to TLR and NOD2 ligands. Despite the small number of subjects with the *XBP1* gene variant used in these experiments, our results demonstrate an important role for the *XBP1* gene variant in regulating innate immune responses. In mice, XBP1 deficiency leads to impaired antimicrobial function of Paneth cells and increased Paneth cell apoptosis.[298] XBP1 deficiency also induces ER stress and increases susceptibility to DSS-induced colitis.[298] TNFα is elevated in colonic tissues of DSS-treated XBP1 deficient mice compared to WT.[298] Furthermore, it has been demonstrated that more than 60% of XBP1-/- mice and 30% of XBP1+/- mice develop spontaneous ileitis associated with ER stress, highlighting a critical role for mono-allelic expression of *XBP1* in the onset of intestinal inflammation.[298] In humans, expression of the spliced variant of XBP1 (*XBP1s*), which is induced during ER stress, is increased in both inflamed and non-inflamed ileal and colonic biopsies from CD and UC patients.[298] The CD-associated *XBP1* rs35873774 gene variant is hypomorphic, causing a partial loss of the gene function, and reduced expression of the UPR and XBP1 target genes.[298] Autophagy is induced as a compensatory mechanism during ER stress to clear misfolded proteins. As such, SNPs in genes that are involved in proper functioning of the ER could result in defective autophagy. This may lead to enhanced bacterial persistence in the LP and intestinal inflammation. In addition, ER stress results in

the release of mitochondrial ROS and potassium (K^+) efflux, which activate the NLRP3 inflammasome, and IL-1β/IL-18 production independent of the UPR.[209] Thus, we speculate that the *XBP1* SNP causes increased ER activity and ER stress, which leads to release of excess amounts of endogenous DAMPs that activate inflammasomes, and lead to increased IL-1β production. The protective effects of the *XBP1* rs35873774 IBD-associated risk variant may be mediated by increased IL-18 production in IECs during ER stress. Previous studies have reported that acute exposure of IECs to DAMPs result in IL-18 production, which stimulates epithelial stem cell differentiation and proliferation, and promotes tissue restitution after injury.[237] In addition, SHIP levels may be reduced in subjects with the *XBP1* rs35873774 IBD-associated risk variant, resulting in increased IL-1β production.

In summary, our data suggest that the association of *ATG16L1* gene variant with increased IL-1β production is PRR specific and that the CD-associated *XBP1* gene variant enhances IL-1β production in response to PAMPs. Furthermore, CD is a polygenic disease, as such, it is most likely that genetic variants that alter autophagy and affect SHIP expression may impact the inflammatory response to commensal microbes, and hence predispose to intestinal inflammation.

Chapter 5: Concluding remarks and future directions

5.1 Concluding remarks

CD may encompass multiple subtypes of disease. An inappropriate or uncontrolled immune response to commensal bacteria and bacterial antigens in genetically susceptible individuals is believed to be crucial for the development of CD. Inappropriate immune responses could be due to an overreaction of the innate immune system to commensal or pathogenic bacteria, or a shift in the balance in favor of T helper cell responses over regulatory T cell responses. IL-1β is a central mediator of inflammatory diseases, but strangely, IL-1 targeted therapy has not been used in the treatment of IBD. This thesis addresses two very important questions related to CD: (1) can macrophage-derived IL-1β contribute to intestinal inflammation and CD, and (2) are there cellular processes and specific cell signalling pathways that link previously identified CD-associated genetic variants to autoinflammation in CD subjects?

