ALTERNATIVELY ACTIVATED MACROPHAGES PROTECT MICE DURING INDUCED INTESTINAL INFLAMMATION

by

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Abstract

Inflammatory bowel disease (IBD) is an idiopathic disease characterized by chronic intestinal inflammation and ulceration. Canada has the highest incidence of IBD in the world with 1 in 150 people affected. While treatment options target symptoms and attempt to dampen down inflammation, an increasing population of patients is refractory to current therapeutic options. Macrophages are heterogeneous in their functions and while it is clear that inflammatory macrophages contribute to inflammation in IBD, multiple lines of evidence suggest that alternatively activated macrophages may offer protection during intestinal inflammation. *In vivo* SHIP deficient mouse macrophages are alternatively activated so SHIP deficient mice provide a unique genetic model of alternative macrophage activation. Using the dextran sodium sulfate (DSS)-induced model of colitis, I found that SHIP-/- mice are protected during induced intestinal inflammation, the protection is macrophage mediated, and can be conferred to a susceptible host. To determine how SHIP contributes to alternative activation of macrophages, I demonstrate that SHIP-deficient murine macrophages are more sensitive to IL-4-mediated skewing to an alternatively activated phenotype. Moreover, SHIP levels are reduced in alternatively activated macrophages and this is required for alternative activation because it is dependent on PI3K activity. Arginase (ArgI) induction is specifically dependent on the PI3Kp110δ isoform of class IA PI3K. As such, mice deficient in PI3Kp110δ catalytic subunit activity have increased clinical disease activity and histological damage during DSS-induced colitis. Colitis severity correlates with reduced numbers of $ArgI^+$ M2 macrophages in the colon, increased nitric oxide production, and is macrophage-dependent. Importantly, adoptive transfer of IL-4-treated macrophages from wild type mice, but not from PI3Kp110δ deficient mice, protects mice during DSS-induced colitis. Protection

is lost when mice are treated with inhibitors that block arginase activity showing that ArgI activity is required for M2 macrophage-mediated protection from intestinal inflammation. These findings identify SHIP and the PI3K pathway as critical regulators of alternative macrophage activation and as potential targets for manipulation in IBD. In addition, adoptive transfer of alternatively activated macrophages to patients with IBD also offers a promising, new strategy for treatment that may be particularly useful in patients who are refractory to conventional therapies.

Preface

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Chapter 2: A version of this material has been published as Weisser SB, Brugger HK, Voglmaier NS, McLarren KW, van Rooijen N, Sly LM. SHIP-deficient, alternatively activated macrophages protect mice during DSS-induced colitis. *J Leukoc Biol*. 2011; 90(3):483-92. I performed all of the experiments, data analysis, and manuscript writing with the exception of the following contributions from additional authors: Hayley Brugger and Nicole Voglmaier helped with monitoring mice and acted as second scorers for histological damage. Dr. Keith McLarren performed some of the histological staining and macrophage counting. Dr. Nico van Rooijen provided the PBS-containing liposomes and clodronate-containing liposomes. Dr. Laura Sly and I conceived of the experiments and wrote the manuscript for the published paper.

Chapter 3. A version of this material has been published as Weisser SB, McLarren KW, Voglmaier N, van Netten-Thomas CJ, Antov A, Flavell RA, Sly LM. Alternative activation of macrophages by IL-4 requires SHIP degradation. *Eur J Immunol*. 2011; 41(6):1742-53. I performed all of the experiments, data analysis, and manuscript writing with the exception of the following contributions from additional authors: Dr. Keith McLarren helped with data analysis and manuscript editing. Nicole Voglmaier performed the pSTAT6 analysis seen in Figure 3.5 B, C, and D. Christina van Netten-Thomas performed replicates of Figures 1.2 and 1.3. She also performed Western blots of the IL-4 and IL-10 co-stimulation studies in Figure 3.3A and isoform specific inhibitors in Figure 3.4C. Andrey Antov harvested bone marrow from the STAT6-/- mice provided by Dr. Richard Flavell. Dr. Laura Sly measured SHIP activity in IL-4 and IL-10 co-stimulation studies in Figure 3.3B and Western blot analysis of additional M2 macrophage models seen in Figures 3.3 C and D and Figure 3.5A. Dr. Laura Sly and I conceived of experiments and wrote the manuscript for the published paper.

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Experiments were performed in compliance with biosafety regulations at the University of British Columbia (biohazard approval certificate number B09-0040). Animal experiments were performed in compliance with institutional requirements at the University of British Columbia (breeding protocol A09-0027 and experimental protocol A09-0032) and in accordance with guidelines set forth by the Canadian Council on Animal Care.

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List of Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
7-AAD	7-Amino-Actinomycin D
ABH	2(S)-Amino-6-Boronohexanoic Acid-NH_4
ArgI	Arginase I
ATG16L1	Autophagy Related 16-Like 1
BEC	*S*-(2-Boronoethyl)-L-Cysteine
BM	Bone Marrow
BSA	Bovine Serum Albumin
CD	Crohn's Disease
DMSO	Dimethyl Sulfoxide
DNBS	Dinitrobenzenesulfonic Acid
DSS	Dextran Sodium Sulfate
ECL	Enhanced Chemiluminescence
FACs	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
H&E	Hematoxylin and Eosin
hr	Hour
IBD	Inflammatory Bowel Disease
IFNγ	Interferon gamma
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
iNOS	Inducible Nitric Oxide Synthase
IRGM	Immunity-Related GTPase Family M Protein
IRS	Insulin Receptor Substrate

ITLN1	Intelectin-1
LPS	Lipopolysaccharide
LY29	LY294002
LY30	LY303511
Mφ	Macrophage
M1	Classically activated
M2	Alternatively activated
mA	milliamp
MCSF	Monocyte Colony-Stimulating Factor
MDP	Muramyldipeptide
mg	milligram
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
mM	Millimolar
MUC	Mucin
nsRNA	non-silent RNA
ng	nanogram
nm	nanometer
NK	Natural Killer
NO	Nitric Oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PDK1	Pyruvate Dehydrogenase Kinase 1
PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
PVDF	Polyvinylidene Difluoride
Rx	Receiving
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SHIP	src homology 2 domain-containing inositol 5´-phosphatase

siRNA	small interfering RNA
STAT	Signal Transducer and Activator of Transcription
TAMs	Tumor Associated Macrophages
TCR	T Cell Receptor
TG	Thioglycφollate
TGF	Transforφing Growth Factor
T_H	T helper cell
T_{reg}	T regulatory cell
TREM2	Triggering receptor expressed on myeloid cells 2
TNBS	Trinitrobenzene Sulphonic Acid
TNFα	Tumor Necrosis Factor alpha
UC	Ulcerative Colitis
WCL(s)	Whole Cell Lysate(s)

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For my beautiful daughter, Alexandria Sophia Weisser

With dedication and hard work, you can achieve your dreams

Thank you for filling my heart and making me complete

I love you

1 Chapter: Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a progressive, chronic, or relapsing and remitting disorder characterized by intestinal inflammation and ulceration.[1] Symptoms include abdominal pain, vomiting, diarrhea, rectal bleeding, and fatigue.[2] While IBD can occur at any age, it is most commonly diagnosed in the second or third decade of life.[2] There is a slight gender-related difference in prevalence among those with the IBD subtype, Crohn's Disease (CD), with females being diagnosed in a 1.1 - 1.4:1 ratio over males.[3, 4] Interestingly, Canada has the highest prevalence of IBD in the world with up to 1 in 150 individuals affected.[5, 6]

Personal and economic burdens are associated with IBD. The high prevalence in Canada results in an extremely high economic burden with costs estimated to be approximately $2.8 billion in 2012. This includes both direct medical costs of $1.2 billion; including medication, hospitalization, and physician visits; as well as indirect costs of $1.6 billion, attributed primarily to long-term work losses.[5] In addition, parents and families experience personal costs. Beyond uncomfortable and painful symptoms, additional challenges include a lack of awareness of IBD as a chronic disease, late or incorrect diagnosis, unequal access to health care services, and diminished career prospects.[5]

1.1.1 Disease presentation

IBD encompasses both Crohn's Disease (CD) and ulcerative colitis (UC), which have distinct clinical and histological characteristics. Inflammation in CD is typically

discontinuous and can involve any part of the gastrointestinal tract from the mouth to the anus. Inflammation is often transmural with the ileocecal region most commonly affected, followed by the terminal ileum alone, the diffuse small bowel, and the isolated colon.[2] Disease can be complicated by the occurrence of fistula, commonly in the perianal region, or the development of strictures which require surgical intervention in one third of all patients.[7] Histologically, CD is often characterized by ulceration, inflammation extending to the submucosa, and granuloma formation.[2] Conversely, UC is a continuous mucosal inflammation that is restricted to the colon and rectum with occasional involvement of the cecum.[8] In rare cases, patients may develop toxic megacolon where dilation and ischemia of the gut wall can become lethal.[7] Histologically, UC is characterized by disruption of colonic crypt architecture, inflammatory cell infiltration, edema, and goblet cell depletion.[2] Both CD and UC are accompanied by an increased long term-risk of developing cancer.[9, 10]

1.1.2 Pathogenesis

Currently, the cause of IBD is unknown. Evidence suggests that it is a multifactorial condition involving four separate components in order for clinical disease to become apparent. These interacting factors include genetic susceptibility, environmental influences, barrier dysfunction allowing stimulation by the luminal microbiota, and a dysfunctional immune response.[11]

Genetic analyses have demonstrated that genes and genetic loci play an important role in IBD pathogenesis. Analyses used include linkage analysis, genome-wide association studies, and mouse models.[11, 12] Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is an intracellular pattern recognition receptor that causes an inflammatory

response upon recognition of its ligand, muramyl dipeptide, a bacterial component. It was the first, and best studied, gene associated with IBD and is considered a landmark discovery in the genetics of complex diseases.[13] With advances in technology, a total of 163 IBD associated genes have been identified, much more than any other complex disease.[14] Genes that have been identified link disease to specific biological processes. For example, genes encoding autophagy related 16-like 1 (ATG16L1) and immunity-related GTPase family M protein (IRGM) have been associated with autophagy, intelectin-1 (ITLN1) and mucin (MUC) 19 are associated with epithelial barrier function, and interleukin (IL)-10 and IL-23R are involved in the immune response.[15, 16] Since the concordance rate of CD in identical twins is only 42-58%, and only 6-17% in UC, other triggers must also be necessary to initiate the onset of disease.[3]

To date, a variety of environmental factors have been implicated in predisposing, triggering, or modulating IBD. These include smoking, diet, geographical and social status, stress, and microbial agents.[3, 17-19] Interestingly, tobacco use plays disparate roles in IBD acting as a risk factor for CD, while being protective in UC.[17] Currently, the 'hygiene hypothesis' is a popular explanation of environmental influence based on the observation that IBD prevalence is extremely low in developing countries compared to westernized nations. It proposes that western nations have developed an extremely 'clean' lifestyle with regards to low microbial and parasite exposure, safer food and water, antibiotics, and vaccinations; which leaves our immune systems unprepared for challenges, including a lack of tolerance to our own intestinal bacteria.[20]

Commensal and pathogenic bacteria in the gut play an important role in the pathogenesis of IBD. The gut has a 300-400 m^2 epithelial cell barrier that separates the

underlying tissue from approximately 10^{13}-10^{14} microorganisms.[21, 22] Mouse models have demonstrated that the microbiota can drive chronic inflammation, as mice in germ-free conditions do not develop intestinal inflammation until colonized with commensal bacteria.[22, 23] The bacterial composition could be a contributing factor to IBD pathogenesis. Studies have indicated that dysbiosis, an imbalance of commensal bacteria, could contribute to disease. There is a 30-50% decrease in bacterial biodiversity in IBD patients, specifically due to loss of normal anaerobic bacteria including *Bacteroides*, *Eubacterium*, and *Lactobacillus* species, as well as a significant reduction in *Firmicutes*.[24, 25] Also, an increase in certain pathogens is associated with IBD development, some of which include *Pectinatus*, *Sutterella*, *Fusobacterium*, *Mycobacterium paratuberculosis*, and *Helicobacter hepaticus.*[25] While changes in microbial content may be a result of intestinal inflammation rather than a cause, instances have been reported where mice and humans respond favorably to antibiotic and probiotic treatment.[11, 26, 27] With increasing evidence that commensal bacteria play a significant role in IBD, fecal microbiota transplantation has shown some promise as a new therapeutic option in mouse models and a limited number of patient studies.[28]

Defects in the epithelial barrier can lead to immune cell exposure to commensal microorganisms. IBD patients have been shown to have an increase in mucosal permeability which precedes clinical onset of disease.[29, 30] Also, mouse models such as those expressing a dominant-negative N-cadherin transgene in the epithelium have defective or ‘leaky’ epithelial tight junctions and display severe intestinal inflammation.[23, 31] In addition, functional defects in the specialized epithelial cells, paneth cells and goblet cells, have also shown to be associated with IBD. Paneth cells in mouse models and CD patients have diminished expression of the antimicrobial molecules, defensins, and subsequently fail to

eradicate pathogens.[32] IBD patients also display defects in production of the protective mucus layer by goblet cells.[33] Specifically, Muc2, an intestinal secretory mucin and major component of the mucus layer, has been shown to be critical for host defense from infectious colitis.[34] While it is still unclear whether barrier dysfunction is a cause or consequence of inflammation, studies have indicated that increased intestinal permeability can precede clinical relapse and therefore some therapeutic strategies are focused on re-establishing intestinal barrier function.[35, 36] It is increasingly evident that barrier dysfunction alone is insufficient to cause disease, therefore immunoregulatory mechanisms must also play a role in perpetuating inflammation.[37]

Abnormalities in both the innate and the adaptive immune system have been shown to contribute to the pathogenesis of IBD. This could be due to constitutive activation, a failure in down-regulation, or continued stimulation resulting from a defective barrier.[1] There are currently two main hypotheses that address the contribution of the innate immune system in IBD pathogenesis. The first suggests that there is a reduced response to microbes that breach the epithelial barrier allowing accumulation of commensals and recruitment of inflammatory B and T cells.[32] The second hypothesis suggests that defects in innate immune cells lead to a loss of tolerance to the microbiota and will initiate an inappropriate inflammatory response, recruiting additional innate cells as well as cells from the adaptive immune system.[32] In terms of adaptive immunity, it is typically reported that CD produces a T helper (T_H)1 response while UC exhibits a T_H2 response, but recent studies have demonstrated some overlap.[38] In addition, both T_H17 cells, which produce inflammatory cytokines, and a reduction in T regulatory (T_{reg}) cells, which promote immune tolerance, have been implicated in the

pathogenesis of IBD.[39, 40] Generally, it is accepted that inflammation occurs through either excessive effector T cell function or deficient regulatory T cell function.[41]

In summary, the etiology of IBD is extremely complicated with many contributing factors. Genetic susceptibility can predispose individuals to IBD with the appropriate triggers. Environmental factors may influence disease presentation and severity. A defective epithelial barrier may allow commensal organisms to activate the immune response. And finally, intestinal inflammation can be promoted by hyper-inflammatory immune cells or can be initiated by a loss of tolerance to the microbiota.

1.1.3 Treatment

As the specific cause of IBD is unknown, therapeutic options are limited to reducing clinical symptoms and inducing and maintaining disease remission. Medical treatment aims to dampen down inflammation in a step-up approach beginning with non-steroidal anti-inflammatories such as sulfasalazine or 5-aminosalicylic acid, followed by corticosteroids, and finally biological therapies, such as infliximab, an antibody that binds to tumor necrosis factor α (TNFα) and blocks its' activity.[2, 42] Surgery is often needed to control the disease. Approximately 25 to 40% of patients with severe colitis will require a colectomy, while 70-80% of CD patients will require surgery during the course of their disease to remove fibrotic tissue.[2, 43] Unfortunately, there is a population of patients who remain refractory to available treatments, therefore new treatment options are desperately needed.[42]

1.2 Mouse models of intestinal inflammation

Mouse models are a valuable tool used to study intestinal inflammation. Although none of the current models perfectly recapitulates the features of human IBD, they have been indispensable for investigating the involvement of different factors in pathogenesis. Models of intestinal inflammation generally fall into four main categories: spontaneous models, adoptive transfer models in immunocompromised hosts, genetically engineered models, and inducible models.

The first main category of models of intestinal inflammation is those that occur spontaneously. The SAMP1/YitFc mice are a spontaneous model of CD where inflammation is restricted to the small intestine, is discontinuous, and displays granulomata in the tissue.[44] Ileitis in this model is mediated by a T_H1 type response that can be largely treated with anti-TNFα, much like with human CD.[44, 45] Conversely, C3H/HeJBir mice are a spontaneous model of colitis. Inflammation is found in the cecum and right colon and histologically characterized by inflammation, ulceration, and crypt abscesses.[46] These mice have increased B cell and T cell activation by antigens of the commensal bacteria.[47] As there are so few spontaneous models, the use of models that have undergone manipulation is necessary to identify factors that play a role in this complex disease.

The second category of models characterized by inducing inflammation by transferring a specific cell population to a neutral host lacking lymphoid tissue, such as *scid* or *Rag-/-* mice.[48] These adoptive transfer studies have indicated that the $CD4^+$ $CD45RB^{hi}$ T cell subset plays a key role in the pathogenesis of intestinal inflammation as recipient mice develop a wasting syndrome with transmural inflammation primarily in the colon.[49] Interestingly, the co-transfer of $CD4^+$ $CD45RB^{lo}$, specifically T_{reg} cells, prevents the

induction of colitis.[48, 49] While these models show that defects in the adaptive immune system play a role in disease pathogenesis, they do not identify what specific cellular defects are responsible for the resulting intestinal inflammation.

Another category of mouse models of intestinal inflammation is the genetically engineered models. This group, including both knock out and transgenic mice, is responsible for many advances in understanding mucosal inflammation and is useful for identifying specific therapeutic targets and strategies.[50] This is a large group where genetic manipulation can target cytokine function (eg. *il10*-/-, transforming growth factor (TGF)β-/-, or signal transducer and activator of transcription (STAT)4 transgenic mice), T cell function (eg. T cell receptor (TCR)α-/- or major histocompatibility complex (MHC) class II-/-), or epithelial barrier function (eg. *mdr1a*-/- or N-cadherin dominant negative).[48] Mutant strains can be crossed or can be coupled with induced models to further our understanding of mechanisms underlying intestinal inflammation.

The last category is comprised of those models where acute or chronic intestinal inflammation is induced by exposure to an exogenous agent. These include administration of compounds such as trinitrobenzene sulphonic acid (TNBS), oxazolone, acetic acid, or dextran sodium sulfate (DSS).[51] Administration is either oral or by enema and is associated with a mechanical or chemical disruption of the epithelial barrier.[50, 51] Chemically induced models, such as DSS, have been used for over two decades and are some of the most commonly used models because it is easy to control the onset, duration, and severity of inflammation in these models.[52]

1.3 Immune response in inflammatory bowel disease

The gastrointestinal tract is a complex system where specialized cells coordinate to achieve digestion and nutrient uptake while promoting tolerance to and protection from intestinal microorganisms (Figure 1.1). Intestinal tissue is composed of a single cell epithelial layer that acts as a barrier between the gut content (lumen) and the underlying connective tissue (lamina propria), preventing antigen from encountering the immune system.[25, 53] Some of these epithelial cells are further specialized in order to enhance their protective function. Goblet cells produce mucus which acts as a protective coat over the epithelium.[34] Paneth cells produce antimicrobial proteins that regulate microbial density and protect adjacent stem cells.[32] Villous microfold (M) cells are another specialized epithelial cell which channels antigen across the barrier for sampling by immune cells within lymphatic nodules called Peyer's patches.[33] Together, these epithelial cell types cooperate to shore up the epithelial barrier.

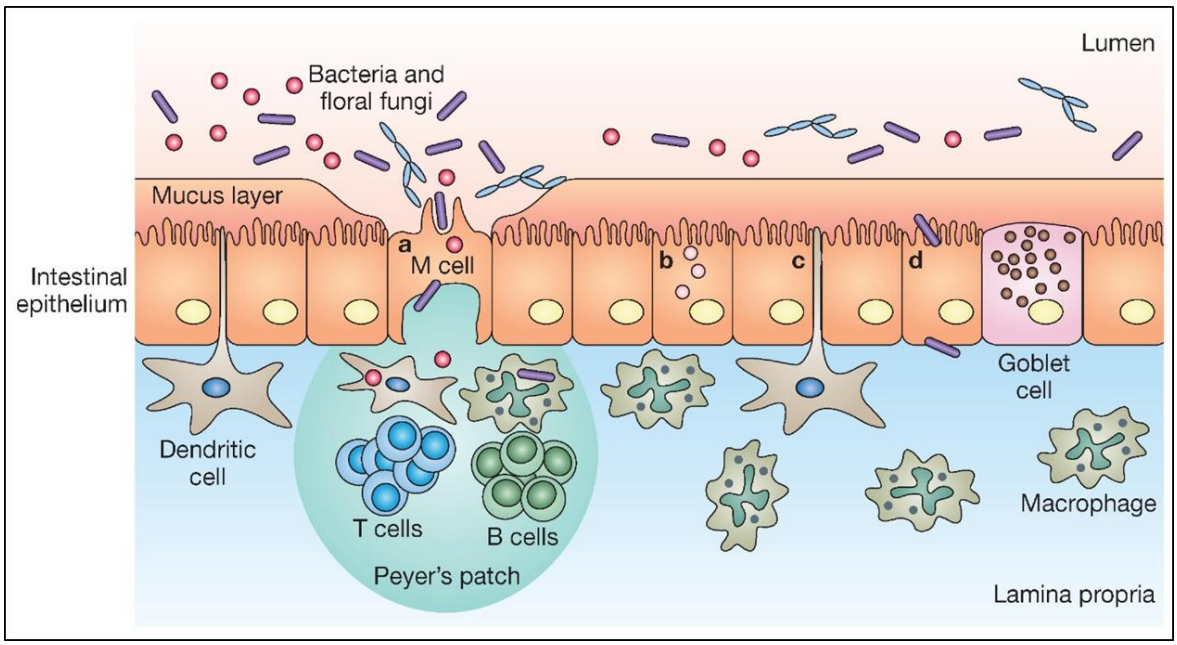


Figure 1.1 Epithelial and immune cell location within the intestine
A single cell epithelial barrier separates the gut content (lumen) from the underlying immune cells within the lamina propria. (Reprinted with permission from Cambridge University Press: Expert Reviews in Molecular Medicine, Heinsbroek, S. & Gordon, S. 2009[54])

Underlying the epithelial barrier are immune cells that facilitate intestinal homeostasis but are also poised to initiate an inflammatory response should infection occur. Dendritic cells are located within Peyer's patches and along the epithelial barrier. They can project through the epithelial layer to sample antigen from the commensal milieu.[33] Macrophages can be found directly beneath the epithelium and within Peyer's patches and dispose of cellular debris or microbes that have breached the barrier.[54] Finally, both B and T cells can be found within Peyer's patches and will perpetuate an adaptive immune response to microbial infection.[54] Defects in immune cells and the inflammatory response have been associated with IBD.

IBD is believed to result from an inappropriate immune response to intestinal microorganisms. As a result, inflammatory cells infiltrate the tissue and this is a histological

characteristic of IBD.[1] In fact, both CD and UC patients have activated innate and adaptive immune responses and loss of tolerance to the intestinal microflora.[11] Each of these responses will be considered separately here.

1.3.1 Innate immune response in IBD

Intestinal inflammation in IBD may be due to increased activity of the innate immune system. Mouse models suggest that dendritic cells incorrectly recognize commensal bacteria and as a result induce an inappropriate adaptive immune response.[55] In fact, increased numbers of activated macrophages and dendritic cells within the lamina propria have been reported in patients with either CD or UC correlating with increased expression of pro-inflammatory cytokines and chemokines.[11, 32] Finally, IL-10-/- mice develop chronic intestinal inflammation where their antigen presenting cells have been reported to be potent activators of a T_H1 response.[56] Interestingly, depleting phagocytic cells in this model prevents chronic colitis.[57] These studies suggest that inappropriate inflammatory responses and accumulation of innate immune cells are involved in the pathogenesis of IBD and subsequent signalling will direct an adaptive immune response.

The intestinal inflammation seen in IBD may be due to an inadequate innate immune response to microbial insult. Mutations in the gene encoding the pattern recognition receptor NOD2 have been found in one-third of patients with CD.[58] Patients with this mutation have defective NOD2 signalling in macrophages and reduced secretion of the cytokines IL-1β and IL-8. This results in diminished neutrophil recruitment, suboptimal clearance of bacteria, and a reduced acute inflammatory response.[32, 59] In addition, dendritic cells from CD patients with a NOD2 mutation have defective cytokine secretion in response to bacterial

muramyldipeptide (MDP) stimulation.[60] CD patients have also been shown to have neutrophil dysfunctions including impairment in recruitment, decreased bactericidal and phagocytic activity, and deficient superoxide generation.[61] Taken together, these studies suggest that an impaired inflammatory response by the innate immune system may initiate IBD due to insufficient clearance of commensal microorganisms by allowing establishment of chronic inflammation and by activating the adaptive immune response.

1.3.2 Adaptive immune response in IBD

Within the adaptive immune response, T cells have been extensively studied and have been shown to play a critical role in IBD progression. T cells from IBD patients are more resistant to apoptosis leading to an inappropriate expansion of T cells upon stimulation by antigen presenting cells.[62] Specifically, a T_H1 response is generally associated with CD and is mediated by IL-12, IFN-γ, and TNF.[58, 63] In addition, lamina propria T cells from CD patients display a loss of tolerance to commensal bacteria and perpetuate an inflammatory response to enteric flora.[25]

The balance of T_{reg} and T_H17 cells may be disrupted in IBD pathogenesis. T_{regs} are involved in suppressing inflammation and maintaining self-tolerance while T_H17 cells are involved in inflammation.[64] Patients with IBD have significantly fewer T_{regs} in their peripheral blood compared to healthy controls, while the opposite was found in mucosal tissue.[65, 66] Insufficient numbers of peripheral blood T_{regs} has been associated with the recurrence of IBD.[64] T_H17 cells express IL-17 in response to IL-23 signalling which subsequently stimulates the production of pro-inflammatory cytokines IL-1, IL-6, and TNF by macrophages and epithelial cells.[25, 38] IL-23p19 transgenic mice spontaneously develop

chronic colitis.[67] In addition, transferring T_H17 cells to immunocompromised mice induced colitis which was inhibited by anti-IL-23p19 monoclonal antibody treatment.[68] Studies of CD patients have shown an increase of both IL-23 and IL-17 in their intestinal tissue.[69, 70] Recently, a distinct population of T_{regs} has been identified in CD patients that also produce pro-inflammatory IL-17 and IFN-γ.[71] These cells have been implicated in both inflammatory and regulatory functions.[64, 72] While this evidence suggests that IL-17 may contribute to disease pathogenesis, an anti-IL-17 monoclonal antibody has not been protective when used to treat IBD patients.[68]

The role of B cells in IBD is less well studied than T cells but there is some evidence that they play a protective role in murine intestinal inflammation. The chronic colitis displayed in TCRα-/- mice was worse in the absence of B cells.[73] In fact, a B cell subset has been shown to produce IL-10 and suppress intestinal inflammation.[74] With regards to human IBD, the role of B cells remains unclear. Both pro-inflammatory and immune-regulatory B cells may exist in the human intestine.[68] This is reflected in results from B cell targeted therapy where the anti-CD20 monoclonal antibody, rituximab, showed short term improvement in colitis in some studies.[75-77]

1.4 Macrophages

Macrophages develop from precursor monocytes which also give rise to dendritic cells and osteoclasts.[53] Monocytes originate in the bone marrow and subsequently move into circulation where they make up 5-10% of peripheral blood leukocytes.[53] Once in the peripheral blood, monocytes will either differentiate into $Ly6C^+$ cells, which will become macrophages involved in the inflammatory response, or $Ly6C^-$ monocytes, which give rise to

tissue-resident macrophages.[78]

Macrophages are essential components of the immune system and are involved in many different biological processes. They play a critical role in the innate immune system by initiating and resolving the inflammatory response and also drive the adaptive immune response to intracellular and extracellular pathogens.[79] To accomplish this, macrophages are equipped with a broad range of pathogen-recognition receptors that make them efficient at recognizing, engulfing, and killing microorganisms and multicellular parasites.[78, 79] In addition, macrophages also produce inflammatory cytokines and present antigen to T and B lymphocytes.[53] They are also crucial for maintaining homeostasis by clearing away microorganisms and tissue debris.[78]

1.4.1 Macrophage phenotype

Macrophages have a high degree of heterogeneity due to their specialized roles at different anatomical locations and in biological processes. In steady-state, tissue macrophages have intrinsic anti-inflammatory functions to ensure that homeostasis is maintained.[80] In this role, macrophages have very specific functions depending on their location within the body. For example, bone macrophages, or osteoclasts, are responsible for bone resorption and remodeling.[53] Alveolar macrophages of the lung phagocytose inhaled particles such as dust or allergens, as well as microorganisms.[80] Kupffer cells, or liver macrophages, are critical for clearance of pathogens and toxins from the blood.[81] Splenic macrophages clear senescent red blood cells and blood-born antigens and macrophages in lymph nodes capture antigen and present it to B cells.[80] While the specific functions of these macrophages may be different, they are all involved in maintaining tissue homeostasis

because they perform these effector functions in the absence of mounting an inflammatory response.

When homeostasis is disrupted, macrophage activation can be altered in response to signals from the microenvironment producing macrophages with very different properties and functions.[53] Classically activated (M1) macrophages are typically associated with initiating a pro-inflammatory immune response while alternatively activated (M2) macrophages are involved in resolution of inflammation and tissue remodeling (Figure 1.2).[82] Alternatively activated macrophages can be further divided into three subtypes according to their inducing stimulus, but they all share the common feature of producing cytokines at a relatively low IL-12/IL-10 ratio in response to inflammatory stimuli (Fig. 1.2). M2a macrophages are activated by IL-4 or IL-13 and induce Th2 immune responses; M2b macrophages are activated by immune complexes and toll-like receptor (TLR) ligands; and M2c macrophages are activated by IL-10 and are important for immunosuppression and tissue remodeling.[83, 84] M1 and M2 macrophages are often considered to be opposite ends of a spectrum yet it has been shown that macrophages stimulated towards a specific phenotype can return to a quiescent state after signal arrest or can switch their phenotype rapidly upon counterstimulation.[82] This plasticity is particularly relevant when considering the initiation and resolution of the inflammatory response. Of note, this thesis focuses on the role that M2a macrophages play in dampening down the inflammatory response and therefore subsequent referral to alternatively activated or M2 macrophages refer to this specific phenotypic subset.

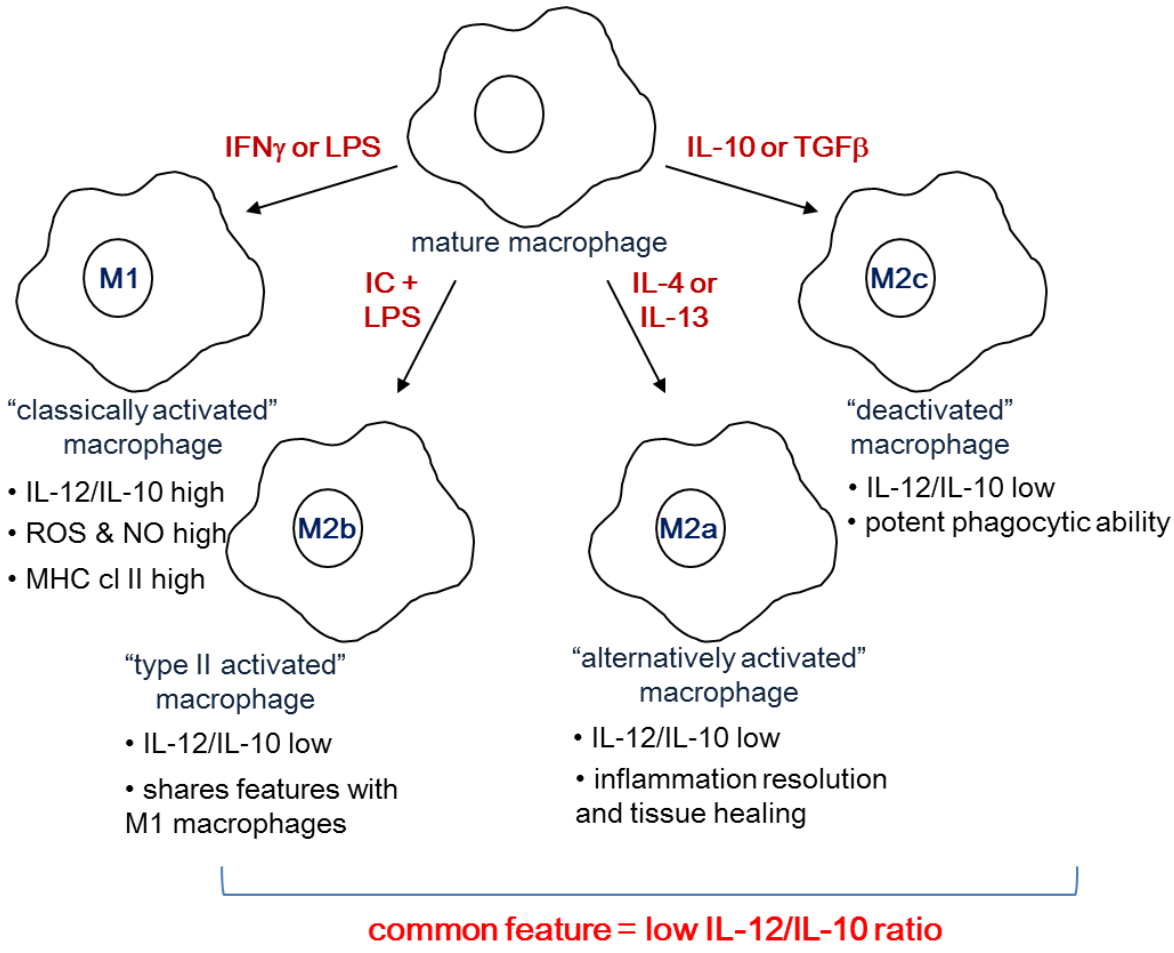


Figure 1.2 Macrophage subtypes
Macrophage phenotype is plastic and dependent on signals from the microenvironment. Classically activated (M1) and alternatively activated (M2a, M2b, and M2c) subtypes are classified based on inducing stimuli and responses.

1.4.2 Classically activated macrophages

Classically activated (M1) macrophages are typically associated with the initiation and perpetuation of inflammation. They are activated in response to injury or infection and exhibit an inflammatory phenotype.[80] Activation can occur in response to two signals (Fig. 1.3). The first signal is interferon-γ (IFNγ) which is provided by natural killer or natural killer T cells in early response and T_H1 T cells in later response.[85] The second signal comes from exposure to a pathogen associated molecular pattern (PAMP), such as

lipopolysaccharide (LPS), found on microbes.[86] Upon activation, M1 macrophages will produce a variety of inflammatory signals to initiate and direct an immune response. They are characterized by a high IL12/IL-10 ratio implying that they promote a strong T_H1 immune response which further drives the inflammatory response forward.[84] In addition, they also express high levels of the pro-inflammatory cytokines IL-23, IL-6, and IL-1.[87] Importantly, a defining characteristic of M1 macrophages in mice is the activity of inducible nitric oxide synthase (iNOS), which converts L-arginine to nitric oxide (NO) and L-citrulline.[87] Reactive oxygen and nitrogen intermediates, including NO, are toxic to invading microorganism and are therefore key mediators of the inflammatory response in mice.[80]

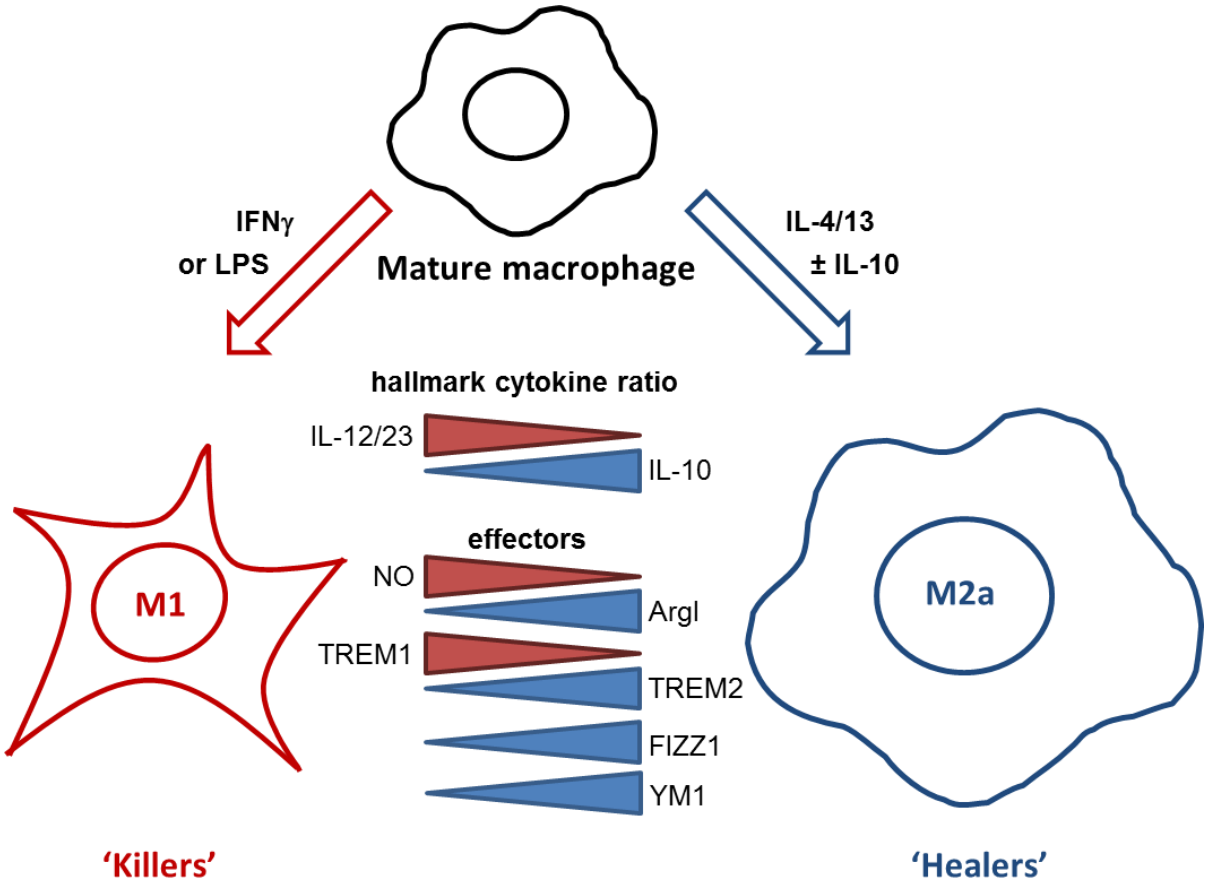


Figure 1.3 Classically activated (M1) versus alternatively activated (M2) macrophage phenotypes
Classically activated (M1) macrophages produce pro-inflammatory signals in response to IFNγ or LPS activation while and alternatively activated (M2) macrophages express anti-inflammatory signals following exposure to IL-4 or IL-13.

Classically activated macrophages play a critical role in protection from infectious diseases. As such, they have a strong microbicidal activity and augmented complement-mediated phagocytosis.[88, 89] They also have an increased antigen presenting capacity allowing the induction of the adaptive immune response.[89] This persistence of inflammation can be detrimental to tissue, therefore it must be tightly controlled to prevent damage following an inflammatory response.

1.4.3 Alternatively activated macrophages

Resolution of an inflammatory response is vitally important in repairing affected tissue and restoring homeostasis. Alternatively activated (M2) macrophages, in particular, play a key role in dampening down inflammation and promoting wound healing.[85] These macrophages are activated by the cytokines, IL-4 or IL-13 (Fig. 1.3).[90] IL-4 is produced by basophils and the T_H2 subpopulation of $CD4^+$ T lymphocytes.[79, 91] Activation of M2 macrophages results in high expression of the anti-inflammatory cytokine, IL-10, and low levels of IL-12 and IL-23.[87] They also produce triggering receptor expressed on myeloid cells 2 (TREM2), which has been shown to inhibit cytokine production.[79] In addition, M2 macrophages express the chitinase-like molecule, Ym1, and the secreted protein, FIZZ1, also known as Relmα.[90] Importantly, arginase I (ArgI) is expressed by M2 macrophages. It metabolizes L-arginine producing polyamines, which are important in cell growth and division, and L-proline, an amino acid required for collagen biosynthesis (Fig. 1.4).[86, 92] ArgI also competes with iNOS for their common substrate, L-arginine, and limits NO production in response to inflammatory stimuli, thereby limiting inflammation.

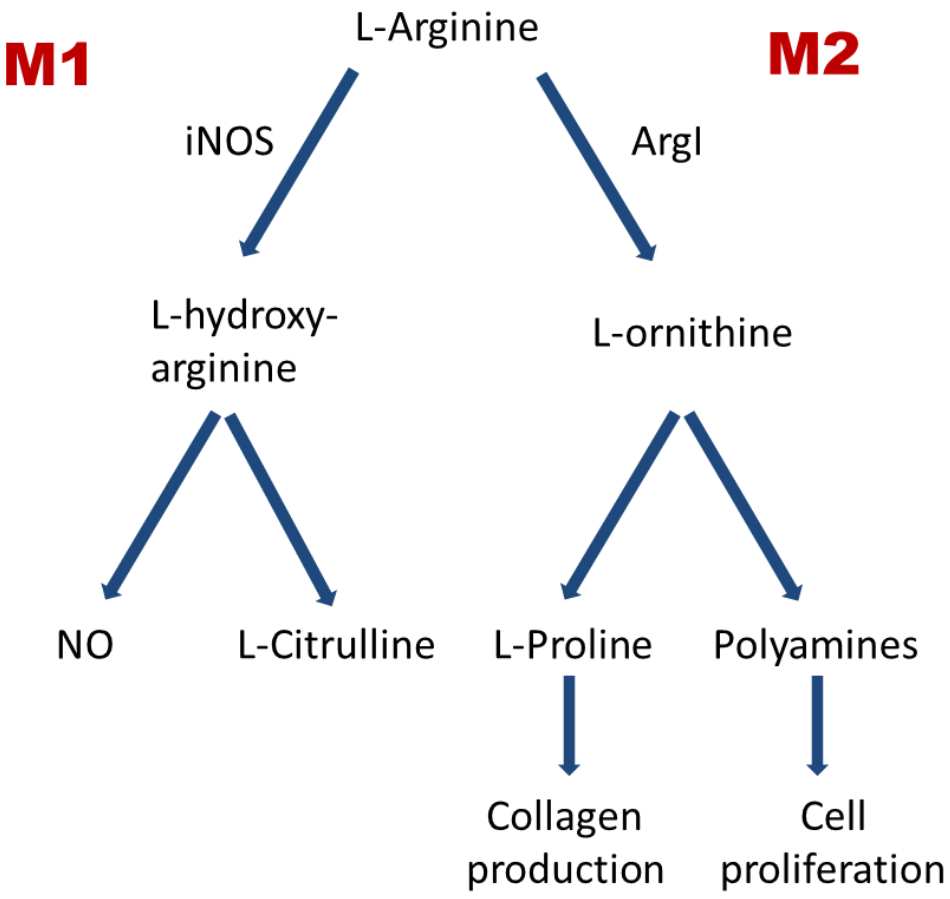


Figure 1.4 iNOS and ArgI compete for the common substrate, L-arginine
Classically activated (M1) macrophages utilize L-arginine to produce pro-inflammatory NO and citrulline while ArgI expression in alternatively activated macrophages (M2) competes for this common substrate to produce proline and polyamines.

M2 macrophages are implicated in host protection by negatively regulating inflammation. M2 macrophages have also been identified in the placenta and lung where they protect from unwanted inflammation and immune reactivity, as well as in acute inflammation and chronic inflammatory diseases, such as rheumatoid arthritis, where they are implicated in repair.[79] Following an inflammatory response that is initiated and directed by M1 macrophages, macrophages will skew to an M2 phenotype to reduce inflammation and promote healing of affected tissue.

To contribute to healing, M2 macrophages are involved in angiogenesis, debris scavenging, wound healing and tissue remodeling.[86, 93] Inhibiting macrophage infiltration during an immune response delays the resolution of inflammation and wound closure suggesting that infiltrating macrophages are physiologically different than the M1 macrophages that are originally found in the inflammatory site.[85] In addition, depleting macrophages prior to injury leads to a failure in clearing dead and damaged cells and other debris, resulting in a delay of wound healing.[94]

Human and mouse macrophages exhibit similar macrophage polarization during inflammation and resolution but there are differences in the signals that drive polarization. Human M1 macrophages are derived by treatment with granulocyte-monocyte colony-stimulating factor (GM-CSF) while exposure to monocyte-colony stimulating factor (MCSF) produces M2 macrophages.[95] Conversely, in mice GM-CSF or IL-3 treatment results in the expression of the M2 markers, Ym1 and ArgI, while MCSF-derived macrophages do not express these markers.[95, 96] In addition, human macrophages do not have homologs to the murine M2 macrophage markers, Ym1 or FIZZ1.[89] Finally, murine M2 macrophages are a key source of arginase activity that regulates NO production, whereas human M1 macrophages are not strong producers of NO and human M2 macrophages do not induce ArgI.[97, 98] In humans, neutrophils are likely the source of arginase activity during wound healing and tissue repair.[98]

1.4.4 The role of macrophages in inflammatory bowel disease

Intestinal macrophages have a unique phenotype and function. The intestinal tract consists of up to 10^{14} commensal bacteria of approximately 500 different species.[99] Together

with the high antigenic load from the diet, intestinal macrophages encounter more antigen than any other part of the body.[100] They are highly effective at clearing enteric bacteria that breach the epithelial barrier but do not mount an inflammatory response.[87] Intestinal macrophages do not act as antigen presenting cells and therefore have low expression of the cell surface molecules CD40, CD80, and CD86. In addition, they also lack Fc and complement receptors that mediate activation and secretion of pro-inflammatory cytokines such as TNF, IL1β, IL-6 and IL-12.[87] Human intestinal macrophages have a high phagocytic and bactericidal activity yet do not express innate response receptors or produce pro-inflammatory cytokines.[101] The function and phenotype of these macrophages is dependent on the intestinal milieu as treating blood monocytes with intestinal stromal cell-conditioned media recapitulates this tolerized state.[102] This anergic state of intestinal macrophages is critically important in maintaining immune homeostasis.

In IBD, gut homeostasis is disrupted and is replaced by an inappropriate inflammatory response. Following an inflammatory signal, monocytes migrate to the intestinal mucosa and, unlike resident macrophages, rapidly respond to luminal microbial triggers.[54] While this perpetuation of inflammation is expected in an immune response, in IBD, the question remains as to what signal or process is responsible for the inappropriate inflammation. Macrophages, critical for tissue homeostasis, can contribute to disease pathogenesis in two ways. First, a defect in microbial handling may cause an accumulation of microbes within the lamina propria, triggering an immune response. Another way that macrophages might contribute to disease in IBD is by an improper increase in activation and production of pro-inflammatory signals triggering an inappropriate inflammatory response. There is accumulating evidence to support both of these scenarios.

The first defect in macrophage function that is associated with IBD is a defect in microbial handling. The specialization of intestinal macrophages includes a high bactericidal and phagocytic activity allowing the efficient destruction of microbes that breach the epithelial barrier. In patients with IBD, macrophages show a dramatic reduction in phagocytosis and antibacterial activity.[103] In fact, CD patients have a high level of adherent/invasive *E. coli* that survived within macrophages that induced expression of high levels of TNFα compared to control subjects.[104] In addition, macrophages derived from blood monocytes of CD patients have an abnormal proportion of degraded pro-inflammatory cytokines and chemokines, which may result in an insufficient inflammatory response and impaired clearance of bacteria.[105, 106] While failure to properly handle and eradicate invading commensals can result in an uncontrolled abundance of microbes within the lamina propria, macrophages may also be responsible for initiating and driving an inappropriate immune response.

Evidence from mouse models and patients has supported the theory that macrophages contribute to disease in IBD through an increased number of activated macrophages at sites of inflammation and/or an increase in pro-inflammatory signalling. IL10-/- mice develop intestinal inflammation where macrophages differentiate into a pro-inflammatory phenotype producing large amounts of IL-12 and IL-23.[107] Depleting macrophages in IL-10-deficient mice suppressed the development of chronic colitis suggesting that macrophages are key mediators of disease in this animal model.[57] Also, during IBD, patients have an increased number of activated macrophages expressing high levels of the co-stimulatory molecules, CD80 and CD86, as well as a pro-inflammatory cytokine profile.[108] An increase in intestinal CD14-expressing macrophages has also been observed in CD patients stimulating increased

levels of IL-6, IL-1β, IFN-γ, IL-23, and TNFα.[109, 110] While these studies show that macrophages play a role in determining disease severity, considering the phenotypic plasticity of macrophages, it is important to understand which subset of macrophage is involved when targeting these cells for potential new therapies. To understand and target macrophage phenotype in IBD, we must first understand the signalling pathways involved in macrophage activation and skewing.

1.5 The phosphoinositide 3-kinase pathway

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases involved in intracellular signal transduction.[111] There are three classes of PI3Ks based on sequence homology and substrate specificity.[112] Class I PI3Ks are the most extensively studied group and are associated with growth factor and immune responses.[113] Four members have been identified and these have been further divided into Class IA and Class IB categories based on their mechanism of activation.[114] These PI3Ks are heterodimeric with different combinations of a catalytic subunit and regulatory subunit. For Class IA, catalytic subunits include p110α, p110β, and p110δ, which associate with the p85 regulatory subunit of which there are 8 isoforms encoded by three genes.[115] Conversely, Class IB has one catalytic subunit, p110γ, which associates with the regulatory subunit p101 (Table 1.1). All Class IA PI3Ks are activated through tyrosine kinase signalling pathways, whereas Class IB signals downstream of G-protein-coupled receptors.[115] Finally, while most Class I PI3Ks are ubiquitously expressed or have a broad tissue distribution, p110γ and p110δ are highly enriched in leukocytes.[115]

Table 1.1 PI3K family members

Class	Catalytic Subunits	Regulatory Subunits	Lipid Product
IA	p110α, p110β, p110δ	p85	$PI(3,4,5)P_3$
IB	p110γ	p101	$PI(3,4,5)P_3$
II	PI3K-C2α, PI3K-C2β, PIK3C2γ		PI(3)P
III	Vps34	Vps15	PI(3)P

Class II and Class III PI3Ks are not as well characterized as Class I and as they are not the focus of this thesis, they will only be mentioned briefly here. Class II consists of three members including, PI3K-C2α, PI3K-C2β, and PIK3C2γ with no known regulatory subunits.[114] Class II enzymes are typically membrane bound and activated by receptors such as receptor tyrosine kinases and integrins.[114, 115] While little is known about the physiological function of Class II PI3Ks, they have been shown to play an important role in cell migration.[116] Class III contains only 1 known enzyme, Vps34 with the Vps15 regulatory subunit.[117] This enzyme is responsible for producing the majority of PI-3-P and is associated with endocytosis, autophagy, and nutrient signalling.[118]

1.5.1 Enzymatic activity

Activation of PI3Ks by extracellular agonists involves their translocation to the plasma membrane to phosphorylate lipid substrates.[117] They do this by transferring the γ-phosphate group of ATP to the 3-OH group of inositol membrane lipids.[119] Class I PI3Ks exclusively

phosphorylate the 3-OH group of PI(4,5)P_2 to produce the critical second messenger, PI(3,4,5)P_3 *in vivo* (Fig. 1.5). The presence of PI(3,4,5)P_3 at the membrane triggers a number of possible signalling cascades.

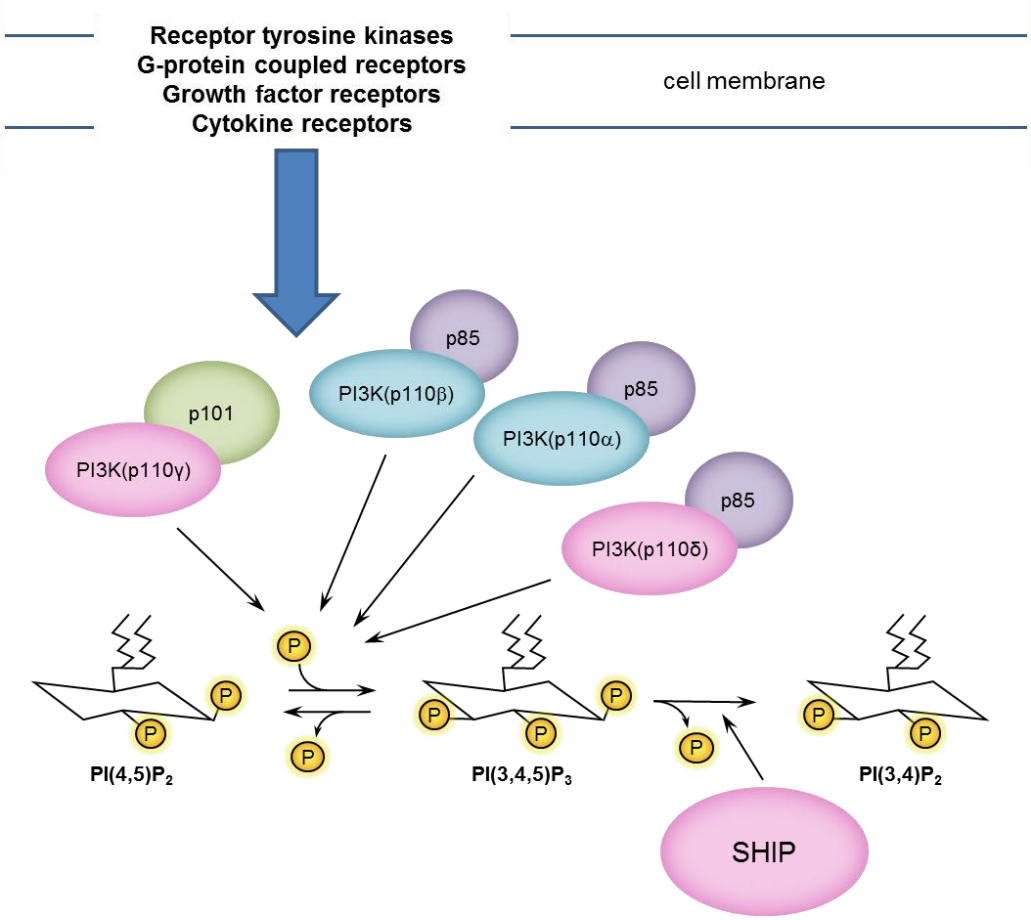


Figure 1.5 Class I PI3Ks
Class I PI3Ks catalyze the phosphorylation of PI(4,5)P_2 to PI(3,4,5)P_3, a critical second messenger. There are four distinct catalytic subunits of Class I PI3Ks; p110α, p110β, p110γ, and p110δ. SHIP dephosphorylates PI(3,4,5)P_3 to generate PI(3,4)P_2 limiting class I PI3K activity. Hematopoietic specific subunits and enzymes, p110γ, p110δ, and SHIP are shown in pink.

1.5.2 Class I PI3K function

Class I PI3Ks have been associated with a number of cellular processes including cell growth, proliferation, metabolism, and survival.[120] Once PI(3,4,5)P_3 is generated, it acts as a docking site at the plasma membrane that recruits and activates pleckstrin-homology (PH) domain-containing signalling effectors such as Akt (PKB) and pyruvate dehydrogenase kinase (PDK1).[112, 120] Akt has been shown to mediate many of the downstream effects of PI3K by phosphorylating a variety of substrates involved in the regulation of these cellular processes.[114] For example, PI3K/Akt regulates apoptosis through target proteins such as BAD, FoxO, and GSK-3, or cell survival, through IKK and subsequent NF-κB activation.[121] While PI3K activity is important in cell growth and survival, it also plays a key role in the immune system.

PI3K activity has been implicated in most cells of the innate and adaptive immune system. Although leukocytes express all Class I PI3Ks, the isoforms PI3Kδ and PI3Kγ are preferentially expressed in immune cells.[111] Specifically, these isoforms play an important role in the inflammatory response.[112] Studies from PI3Kp110δ and PI3Kp110γ deficient mice show disruptions in development, maturation, expansion, and migration in virtually all innate and adaptive immune cells.[111, 122] Due to the involvement in inflammatory mechanisms, manipulation of these PI3K isoforms has therapeutic potential for diseases involving dysregulated immune responses.

1.5.3 PI3K in IBD

PI3Ks have been shown to play a role in inflammatory cytokine production and have been implicated in IBD pathogenesis. In fact, mice given the PI3K inhibitor, wortmannin,

during DSS-induced colitis had significantly reduced clinical disease symptoms and histological damage compared to controls.[123] In addition, treatment with PIK-75, a PI3Kp110α/γ inhibitor, dramatically downregulates the production of pro-inflammatory cytokines and has been shown to be protective during DSS-induced colitis.[124] PI3Kp110γ has been shown to be involved in exacerbating disease, where studies using PI3Kp110γ-deficient mice or isoform specific inhibition have shown amelioration of disease.[125, 126] Since PI3K plays a role in inflammation and IBD pathogenesis, manipulating specific isoforms, or regulators of its activity, may provide novel therapeutic options for treatment of IBD.

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

The src homology 2 domain-containing inositol 5´-phosphatase (SHIP) is a negative regulator of the PI3K pathway. Its expression is hematopoietic specific and is first detectable in 7.5 day embryos, coinciding with the onset of hematopoiesis.[127] Alternative splicing and C-terminal truncation produces four SHIP isoforms detectable by Western blot (145, 135, 125, and 110kDa).[128] In addition to SHIP, which is sometimes referred to as SHIP1, two additional PI(3,4,5)P_3 5′ phosphatases have been described. sSHIP (104kDa) is a splice variant initiated at a promoter within the intron between exons 5 and 6 of SHIP, which produces a protein missing the SH2 domain.[129] Expression of sSHIP is restricted to murine embryonic stem cells and hematopoietic stem cells.[130] SHIP2 (155kDa) is transcribed from a separate gene and, while it has a similar function and structure to SHIP, its proline-rich C-terminus is different and it cannot bind to the adaptor, Grb2, for translocation to the membrane.[129, 131-133]

1.6.1 Enzymatic activity

SHIP negatively regulates PI3K by dephosphorylating its product, $PI(3,4,5)P_3$, removing this second messenger from the membrane and stopping PI3K activation. SHIP is recruited to the plasma membrane, where it dephosphorylates the 5´-phosphate of $PI(3,4,5)P_3$ to generate $PI(3,4)P_2$ (Fig.1.5).[134] Interestingly, the product of this reaction, $PI(3,4)P_2$ has been shown to play a critical role in Akt activity. As previously mentioned, the PI3K reaction product, $PI(3,4,5)P_3$ is required for activation of Akt by triggering its phosphorylation. More recent studies have shown that $PI(3,4,5)P_3$ is insufficient for Akt activation and that $PI(3,4)P_2$ also contributes to Akt activity.[113] Both of these second messengers are required for Akt activation. $PI(3,4,5)P_3$ levels correlate with phosphorylation at the Thr308 position, while $PI(3,4)P_2$ levels correlate with phosphorylation at the Ser473 position.[113, 135]

1.6.2 SHIP function

SHIP plays an important role in cells of the hematopoietic lineage by negatively regulating PI3K signalling. SHIP has been implicated as a negative regulator of growth factor induced survival and proliferation.[136] It has also been shown to inhibit immune receptor activation in mast cells and B cells by binding to the inhibitory coreceptor FcγRIIB, preventing degranulation and proliferation.[136] In bone marrow derived macrophages and mast cells, LPS exposure results in an upregulation of SHIP which is essential for endotoxin tolerance and can be blocked by SHIP antisense oligonucleotides.[137] SHIP has also been shown to prevent LPS from triggering an antiviral response in mice.[138] Taken together, these results indicate that SHIP plays a critical role in regulating the inflammatory response and preventing damage from over-exposure to pro-inflammatory mediators.