In Chapter 2 we show that SHIP suppresses intestinal macrophage-derived IL-1β production and SHIP deficiency contributes to ileal inflammation. Moreover, depleting intestinal macrophages prevents development of intestinal inflammation in the SHIP-/- mice. Furthermore, we demonstrate that prophylactic treatment with anakinra reduced active caspase-1 and IL-1β production, and prevented development of intestinal pathology in SHIP-/- mice. In Chapter 3, we show that homozygosity for the CD-associated ATG16L1 T300A-encoding gene variant was associated with reduced SHIP mRNA and activity, and increased IL-1β production in healthy control subjects and CD subjects. We also show that autophagy up-regulates SHIP expression, which was compromised in the presence of the

T300A-encoding gene variant. SHIP is a critical negative regulator of inflammatory cytokine production including IL-1β, and SHIP deficiency results in increased pro-inflammatory cytokine production. Data from Chapter 3 suggest that, IL-1β is increased in subjects homozygous for the T300A-encoding gene variant because they fail to up-regulate the negative regulator, SHIP. Finally, in Chapter 4, we confirm the association between homozygosity for the *ATG16L1* CD-associated gene variant and increased IL-1β production, and identify a possible association between the *XBP1* rs35875774 gene variant and increased IL-1β production. Indeed, the *XBP1* rs35875774 gene variant was associated with increased IL-1β production in response to several intestinal microbial-associated molecular patterns (MAMPs) that activate different PRRs in the presence of ATP.

SNPs in genes encoding proteins that play key roles in the regulation of both innate and adaptive immune responses have been associated with IBD. We have shown that these SNPs could serve as important elements in the identification of different subtypes of CD. We have identified a unique disease phenotype in CD patients that is associated with homozygosity for the ATG16L1 T300A-encoding gene variant. Recent studies have shown that the presence of the CD risk variant in ATG16L1 increases its degradation by Caspase 3/7 activated during metabolic stress, death receptor ligation or bacterial infection, [254,255] and result in defective autophagy. We have shown that subjects homozygous for the CD risk variant in ATG16L1 not only have defective autophagy, but also have reduced SHIP expression and activity. The T300A-encoding gene variant and defective autophagy have been associated with increased IL-1β production in both mice and humans.[48,250-252,261] Hence, subjects homozygous for the CD-associated *ATG16L1* gene variant have defective

autophagy, which may lead to reduced SHIP expression and activity, and in turn increase IL-1β production.

CD has been included among polygenic diseases associated with autoinflammation due to overlapping features, which include its periodicity, strong association with environmental triggers, and a failure to respond to therapy directed against TNFα.[352] Identification of disease subtypes for personalized therapeutic intervention is the new goal of current IBD research. In the past two decades, biological therapies, such as infliximab and adalimumab, which are monoclonal antibodies against TNFα, have been used to manage IBD. Studies have shown that, treating CD patients early in the course of disease with infliximab is the most efficacious way to induce mucosal healing in patients.[400] In addition, adult IBD patients, who not responsive to infliximab, have been treated with adalimumab, which can induce and maintain remission.[401] However, the use of anti-TNFα drugs increases the possibility of opportunistic infections, since anti-TNFα may reduce the body's ability to fight infectious diseases. Also, the large number (30%) of CD patients that are predicted to become refractory to current therapy and the fact that some patients develop antibodies against these biological therapies, underscores the urgent need to develop novel therapeutic strategies to target intestinal inflammation in CD.

IL-1 antagonism with anakinra, the synthetic IL-1 receptor antagonist, rilonacept, the soluble IL-1 decoy receptor, or canakinumab, a human monoclonal Ab against IL-1 has been used to treat many different autoinflammatory diseases in humans, but not CD. Anakinra is currently used to treat autoinflammatory diseases that I have previously discussed in Chapter 1, but has a very short half-life and causes transient injection site reactions in most patients. It was approved by the US Food and Drug Administration (FDA) in 2001 for the treatment of