1.6.3 Role of SHIP in macrophage polarization

SHIP activity has been implicated as a key factor in controlling the inflammatory response by determining macrophage phenotype. Murine studies have shown that *in vivo*-differentiated SHIP-/- macrophages are profoundly M2 skewed, expressing high levels of the M2 macrophage marker ArgI. This activation results in minimal NO production upon LPS stimulation.[139] They also express Ym1, another M2 macrophage marker, and significantly lower levels of pro-inflammatory cytokines such as IL-6, IL-12, and TNFα, and higher levels of anti-inflammatory IL-10 and TGFβ, in response to LPS.[139] In addition, bone marrow progenitors from SHIP-deficient mice differentiated with GM-CSF or IL-3 are more strongly M2-skewed than their wild type counterparts.[140] Interestingly, despite their M2 phenotype, SHIP-deficient *in vitro*-derived bone marrow macrophages are hyper-responsive to growth factors and TLR stimulation.[134, 138, 140] In fact, studies have shown that SHIP restrains macrophage production of pro-inflammatory cytokines and NO in response to LPS and this may be critical for protection from excessive exposure that could contribute to chronic inflammation.[141]

1.7 Overall objectives and hypothesis

It is widely accepted that IBD involves an inappropriate initiation and perpetuation of the inflammatory response to commensal microorganisms that invade the lamina propria. Macrophages are strategically placed beneath the intestinal epithelial barrier to clear any invading microbes. Typically, these macrophages are anergic and play an important role in intestinal homeostasis. In the case of IBD, however, macrophages may be defective in microbial handling or mount an inappropriate inflammatory response to these commensals.

In this case, they could be responsible for driving the chronic inflammation seen in IBD. Currently, results from macrophage depletion studies show disparate roles for macrophages in IBD. This discrepancy may be explained when considering macrophage phenotype. IL-10-/- macrophages are profoundly M1 skewed as they produce elevated levels of NO and inflammatory cytokines due to the loss of inhibitory IL-10.[142] As such, depletion of these hyper-inflammatory macrophages is expected to be protective in IBD.[57] Conversely, as mentioned, macrophage depletion worsened DSS-induced colitis in BALB/c and C57BL/6 mice with a greater effect in BALB/c mice. This correlates with BALB/c mice being M2 skewed relative to C57BL/6 mice and therefore depleting more M2 skewed macrophages made disease worse.[143] Since M2 macrophages play an important role in dampening inflammation and tissue healing, I hypothesize that M2 macrophages would be protective during induced intestinal inflammation. If M2 macrophages are protective during intestinal inflammation, we may be able to dampen inflammation and promote healing in IBD by providing patients with M2 macrophages or by manipulating macrophage phenotype within the inflammatory environment of the diseased tissue.

The goal of Chapter 2 was to determine how macrophage phenotype affects disease severity. To address this, intestinal inflammation was induced in SHIP deficient mice which have more M2 skewed macrophages. I also determined whether differences in disease severity were macrophage mediated and if protection could be conferred to susceptible hosts through macrophage reconstitution.

SHIP has been shown to be involved in determining macrophage phenotype. The goal of Chapter 3 was to identify the role that SHIP plays in M2 skewing in response to IL-4. Using additional sources of alternatively activated macrophages, inhibition of SHIP-

interacting proteins, and manipulating SHIP expression, I develop and propose a model of SHIP's role in M2 macrophage skewing.

Finally, to fully examine the role that macrophages play in colitis, Chapter 4 focuses on disease severity in PI3Kp110δ deficient mice that are defective in M2 macrophage skewing and whether M2 macrophages can confer protection in these mice. I further examined differences in the expression and activity of cytokines and M2 markers between wild type and PI3Kp110δ deficient macrophages following IL-4 treatment. Through these analyses I determined that ArgI activity is critical for M2 macrophage-mediated protection during induced intestinal inflammation in mice.

2 Chapter: SHIP-deficient alternatively activated macrophages protect mice during DSS-induced colitis

2.1 Introduction and rationale

Alternatively activated macrophages have been associated with dampening down inflammation and promoting tissue repair and therefore may be protective during intestinal inflammation. Unlike inflammatory, classically activated macrophages, they fail to produce NO which limits their ability to kill invading microbes.[86] In addition, studies have shown that they express relatively lower amounts of pro-inflammatory cytokines in response to toll-like receptor ligation.[89, 144] M2 macrophages are also poor antigen presenting cells and can inhibit T cell proliferation and responses.[86, 145] They mediate wound healing, partially due to biosynthesis of polyamine and proline, leading to cell growth, and collagen deposition, which facilitates tissue repair.[86] Importantly, parasite infection confers macrophage-dependent protection in two acute, chemically-induced murine colitis models.[146, 147] Whereas, protection via infection with *Schistosoma mansoni* was not attributable to the canonical IL-4 derived macrophage phenotype (M2),[146] *Hymenolepis diminuta*-mediated protection correlated with the induction of M2 macrophage markers.[147] This suggests that M2 macrophages may protect mice from induced intestinal inflammation.

SHIP-/- mice were developed by replacing the entire first exon of the gene with a neomycin resistance cassette resulting in alterations to macrophage proliferation and phenotype.[148] As SHIP negatively regulates survival and proliferation, SHIP-/- mice have an increased number of monocytes/macrophages which differentiate more quickly compared to wild type mice.[134] In addition, *in vivo* differentiated SHIP-/- mouse macrophages are

profoundly M2 skewed.[91, 139, 140, 148] Compared to their wild type counterparts, they express low levels of pro-inflammatory cytokines and high levels of IL-10 in response to stimulation with LPS + IFNγ and they also express constitutively high levels of the M2 macrophage markers, ArgI and Ym1.[139] The profound M2 phenotype in SHIP-/- mouse macrophages can be attributed to a key extrinsic effect. SHIP-/- mice have hyper-active IL-4 secreting basophils signalling macrophages to skew to an M2 phenotype.[140] These properties make SHIP-/- mice a valuable genetic model to study the role of M2 macrophages in IBD. Importantly, the SHIP-/- M2 macrophage phenotype can be recapitulated *in vitro* by differentiating SHIP-/- bone marrow aspirates in the presence of GM-CSF.[140] One of the unique features of SHIP-/- M2 macrophages is that once they have differentiated *in vivo* or *ex vivo* to an M2 phenotype, that phenotype is not reversible.[139, 140]

DSS-induced colitis is a widely used mouse model of colonic inflammation. It mimics clinical and hisopathological features of human acute phase UC, including massive granulocyte and macrophage infiltration.[48, 52] Typically, DSS is added to the drinking water of mice which damages epithelial cells of the colon and affects the integrity of the mucosal barrier.[52] Since T-and B-cell-deficient C.B-17^{scid} or Rag1$^{-/-}$ mice develop severe intestinal inflammation, it is especially useful in examining the role that the innate immune system plays in pathogenesis.[51] For these reasons, DSS is used in these studies to induce intestinal inflammation in mice.

Since SHIP-/- mice have predominantly M2 skewed macrophages and M2 macrophages have the potential to protect mice against intestinal inflammation, I hypothesized that SHIP-/- mice have increased M2 macrophage numbers and will be protected during DSS-induced intestinal inflammation. To investigate this, both wild type

and SHIP-/- mice were given DSS in their drinking water for 7 days to induce colitis. Clinical disease severity was measured daily by monitoring rectal bleeding and weight loss. In addition, colon length and histological damage were documented following the experiment. We further examined the role that macrophages play in induced colitis by depleting and reconstituting macrophages during DSS treatment. Our results show that M2 colonic macrophages are protective during DSS-induced intestinal inflammation.

2.2 Materials and methods

Mice. SHIP-/- mice were created in 1998 [148] and have been maintained on a mixed C57BL/6 × 129Sv background. Brother-sister matings of SHIP heterozygotes have been used to generate a line of SHIP mice and to generate SHIP+/+ and SHIP-/- littermates for experiments. All mice used were between 8 and 12 weeks of age. Mice were maintained in the Animal Research Center at the Child & Family Research Institute and experimentation was performed in accordance with institutional and Canadian Council on Animal Care guidelines.

Immunohistochemistry. For detection of F4/80, ArgI, Ym1, and nitrotyrosine, slide-mounted 5μm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. For F4/80 detection, enzyme induced epitope retrieval was performed by incubating samples with 20μg/ml proteinase K in phosphate buffered saline (PBS) for 15 minutes (min) at room temperature. For ArgI, Ym1, and nitrotyrosine detection, heat-induced epitope retrieval was performed by immersing the slides in sodium citrate buffer pH6.0 at 95°C for 20 min and allowing slides to cool to room temperature. All slides were

rinsed thoroughly in Tris-buffered saline with 0.1% Tween-20. Endogenous peroxidase activity was blocked with 1.5% H_2O_2 in PBS for 10 min. Endogenous avidin and biotin were blocked with an avidin-biotin blocking kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Primary antibodies including rat anti-F4/80 (AbD Serotec, Oxford, UK), mouse anti-ArgI (BD Biosciences, Mississauga, Canada), mouse anti-Ym1 (StemCell Technologies, Vancouver, Canada), and mouse anti-nitrotyrosine (Millipore, Billerica, MA) were used. Blocking buffers, biotinylated secondary antibodies and avidin-biotin-HRP or alkaline phsophatase complexes were prepared and used from rabbit IgG, rat IgG or "Mouse-on-Mouse" immunohistochemical detection kits according to manufacturer's instructions (Vector Laboratories). Signal was detected with diaminobenzidine chromogen system (Dako, Carpinteria, CA) and developed sections were counterstained with Harris' hematoxylin (Sigma, St. Louis, MO). Images were acquired and analyzed using a Zeiss Axiovert 200 microscope, a Zeiss AxiocamHR camera, and the Zeiss Axiovision 4.0 software imaging system. Total macrophages were quantified by counting F4/80-positive cells from six representative fields at 40× magnification from six tissue sections for six mice per group by two individuals blinded to experimental condition. M2 macrophages were quantified using serial sections to identify ArgI-positive cells co-localized with F4/80 positive cells in the same fields.

DSS-induced colitis and macroscopic assessments of pathology. 5% DSS (M.W. 36-50,000; MP Biomedicals, Solon, OH) was dissolved in the drinking water of mice. Mice were treated for 7 days during which time they were monitored daily for weight loss and fecal blood. Rectal bleeding scores were assigned on a scale from 0-4 as seen in Table 2.1.

Hemocult paper was from Beckman Coulter, Mississauga, Canada. Rectal bleeding is reported as the median score from the mice examined in each experiment. Colons were excised upon autopsy and colon lengths were measured.

Table 2.1 Rectal bleeding scoring

Score	Rectal Bleeding
0	None
1	Detectable on Hemocult Paper
2	Visible blood in stool
3	Large amount of blood in stool
4	Extensive blood in stool and blood visible at the anus

Histological analyses. Mice were euthanized and colons were removed and fixed in PBS-buffered 10% formalin. Samples were embedded in paraffin and cross-sections were stained with hematoxylin and eosin (H&E). Histological damage was scored using a 16 point scale by two individuals blinded to experimental condition as described in Table 2.2. [149, 150]

Table 2.2 Histological damage scoring

Damage Component	Score
Loss of architecture	0 = none
	1 = <25% loss
	2 = 25%-50% loss
	3 = 50%-75% loss
	4 = >75% loss
Immune cell infiltration	0 = none
	1 = occasional immune cell in lamina propria
	2 = increased immune cells in lamina propria
	3 = confluent immune cells in lamina propria and breaching mucosa
	4: immune cell infiltration throughout the section
Goblet cell depletion	0 = none;
	1 = <50% depletion
	2 = >50% depletion
Ulceration	0 = none
	1 = intermediate ulceration
	2 = substantial ulceration
Edema	0 = none
	1 = <50% of section
	2 = >50% of section
Muscle thickening	0 = none
	1 = intermediate thickening
	2 = substantial thickening

Macrophage depletion *in vitro* and *in vivo.* For *in vitro* macrophage depletion experiments, macrophages were derived in either MCSF or GM-CSF (StemCell Technologies Inc.) from bone marrow aspirates of femurs and tibias from SHIP+/+ and SHIP-/- mice. Following adherence depletion for 2 hours (hr) at 37°C, macrophages were derived by plating progenitors at $0.5x10^6$ nucleated cells/ml in Iscove's Modified Dulbecco's Media (IMDM) (StemCell Technologies), 10% fetal calf serum (FCS) (Invitrogen), 150μM monothioglycerol (Sigma) and 10ng/ml MCSF or GM-CSF (StemCell Technologies). Complete media changes were performed after 4 and 7 days discarding non-adherent cells. After 10 days, adherent cells were removed from flasks by incubating for 5 min at 21°C in Cell Dissociation Buffer (Invitrogen, Burlington, Canada) and macrophages were > 95% positive for Mac-1 and F4/80 expression. Mature macrophages (5×10^4) were seeded in a 96 well plate and treated with 30μl of PBS, PBS-containing liposomes, or clodronate-containing liposomes for 8, 24, or 48 hours at 37ºC, 5% CO_2. Cell viability was determined using the WST-1 cell assay according to the manufacturer's instructions (Clontech, Mountain View, CA).

In vivo macrophage depletion from mouse colons using clodronate-containing liposomes was performed by injecting clodronate-containing liposomes (200μl) intraperitoneally into mice 4 days prior to treatment and at days 0, 2, 4, and 6 during DSS treatment (clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany, and encapsulated in liposomes, as described[151]).[143] Controls included intraperitoneal injections of 200μl of PBS or 200μl of PBS-containing liposomes.

Macrophage reconstitution experiments. Macrophages were derived from bone marrow aspirates from femurs and tibias of SHIP-/- mice. SHIP-/- MCSF or GM-CSF-derived macrophages (10^6) were injected into the tail vein of mice on day 0 and 4 of DSS treatment.

Tissue dissociation and fluorescence-activated cell sorting (FACS) analysis. Colon homogenates were digested with Liberase Blendzyme at 37°C for 1.5 hr according to manufacturer's instructions (Roche Diagnostics). Isolated cells were pelleted in 30% Percoll (Sigma) and resuspended in PBS (Invitrogen) with 1% bovine serum albumin (BSA) (EMD Chemicals, Gibbstown, NJ) at a concentration of 1.0×10^5 cells/ml. Non-specific antibody binding was blocked with 2.4G2 (AbLab, University of British Columbia, Vancouver, Canada), cells were stained with F4/80-PE (eBioscience, San Diego, CA) and 7-amino-actinomycin D (7-AAD; EMD Bioscience), and analyzed by flow cytometry. Data was processed using FlowJo software (Tree Star Inc, Ashland, OR).

SDS-PAGE and Western blotting. Whole cell lysates (WCLs) were prepared for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) by lysing in 1× Laemmli's digestion mix, sheering DNA using a 26 gauge needle, and boiling for 1min. Lysates were loaded onto a 12% polyacrylamide gel and Western blotting was carried out by transferring protein onto an Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, ON) using 600 mA for 4 hr at 23°C. Blots were blocked, incubated with antibodies and then with horseradish peroxidase-conjugated secondary antibody (BioLegend, San Diego, CA) before adding enhanced chemiluminescence (ECL) substrate solution (Invitrogen) and exposing to Kodak X-Omat film (PerkinElmer Life Sciences, Woodbridge,

ON). The following antibodies were used for Western blot analyses: anti-ArgI (BD Biosciences), anti-Ym1 (StemCell Technologies), and anti-GAPDH (Fitzgerald Industries International, Acton, MA).

Griess assays. Macrophages were treated with 10 ng/ml of LPS for 24 hr. NO production was determined indirectly by measuring the accumulation of nitrite in tissue culture supernatants using the Griess Reagent Kit (Invitrogen) according to manufacturer instructions.[139]

Statistical analyses. Repeated measures ANOVA, unpaired one-tailed Student's *t* tests, and Mann-Whitney tests were performed where indicated using GraphPad Prism version 5 (GraphPad Software Incorporated, San Diego, CA). Differences were considered significant at $P \leq 0.05$.

2.3 Results

SHIP-/- mice had increased numbers of alternatively activated macrophagesRecent *in vivo* studies have shown that SHIP-/- mice have profoundly M2 skewed macrophages.[139, 140] Peritoneal and alveolar macrophages were shown to express high levels of the M2 macrophage markers, ArgI and Ym1.[139] Based on these findings, I asked if colonic macrophages from SHIP-/- mice are also M2 skewed. To address this question I stained serial cross sections of colons from both SHIP+/+ and SHIP-/- littermates with F4/80 to detect macrophages and for the M2 macrophage markers, ArgI and Ym1 (Fig. 2.1A). Two individuals blinded to experimental conditions counted the number of $F4/80^{+}$ macrophages and $F4/80^{+}ArgI^{+}$ macrophages in serial sections (Fig. 2.1B). There were comparable numbers of macrophages in SHIP+/+ and SHIP-/- mice, but SHIP-/- mice had 1.8-fold more ArgI-expressing (M2) macrophages.

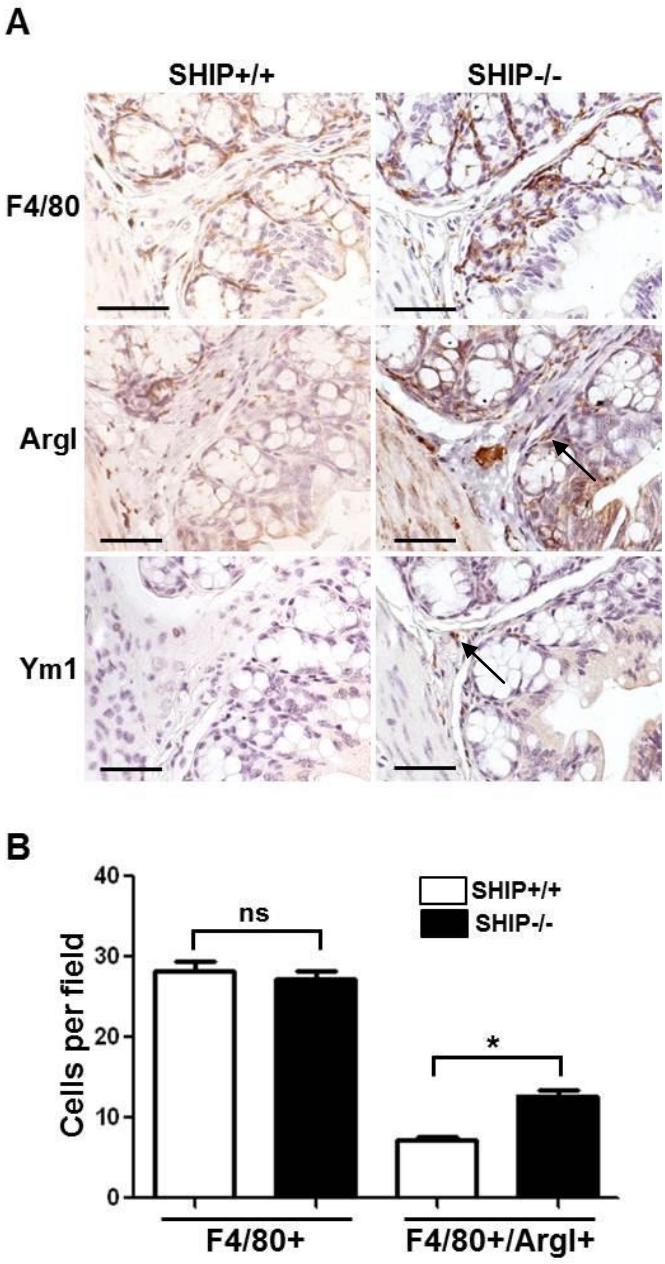


Figure 2.1 SHIP-/- mice have more alternatively activated macrophages in their colons than their wild type counterparts

(A) SHIP+/+ (left) and SHIP-/- (right) colon serial sections stained by immunohistochemistry for mature macrophages (F4/80; top) and M2 macrophage markers, ArgI (middle) and Ym1 (bottom). Scale bars = 50μm. Data are representative of similar staining from N = 3 mice for each group. (B) Quantitation of $F4/80^+$ and $F4/80^+ArgI^+$ macrophages in SHIP+/+ (white bars) and SHIP-/- mice (black bars). Data are represented as means ± SD. Positively stained cells were counted at 40× magnification in 6 fields, from 6 sections per mouse separated by ≥50 μm, and from 6 mice per group. ns=not significantly different and *P<0.01.

2.3.1 SHIP-/- mice had decreased clinical disease symptoms during dextran sodium sulfate-induced colitis

It has been shown that colonic infection with parasitic nematodes skews intestinal macrophages to an M2 phenotype.[152] This increase of M2 macrophages in the gut has been correlated with reduced colonic inflammation and disease severity in mice with dinitrobenzenesulfonic acid DNBS-induced colitis.[147] Since SHIP-/- mice have more $ArgI^{+}$ M2 colonic macrophages, I asked whether they would also have reduced intestinal inflammation during DSS-induced colitis. To examine this, I treated SHIP+/+ and SHIP-/- mice with 5% DSS in their drinking water for 7 days and monitored their weight and rectal bleeding daily. I found that SHIP-/- mice lost significantly less weight during this period (Fig. 2.2A). Onset of rectal bleeding was delayed in SHIP-/- mice and significantly lower rectal bleeding scores were observed compared to wild type littermates (Fig. 2.2B). Colon length is decreased during DSS-induced colitis. On day 7, mice were euthanized and excised colons were measured. A significantly decreased colon length was observed in SHIP+/+ mice compared to SHIP-/- littermates (Fig. 2.2C).

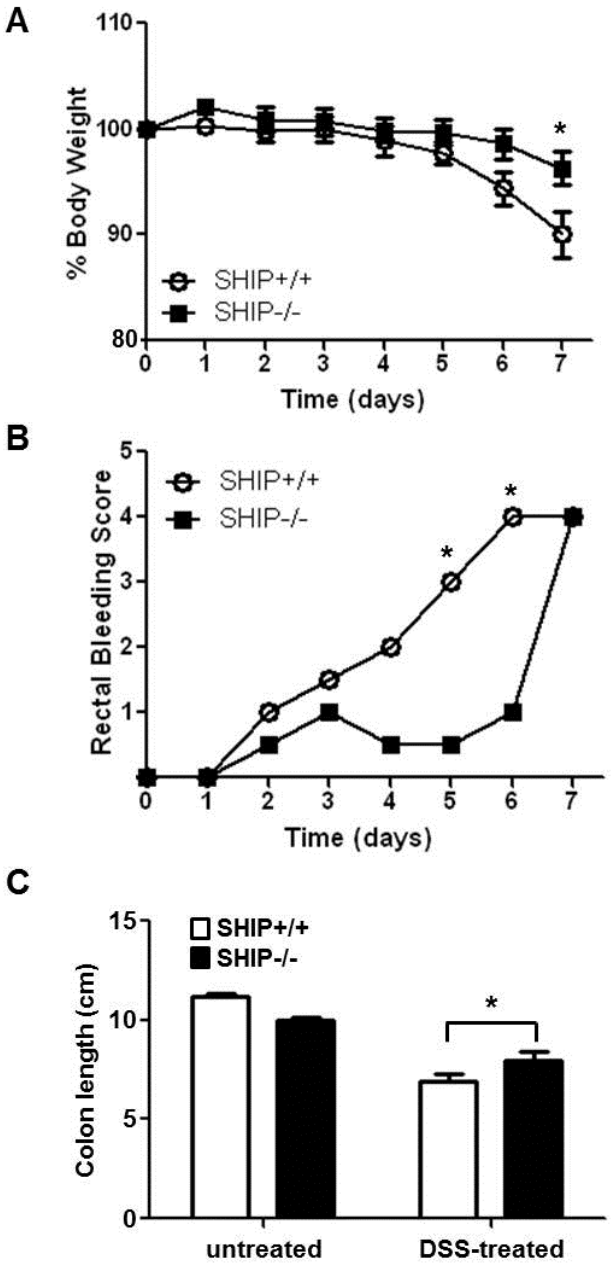


Figure 2.2 SHIP-/- mice are protected during DSS-induced colitis
SHIP+/+ and SHIP-/- mice were treated with 5% DSS in their drinking water for 7 days. (A) Weight loss was measured daily during treatment and is expressed as the average percentage of initial body weight ± SD. (B) Rectal bleeding was scored daily during treatment. The median score is reported for each group of mice. (C) Control mice (untreated) or DSS-treated mice were euthanized on day 7 and colons were removed and measured. Results are expressed as the mean colon length ± SD. *$P < 0.05$ for N = 9 mice per group in 3 independent experiments for A (repeated measures ANOVA) and B (Mann-Whitney test) and for N=9 mice per group in 2 independent experiments for C (student's t-test).

2.3.2 SHIP-/- mice had less histological damage during dextran sodium sulfate-induced colitis

I also asked if SHIP-/- mice were protected from histological damage during DSS-induced colitis relative to SHIP+/+ littermate controls. After the 7 day DSS treatment, the distal colon was fixed for histological assessment. H&E staining revealed severe damage in SHIP+/+ colonic tissue compared to that seen in tissue from SHIP-/- mice (Fig. 2.3A). Histological damage was scored based on the parameters outlined in Table 2.2. SHIP+/+ mice had significantly worse histological damage scores when compared to SHIP-/- mice (Fig. 2.3B). SHIP+/+ and SHIP-/- mice had a 4.6-fold increase in total $F4/80^{+}$ macrophages in colon sections during DSS-treatment (compare Fig. 2.3C to 2.1B). Importantly, SHIP-/- mice had 2.5-fold more $ArgI^{+}$ macrophages in the colon after DSS treatment compared to their wild type littermates (Fig. 2.3C). Taken together, these data show that SHIP-/- mice have reduced clinical disease and histological damage compared to SHIP+/+ littermates and protection during DSS-induced colitis correlated with increased numbers of M2 macrophages in the colon.

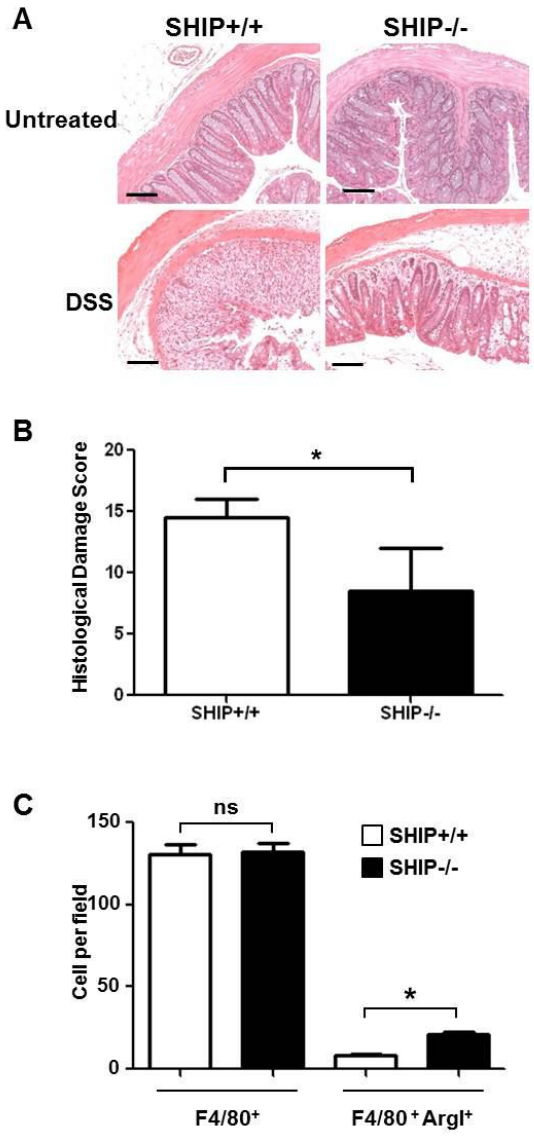


Figure 2.3 SHIP-/- mice have reduced histological damage during DSS-induced colitis SHIP+/+ and SHIP-/- mice were treated with 5% DSS in their drinking water for 7 days after which distal colon sections were collected for histological analysis. (A) Representative H&E stained sections of SHIP+/+ (left) and SHIP-/- (right) colons from mice that were not treated (top) or treated with DSS (bottom). Scale bars = 100μm. (B) Histological damage was scored and is expressed as median score ± range. *$P < 0.05$ for N = 6 mice per group in 2 independent experiments (Mann-Whitney test). (C) Quantitation of $F4/80^+$ and $F4/80^+ArgI^+$ macrophages in DSS-treated SHIP+/+ (white bars) and SHIP-/- mice (black bars). Data are represented as means ± SD. Positively stained cells were counted at 40× magnification in 6 fields, from 6 sections per mouse separated by ≥50 μm, and from 6 mice per group by two individuals blinded to experimental conditions. ns=not significantly different and *$P<0.01$.

2.3.3 Macrophage depletion eliminated protection in SHIP-/- mice during dextran sodium sulfate-induced colitis

To determine if the protection seen in SHIP-/- mice during disease progression was due to M2 macrophages in the colon, I eliminated them from the gut and monitored disease severity. Clodronate-containing liposome injections have been shown previously to deplete macrophages in mouse colons[143, 147] so I used this method to deplete colonic macrophages in mice. SHIP+/+ and SHIP-/- mice were given 5% DSS for 7 days to induce colitis. Mice were given PBS as an injection control, PBS-containing liposomes as a liposome injection control, and clodronate-containing liposomes to deplete macrophages. Intraperitoneal injections were given 4 days prior to DSS treatment as well as at days 0, 2, 4, and 6 of DSS treatment. In SHIP+/+ mice, macrophage depletion did not significantly impact weight loss or rectal bleeding scores (Fig. 2.4, left). In contrast, clodronate-containing liposome depletion of macrophages in SHIP-/- mice increased DSS-induced weight loss (Fig. 2.4A, right) and rectal bleeding scores (Fig 2.4B, right). Injection of PBS-containing liposomes, caused a similar increase in disease severity in SHIP-/- mice (Fig. 2.4, right).

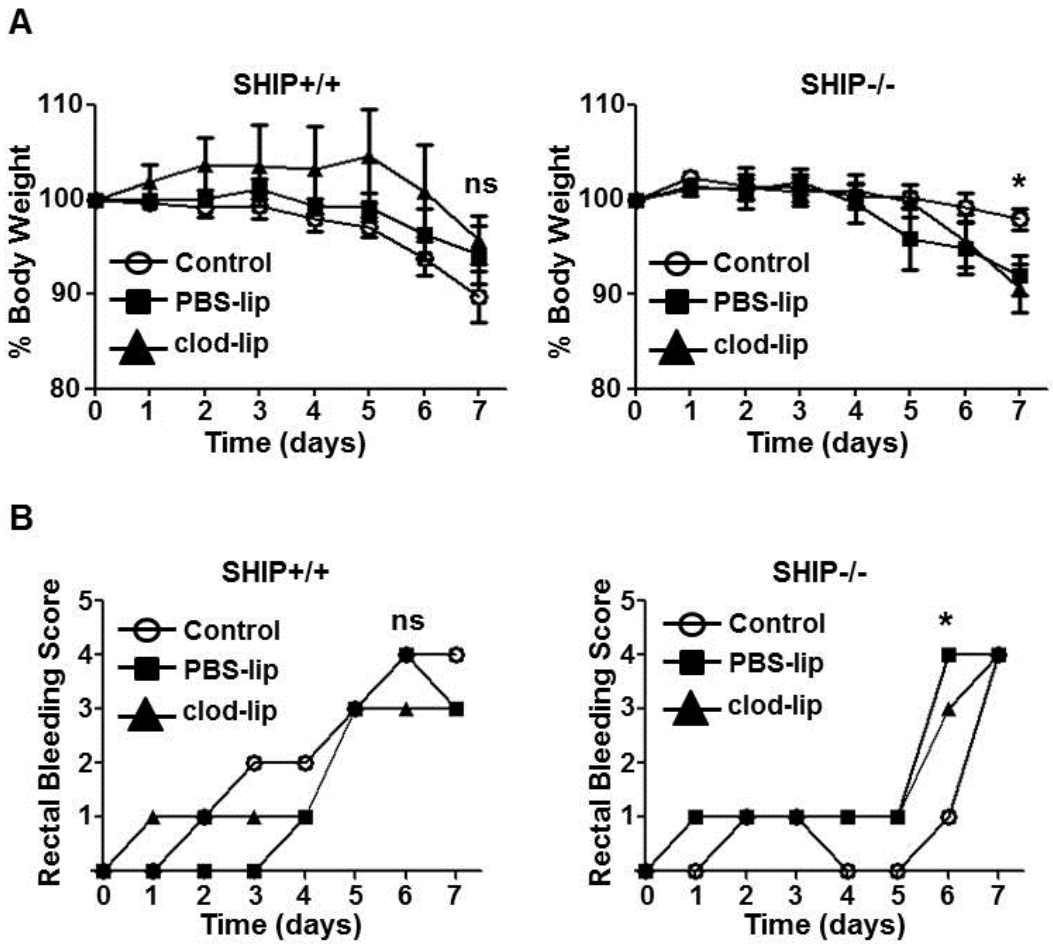


Figure 2.4 Macrophage depletion by clodronate-containing liposomes eliminates protection of SHIP-/- mice during DSS-induced colitis

SHIP+/+ and SHIP-/- mice were treated with 5% DSS in their drinking water for 7 days. DSS-treated control mice were compared to mice receiving either PBS-containing liposome injections (PBS-lip) or clodronate-containing liposome injections (clod-lip) 4 days prior to DSS treatment and on day 0, 2, 4, and 6 during DSS treatment. (A) Weight loss during treatment for SHIP+/+ mice (left) and SHIP-/- mice (right) is expressed as average percentage of initial body weight ± SD. (B) Rectal bleeding was monitored and scored daily during treatment for SHIP+/+ mice (left) and SHIP-/- mice (right). The median score is reported for each group of mice. *$P < 0.05$ for N = 6 mice per group in 2 independent experiments (Repeated measures ANOVA for A and Mann-Whitney test for B). ns = not significantly different.

2.3.4 Macrophage depletion reduced histologically apparent disease in SHIP+/+ mice and exacerbated disease in SHIP-/- mice

In SHIP+/+ mice, treatment with both PBS- and clodronate-containing liposomes reduced histological damage (Fig. 2.5A, top) and histological damage scores (Fig 2.5B, top). This suggests that macrophages in SHIP+/+ mice contribute to pathology despite no change in weight loss or rectal bleeding. In contrast, SHIP-/- mice had increased tissue damage when treated with either PBS- or clodronate-containing liposomes during treatment compared with DSS-treated controls (Fig. 2.5A, bottom). Histological damage in SHIP+/+ mice, was reduced in the PBS- or clodronate-containing liposome injected groups compared with controls (Fig. 2.5B, top), yet no difference was seen between mice injected with PBS- or with clodronate-containing liposomes. In SHIP-/- mice, groups injected with PBS- or clodronate-containing liposomes had significantly worse histological damage when compared with PBS injected controls (Fig. 2.5B, bottom). Again, there was no difference between groups injected with PBS- or clodronate-containing liposomes. Taken together, these data suggest that liposome treatment ameliorates disease in wild type (SHIP+/+) mice by removing inflammatory, disease-causing macrophages but exacerbates disease in SHIP-/- mice by removing protective M2 skewed macrophages.

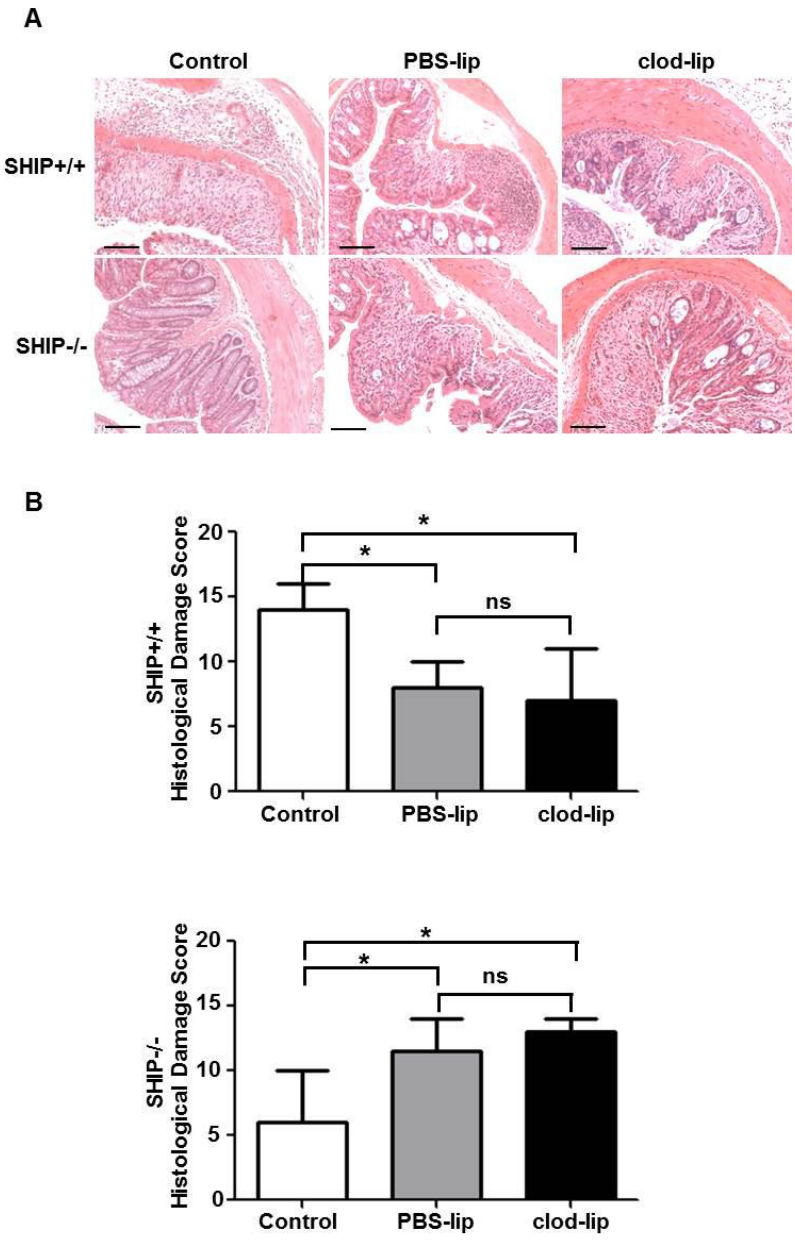
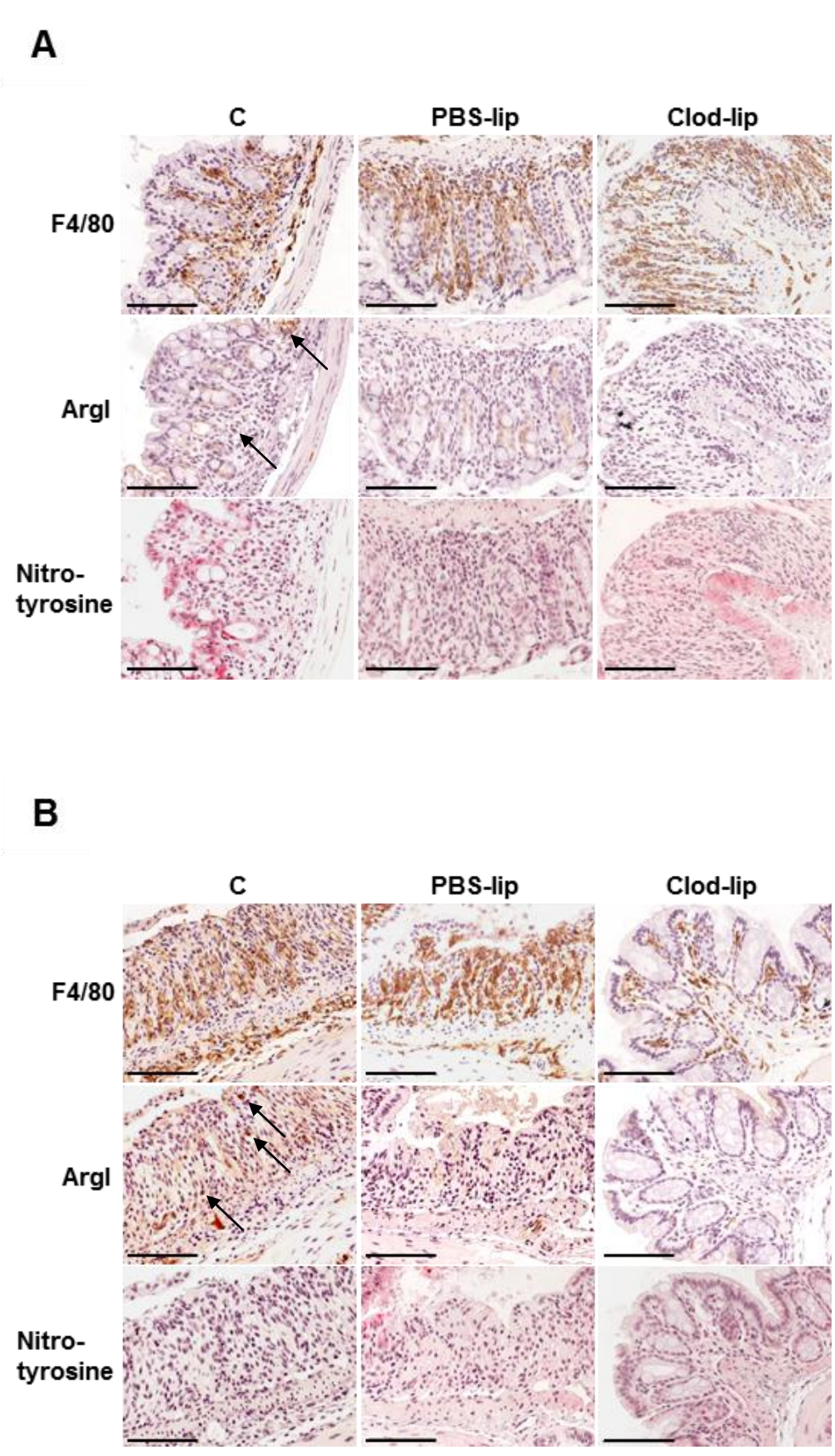


Figure 2.5 Liposome treatment reduces histological damage in SHIP+/+ mice and exacerbates histological damage in SHIP-/- mice during DSS-induced colitis
SHIP+/+ and SHIP-/- mice were given 5% DSS for 7 days ± treatment with PBS, PBS-containing liposomes or clodronate-containing liposomes injected 4 days prior to DSS treatment and on day 0, 2, 4, and 6 during DSS treatment. After 7 days, mice were euthanized and colon sections were taken for histology. (A) Representative H&E stained sections of SHIP+/+ (top) and SHIP-/- (bottom) colons from mice that were not treated with liposomes (left), treated with PBS-containing liposomes (middle) or clodronate-containing liposomes (right). Scale bars = 100μm. (B) Histological damage scores for SHIP+/+ (top) and SHIP-/- mice (bottom) expressed as median score with range. *$P < 0.05$ for N = 6 mice per group in 2 independent experiments (Mann-Whitney test). ns = not significantly different.

2.3.5 PBS- and clodronate-containing liposomes effectively depleted colonic M1 and M2 macrophages

When performing the macrophage depletion experiments, I was surprised to see that injecting the mice with PBS-containing liposomes as a control had the same impact on disease severity as injecting them with clodronate-containing liposomes, which affected disease severity as hypothesized for both SHIP+/+ and SHIP-/- mice. PBS-containing liposomes have been reported to cause macrophage depletion in colons previously [143] so I asked whether colonic macrophages were being depleted by both liposome treatments in our model. SHIP-/- mice were treated with 5% DSS for 7 days with intraperitoneal injections of PBS, PBS-containing liposomes, or clodronate-containing liposomes 4 days prior to treatment as well as at days 0, 2, 4, and 6 during DSS treatment. Following treatment, colons were harvested and fixed for immunohistochemical analyses. Sections were stained for F4/80, ArgI, or nitrotyrosine (Fig. 2.6A and B). SHIP-/- mice have more $ArgI^+$ macrophages and lower nitrotyrosine staining than their wild type counterparts (Fig. 2.6A and B, left panels). In addition, treatment with either PBS-containing liposomes or clodronate-containing liposomes reduced $ArgI^+$ macrophages in SHIP-/- colon sections (Fig. 2.6B, middle row) and reduced nitrotyrosine staining in SHIP+/+ colon sections (Fig. 2.6A, bottom). $F4/80^+$ macrophages and $F4/80^+ArgI^+$ macrophages were counted in serial colon sections (Fig. 2.6C). PBS- or clodronate-containing liposomes effectively depleted total $F4/80^+$ macrophages in both SHIP+/+ and SHIP-/- mouse colons by 55%. In addition, ArgI+ macrophages were also depleted by 51% in SHIP+/+ mice and 45% in SHIP-/- mice. Sham PBS injection alone had no impact on clinical or histological disease when compared to DSS-treated control mice and had no effect on total $F4/80^+$ or $F4/80^+ArgI^+$ cell numbers in colons. To confirm these results,

following treatment I harvested full length colons from SHIP+/+ mice for homogenization and enzymatic digestion. I then probed the samples for the macrophage marker F4/80 and analyzed them by flow cytometry. These results also indicated that half of the total colonic macrophage number was depleted by either PBS- or clodronate-containing liposomes compared to controls (Fig. 2.6D). Dead cells were eliminated from the analysis by staining with 7-AAD and excluding 7-AAD positive cells from our analyses.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C
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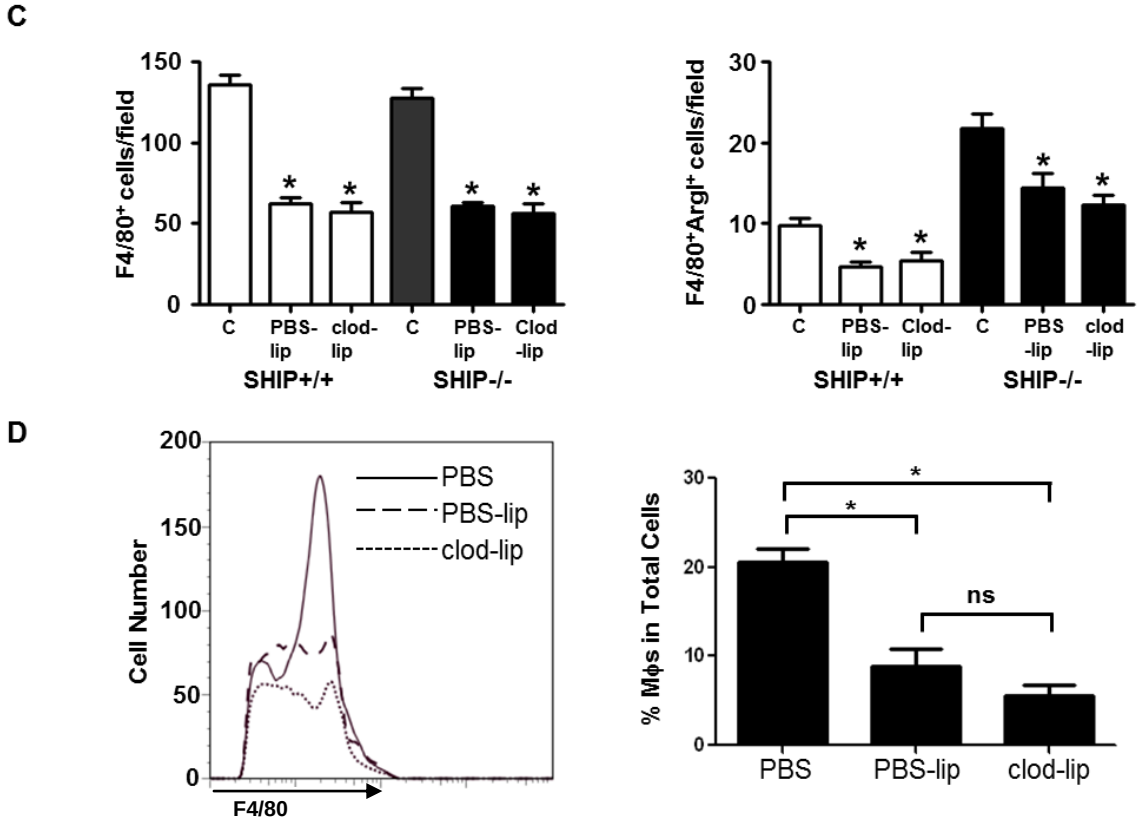

Figure 2.6 Clodronate- and PBS-containing liposomes effectively deplete macrophages in mouse colons

SHIP-/- mice were given 5% DSS for 7 days and injected intraperitoneally with either PBS (C), PBS-containing liposomes (PBS-lip), or clodronate-containing liposomes (clod-lip) 4 days prior to DSS treatment and on day 0, 2, 4, and 6 during DSS treatment. Following treatment, colons were removed, tissue was fixed, and sections were stained by immunohistochemistry. SHIP+/+ (A) and SHIP-/- (B) colon sections stained for mature macrophages (F4/80; top), M2 macrophage marker, ArgI (middle) and nitrotyrosine (bottom). Scale bars = 50μm. Data are representative of similar staining from N=6 mice for each group. (C) Quantitation of $F4/80^+$ and $F4/80^+ArgI^+$ macrophages in SHIP+/+ (white bars) and SHIP-/- mice (black bars). Data are represented as means ± SD. Positively stained cells were counted at 40× magnification in 6 fields, from 6 sections per mouse separated by ≥50 μm, and from 6 mice per group. (D) Following treatment, excised colons from SHIP+/+ mice were homogenized and enzymatically digested, stained with the macrophage marker F4/80, and analyzed by flow cytometry. Data are represented as means ± SD. *P<0.01 comparing PBS-containing liposome or clodronate-containing liposome treatment with sham injected, control mice.

2.3.6 Clodronate-containing liposomes deplete M1 and M2 macrophages equally

To ensure that loss of protection by macrophage depletion was due to a decrease in M2 macrophages, I wanted to verify that clodronate-containing liposomes did not preferentially deplete M1 or M2 macrophages. I derived macrophages from bone marrow aspirates from SHIP+/+ and SHIP-/- mice in either MCSF (M1) or GM-CSF (M2). Mature macrophages were treated with PBS, PBS-containing liposomes, or clodronate-containing liposomes for 8, 24, or 48 hours. Following treatment, cell viability was determined using the colorimetric WST-1 assay whereby viable cells break down a tetrazolium dye. Clodronate-containing liposomes were equally effective at depleting SHIP+/+ and SHIP-/- macrophages (Fig. 2.7). In addition, SHIP-/- macrophages that were differentiated in the presence of MCSF and GM-CSF were depleted equally by clodronate-containing liposomes. Finally, PBS-containing liposomes did cause macrophage depletion, albeit at lower levels than clodronate-containing liposomes providing additional support for our *in vivo* data.

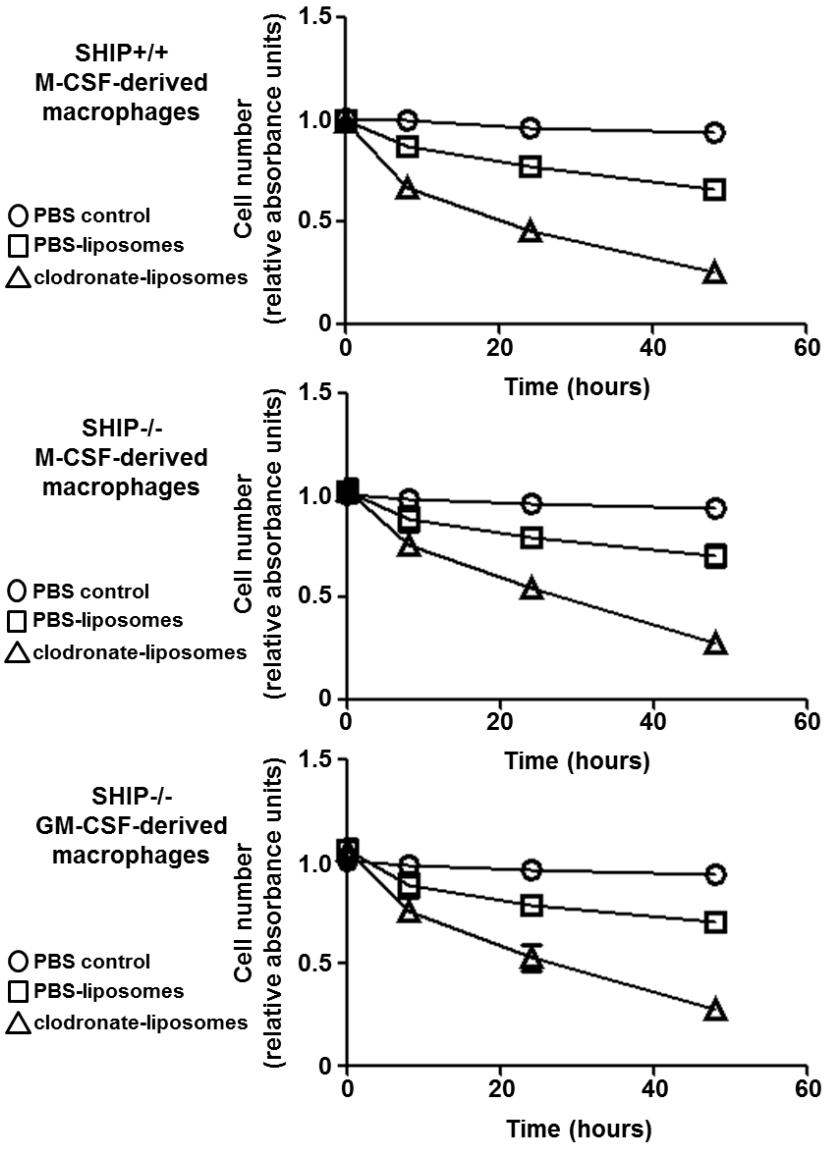

Figure 2.7 Clodronate- and PBS-containing liposomes deplete different macrophage phenotypes equally well

SHIP+/+ MCSF-derived macrophages (M1; top graph), SHIP-/- MCSF-derived macrophages (M1; middle graph), or SHIP-/- GM-CSF-derived macrophages (M2, bottom graph) were treated with PBS (control, circles), PBS-containing liposomes (squares), or clodronate-containing liposomes (triangles). Wst-1 assays were performed after 8, 24 and 48 hours. Data shown are represented as means ± SD and are representative of 3 experiments.

2.3.7 Adoptive transfer of SHIP-/- M2 macrophages conferred protection to wild type mice during dextran sodium sulfate-induced colitis

It has been shown that deriving SHIP-/- macrophages from bone marrow in MCSF or GM-CSF can produce M1 or M2 macrophages, respectively.[140] Although macrophage phenotype is plastic and macrophages can skew according to signals provided by their local microenvironment[153], it has been shown that SHIP-/- macrophages derived in GM-CSF will remain M2 despite pro-inflammatory signalling.[139, 140] For these reasons, I asked whether providing SHIP-/- GM-CSF-derived M2 macrophages to SHIP+/+ mice could confer protection during DSS-induced colitis. Mature bone marrow macrophages were derived from SHIP-/- mice using either MCSF, to generate M1 macrophages, or GM-CSF, to generate M2 macrophages. After 10 days, macrophage phenotype was assessed by Western blotting and by Griess assays on cell supernatants after LPS stimulation. SHIP-/- macrophages derived in MCSF did not expressed M2 macrophage markers, ArgI or Ym1, and produced large amounts of NO in response to LPS stimulation. In contrast, SHIP-/- macrophages derived in GM-CSF expressed high levels of M2 macrophage markers, ArgI and Ym1, and produced relatively lower amounts of NO in response to LPS stimulation (Fig. 2.8A). To determine their effect on disease severity, SHIP+/+ mice were given 5% DSS for 6 days with MCSF or GM-CSF derived macrophages (10^6) injected by tail vein on day 0 and 4 of DSS treatment. I found that rectal bleeding scores for GM-CSF derived macrophage-injected mice were significantly lower than either the control group or the MCSF derived macrophage injection group (Fig. 2.8B). In addition, H&E stained histological sections of colons post-treatment showed less histological damage in colons from mice injected with GM-CSF derived macrophages compared with those injected with either PBS or MCSF

derived macrophages (Figs. 2.8C). MCSF derived macrophages did not show a statistically significant reduction in histological damage when injected into SHIP+/+ mice (Fig. 2.8D). These data demonstrate that macrophage SHIP deficiency is not sufficient to confer protection from intestinal inflammation, but rather, the M2 macrophage phenotype of SHIP-/- mouse macrophages provides protection during DSS-induced colitis.

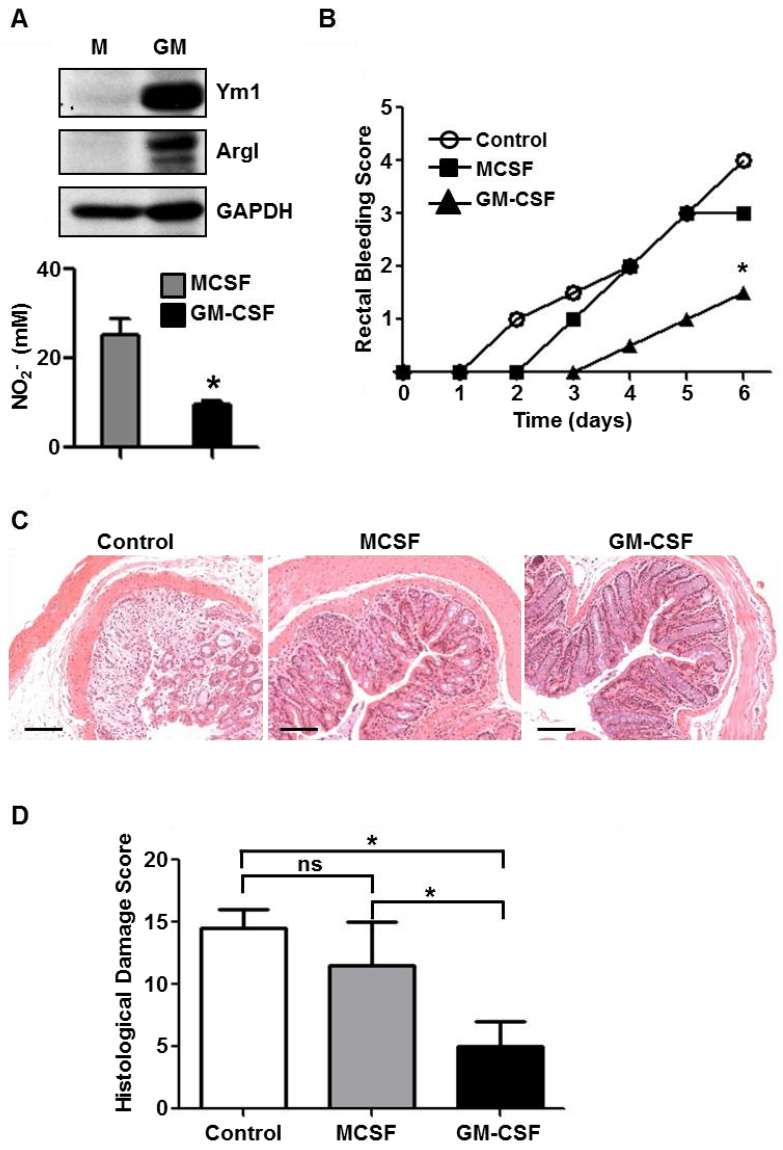

Figure 2.8 SHIP-/- M2 macrophages derived *in vitro* confer protection to susceptible wild type (SHIP+/+) mice during DSS-induced colitis

SHIP+/+ mice were given 5% DSS in their drinking water for 6 days and either MCSF or GM-CSF (M2) derived macrophages were injected intravenously into mice on days 0 and 4 of DSS treatment. (A) SHIP-/- bone marrow macrophages used for injection were derived for 10 days in 10ng/ml of MCSF (M) or GM-CSF (GM). Whole cell lysates were analyzed for M2 macrophage markers, Ym1 and ArgI, and GAPDH (as a loading control) by Western blotting. Macrophages were treated with LPS (10 ng/ml) and nitrate was measured in cell supernatants after 24 hrs using the Griess assay, as a marker of M1 macrophage phenotype. Data are represented as mean ± SD. (B) Rectal bleeding was monitored and scored during treatment and is expressed as the median score. (C) Representative H&E stained sections of colons. Scale bars = 100µm. (D) Histological damage expressed as median score with range. *$P < 0.05$ for N = 6 mice per group in 2 independent experiments (Mann-Whitney test). ns = not statistically significant.