rheumatoid arthritis. Rilonacept, on the other hand, is a soluble decoy receptor that binds IL-1, and prevents IL-1 signalling events. The US FDA approved the use of this drug in 2008 for the treatment of CAPS in both adults and children, 12 years and older. Canakinumab is a human monoclonal antibody that specifically binds free floating human IL-1β, forming a complex that does not bind the IL-1 receptor, and hence prevents IL-1β-dependent signalling. It was approved in 2009 by the US FDA for the treatment of CAPS in both adults and children. Canakinumab has a long half-life and does not require daily or weekly injections, as is the case with the other approved anti-IL-1β therapies. IL-1-targeted therapy could serve as an important option for CD patients with the T300A gene variant. As shown in our *in vivo* studies in SHIP-/- mice, anakinra prevents CD-like intestinal pathologies by directly targeting IL-1 signalling, reducing up-regulation of IL-1 mRNA, IL-1β, and IL-6. In addition, evidence suggests that anti-IL-1β therapies reduce neutrophil recruitment, and accumulation of IL-17-producing cells in the inflamed mucosal tissue.[241] A recent study by Antonella de Luca *et al* demonstrated that anakinra restores autophagy and significantly reduces IL-1β production, and inflammation in chronic granulomatous disease (CGD) patients and in mice.[220] Our results indicate that active caspase 1 is reduced in ilea from SHIP-/- mice treated with anakinra, suggesting that this reduction might be associated with increased autophagy. Previous studies have shown that autophagy increases sequestration and degradation of inflammasomes and caspase-1 in autophagosomes when they fuse with lysosomes.[250,251] Clinically, anakinra treatment could also be useful for CD patients, who have undergone small bowel resection for segmental disease, and, who are at high risk for recurrence. In this group of patients, anakinra could delay or prevent recurrence. Further

studies and clinical trials in subjects, who have failed current biological therapies, will shed more light on the future use of IL-1-targeted therapy in the treatment of CD.

Owing to the central role of inflammasomes in the pathogenesis of IBD, therapy that targets these multi-protein complexes may serve as therapeutic options for the treatment of these diseases. Our *in vitro* data suggest that, blocking the NLRP3 inflammasomes significantly reduces macrophage-derived IL-1β production, and could be a therapeutic target to reduce intestinal pathology. While this may be an important therapeutic target, identifying the specific inflammasome involved in IL-1β production during IBD will help increase the efficacy of a therapy of this nature.

We also identify a reduction in SHIP expression and activity as an important immune-regulatory defect associated with the *ATG16L1* CD SNP. Intriguingly, despite its up-regulation by autophagy, SHIP is also required for effective autophagy, suggesting that, enhancing SHIP expression, and/or activity could also be a possible therapeutic strategy to control IL-1β production and reduce intestinal inflammation in CD subjects with the T300A-encoding gene variant. SHIP agonists, such as Pelerol have been developed and enhance SHIP activity by two fold.[402] Despite these findings, much still needs to be done to test the efficacy of these SHIP agonists at reducing intestinal inflammation.

We have demonstrated that depletion of phagocytic cells was sufficient to prevent development of CD-like pathology in SHIP deficient mice. Phagocytic cells are the main source of IL-1β that promotes intestinal inflammation. As such, specifically targeting these cells may serve as a therapeutic option for the treatment of IBD. Histological analysis of mucosal biopsies from IBD patients has shown that, the presence of massive granulocyte infiltrates correlates with disease severity.[403] Leukocyte apheresis, which involves

extracorporeal purification of the blood by selectively removing undesired granulocytes and monocytes [granulocyte and monocyte adsorptive (GMA) apheresis] from the blood stream has been successfully used to treat IBD patients.[404-406] Patients had relatively fewer side effects and significantly higher remission rates compared to patients on conventional IBD therapy.[407] Further studies are required to fully determine if this therapeutic option could be used for patients with severe and/or refractory CD.

Targeting Caspase activity, especially caspase-1, -3, or -7, could serve as a therapeutic option for treatment of IBD. Uncontrolled caspase activity is associated with development of IBD and colorectal cancer.[408] We have shown that, increased caspase-1 activity in the inflamed ileum correlates with intestinal pathology in the SHIP deficient mice. Therapy that regulates caspase-1 activity could be an important therapeutic option for IBD patients. Furthermore, caspase 3/7 is required for the degradation of ATG16L1 containing the T300A-encoding gene variant, resulting in defective autophagy, pathogen expansion, and intestinal inflammation.[254,255] As such, targeting caspase 3 or 7 could restore autophagy in subjects with the *ATG16L1* gene variant, increase pathogen clearance, and prevent development of CD. In this regard, current biological therapy for IBD that targets TNFα, prevent caspase activation, and IEC and Paneth cell death.[409] Pralnacasan, a potent non-peptide inhibitor of caspase-1 was shown to pharmacologically block caspase-1 activity during DSS-induced colitis, effectively reducing clinical disease in these mice.[370,410] Nevertheless, owing to demonstrated liver abnormalities in certain animal studies, phase II clinical trials to test the efficacy of this drug was halted by the producing companies. Furthermore, current inhibitors of caspase activity are non-specific and may also inhibit the activity of caspases required for certain organ development. Future research could be aimed at developing small molecule

inhibitors that specifically target inflammatory caspases, such as caspase-1, -3, or -7 that have been strongly implicated in the development of CD.

Finally, our data identify defective autophagy as central for the development of auto-inflammatory CD. Drugs that induce autophagy have been used to prevent or delay the onset of neurodegenerative diseases, such as Huntington's disease.[411] Treatment options aimed at inducing autophagy in cells may prove clinically useful in the treatment of CD patients with the *ATG16L1* gene variant. Increasing autophagy in these patients will increase bacterial clearance, and reduce the presence of inflammasomes, caspase 1, and IL-1β production, and may reduce inflammation. Recently, sirolimus (rapamycin), which induces autophagy, has been shown to induce clinical remission and mucosal healing in children with severe IBD that were refractory to conventional anti-TNFα therapy.[412]

Taken together, data in this thesis suggest that, SHIP is reduced in CD subjects with the *ATG16L1* gene variant because they have defective autophagy. Our data also demonstrate that autophagy up-regulates SHIP, and as such, defective autophagy compromises SHIP up-regulation in CD subjects. Furthermore, reduced SHIP expression results in increased IL-1β production, which drives intestinal auto-inflammation. Blocking IL-1 signalling with an IL-1Ra, anakinra, prevents development of intestinal pathology. Figure 5.1 summarizes our model of how subjects with the *ATG16L1* gene variant develop intestinal inflammation in CD and how anakinra can be used to reduce inflammation.

5.2 Future directions

Future studies will focus on examining the impact of additional autophagy-related CD susceptibility SNPs on autophagy, SHIP mRNA expression/activity, and IL-1β production in

a larger cohort to confirm this CD phenotype that is associated with defective autophagy and also identify subjects that could be amenable to treatment with IL-1 targeted therapy. We will also examine the impact of the *ORMDL3* rs2872507 gene variant on cytokine production in our cohort. Furthermore, it will be interesting to determine if some of the trends seen in this thesis are confirmed in a larger cohort with many subjects having the *NOD2* (rs2066844), and *XBP1* (rs35873774) gene variants. I demonstrated in Chapter 3 that the homozygosity for the ATG16L1 T300A-encoding gene variant is associated with reduced SHIP mRNA expression and activity, and that starvation-induced autophagy increases protein levels of SHIP. It will be important to determine if SHIP mRNA expression and protein levels, as well as SHIP activity, are affected by the presence of other CD-associated genetic variants. Autophagy will be determined by looking at LC3-II protein levels amongst the genotypes of the different SNPs to establish if defective autophagy could be associated with increased IL-1β as previously shown. The results of these studies will be a significant step forward in the identification of specific CD phenotypes necessary for development of personalized therapeutic approaches.