2.4 Discussion

Intestinal macrophages reside in the lamina propria and are poised underneath the epithelial cells lining the gut.[54] Colonic macrophages contribute to intestinal homeostasis by maintaining potent phagocytic and antimicrobial ability while remaining anergic to inflammatory stimuli.[101, 102] During intestinal inflammation, monocytes are recruited from circulation to the gastrointestinal tract and mature into inflammatory macrophages that aggravate inflammation.[102] The murine DSS-induced colitis model is particularly useful in the study of innate immune driven colitis where monocytes are recruited to the colon during DSS-induced inflammation mimicking this important element of human disease [154-157] and play a central role in disease pathogenesis.[158, 159] Consistent with this, I see a 4.6-fold increase in F4/80^{+} cells in mouse colons during DSS-treatment. Macrophage depletion by clodronate-containing liposomes during DSS-induced colitis demonstrates that inflammatory macrophages contribute to disease in this model.[57, 160] Macrophage depletion did not significantly reduce weight loss or rectal bleeding in SHIP+/+ mice but did improve histological appearance of disease. Histological analyses may detect more subtle changes in disease pathology and our results are consistent with inflammatory macrophages contributing to disease in the SHIP+/+ mice.

SHIP is a hematopoietic-specific negative regulator of the PI3K pathway. *In vivo* differentiated SHIP-/- macrophages are profoundly M2 skewed [139] and this has been cited as important in their response to the enteric pathogen *Salmonella enterica* serovar Typhimurium.[161] I found that SHIP-/- colonic macrophages also express high level of M2 macrophage markers, ArgI and Ym1, relative to those from SHIP+/+ littermate controls. Macrophages skewed to an alternatively activated phenotype by parasite infection are

protective in a murine model of colitis and *ex vivo* IL-4-skewed M2 macrophages are protective during DNBS-induced colitis [147] so I hypothesized that SHIP-/- mice might be protected in a colitis model by their M2 macrophages. Herein, I demonstrate that SHIP-/- mice are protected during DSS-induced colitis with respect to the clinical presentation of disease (weight loss and rectal bleeding) and the histological damage observed. SHIP-/- mice do develop disease in response to DSS but disease onset is delayed and severity of disease is reduced at the time points examined. Assessment of histological damage included examination of immune cell infiltrates which were consistently reduced in SHIP-/- mice suggesting that SHIP deficiency did not increase PI3Kp110γ-mediated activation of immune cell chemotaxis. Clodronate depletion experiments demonstrate that protection was mediated by SHIP-/- macrophages. Liposome-encapsulated PBS also caused macrophage depletion in our model as has been described previously [143] so macrophage depletion experiments were compared to a PBS injection control, wherein disease activity and histological damage was comparable to DSS-treated mice without injection.

Bone marrow derived macrophages from SHIP-/- mice can be skewed to an M2 phenotype by differentiation with GM-CSF [140] and importantly, when SHIP-/- macrophages are skewed to an M2 phenotype during differentiation, they do not revert to an M1 phenotype.[139, 140] I differentiated SHIP-/- bone marrow macrophages in the presence of MCSF, to generate M1 macrophages, or GM-CSF, to generate M2 macrophages, and injected them via tail vein into SHIP+/+ mice during the development of colitis. GM-CSF-derived SHIP-/- M2 macrophages, but not MCSF-derived (M1 control), conferred protection to wild type mice (SHIP+/+) during DSS-induced colitis. This demonstrates that SHIP deficiency in

macrophages is not sufficient to protect mice from intestinal inflammation, but rather, the M2 macrophage phenotype is responsible for protection.

Macrophage skewing to a protective M2 phenotype has tremendous potential in dampening down acute inflammation. However, M1 macrophages are critical in our bodies' recognition and response to infectious organisms and foreign tumor cells.[89, 90, 162] In addition, the practical application of M2 macrophages in dampening down inflammation is limited in chronic inflammatory diseases like inflammatory bowel diseases because when IL-4 is removed or when an M2 macrophage encounters an inflammatory environment, its protective M2 phenotype will be lost and it will acquire properties of inflammatory macrophages that would be expected to exacerbate inflammation.[79, 139] The ability to lock macrophages into an M2 phenotype by SHIP depletion, may provide a novel approach to therapeutically dampen down intestinal inflammation in people with IBD. Knocking down SHIP may be a viable strategy to skew macrophages to an M2 phenotype *in vivo* in IBD patients, however, a key question that needs to be answered is how SHIP deficiency leads to alternative activation of macrophages. To address this question, we sought to determine the role of SHIP in the canonical IL-4 induced alternative activation of macrophages.

3 Chapter: Alternative activation of macrophages by IL-4 requires SHIP degradation

3.1 Introduction and rationale

Macrophage phenotype is caused by signals from the microenvironment that are encountered during development, inflammation, and resolution of inflammation.[90] In human macrophages, MCSF and GM-CSF lead to the development of M2 and M1 macrophages, respectively[95] but in the murine system, GM-CSF derivation leads to induction of the murine alternatively activated macrophage marker, ArgI,[140] which dampens down inflammation by competing with iNOS for L-arginine.[139, 140] Mature macrophages can be alternatively activated by treatment with IL-4 or IL-13 and this is enhanced by co-treatment with IL-10.[90] Murine GM-CSF-derived or IL-4-induced M2 macrophages also express Ym1, a mammalian chitinase-like molecule.[163]

In vivo-differentiated SHIP-/- macrophages are M2 skewed[139] and bone marrow progenitors from SHIP-deficient mice differentiated with GM-CSF or IL-3 are more strongly M2 skewed than their wild type counterparts.[140] Based on these observations, and our data showing SHIP-/- GM-CSF-derived M2 macrophages are protective during DSS-induced intestinal inflammation, I investigated whether SHIP played a role in canonical IL-4-induced alternative activation of macrophages.

3.2 Materials and methods

Mice. SHIP heterozygotes were maintained on a C57BL/6×129Sv mixed background as previously described.[148] STAT6-/- mice were on a C57BL/6 background. Mice were housed in the Animal Research Center at the Child & Family Research Institute and experimentation

was performed in accordance with institutional and Canadian Council on Animal Care guidelines.

Reagents. *E. coli* serotype 0127:B8 LPS was from Sigma Aldrich (St Louis, MO). IL-4, IL-10, GM-CSF and MCSF were from StemCell Technologies (Vancouver, Canada). MG132, LY294002 (LY29) and LY303511 (LY30) were from Calbiochem (San Diego,CA). PI3Kp110 isoform specific inhibitors PIK-90, TGX-221, SW18, SW30, SW14, and AS605240 were provided by our collaborator, Dr. Kevan Shokat. Synthesis was performed as he described previously.[164] Table 3.1 lists the IC_{50} (μM) for each of the inhibitors used in this study against purified p110 isoforms. IC_{50} values for inhibitors in whole cells are 10-100-fold higher.[165] Anti-Ym1, anti-SHIP2 were from StemCell Technologies; anti-ArgI, anti-Shc were from BD Biosciences, anti-GAPDH was from Fitzgerald Industries International; anti-PTEN, anti-SHIP, anti-pSTAT6 were from Santa Cruz Biotechnology, Inc.

Table 3.1 IC_{50} (μM) values of the p110 isoform-specific PI3K inhibitors against pure p110 enzymes

		IC_{50} (μM)			
Inhibitor	Abbreviation	p110α	p110β	p110δ	p110γ
PIK-90	A	0.011	0.35	0.058	0.018
TGX-221	B	5.0	0.005	0.1	>10.0
SW18	δA	6.7	2.4	0.005	0.038
SW30	δB	85	0.74	0.007	1.3
SW14	δC	8.9	0.697	0.009	0.021
AS605240	Γ	0.06	0.27	0.3	0.008

Macrophage derivation and purification of tumor associated macrophages and thioglycollate-elicited peritoneal macrophages. Bone marrow (BM) macrophages were derived from bone marrow aspirates of femurs and tibias as previously described.[138] MCSF and GM-CSF BM macrophages cultures were >95% Mac-1^+ and F4/80^+ after 10 days in culture. Tumor associated macrophages (TAMs) were labeled with F4/80-PE (eBiosciences) and purified using an EasySep™ PE selection kit (StemCell Technologies) from M27 Lewis lung carcinoma tumors grown subcutaneously on the hind flanks of C57BL/6 mice as described previously.[139] Thioglycollate (TG)-elicited peritoneal macrophages were prepared by injecting mice intra-peritoneally with 2ml of 3% thioglycollate in sterile PBS. After 4 days, mice were euthanized and peritoneal macrophages were harvested by lavaging the peritoneal cavity with 3×5mL of complete medium.

***In vitro* macrophage stimulations.** Macrophages were stimulated ±IL-4 or IL-4+IL-10 (10ng/ml) for 1-5 days and WCLs were harvested for Western blot analysis (0.5-1.0×10^6cells), SHIP, or arginase assays. For NO analysis, cells were treated for 24 hr with LPS (10 ng/ml) or LPS+L-Arg (2 mM) and clarified cell supernatants were collected for Griess assays. For inhibitor experiments, cells were pre-incubated with inhibitor, control, or vehicle for 15 min.

SDS-PAGE and Western blotting. WCLs were prepared for SDS-PAGE by lysing in 1× Laemmli's digestion mix, sheering DNA using a 26 gauge needle, and boiling for 1 min. Lysates were loaded onto a 12% polyacrylamide gel and Western blotting was carried out by transferring protein onto an Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-

Rad, Mississauga, ON) using 600 mA for 4 hr at 23°C. Blots were blocked, incubated with antibodies and then with horseradish peroxidase-conjugated secondary antibody (BioLegend, San Diego, CA) before adding ECL substrate solution (Invitrogen) and exposing to Kodak X-Omat film (PerkinElmer Life Sciences, Woodbridge, ON). Densitometry was performed using ImageJ software (National Institute of Health, USA).

SHIP activity assays. To measure SHIP activity in BM macrophages, SHIP was immunoprecipitated with anti-SHIP-P1C1-agarose conjugate (Santa Cruz) as described previously.[166] Resultant immunoprecipitates were washed 3 times and equivalent numbers of input BM macrophages were compared in SHIP activity assays. Substrate, 100µM inositol-1,3,4,5-tetrakisphosphate (IP4; Echelon Biosciences, Salt Lake City, UT), was incubated with immunoprecipitates for 20 min and the reaction was stopped by heating to 80°C for 5 min. Inorganic phosphate released was measured by the addition of Malachite Green reagent (Echelon Biosciences) and absorbance was read at 650 nm and compared to a standard curve.

Arginase assays. Arginase activity was determined indirectly by measuring the concentration of urea generated by the arginase-dependent hydrolysis of L-arginine. Cells (10^6) were lysed with 400 µl of cell lysis buffer (0.1% Triton X-100 (BDH), 25 mM Tris-Cl (Bio Basic Inc., Markham, ON), pH 8.0). Following lysis, 10 µl of 10 mM $MnCl_2$ was added to each sample which consisted of 10µg of protein in 100 µl of buffer. The enzyme was activated by heating for 10 min at 55°C. Arginine hydrolysis was conducted by incubating the lysates with 100 µl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 800 µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The urea

concentration was measured at 550 nm after addition of 40 µl of α-isonitrosopropiophenone (Sigma) (dissolved in 100% ethanol), followed by heating at 100°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol urea/min.[167]

Nitric oxide assays. NO production was determined indirectly by measuring the accumulation of nitrite in tissue culture supernatants using the Griess Reagent Kit, according to manufacturers' instructions (Invitrogen).[137]

SHIP knockdown in RAW264.7 cells and bone marrow-derived macrophages and SHIP over-expression in RAW264.7 cells. Cells were transfected with SHIP small interfering RNA (siRNA) or non-silent RNA (nsRNA).[138] For SHIP over-expression experiments, RAW264.7 cells were transfected with a MSCV vector containing the full length SHIP cDNA[132] or empty vector control using the NanoJuice Transfection Kit (EMD Chemicals Inc.), according to the manufacturer's instructions.

STAT6-luciferase reporter assays. RAW264.7 cells (10^4/100µl) were transiently transfected with plasmid encoding STAT6-luciferase as described previously[168] or STAT5-luciferase as a control. Transfections were performed for inhibitor experiments or in the presence of SHIP siRNA or nsRNA. Transfected cells were allowed to recover for 48 hrs and then were stimulated ±50 ng/ml IL-4 for 24 hrs. Cells were lysed and luciferase activity was assessed using the Dual Luciferase Reporter Assay System, according to the

manufacturer's protocol (Promega, Madison, Wisconsin USA), and read on the VictorX multimode plate reader (Perkin Elmer).

Statistical analyses. Unpaired, two-tailed Student's *t* tests were performed using GraphPad Prism version 5 (GraphPad Software Incorporated).

3.3 Results

3.3.1 SHIP blocked IL-4-induced M2 macrophage skewing

SHIP-/- mouse macrophages are profoundly M2 skewed. They express high levels of the M2 markers, ArgI and Ym1, and have impaired LPS-induced NO production.[139] While IL-3 and GM-CSF-induced alternative macrophage activation is enhanced in SHIP-/- progenitors, progenitors are not more sensitive to IL-4-induced skewing.[140] Based on these findings, I asked whether SHIP affects IL-4-induced alternative macrophage activation. SHIP+/+ and SHIP-/- BM macrophages were treated for 3 days with IL-4 and WCLs were analyzed by Western blot for M2 macrophage marker expression. SHIP+/+ macrophages expressed low levels of Ym1 and ArgI at a dose of 3 ng/ml IL-4 and higher levels when treated with 30 ng/ml IL-4. SHIP-/- BM macrophages expressed these markers at a 10-fold lower dose of IL-4 (0.3 ng/ml) and showed higher expression when treated with 3 ng/ml and 30 ng/ml of IL-4 (Fig. 3.1A). SHIP+/+ BM macrophage M2 marker expression correlated with loss of the 145kDa isoform of SHIP protein and the appearance of lower molecular weight bands at 110kDa and 90kDa. SHIP immunoprecipitates were assayed for enzymatic activity to see if this correlated with a loss of SHIP activity. SHIP activity was significantly lower after IL-4 treatment for 3 days with 0.3 ng/ml of IL-4 and decreased by 82% when treated with 30

ng/ml of IL-4 (Fig. 3.1B). SHIP-/- BM macrophages had an increase in arginase activity in response to 0.5 ng/ml of IL-4 as opposed to 2 ng/ml that was required for SHIP+/+ BM macrophages (Fig. 3.1C). Arginase activity was significantly higher in SHIP-/- BM macrophages than in SHIP+/+ BM macrophages at all doses. Since ArgI competes with iNOS for L-arginine; nitrite, was measured after LPS stimulation. SHIP+/+ BM macrophages produced higher amounts of NO when skewed to an M2 phenotype and this was augmented by L-arginine supplementation (Fig. 3.1D). IL-4 treated SHIP-/- BM macrophages produced more NO than untreated and more than SHIP+/+ BM macrophages and this was augmented by L-arginine supplementation (Fig. 3.1E). This suggests that IL-4 treatment potentiates iNOS activity in response to LPS, but activity is masked by ArgI activity.

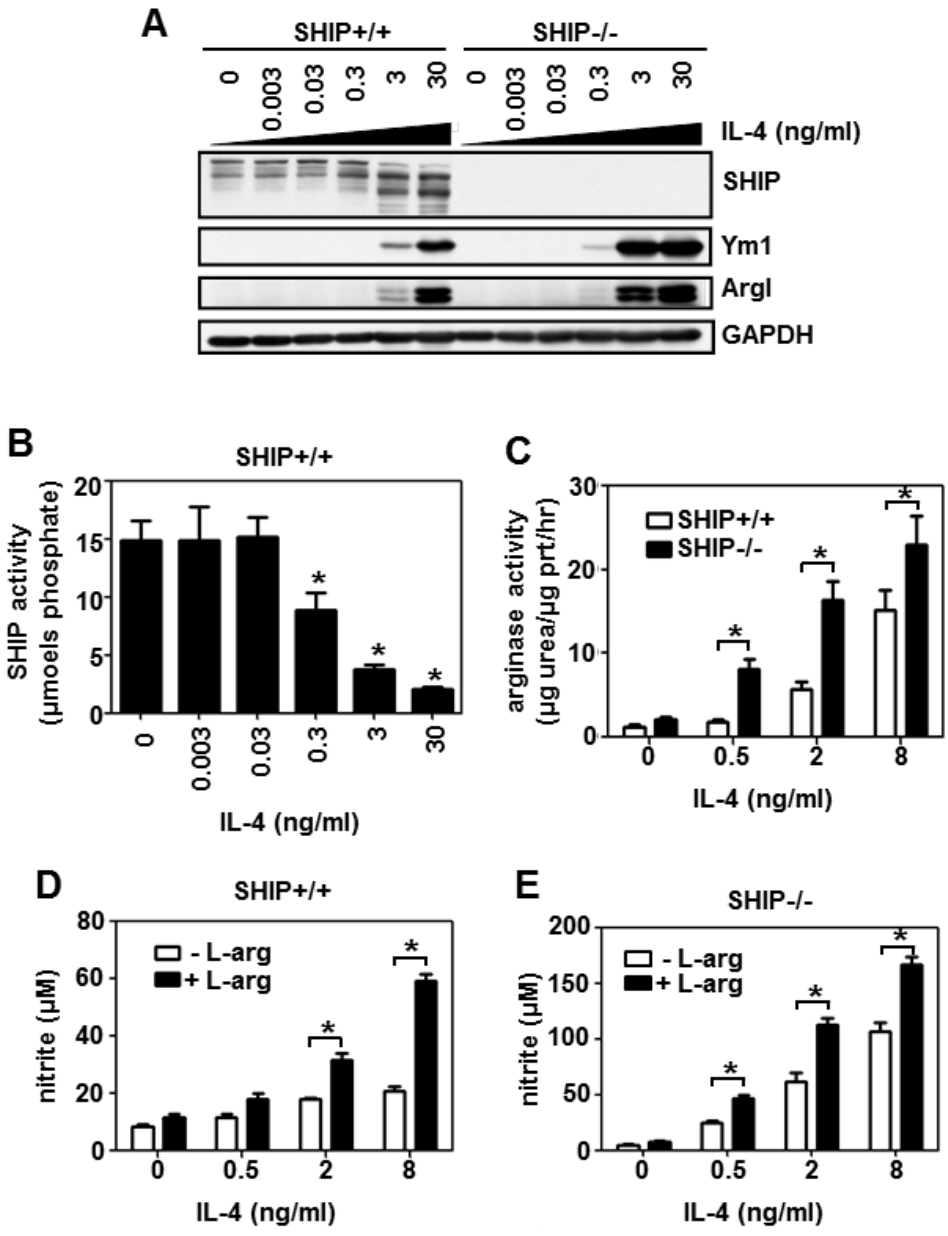

Figure 3.1 SHIP-/- macrophages are more sensitive to IL-4-induced M2 skewing
SHIP+/+ and SHIP-/- BM macrophages were treated with the indicated dose of IL-4 for 3 days. Whole cell lysates (WCLs) were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting (A). SHIP immunoprecipitates were assayed for enzymatic activity (B) and arginase activity (C). SHIP+/+ (D) or SHIP-/- (E) BM macrophages were treated with the indicated concentrations of IL-4, followed by LPS (10ng/ml) for 24h in the absence or presence of L-arginine. Clarified cell supernatants were analyzed for nitrite. Data in (A) are representative of 4 independent experiments. Data are means ± SEM from 4 independent experiments in (B-C) and 3 independent experiments in (D, E). *P<0.05 comparing IL-4-treated to untreated in (B); *P<0.03 comparing SHIP+/+ versus SHIP-/- in (C) and control versus L-arginine supplementation in (D, E) (Student's *t*-test).

3.3.2 IL-4 treatment reduced SHIP protein levels and activity correlating with M2 marker induction

It has been reported that IL-4 treatment of BM macrophages leads to a decrease in SHIP protein levels[169] and that SHIP's phosphorylation triggers its poly-ubiquitination and degradation by the proteasome.[170] I next asked whether SHIP protein levels and activity decrease over time in response to IL-4 treatment and how this impacts M2 macrophage marker expression. Incubating SHIP+/+ BM macrophages with 10 ng/ml of IL-4 over 5 days resulted in decreased SHIP protein expression and activity that correlated with increased expression of M2 macrophage markers and arginase activity (Figs. 3.2A-C). Other inositol phosphatases, SHIP2 and PTEN were unaffected. LPS-induced NO production increased during the first day of IL-4 treatment and decreased in subsequent days, unless supplemented with L-arginine (Fig. 3.2D). Therefore, during IL-4 induced M2 macrophage skewing, decreased SHIP protein expression and loss of activity correlates with increased expression of M2 macrophage markers and decreased NO production, due to ArgI-mediated L-arginine depletion.

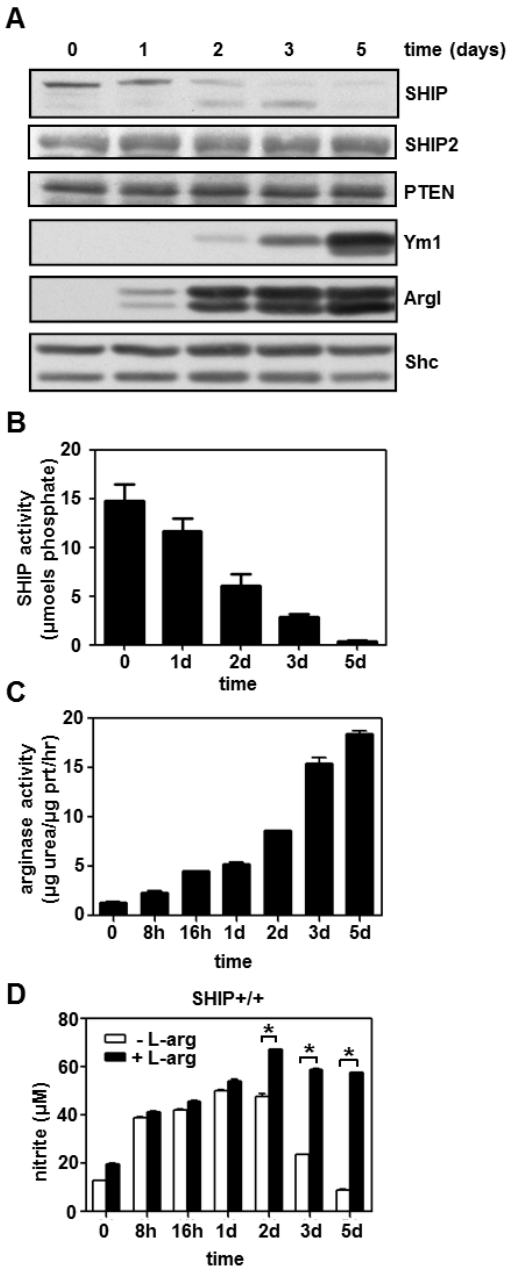

Figure 3.2 SHIP protein and activity levels decrease over time in response to IL-4 and correlate with an increase in M2 macrophage markers

SHIP+/+ BM macrophages were treated with IL-4 (10ng/ml) for the indicated times. WCLs were analyzed for SHIP, SHIP2, PTEN, Ym1, ArgI, and Shc by Western blotting (A), SHIP immunoprecipitates were assayed for enzymatic activity (B) and 10µg protein was used to determine arginase activity (C). (D) SHIP+/+ BM macrophages were IL-4-treated followed by 24h treatment with 10ng/ml LPS, in the absence or presence of L-arginine. Clarified cell supernatants were analyzed for nitrite. Data in (A) are representative of 3 experiments and in (B-D) are the means±SEM of 3 independent experiments. *P<0.002 comparing control versus L-arginine supplementation (Student's *t*-test).

3.3.3 SHIP protein levels were lower in other models of M2 macrophages

SHIP+/+ BM macrophages were treated with IL-4, IL-4+IL-10, or IL-10. WCLs were analyzed by Western blot and SHIP immunoprecipitates were assayed for enzymatic activity. IL-4+IL-10 treatment caused a greater decrease in SHIP protein levels and activity than IL-4 treatment alone (Figs. 3.3A and 3.3B). IL-10 alone did not promote SHIP degradation or M2 skewing. In mice, GM-CSF derived BM macrophages are M2 skewed and produce ArgI thereby repressing NO production[140] so MCSF and GM-CSF derived macrophages were compared to IFNγ treated (M1 polarized) and IL-4 treated (M2 polarized) macrophages. MCSF derived BM macrophages, like M1 polarized controls, expressed SHIP protein and did not express Ym1 or ArgI, whereas GM-CSF derived BM macrophages expressed lower amounts of SHIP protein and expressed both Ym1 and ArgI, like M2 macrophages (Fig. 3.3C). SHIP levels and M2 macrophage marker expression in $F4/80^+$ tumor associated macrophages (TAMs) from C57BL/6 mice were compared with M1 and M2 macrophages, polarized controls, and peritoneal macrophages from non-tumor bearing C57BL/6 mice (Fig. 3.3D). TAMs had intermediate levels of expression for ArgI and SHIP compared to M1 and M2 polarized controls and more ArgI than peritoneal macrophages, but showed no expression of Ym1. Thus, in three additional models of macrophage polarization: skewing with IL-4+IL-10, skewing during differentiation, and in TAMs; SHIP protein levels decreased and were inversely proportional to the expression of the M2 macrophage marker, ArgI.

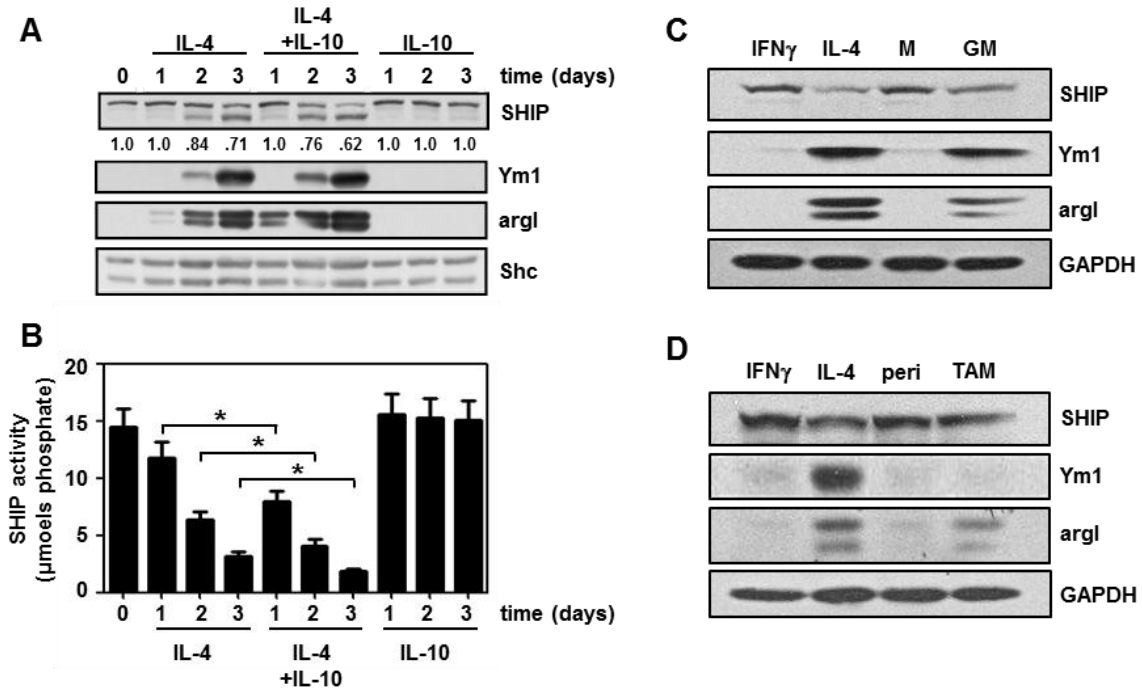

Figure 3.3 SHIP levels are reduced in M2 macrophage models and inversely correlate with M2 marker expression

SHIP+/+ BM macrophages were treated with IL-4, IL-4+IL-10 or IL-10 for 1-3 days. WCLs were analyzed for SHIP, Ym1, ArgI, and Shc by Western blotting (A). Densitometry values for SHIP are relative to loading control and normalized to untreated sample. (B) SHIP immunoprecipitates were assayed for SHIP activity. Data are means ± SEM of 3 independent experiments assayed in duplicate. *P<0.05 comparing IL-4-treated to IL-4+IL-10-treated BM macrophages (Student's *t*-test). (C), SHIP+/+ BM macrophages were derived for 10 days in 5ng/ml of GM-CSF (GM) or MCSF (M) ± treatment with IFNγ or IL-4 for 3 days. (D), BM macrophages treated with IFNγ or IL-4 for 3 days were compared to peritoneal macrophages (peri) from C57BL/6 mice or Lewis lung carcinoma TAMs (TAM) purified by F4/80 positive selection. WCLs were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting. Data shown are representative of 3 experiments.