5.3 Thesis model

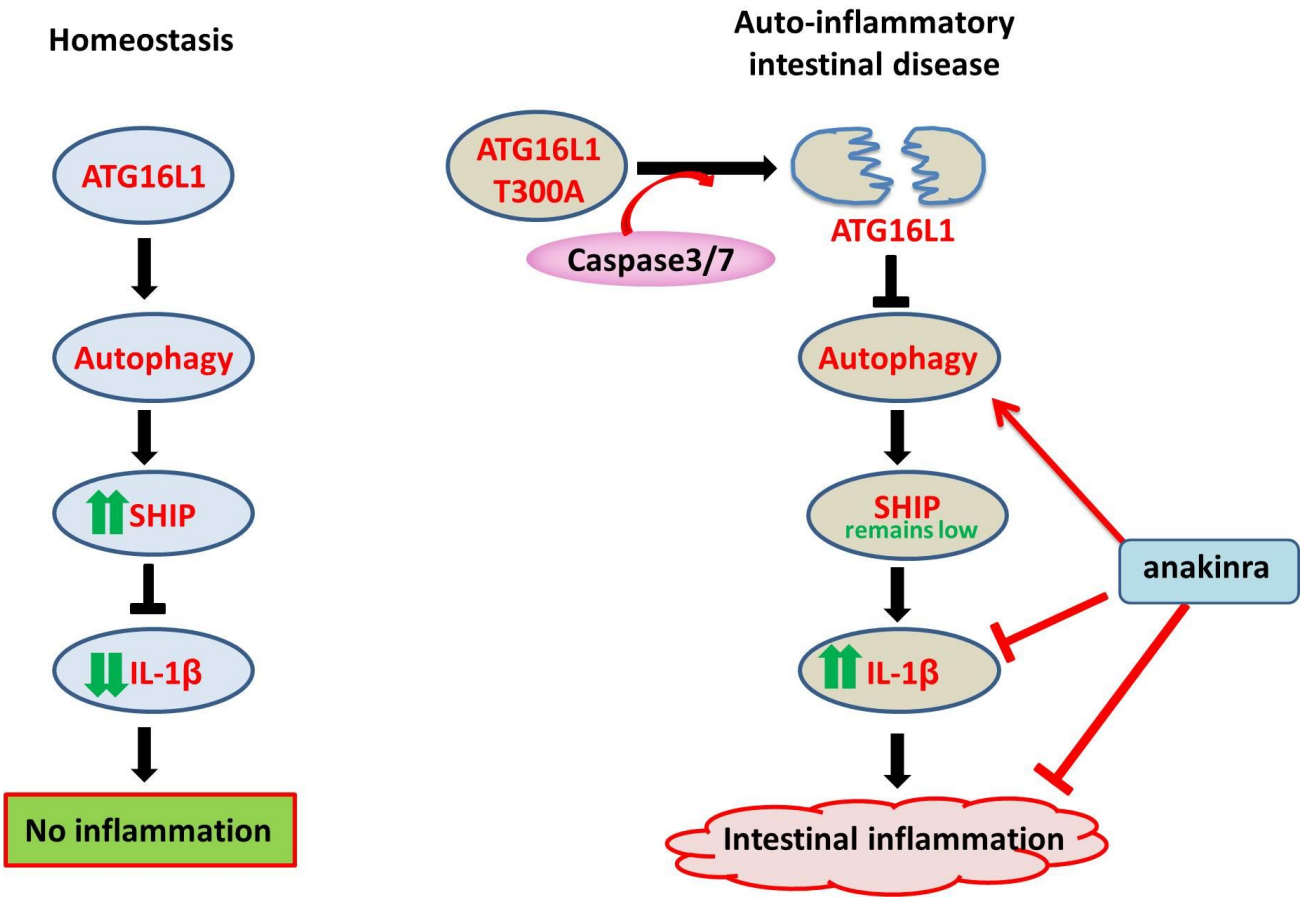

Figure 5.1 Autophagy regulates IL-1β production and intestinal auto-inflammation via SHIP up-regulation.
In subjects without the *ATG16L1* gene variant, ATG16L1 protein is fully functional and sets the platform for autophagy. Autophagy up-regulates SHIP protein expression, which in turn blocks IL-1β production, and inflammation. In contrast, in subjects with the T300A-encoding gene variant, ATG16L1 protein is prone to caspase-3/7-mediated cleavage, resulting in defective autophagy. Defective autophagy results in reduced SHIP expression and increased IL-1β production. Increased IL-1β drives intestinal auto-inflammation and results in CD. Anakinra (IL-1Ra) restores defective autophagy, and blocks IL-1 signalling and intestinal auto-inflammation.

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