3.3.4 PI3K activity was required for decreased SHIP levels and STAT6-driven transcription

The role of SHIP in macrophages has been attributed to its ability to inhibit the PI3K pathway[137-139] and the decrease in SHIP activity in IL-4-treated BM macrophages led me to ask whether PI3K activity was required for M2 skewing. SHIP+/+ BM macrophages were

pre-treated with vehicle (dimethyl sulfoxide (DMSO)), the PI3K inhibitor, LY29, or its inactive analog, LY30, followed by IL-4 (10 ng/ml) for 3 days. WCLs were harvested and analyzed by Western blot for SHIP, Ym1, ArgI, and GAPDH, and SHIP immunoprecipitates were assayed for SHIP activity. The PI3K inhibitor, LY29, but not its inactive analogue, LY30, blocked IL-4 induction of Ym1, ArgI, and reduction of SHIP protein levels and activity (Figs. 3.4A and 3.4B). There are four catalytic subunits of PI3Kp110 that generate SHIP's substrate, $PI(3,4,5)P_3$[134]. Isoform specific inhibitors have been developed that allow determination of the isoform(s) involved in biological processes.[138] SHIP+/+ BM macrophages were treated with 10 μM isoform-specific inhibitors for 15 min prior to treatment with 10 ng/ml IL-4 for 3 days and analyzed for SHIP protein levels and M2 macrophage marker expression (Fig. 3.4C). Isoform-specific inhibitors had no effect on Ym1 expression or SHIP degradation suggesting that multiple PI3K isoforms may contribute to these effects and their activity may compensate for one another. PI3Kp110δ inhibitors, SW18, SW30 and SW14, but not p110α, p110β, or p110γ inhibitors (PIK-90, TGX-221, or AS605240), suppressed induction of ArgI. To determine whether PI3K activity induced STAT6-driven transcription, a P_{STAT6}-luciferase assay was performed in the presence of the pan-PI3K inhibitor (LY29) or control (LY30). Inhibiting PI3Ks prevented IL-4-induced STAT6-dependent luciferase production (Fig. 3.4D).

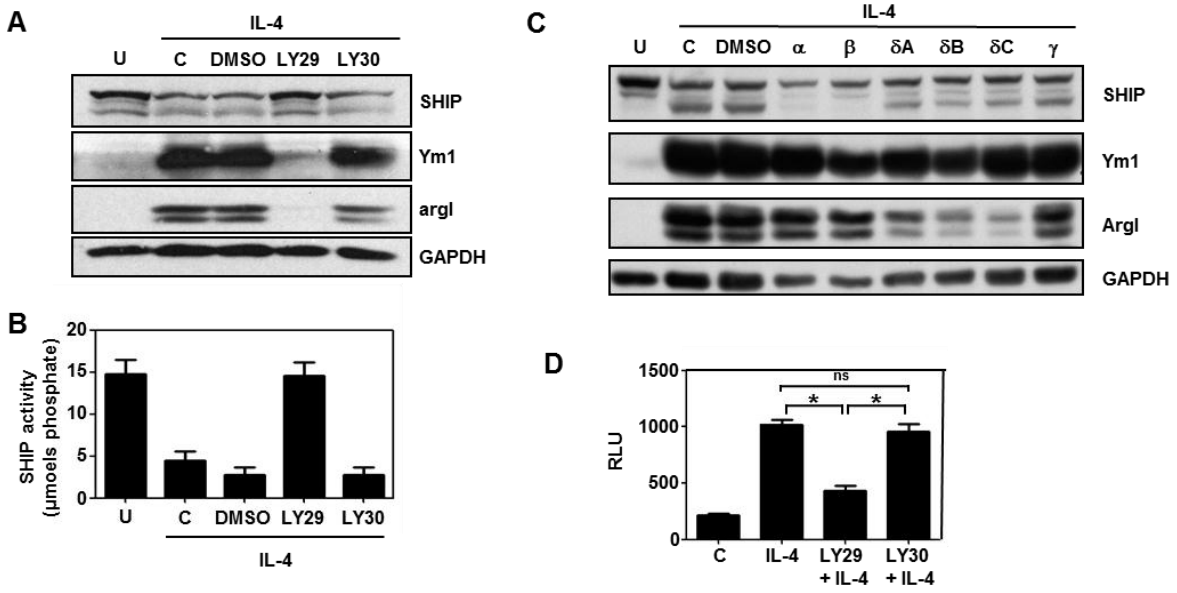

Figure 3.4 PI3K activity is required for decreased SHIP protein levels, increased M2 marker expression, and STAT6 transcription

SHIP+/+ BM macrophages were untreated (U), were not-pre-treated (C), or pre-treated with 0.1% DMSO, the PI3K inhibitor LY29 or its inactive analog LY30 followed by IL-4 treatment. WCLs were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting (A). SHIP immunoprecipitates were assayed for enzymatic activity (B). (C) SHIP+/+ BM macrophages were untreated (U), not pre-treated (C), or pre-treated with 0.1% DMSO, or the isoform specific PI3K inhibitors PIK-90(α), TGX-221(β), SW18(δA), SW30(δB), SW14(δC), or AS605240(γ) followed by IL-4 treatment. WCLs were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting. (D) RAW264.7 cells were transfected with pSTAT6-luciferase. After 48hrs, cells were treated with or without LY29 or LY30 and then IL-4-stimulated for 24hrs. WCLs were analyzed for luciferase activity. In (A, C), data are representative of 3 experiments and in (B, D) data are the means ± SEM of 3 independent experiments. ns=not significant; *P<0.001 comparing LY29 with untreated or with LY30 (Student's *t*-test).

3.3.5 STAT6 was required for SHIP degradation but SHIP did not limit STAT6 phosphorylation

Since IL-4 activates STAT6,[144] I examined cross-talk between the STAT6 and PI3K pathways. TG-elicited peritoneal macrophages from STAT6+/+ and STAT6-/- mice were treated with IL-4 (Fig. 3.5A). SHIP levels did not decrease in STAT6-/- macrophages in response to IL-4, and ArgI and Ym1 were not induced. SHIP+/+ BM macrophages were

treated ± IL-4 for 3 days to reduce SHIP protein levels. BM macrophages were incubated overnight in cytokine free media (starved), stimulated with 50 ng/ml IL-4, and STAT6 phosphorylation was examined (Fig. 3.5B). There was only a modest increase in total STAT6 phosphorylation in response to IL-4 but there was higher constitutive phosphorylation in BM macrophages pre-treated with IL-4. IL-4-induced STAT6 phosphorylation was compared in SHIP+/+ and SHIP-/- BM macrophages. The absence of SHIP expression did not increase total STAT6 phosphorylation but there was higher constitutive phosphorylation of STAT6 (Fig. 3.5C). Lastly, SHIP+/+ BM macrophages were treated for 3 days with siRNA to SHIP protein, to reduce SHIP levels, or nsRNA, as a control. Treated BM macrophages were starved and stimulated with IL-4, and STAT6 phosphorylation was examined. There was no change in STAT6 phosphorylation when SHIP protein levels were reduced (Fig. 3.5D). In summary, STAT6 and PI3K activity are required for IL-4-induced reduction of SHIP protein levels and PI3K activity is required for Ym1 and ArgI expression. STAT6 and PI3Kp110δ are both required for ArgI expression. PI3K enhances IL-4/STAT6-driven transcription but does not affect total phosphorylation of STAT6.

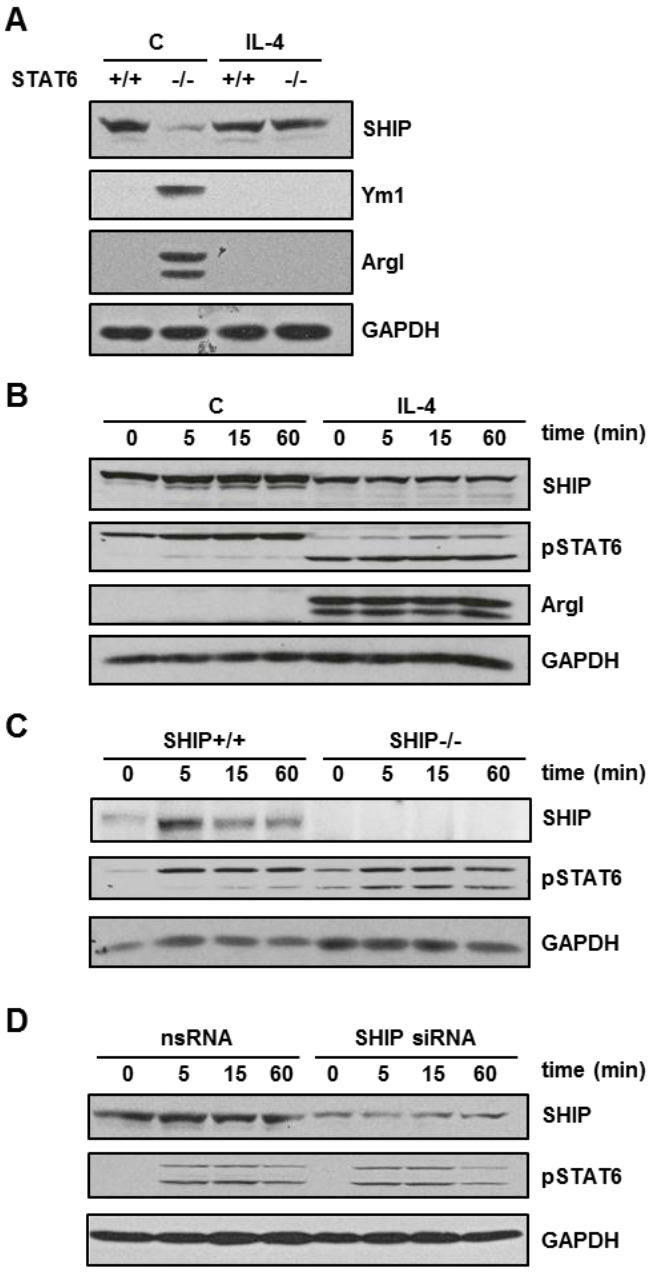

Figure 3.5 STAT6 is required for decreased SHIP expression and increased ArgI expression, but SHIP does not block STAT6 phosphorylation
(A) Thioglycollate-elicited peritoneal macrophages from STAT6+/+ and STAT6-/- mice were either untreated (C) or treated with IL-4. WCLs were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting. (B) SHIP+/+ BM macrophages were either untreated (C) or treated with IL-4 for 3 days followed by cytokine starvation overnight. Cells were then stimulated with 50ng/ml IL-4 for the indicated times. (C) SHIP+/+ and SHIP-/-BM macrophages were starved overnight and IL-4 stimulated. (D) SHIP+/+ BM macrophages were treated with SHIP siRNA or nsRNA for 3 days, starved overnight and stimulated with IL-4. In (B-D), WCLs were analyzed for SHIP, pSTAT6, and GAPDH by Western blotting. Data shown are representative of 3 experiments with similar results.

3.3.6 siRNA knockdown of SHIP increased IL-4-induced ArgI expression and activity

To establish whether SHIP levels could be manipulated to affect M2 skewing, SHIP+/+ and SHIP+/- BM macrophages were compared. SHIP+/- BM macrophages expressed SHIP in a gene dose-dependent manner, IL-4-induced Ym1 was modestly higher, and IL-4-induced ArgI expression was much higher (Fig. 3.6A). SHIP was knocked down by siRNA in RAW264.7 cells and arginase activity was measured as a surrogate for ArgI induction. SHIP siRNA caused an 86% reduction of SHIP protein levels (Fig. 3.6B) and dramatically enhanced IL-4-induced ArgI activity compared to nsRNA (Fig. 3.6C). IL-4-induced P_{STAT6}-luciferase activity was higher when SHIP levels were decreased (Fig. 3.6D). Taken together, these data demonstrate that lower SHIP levels or artificially reducing SHIP levels, enhances IL-4 responsiveness, specifically in terms of ArgI and P_{STAT6}-luciferase activity.

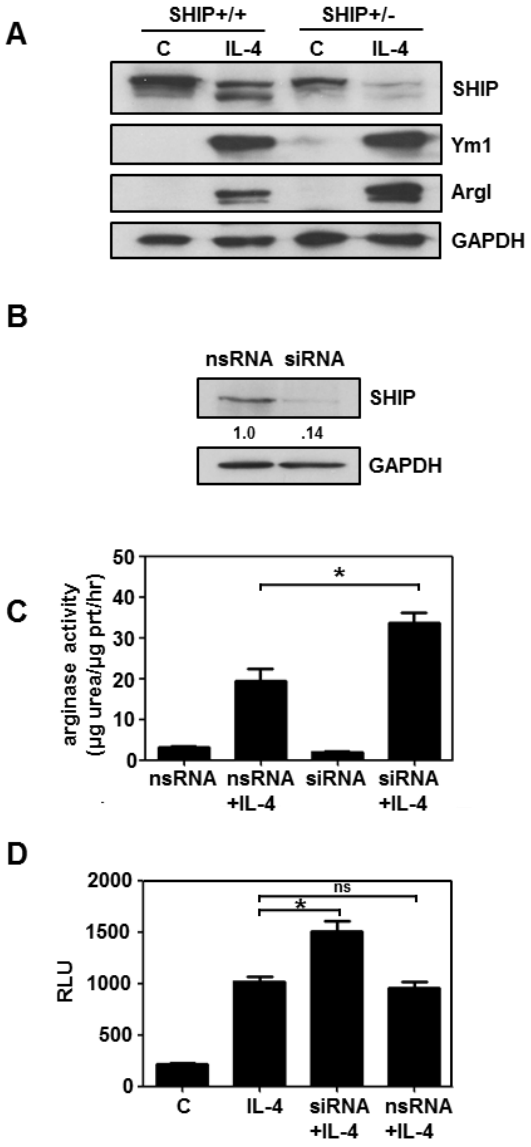

Figure 3.6 Reducing SHIP protein levels increases ArgI expression and activity in response to IL-4 and enhances STAT6-driven transcription

(A) SHIP+/+ and SHIP+/- BM macrophages were either untreated (C) or treated with IL-4. WCLs were analyzed for SHIP, Ym1, ArgI, and GAPDH by Western blotting. Data shown are representative of 3 experiments with similar results. (B) RAW264.7 cells were treated with SHIP siRNA or nsRNA for 3 days. WCLs were analyzed for SHIP and GAPDH by Western blotting. Densitometry values are relative to GAPDH and normalized to nsRNA control. Data shown are representative of 4 experiments with similar results. (C) RAW264.7 cells were treated with SHIP siRNA or nsRNA for 6h and were treated or not with IL-4. WCLs were collected and 10µg of protein was assayed for arginase activity. (D) RAW264.7 cells were transfected with pSTAT6-luciferase in the presence of SHIP siRNA or nsRNA. After 48h, cells were stimulated ±IL-4 for 24h. WCLs were analyzed for luciferase activity. Data in (C, D) are the means ± SEM of 3 independent experiments assayed in duplicate. ns=not significant; *P<0.05 comparing SHIP siRNA with nsRNA (Student's *t*-test).

3.3.7 Overexpressing SHIP protein levels reduced IL-4-induced M2 skewing

The proteosome inhibitor, MG132, blocks IL-4-induced SHIP degradation[170]. To increase SHIP protein levels, MG132 was used at concentrations that did not affect BM macrophage viability. MG132 prevented IL-4-induced SHIP degradation, reduced Ym1 expression and dramatically reduced ArgI induction (Fig 3.7A). SHIP was also over-expressed (1.68-fold) in RAW264.7 cells (Fig. 3.7B). Control and SHIP over-expressing cells were treated with IL-4 for 3 days and arginase activity was measured. Over-expression of SHIP resulted in a significant decrease in IL-4-induced arginase activity (Fig. 3.7C). These data demonstrate that higher SHIP levels reduce IL-4 responsiveness of BM macrophages, with respect to Ym1 and ArgI induction.

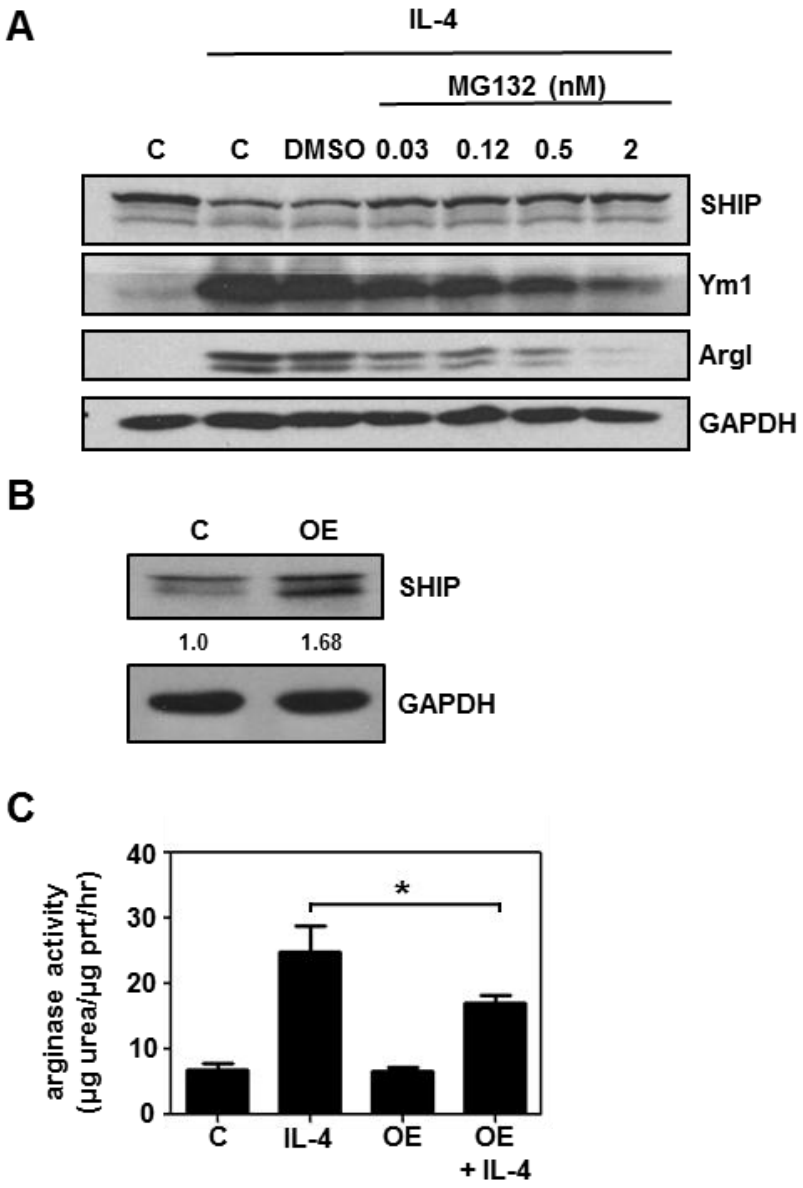

Figure 3.7 Reducing SHIP protein levels increases ArgI expression and activity in response to IL-4 and enhances STAT6-driven transcription.
(A) SHIP+/+ BM macrophages were untreated or treated with IL-4 with the indicated dose of the proteasome inhibitor, MG132. WCLs were analyzed for SHIP, Ym1, ArgI and GAPDH by Western blotting. Data shown are representative of 3 experiments. (B) RAW264.7 cells were transfected with empty vector (C) or vector encoding SHIP (OE) for 3 days. WCLs were analyzed for SHIP and GAPDH by Western blotting. SHIP densitometry values are relative to GAPDH and normalized to empty vector. Data shown are representative of 4 experiments with similar results. (C) Control (C) and transfected RAW264.7 cells were treated or not with IL-4 and protein lysates were assayed for arginase activity. Data are the means ± SEM of 3 independent experiments assayed in duplicate. *P<0.05 comparing IL-4-induced arginase activity in control versus SHIP over-expressing cells (Student's *t*-test).

3.4 Discussion

SHIP plays a pleiotropic role in macrophage activation by limiting PI3K activity in response to multiple immune stimuli. By limiting PI3K activity downstream of innate immune activation, SHIP-deficient macrophages are hyper-inflammatory.[134, 137, 138] Herein, I demonstrate a critical role for SHIP in IL-4-induced alternative macrophage activation where SHIP limits PI3Kp110δ activity downstream of IL-4 thereby attenuating IL-4-induced M2-skewing. PI3K and its downstream target, Akt, are activated downstream of the type I IL-4R in Natural Killer (NK) cells, B cells, and T cells[165, 171, 172] and has recently been shown to be recruited to the type I IL-4 receptor in macrophages.[172] *In vivo* SHIP knockout macrophages are profoundly M2 skewed.[139, 148] Their phenotype is recapitulated in Hck/Lyn double knockout mice and rescued by expression of membrane bound SHIP.[173] GM-CSF and IL-3 derivation skews macrophages to an M2 phenotype and is more pronounced in SHIP-deficient macrophages due to enhanced IL-4 production by SHIP-deficient basophils in BM cultures.[140] Consistent with this, Hck/Lyn double knockout and SHIP-/- GM-CSF- and IL-3-derived BM macrophage phenotypes were dependent on STAT5 activation, which acts downstream of IL-3 and GM-CSF.[140, 173] SHIP-/- macrophages are inherently more susceptible to multiple inflammatory stimuli[134], but SHIP-/- progenitors were not more susceptible to IL-4 treatment.[140] This is expected as resting myeloid progenitors do not express the IL-4 receptor.[174] Based upon these observations, I asked whether SHIP-/- BM macrophages were more sensitive to IL-4 skewing directly.

My data is consistent with a model (Fig. 3.8) in which IL-4 acts through the IL-4R to activate STAT6. The PI3Kp110δ catalytic subunit promotes STAT6-driven transcription but not through direct effects on STAT6 phosphorylation. Both STAT6 and PI3K activity are

required for the degradation of SHIP protein and loss of SHIP activity, creating an environment that potentiates expression of STAT6-driven alternative macrophage activation. SHIP activity normally restricts the PI3K-mediated activation of STAT6-driven transcription and so is removed for alternative activation. Alternatively activated macrophages lose SHIP protein and activity mimicking SHIP-/- macrophages. Loss of SHIP protein expression and activity may be a novel marker of IL-4-induced alternatively activated macrophages and $PI(3,4)P_2$, the lipid product of SHIP's enzymatic activity, has been reported to be required for M1 macrophage effector functions.[175]

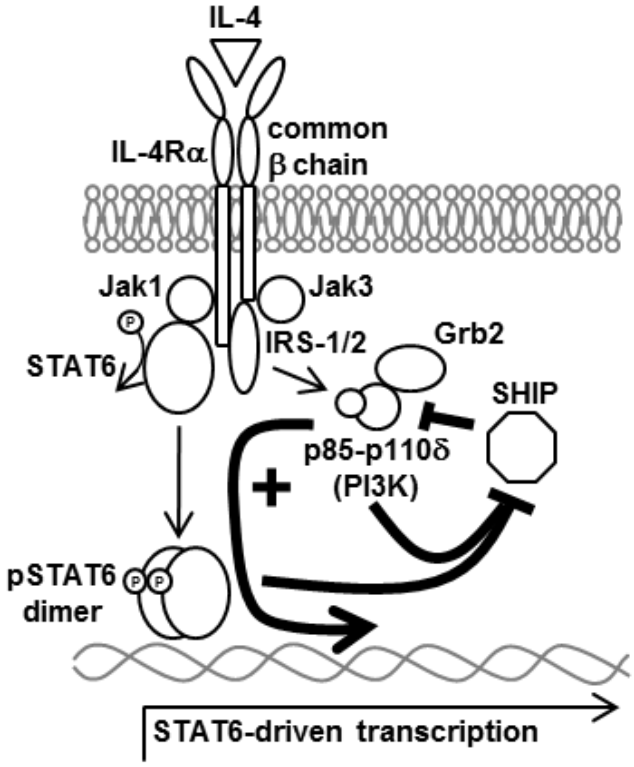

Figure 3.8 Model describing SHIP's role in IL-4 signalling
IL-4 acts through the IL-4R (type I shown here) to trigger phosphorylation, dimerization, and transcription by STAT6. The IL-4R also activates PI3K, specifically the PI3Kp110δ catalytic subunit, which is required for STAT6-driven transcription. SHIP limits PI3K signalling, attenuating expression of STAT6 responsive genes. Both STAT6 and PI3K are required to turn off SHIP activity by reducing SHIP protein levels.

IL-4 is critical in Th2 cell differentiation and expansion, B cell Ig class switching, and has been implicated in autoimmunity, particularly allergic inflammation[176]. IL-4 acts through STAT6 but also recruits and activates insulin receptor substrate (IRS)-2 that cooperates in IL-4-mediated effects.[172, 176] Heller *et al.*[172] have recently demonstrated a similar role for PI3K binding to IRS-2 in IL-4/STAT6 induction of FIZZ1[172]. Herein, I extend these findings demonstrating that IL-4-induced ArgI and Ym1 expression are PI3K-dependent. ArgI induction required PI3Kp110δ activity. Perhaps PI3K-dependence of ArgI and Ym1 induction was not reported previously because wortmannin, the PI3K inhibitor used, is rapidly degraded in tissue culture medium.[177] My studies show the importance of the PI3K pathway in ArgI and Ym1 expression when SHIP activity is lost (PI3K high), during the over-expression of SHIP (limiting PI3K) using the PI3K inhibitor, LY29, and in the case of ArgI, using a panel of PI3Kp110δ inhibitors. My results, together with Heller *et al.*[172], support the notion that expression of M2 macrophage markers are differentially regulated. From data presented here, this is evident for ArgI and Ym1, where PI3Kp110δ is not required for Ym1 expression and Ym1 is not expressed in TAMs, despite low SHIP levels and increased ArgI expression.

I demonstrate that SHIP protein levels and activity are reduced in response to IL-4 in a dose- and time-dependent manner. SHIP protein levels and activity were inversely proportional to M2 macrophage marker expression and arginase activity. Furthermore, arginase limited the production of pro-inflammatory NO in response to LPS. This was not due to limited iNOS activity because L-arginine supplementation enhanced NO production suggesting that the effect of IL-4 limiting NO production was due to competition between ArgI and iNOS. This is consistent with previous reports on the mechanism of IL-4 reducing

NO production in murine macrophages.[139, 178] However, an emerging paradigm is that alternatively activated macrophages express lower amounts of iNOS because IL-4 blocks transcription of iNOS.[179] Our results show that IL-4 treatment potentiates NO production by MCSF-derived macrophages and this is exacerbated in SHIP-deficient macrophages. Induction of iNOS activity in MCSF derived BM macrophages, the most commonly used model in the study of murine macrophage biology, was evident by 8 hours after IL-4 treatment.[95] IL-4 has been shown to stimulate the expression of iNOS in endothelial cells and eosinophils[180, 181] so it is important to define the effect of IL-4 on the cell and macrophage type being studied. There has been very limited study of macrophage responses to mixed signals that may be encountered *in vivo*[79] and the potentiation of iNOS expression in response to LPS may not be limited to IL-4-treated, MCSF-derived macrophages.

There is cross-talk between the STAT6 and PI3K pathways required for IL-4-induced alternative macrophage activation because STAT6 was required for SHIP degradation and reduced SHIP levels did not augment IL-4-mediated STAT6 phosphorylation. PI3K inhibition reduced, and SHIP knockdown enhanced, STAT6-driven transcription in a luciferase reporter assay suggesting that STAT6 and PI3K pathways act in parallel to drive transcription of STAT6 responsive genes. The type I IL-4Rγc chain phosphorylates and activates the IRS-2, which in turn recruits the p85 subunit of PI3K.[172] My data supports activation of the PI3K pathway acting downstream of the type I IL-4R as a potential mechanism accounting for differences in the strength of expression of M2 macrophage phenotype downstream of IL-4 versus IL-13, which only acts through the type II receptor.[171, 172] Further study is required to investigate the downstream targets of PI3K that lead to enhanced STAT6-driven transcription, but one can speculate about the potential pathway

involved. STAT6-mediated transcription requires several signalling pathways and STAT6 can cooperate with other transcription factors including C/EBPβ[172, 182] ArgI and FIZZ1 promoters have C/EBPβ binding sites[183] and C/EBPβ has been shown to be required for IL-4-induced ArgI transcription in macrophages via CREB activation.[183, 184] PI3K activation leads to the phosphorylation/inactivation of GSK3β and this positively regulates CREB[185] and C/EBPβ[186] providing a possible link between PI3K activation and enhancement of STAT6-mediated transcription.

A significant finding of this study is that PI3K activity, specifically the p110δ isoform, is required for SHIP degradation, STAT6 mediated transcription, and expression of the M2 macrophage marker, ArgI. Since SHIP-/- M2 macrophages confer protection during DSS-induced intestinal inflammation, I next asked whether PI3Kp110δ deficiency would have the opposite effect and exacerbate disease severity during DSS-induced colitis by blocking protective alternatively activated macrophage functions.

4 Chapter: Arginase activity in alternatively activated macrophages protects mice from dextran sodium sulfate-induced intestinal inflammation

4.1 Introduction and rationale

A failure in macrophage skewing to an M2 phenotype may promote an inappropriate inflammatory response and exacerbate inflammation. Macrophages are heterogeneous and their phenotype is dependent on signals from the microenvironment.[89] Classically activated (M1) macrophages have been implicated in IBD pathogenesis.[54] They are activated by LPS or IFNγ and promote inflammation by producing pro-inflammatory cytokines, including TNFα, IL-12, and IL-23, and reactive oxygen species.[88] Conversely, I and others have shown that alternatively activated (M2) macrophages are protective in mouse models of intestinal inflammation.[147, 187] M2 macrophages help dampen down inflammation by producing relatively higher levels of the anti-inflammatory cytokine, IL-10, and lower levels of pro-inflammatory cytokines in response to inflammatory stimuli. Murine M2 macrophages also up-regulate the enzyme ArgI, which competes with iNOS for L-arginine, thereby limiting pro-inflammatory NO production.[188] Macrophages that cannot convert to an M2 phenotype may cause exacerbated inflammatory responses and increase inflammation in mouse models of intestinal inflammation such as DSS-induced colitis.

Class I PI3Ks are lipid kinases that act as important mediators in signalling cascades that regulate the inflammatory response.[111] I have demonstrated that PI3Kp110δ is required for STAT6 driven transcription, including expression of the M2 macrophage marker, ArgI. Expression levels of class IA PI3K catalytic subunits affects the expression of others so Okkenhaug *et al.* (2002) created a knock-in mouse with a point mutation in the active site of

p110δ.[189] The PI3Kp110δ$^{D910A/D910A}$ mice develop a mild, spontaneous colitis that has been attributed to macrophage function because macrophages from these mice are defective in bactericidal activity and have increased inflammatory responses to toll-like receptor signalling *in vivo*.[189, 190] Additional evidence suggests that PI3Kp110δ$^{D910A/D910A}$ mice are defective in their ability to skew to an M2 phenotype. They have exaggerated mucosal and systemic pro-inflammatory cytokines and they lack giant cells in their lymph nodes and spleens, which may reflect defects in M2 macrophage fusion events.[190, 191]

Based on this, I hypothesized that PI3Kp110δ$^{D910A/D910A}$ mice are deficient in ArgI-expressing M2 macrophages and this will lead to macrophage-mediated exacerbation of DSS-induced colitis. To investigate this, both wild type and PI3Kp110δ$^{D910A/D910A}$ mice were given DSS in their drinking water for 7 days to induce colitis. Clinical disease severity was measured daily by monitoring weight loss, rectal bleeding, and stool consistency. In addition, colon length and histological damage were recorded following the experiment. We further examined the role that macrophages play in induced colitis by depleting and reconstituting macrophages during DSS treatment. Finally, we explored differences in expression of M2 markers when both wild type and PI3Kp110δ$^{D910A/D910A}$ macrophages were treated with IL-4 and used this information to determine that ArgI activity is critical for M2 macrophage-mediated protection during DSS-induced intestinal inflammation.

4.2 Materials and methods

Mice. Mice that are deficient in PI3Kp110δ activity due to an inactivating homozygous point mutation (PI3Kp110δ$^{D910A/D910A}$) were created in 2002 and were obtained from the Ludwig Cancer Research Institute.[189] Mice were maintained on a C57BL/6 background and brother-

sister mating of heterozygotes was used to generate wild type and PI3Kp110δ$^{D910A/D910A}$ littermates for experiments. All mice used for experiments were between 8 and 12 weeks of age. Mice were maintained in the Animal Research Center at the Child & Family Research Institute (Vancouver, Canada), and experimentation was performed in accordance with institutional and Canadian Council on Animal Care guidelines.

Dextran sodium sulfate-induced colitis and macroscopic assessment of pathology. Dextran sodium sulfate (DSS) (2.5%; MW 36–50,000; MP Biomedicals, Solon, OH, USA) was dissolved in drinking water and provided to mice *ad libitum*. Treatment was given for 6-7 days with daily monitoring of disease activity indices (DAI) including weight loss, stool consistency, and rectal bleeding. DAI scores were assigned on a scale of 0 - 4 as outlined in Table 4.1. Scores for the different parameters were combined to give an overall DAI score out of 12 points for each mouse on each day of the experiment. Hemocult paper was from Beckman Coulter, Mississauga, Canada. Colons were excised upon autopsy and colon lengths were measured.

Table 4.1 Disease activity index scoring

Score	Weight Loss	Stool Consistency	Rectal Bleeding
0	0%	Normal	none
1	1-3%	loose stool	detectable by hemdetect paper
2	3-6%	very loose stool	visible blood in stool
3	6-9%	Diarrhea	large amount of blood in stool
4	>9%	no formed stool	extensive blood in stool and blood visible at the anus

Histological analyses. Mice were sacrificed and colons were removed and fixed in PBS-buffered 10% formalin (Fisher Scientific, Ottawa, Canada). Samples were embedded in paraffin, and cross-sections were stained with H&E at the Child & Family Research Institute histology facility. Histological damage was scored using a 16 point scale (Table 2.2) by two individuals blinded to experimental condition as described previously.[149, 150]

Macrophage depletion *in vivo.* Macrophages were depleted from mouse colons using clodronate-containing liposomes.[187] 200 µl of clodronate-containing liposomes were administered to mice by intraperitoneal injection 4 days prior to treatment and at days 0, 2, 4, and 6 during DSS treatment. Intraperitoneal injections of 200 µl of PBS were used as an injection control. Clodronate-containing liposomes were prepared by our collaborator, Dr. Nico van Rooijen, as he has described before.[151]

Adoptive transfer of macrophages into mice. Macrophages were derived from bone marrow aspirates from femurs and tibias of wild type and PI3Kp110$\delta^{D910A/D910A}$ mice. Following adherence depletion for 2 hr at 37°C, macrophages were derived by plating progenitors at 0.5×10^6 nucleated cells/ml in IMDM (StemCell Technologies, Vancouver, Canada), 10% FCS (Invitrogen, Burlington, Canada), 150 μM monothioglycerol (Sigma-Aldrich, St Louis, MO), and 10 ng/ml MCSF (StemCell Technologies). Complete media changes were performed after 4 and 7 days, discarding nonadherent cells. At 10 days, 10 ng/ml of IL-4 (StemCell Technologies) was added to the media to skew macrophages to an M2 phenotype. Some samples were also given 0.1 mM *S*-(2-boronoethyl)-L-cysteine (BEC) or 0.1 mM 2(S)-Amino-6-boronohexanoic acid-NH_4 (ABH) to inhibit arginase activity at day 0 and 2 of IL-4 treatment. After 13 days, adherent cells were removed from flasks by incubating for 5 min at 21°C in cell dissociation buffer (Invitrogen) and macrophages (10^6) were injected into the tail vein of mice on Days 0 and 4 of DSS treatment. Experiments where arginase activity was inhibited *in vivo*, 100 μl of a filter sterilized solution of 0.2% BEC pH 7.0 solution or 0.04% ABH pH 7.0 solution was given to mice daily by oral gavage during DSS treatment.[192] BEC and ABH were synthesized by our collaborator Dr. Jean-Luc Boucher (Université Paris Descartes, Paris, France), as previously described.[192-194]

Immunohistochemistry. For detection of F4/80, ArgI, and nitrotyrosine, slide-mounted, 5-μm serial sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. For F4/80 detection, enzyme-induced epitope retrieval was performed by incubating samples with 20 μg/ml proteinase K in PBS for 15 min at room temperature. For ArgI and nitrotyrosine detection, heat-induced epitope retrieval was performed by immersing

the slides in 10 mM sodium citrate buffer, pH 6.0, at 95°C for 20 min and allowing slides to cool to room temperature. For all stains, slides were rinsed thoroughly in Tris-buffered saline with 0.1% Tween-20. Endogenous peroxidase activity was blocked with 1.5% H_2O_2 in PBS for 10 min. Endogenous avidin and biotin were blocked with an avidin-biotin blocking kit, according to the manufacturer′s instructions (Vector Laboratories, Burlingame, CA, USA). Primary antibodies, including rat anti-F4/80 (AbD Serotec, Oxford, UK), mouse anti-ArgI (BD Biosciences, Mississauga, Canada), and mouse anti-nitrotyrosine (Millipore, Billerica, MA, USA), were used. Blocking buffers, biotinylated secondary antibodies, and avidin-biotin-HRP or alkaline phosphatase complexes were prepared and used from rabbit IgG, rat IgG, or "Mouse-on-Mouse" immunohistochemical detection kits, according to the manufacturer's instructions (Vector Laboratories). Signal was detected with a diaminobenzidine chromogen system (Dako, Carpinteria, CA, USA) for F4/80 and ArgI, and an alkaline phosphatase substrate kit (Vector Laboratories) for nitrotyrosine. Developed sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Images were acquired and analyzed using a Zeiss Axiovert 200 microscope, a Zeiss AxioCam HR camera, and the Zeiss AxioVision 4.0 software imaging system. Total macrophages were quantified by counting $F4/80^+$ cells from six representative fields at 40× magnification from six tissue sections separated by ≥ 50 μm for six mice/group. M2 macrophages were quantified counting $ArgI^+$ cells that colocalized with $F4/80^+$ cells in corresponding fields in serial sections. Counting was performed by two individuals blinded to experimental condition.

SDS-PAGE and Western blotting. WCLs were prepared for SDS-PAGE by lysing in 1× Laemmli's digestion mix, sheering DNA using a 26-gauge needle, and boiling for 1 min.

Lysates were loaded onto a 12% polyacrylamide gel and Western blotting was carried out by transferring protein onto an Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, ON) using 600 mA for 4 hrs at 23°C. Blots were blocked, incubated with antibodies and then with horseradish peroxidase-conjugated secondary antibody (BioLegend, San Diego, CA) before adding ECL substrate solution (Invitrogen) and exposing to Kodak X-Omat film (PerkinElmer Life Sciences, Woodbridge, ON).[195] The following antibodies were used for Western blot analyses: anti-SHIP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ArgI (BD Biosciences), anti-Ym1 (StemCell Technologies), anti-TREM2 (Abcam, Toronto, ON), anti-FIZZ1 (Abcam), and anti-GAPDH (Fitzgerald Industries International, Acton, MA, USA).

Arginase assay. Macrophages in culture (10^6) or fresh colonic tissue samples were collected and homogenized in 1 mL arginase lysis buffer using a 4 Polytron MR2100 benchtop homogenizer and used to assay arginase activity. Samples were centrifuged at 16,000 rpm for 10 min at 4°C to clarify supernatants. Arginase activity was determined indirectly by measuring the concentration of urea generated by the arginase-dependent hydrolysis of L-arginine. Cells (10^6) were lysed with 400 µl of cell lysis buffer (0.1% Triton X-100 (BDH), 25mM Tris-Cl (Bio Basic Inc., Markham, ON), pH 8.0). Following lysis, 10 µl of 10 mM $MnCl_2$ was added to each sample which consisted of 10µg of protein in 100 µl of buffer. The enzyme was activated by heating for 10 min at 55°C. Arginine hydrolysis was conducted by incubating the lysates with 100 µl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 800 µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The urea concentration was measured at 550 nm after addition of 40 µl of α-isonitrosopropiophenone

(Sigma) (dissolved in 100% ethanol), followed by heating at 100°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min.[167]

Cytokine analyses. Macrophages (0.5×10^6 cells in 1.0 ml of MCSF complete media) were stimulated with 10 ng/ml LPS (Sigma-Aldrich) for 24 hours. IL-12p70, IL-10, and IL-6 were assayed in clarified cell supernatants by ELISA according to the manufacturer's instructions (BD Biosciences).

Statistical analyses. Repeated measures ANOVA, unpaired one-tailed Student's *t* tests, and Mann-Whitney tests were performed where indicated using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P \leq 0.05$.

4.3 Results

4.3.1 PI3Kp110δ$^{D910A/D910A}$ mice did not display clinical or histological disease symptoms at 8-9 weeks of age

PI3Kp110δ$^{D910A/D910A}$ mice have been reported to develop spontaneous colitis.[190] I wanted to ensure that the mice used in our experiments did not have spontaneous colitis which could influence disease severity during DSS-induced intestinal inflammation. To verify this, disease activity was measured daily for 7 days in 8 week old PI3Kp110δ$^{D910A/D910A}$ and wild type mice, the same age as those that would undergo DSS treatment. I found no evidence of clinical or histological disease in PI3Kp110δ$^{D910A/D910A}$ mice at this age (Fig. 4.1).

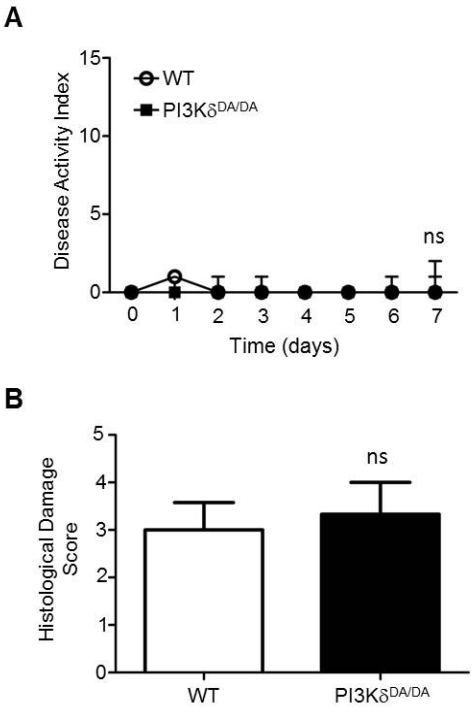

Figure 4.1 PI3Kp110δ$^{D910A/D910A}$ mice did not display clinical or histological disease symptoms at 8-9 weeks of age
(A) Disease activity indices were measured daily for wild type (open circles) and PI3Kp110δ$^{D910A/D910A}$ mice (black squares) for 7 days beginning at 8 weeks of age. The median score ± range is shown. (B) Histological damage was scored and results are expressed as median score ± range. ns = not significant for *n* = 6 mice/group in 2 independent experiments

4.3.2 PI3Kp110δ$^{D910A/D910A}$ mice had reduced colonic macrophages numbers and dramatically reduced numbers of ArgI^{+} macrophages

Although histological colon sections did not show evidence of damage, (Fig. 4.2A, top), it is of interest to note that PI3Kp110δ$^{D910A/D910A}$ mice had fewer F4/80^{+} macrophages in their colon (Fig. 4.2A, 2nd row) and fewer ArgI^{+}F4/80^{+} macrophages (Fig. 4.2A, 3rd row). Correspondingly, there was higher nitrotyrosine staining in colon sections from

PI3Kp110$\delta^{D910A/D910A}$ mice compared to their wild type littermates (Fig. 4.2A, bottom). Since I saw reduced numbers of F4/80^{+} and ArgI^{+} macrophages in the PI3Kp110$\delta^{D910A/D910A}$ mice, F4/80^{+} macrophages and ArgI^{+}F4/80^{+} M2 macrophages in colon sections were counted. There were significantly lower numbers of both macrophages and ArgI^{+} (M2) macrophages in colonic tissue of PI3Kp110$\delta^{D910A/D910A}$ mice compared to wild type mice (Fig. 4.2B).

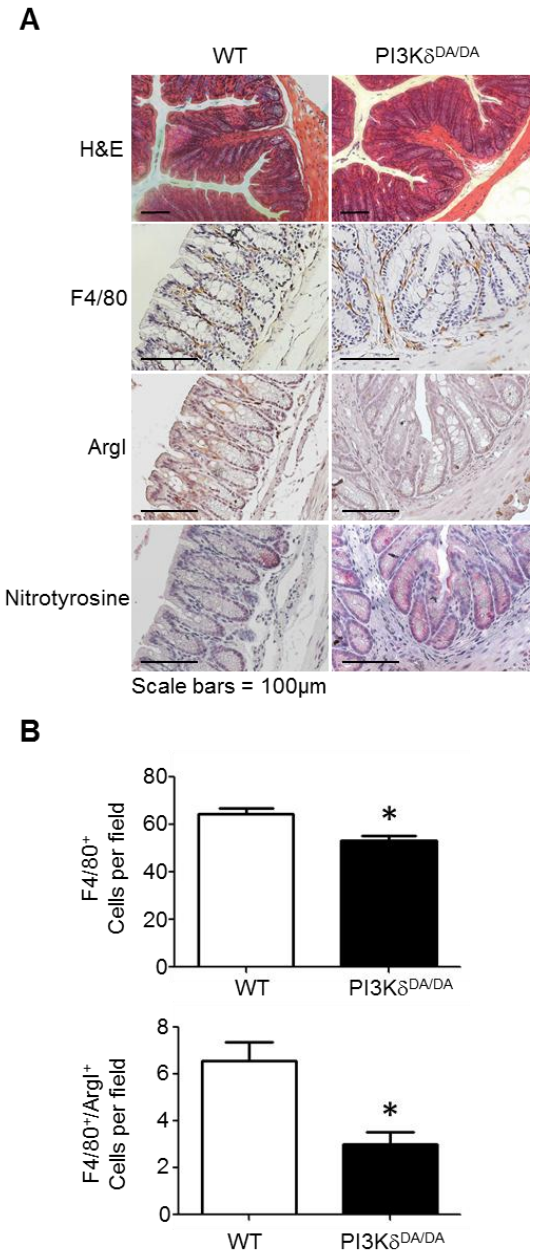

Figure 4.2 PI3Kp110δ$^{D910A/D910A}$ mice have fewer macrophages and fewer ArgI^{+} M2 macrophages in their colons

(A) Wild type (left) and PI3Kp110δ$^{D910A/D910A}$ (right) mouse colon sections stained by H&E (top) and stained by immunohistochemistry for mature macrophages (F4/80; 2nd row), M2 macrophages (ArgI, 3rd row) and nitrotyrosine (bottom). Data are representative of similar staining from n = 6 mice for each group. Scale bars = 100μm. (B) Quantitation of F4/80^{+} and ArgI^{+}F4/80^{+} macrophages in wild type (white bars) and PI3Kp110δ$^{D910A/D910A}$ mice (black bars). Data are represented as means ± SD. Positively stained cells were counted at 40× magnification in 6 fields from 6 sections/mouse, separated by ≥50 μm, and from 6 mice/group by two individuals blinded to experimental conditions; *P<0.05 (Student's t-test).

4.3.3 PI3Kp110δ$^{D910A/D910A}$ mice had exacerbated disease during dextran sodium sulfate-induced colitis

PI3Kp110δ$^{D910A/D910A}$ mice have hyper-responsive macrophages that correlate with development of mild, spontaneous intestinal inflammation.[190] I have shown that M2 macrophages are protective during DSS-induced colitis and that the p110δ subunit of class I PI3K is required for macrophage skewing to an M2 phenotype. Based on this, I asked whether PI3Kp110δ$^{D910A/D910A}$ mice develop exacerbated disease during DSS-induced intestinal inflammation. PI3Kp110δ$^{D910A/D910A}$ mice and their wild type littermates were treated with 2.5% DSS in their drinking water for 7 days. Clinical disease symptoms including weight loss, rectal bleeding, and stool consistency were monitored daily to measure disease activity. I found that PI3Kp110δ$^{D910A/D910A}$ mice developed significantly worse clinical disease in response to DSS treatment (Fig. 4.3A). At day 7, mice were euthanized, colons excised, and colon lengths were measured because shorter colon length is characteristic of colitis.[196] Colon length was significantly shorter in PI3Kp110δ$^{D910A/D910A}$ mice compared to their wild type counterparts (Fig. 4.3B). I also wanted to assess the extent of histological damage caused during DSS-induced colitis. Distal colons were fixed for histological assessment. H&E staining revealed severe damage in PI3Kp110δ$^{D910A/D910A}$ colonic tissue compared to that seen in tissue from wild type mice (Fig. 4.3C, top). Immunohistochemical staining for the macrophage marker F4/80 (Fig. 4.3C, 2nd row) and the M2 macrophage marker ArgI (Fig. 4.3C, 3rd row) demonstrated that PI3Kp110δ$^{D910A/D910A}$ mice had fewer F4/80^{+} macrophages and dramatically fewer ArgI^{+}F4/80^{+} M2 macrophages in tissue sections. Colonic inflammation in mice is associated with increased NO production by iNOS.[197, 198] However, ArgI competes with iNOS for L-arginine, limiting NO production.

To determine whether decreased ArgI staining correlated with increased NO production, tissue sections were stained for nitrotyrosine, a downstream product of NO. Colonic tissue sections from PI3Kp110$\delta^{D910A/D910A}$ mice had increased nitrotyrosine staining compared to those from wild type littermates (Fig. 4.3C, bottom).

Histological damage in H&E stained tissue sections was scored based on loss of tissue architecture, immune cell infiltration, goblet cell loss, muscle thickness, ulceration, and edema, by two individuals blinded to experimental condition. PI3Kp110$\delta^{D910A/D910A}$ mice had significantly higher histological damage scores when compared to wild type mice (Fig. 4.3D). Taken together, these data demonstrate that PI3Kp110$\delta^{D910A/D910A}$ mice have exacerbated clinical disease and histological damage compared to their wild type littermates during DSS-induced colitis. Moreover, this correlates with decreased F4/80^{+}ArgI^{+} M2 macrophages and increased nitrotyrosine staining (NO production) in the colon during inflammation.

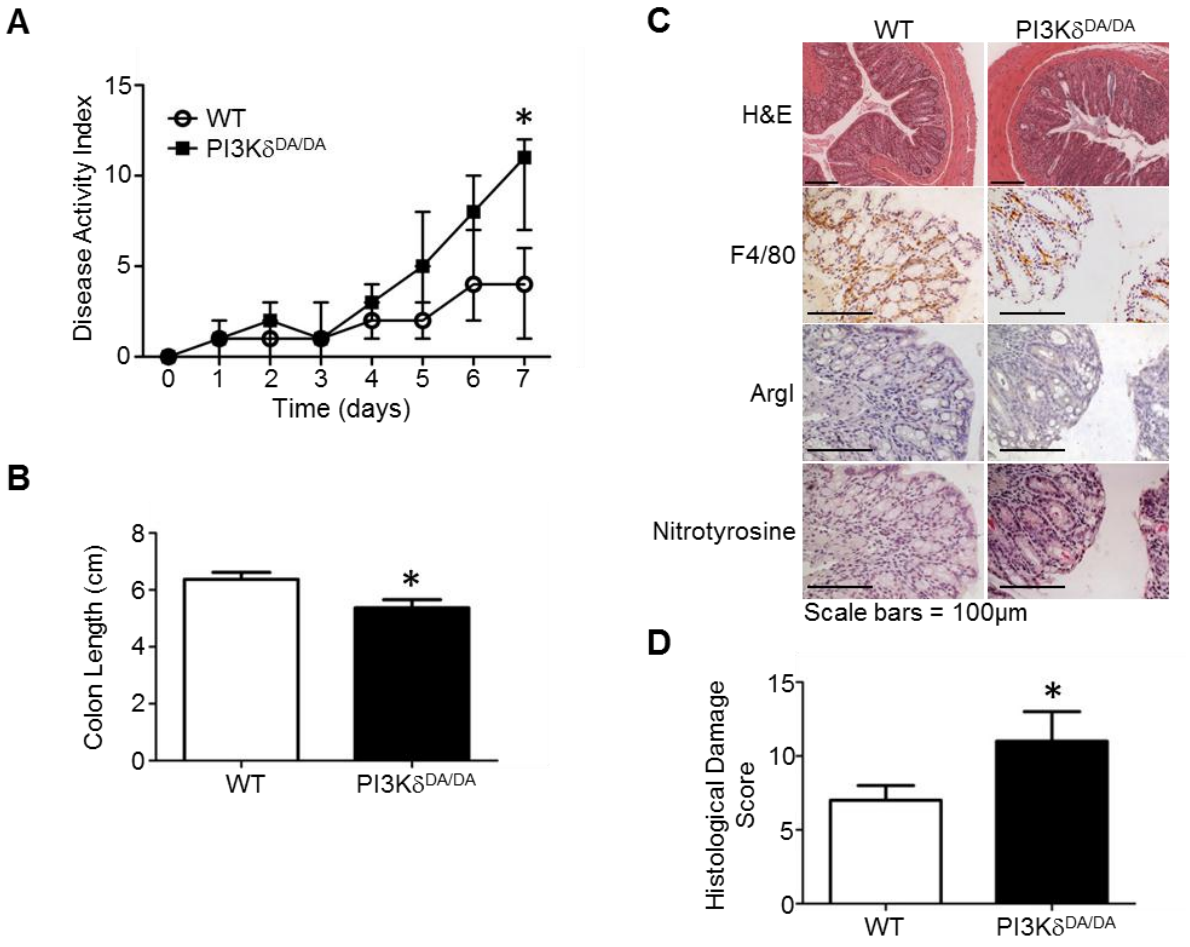

Figure 4.3 PI3Kp110δ$^{D910A/D910A}$ mice have fewer macrophages and fewer M2 macrophages in their colons

PI3Kp110δ$^{D910A/D910A}$ mice and wild type littermates were given 2.5% DSS in their drinking water for 7 days. (A) Disease activity indices were measured daily during treatment for PI3Kp110δ$^{D910A/D910A}$ (black squares) and wild type (open circles) mice and the median score ± range is shown. (B) Mice were sacrificed on Day 7, colons were removed, and colon length was measured. Results are expressed as mean colon length ± SD. (C) Colon sections from wild type mice (left) and PI3Kp110δ$^{D910A/D910A}$ mice (right) were stained with H&E (top) and stained by immunohistochemistry for mature macrophages (F4/80; 2nd row), ArgI (3rd row), and nitrotyrosine (bottom), a downstream product of NO. Scale bars = 100μm. (D) Histological damage was scored and results are expressed as median score ± range. *P <0.05 for n = 9 mice/group in 3 independent experiments (repeated measures ANOVA for A, Student's t-test for B, and Mann-Whitney test for D).

4.3.4 Depleting macrophages reduced disease severity in PI3Kp110δ$^{D910A/D910A}$ mice during dextran sodium sulfate-induced colitis

To determine if the exacerbation of disease seen in PI3Kp110δ$^{D910A/D910A}$ mice was macrophage-mediated, wild type and PI3Kp110δ$^{D910A/D910A}$ mice were treated with clodronate-containing liposomes to deplete colonic macrophages. We, and others, have demonstrated that intraperitoneal injections of clodronate-containing liposomes successfully reduces the number of colonic macrophages.[143, 147, 187, 199] Wild type and PI3Kp110δ$^{D910A/D910A}$ mice were given 2.5% DSS for 7 days to induce colitis. Groups of mice were also given intraperitoneal injections of PBS, as an injection control, or clodronate-containing liposomes 4 days prior to treatment as well as at day 0, 2, 4, and 6 during DSS treatment. In wild type mice, macrophage depletion did not impact disease activity or histological damage (Fig. 4.4).

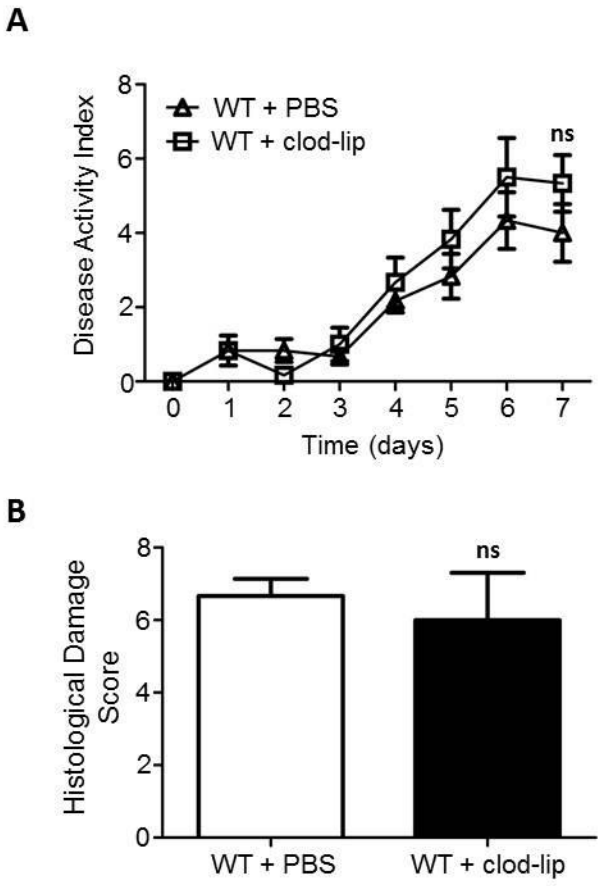

Figure 4.4 Macrophage depletion by clodronate-containing liposomes does not affect disease severity in wild type mice during DSS-induced colitis
Wild type mice were given 2.5% DSS in their drinking water for 7 days. DSS control mice were compared to mice receiving clodronate-liposomes (clod-lip) 4 days prior to DSS treatment and on Days 0, 2, 4, and 6 during DSS treatment. (A) DAIs were measured daily and reported as median disease activity indices ± range. (B) Histological damage was scored and results are expressed as median score ± range. ns = not significant for n = 9 mice/group in 3 independent experiments (repeated measures ANOVA for A and Mann-Whitney test for B).

In contrast, in PI3Kp110$\delta^{D910A/D910A}$ mice, macrophage depletion significantly decreased clinical disease (Fig. 4.5A). Disease activity indices were comparable to that determined for wild type mice. At the end of the experiment (day 7), colons were harvested and measured. Colons were significantly longer in PI3Kp110$\delta^{D910A/D910A}$ mice in which macrophages were

depleted during DSS-treatment compared to PBS-injected controls (Fig. 4.5B). H&E stained colon sections from PI3Kp110$\delta^{D910A/D910A}$ mice revealed less tissue damage in mice treated with clodronate-containing liposomes compared to injection controls (Fig. 4.5C, top). Macrophages were successfully depleted by treatment with clodronate-containing liposomes as seen by a reduced number of F4/80^{+} cells in colonic tissue sections (Fig. 4.5C, 2nd row). In addition, there was also a decrease in the number of ArgI^{+}F4/80^{+} M2 macrophages (Fig.4.5C, 3rd row), and decreased nitrotyrosine (Fig4.5C, bottom) compared to injection control mice. Finally, PI3Kp110$\delta^{D910A/D910A}$ mice treated with clodronate-containing liposomes had significantly less histological damage, comparable to that seen in wild type mice with induced intestinal inflammation (Fig. 4.5D). This data indicates that the exacerbation in clinical and histological disease that PI3Kp110$\delta^{D910A/D910A}$ mice display compared to their wild type littermates is macrophage mediated.

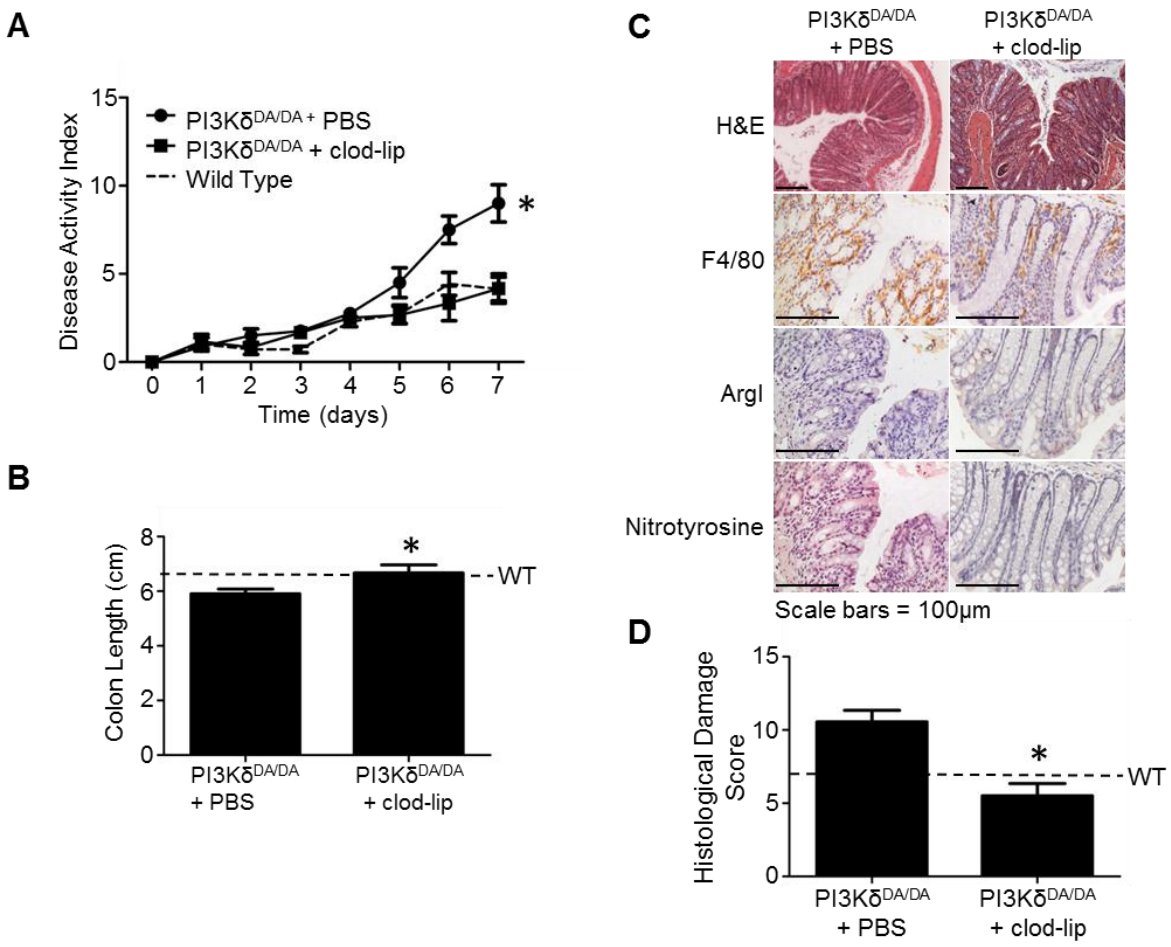

Figure 4.5 Macrophage depletion by clodronate-containing liposomes reduces disease severity in PI3Kp110δ$^{D910A/D910A}$ mice during DSS-induced colitis.
PI3Kp110δ$^{D910A/D910A}$ mice were given 2.5% DSS in their drinking water for 7 days. DSS control mice were compared to mice receiving clodronate-containing liposomes (clod-lip) 4 days prior to DSS treatment and on Days 0, 2, 4, and 6 during DSS treatment. (A) DAIs were measured daily and reported as median disease activity indices ± range. (B) Mice were sacrificed on Day 7, colons were removed and colon length was measured. Results are expressed as mean colon length ± SD. (C) Colon sections of PI3Kp110δ$^{D910A/D910A}$ control mice treated with DSS and receiving a PBS injection (left) and PI3Kp110δ$^{D910A/D910A}$ mice treated with DSS and clod-lip (right) were stained with H&E (top) and stained by immunohistochemistry for mature macrophages (F4/80; 2nd row), M2 macrophages (ArgI, 3rd row), and nitrotyrosine (bottom) a downstream product of NO. Scale bars = 100μm (D) Histological damage was scored and results are expressed as median score ± range. *P<0.05 for n = 9 mice/group in 3 independent experiments (repeated measures ANOVA for A, Student's t-test for B, and Mann-Whitney test for D)

4.3.5 PI3Kp110δ$^{D910A/D910A}$ macrophages were deficient in their ability to skew to an M2 phenotype

Since the exacerbation of disease in PI3Kp110δ$^{D910A/D910A}$ mice was macrophage-mediated and there were reduced numbers of M2 macrophages in the colons of these mice, I next asked whether PI3Kp110δ$^{D910A/D910A}$ macrophages were defective in their ability to skew to an M2 phenotype. Mature bone marrow macrophages from wild type and PI3Kp110δ$^{D910A/D910A}$ mice were derived using MCSF followed by treatment with IL-4 to skew macrophages to an M2 phenotype. To assess M2 macrophage skewing, we assessed M2 macrophage markers, cytokine responses to LPS treatment, and arginase activity. While the expression of some factors was the same between IL-4 treated PI3Kp110δ$^{D910A/D910A}$ and wild type macrophages, some factors were different. Western blot analysis showed that both wild type and PI3Kp110δ$^{D910A/D910A}$ mice degraded SHIP and expressed the M2 macrophage marker, Ym1, following IL-4 treatment (Fig. 4.6A). In addition, I wanted to know whether PI3Kp110δ$^{D910A/D910A}$ macrophages had differences in cytokine expression in response to inflammatory stimuli. MCSF-derived macrophages from PI3Kp110δ$^{D910A/D910A}$ and wild type mice were unpolarized or skewed to an M2 phenotype with IL-4. Macrophages were stimulated with LPS for 24 hours and cytokines were measured in culture supernatants by ELISA. There was no significant difference in expression of the pro-inflammatory cytokines, IL-12p70 or IL-6, or anti-inflammatory IL-10 (Fig. 4.6B) between wild type and PI3Kp110δ$^{D910A/D910A}$ macrophages that were unpolarized or skewed to an M2 phenotype. In contrast, I did see a difference between the IL-4 treated wild type and PI3Kp110δ$^{D910A/D910A}$ macrophages in the expression of the M2 macrophage markers ArgI, TREM2, and FIZZ1 (Fig 4.6C). The PI3Kp110δ$^{D910A/D910A}$ macrophages failed to induce the expression of

TREM2 and FIZZ1 following IL-4 treatment and expressed ArgI poorly, compared to their wild type counterparts. ArgI activity was measured biochemically and was significantly lower in IL-4 treated PI3Kp110$\delta^{D910A/D910A}$ macrophages, compared to IL-4 treated wild type macrophages (Fig. 4.6C). These data demonstrate that PI3Kp110$\delta^{D910A/D910A}$ macrophages are specifically defective in their ability to express TREM2, FIZZ1, and ArgI in response to IL-4.

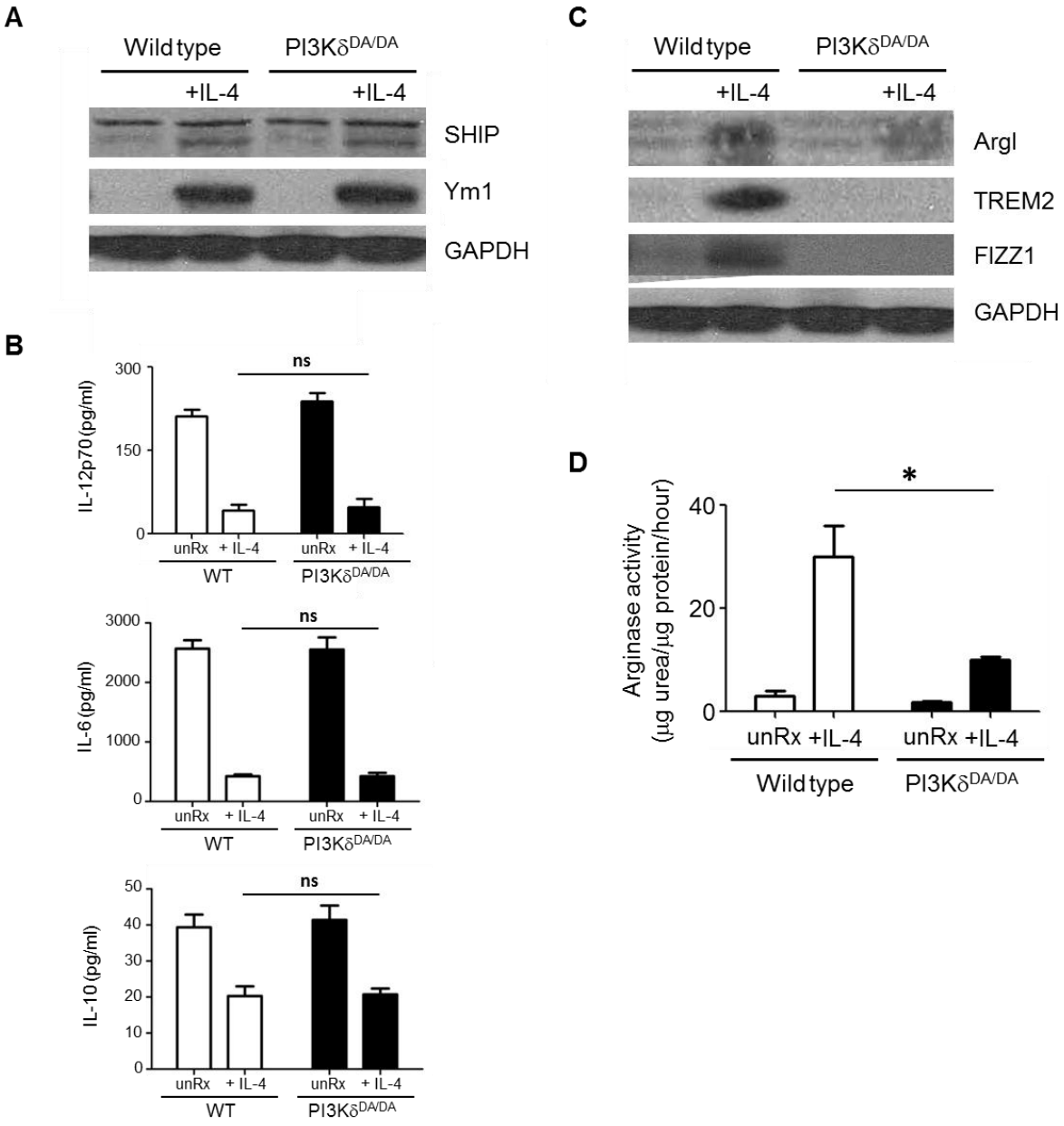

Figure 4.6 PI3Kp110δ$^{D910A/D910A}$ macrophages are defective in their ability to express some characteristics of M2 macrophages in response to IL-4

Bone marrow derived macrophages from wild type and PI3Kp110δ$^{D910A/D910A}$ mice were untreated or treated with IL-4 for 3 days. (A) Whole cell lysates were analyzed for SHIP, Ym1, and GAPDH by Western blotting (B) Macrophages were stimulated with 10ng/ml LPS and clarified supernatants were collected and assayed for IL-12p70, IL-10, and IL-6 by ELISA. (C) Whole cell lysates were analyzed for ArgI, TREM2, FIZZ1, and GAPDH by Western blotting. (D) 10μg of protein from whole cell lysates was used to measure arginase activity. *P<0.05; ns = not significantly different for 3 independent experiments using a Student's t-test for B and D. Data are represented as means ± SD.

4.3.6 IL-4 treated macrophages from wild type mice, but not those from PI3Kp110δ$^{D910A/D910A}$ mice, conferred protection to PI3Kp110δ$^{D910A/D910A}$ mice during dextran sodium sulfate-induced colitis

Since M2 macrophages confer protection during DSS-induced colitis[143, 147, 187] and PI3Kp110δ$^{D910A/D910A}$ macrophages are defective in their ability to express some features of M2 macrophages in response to IL-4, I asked whether *ex vivo* derived IL-4 treated PI3Kp110δ$^{D910A/D910A}$ macrophages could provide protection to PI3Kp110δ$^{D910A/D910A}$ mice during DSS-induced colitis. Mature bone marrow macrophages were derived from wild type and PI3Kp110δ$^{D910A/D910A}$ mice using MCSF and treated with IL-4 to generate M2 polarized macrophages. To determine their effect on disease severity, I injected groups of PI3Kp110δ$^{D910A/D910A}$ mice with either wild type or PI3Kp110δ$^{D910A/D910A}$ IL-4 treated macrophages (10^6) by tail vein on day 0 and 4 of DSS treatment. Mice that received wild type M2 macrophages had a significant improvement in clinical symptoms reflected in lower disease activity index scores compared to injection controls. In contrast, mice that received IL-4 treated PI3Kp110δ$^{D910A/D910A}$ macrophages were not protected in their clinical disease symptoms (Fig. 4.7A). Colons were significantly longer in PI3Kp110δ$^{D910A/D910A}$ mice treated with wild type M2 macrophages and were comparable to measurements of wild type DSS treated mice but mice treated with IL-4 skewed PI3Kp110δ$^{D910A/D910A}$ macrophages were not longer (Fig. 4.7B). H&E stained colon sections from mice treated with wild type M2 macrophages had less histological tissue damage compared to those receiving injections of IL-4 treated PI3Kp110δ$^{D910A/D910A}$ or PBS (Fig. 4.7C, top). Both groups of mice, those receiving either IL-4 treated PI3Kp110δ$^{D910A/D910A}$ macrophages or wild type IL-4 treated macrophages, had more F4/80$^+$ stained macrophages compared to injection controls

(Fig.4.7C, 2nd row). Mice receiving wild type M2 macrophages showed an increase in the M2 marker, ArgI (Fig. 4.7C, 3rd row), and a decrease in nitrotyrosine staining, compared to mice receiving IL-4 treated PI3Kp110$\delta^{D910A/D910A}$ macrophages or injection controls (Fig. 4.7C, bottom). Finally, mice reconstituted with wild type M2 macrophages had significantly lower histological damage scores compared to mice reconstituted with IL-4 treated PI3Kp110$\delta^{D910A/D910A}$ macrophages or PBS injected controls (Fig. 4.7D). These data demonstrate that IL-4 treated PI3Kp110$\delta^{D910A/D910A}$ macrophages are unable to provide protection during DSS-induced colitis.

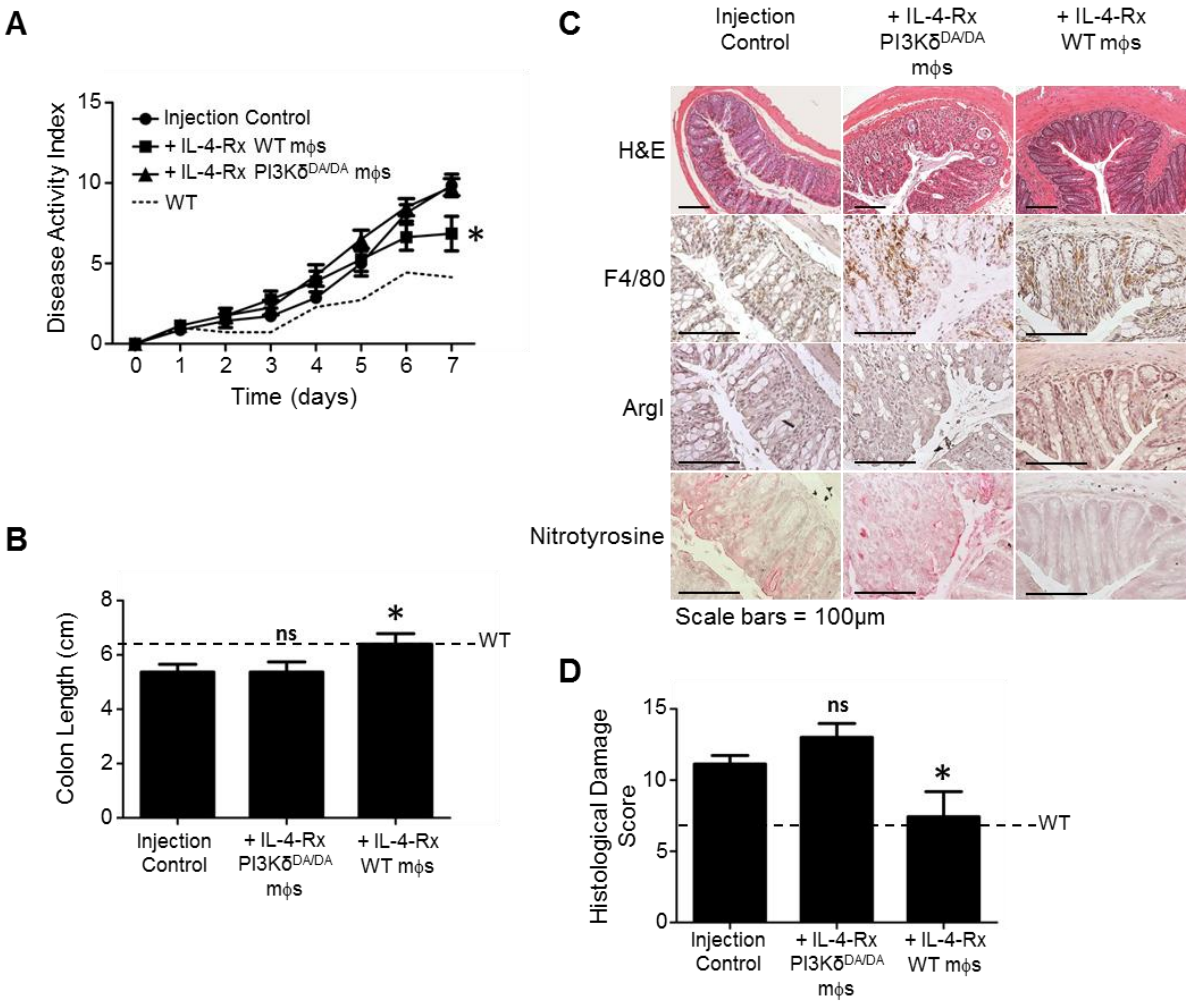

Figure 4.7 Adoptive transfer of IL-4 treated macrophages derived from wild type, but not PI3Kp110δ$^{D910A/D910A}$ mice confer protection to PI3Kp110δ$^{D910A/D910A}$ mice during DSS-induced colitis

PI3Kp110δ$^{D910A/D910A}$ mice were given 2.5% DSS in their drinking water for 7 days. Mice were given PBS injections as a control or IL-4 skewed M2 macrophages derived from wild type mice (+ IL-4 receiving (Rx) WT macrophages (mφs)) or PI3Kp110δ$^{D910A/D910A}$ mice (+ IL-4 Rx PI3Kδ$^{DA/DA}$ mφs). PBS or macrophages were injected intravenously into mice on Day 0 and 4 during DSS treatment. (A) Disease activity indices were measured daily and are reported as median ± range. (B) Mice were sacrificed on Day 7 and colons were removed and colon length was measured. Results are expressed as mean colon length ± SD. (C) Colon sections of PI3Kp110δ$^{D910A/D910A}$ DSS injection control mice (left), PI3Kp110δ$^{D910A/D910A}$ mice treated with M2 macrophages from PI3Kp110δ$^{D910A/D910A}$ mice (center), and PI3Kp110δ$^{D910A/D910A}$ mice treated with M2 macrophages from wild type mice (right) were stained with H&E (top) and stained by immunohistochemistry for mature macrophages (F4/80; 2nd row), M2 macrophages (ArgI, 3rd row) and nitrotyrosine (bottom row). Scale bars = 100μm (D) Histological damage was scored and results are expressed as median score ± range. * P<0.05 for n = 9 mice/group in 3 independent experiments (repeated measures ANOVA for A, Student's t-test for B, and Mann-Whitney test for D).

4.3.7 Arginase activity was required for M2 macrophage mediated protection during dextran sodium sulfate-induced colitis

Because IL-4 treated PI3Kp110δ$^{D910A/D910A}$ macrophages have significantly less arginase activity and are unable to confer protection during DSS-induced colitis, I next asked whether arginase activity is required for protection provided by M2 macrophages. I derived bone marrow macrophages from wild type mice using MCSF and skewed them to a M2 phenotype with IL-4. During the 3 day skewing with IL-4, some were left untreated while others were treated with arginase inhibitors, 0.1mM BEC or 0.1mM ABH. M2 macrophages treated with arginase inhibitors had increased arginase activity compared to untreated controls (Fig. 4.8A). As this was opposite the desired effect, I decided to inhibit arginase *in vivo* as we have done previously.[192] I derived bone marrow macrophages from wild type mice using MCSF and skewed them to a M2 phenotype with IL-4. Macrophages (10^6) were injected by tail vein into PI3Kp110δ$^{D910A/D910A}$ mice on days 0 and 4 of treatment with 2.5% DSS. Groups of mice were untreated (PBS control) or treated with arginase inhibitors, 0.2% BEC or 0.04% ABH daily by oral gavage. I measured arginase activity in full thickness tissue homogenates to determine the impact of arginase inhibitors *in vivo.* Consistent with our previous observations, oral gavage of arginase inhibitors reduced arginase activity in full thickness tissue homogenates from colons (Fig. 4.8B).[192] Mice receiving either BEC or ABH had worse clinical disease symptoms compared to control mice apparent in significantly higher disease activity index scores (Fig. 4.8C). Colon lengths of mice receiving either arginase inhibitor were significantly shorter compared to control mice (Fig. 4.8D). H&E stained colonic tissue from mice receiving either arginase inhibitor had more tissue damage than control mice (Fig. 4.8E, top). PI3Kp110δ$^{D910A/D910A}$ mice receiving either ArgI inhibitor had

similar colonic F4/80^{+} staining macrophages compared to control mice (Fig. 4.8D, 2nd row), but reduced staining for ArgI (Fig. 4.8E, 3rd row). Nitrotyrosine staining was increased in colon tissue sections from mice receiving either BEC or ABH compared to control mice (Fig. 4.8E, bottom). Finally, mice receiving either BEC or ABH had significantly higher histological damage scores compared to control mice (Fig. 4.8F). Taken together, these data indicate that arginase activity is required for M2 macrophage mediated protection during DSS-induced colitis.

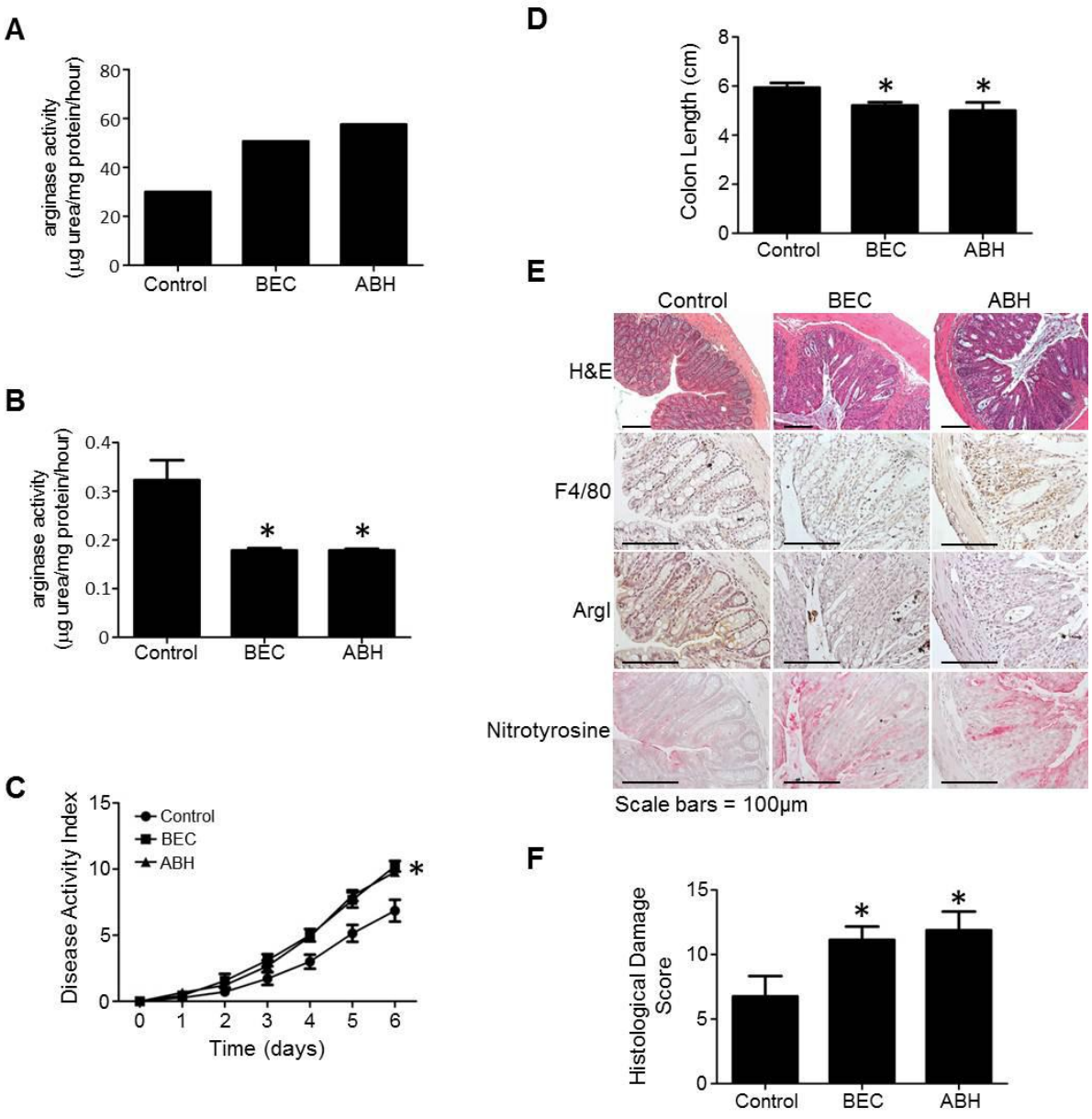

Figure 4.8 Arginase activity is required for M2 macrophage mediated protection during DSS-induced colitis

(A) M2 macrophages were untreated or treated for 3 days with either 0.1mM BEC or ABH to inhibit arginase activity. In addition, M2 macrophages from wild type mice were adoptively transferred into PI3Kp110$\delta^{D910A/D910A}$ mice by intravenous injection on Day 0 and 4 during treatment with 2.5% DSS. Mice were treated daily by oral gavage with 100μl of PBS, as a control, or either 0.2% BEC or 0.04% ABH to inhibit arginase activity. (B) 10μg of protein from colonic lysates was used to measure arginase activity. Data are represented as means ± SD. (C) Disease activity indices were measured daily and are reported as median ± range. (D) Mice were sacrificed on Day 6, colons were removed, and colon length was measured. Results are expressed as mean colon length ± SD. (E) Colon sections of DSS treated PI3Kp110$\delta^{D910A/D910A}$ mice receiving PBS as an injection control (left), BEC (center), or

ABH (right) were stained with H&E (top) and stained by immunohistochemistry for mature macrophages (F4/80; 2nd row), M2 macrophages (ArgI, 3rd row) and nitrotyrosine (bottom row). Scale bars = 100μm. (F) Histological damage was scored and results are expressed as median score ± range. *P<0.05 for n = 9 mice/group in 3 independent experiments (student's t-test for A and C, repeated measures ANOVA for B, and Mann-Whitney test for E).

4.4 Discussion

Herein, I demonstrate a critical role for ArgI in M2 macrophage-mediated protection from intestinal inflammation. In the absence of PI3Kp110δ, mice had modestly, but significantly, reduced numbers of colonic macrophages and significantly reduced numbers of ArgI-expressing M2 macrophages. This correlated with increased pro-inflammatory NO production, increased clinical disease activity, and increased histological damage during DSS-induced colitis. PI3Kp110δ deficient macrophages treated with IL-4 were impaired in their ability to induce the M2 macrophage marker, ArgI, and failed to protect mice during DSS-induced intestinal inflammation. Blockade of arginase activity in mice also prevented wild type $ArgI^+$ M2 macrophages from protecting mice during DSS-induced colitis. This study highlights a critical role for arginase activity in the anti-inflammatory properties of M2 macrophages during intestinal inflammation.

PI3Kp110δ deficient mice are a knock-in mouse model, in which inactivation of the p110δ catalytic subunit of class I PI3-kinase leads to the development of colitis.[189] Colonic inflammation has been reported in these mice at 8 weeks of age and inflammation increases with age.[190] Colonic macrophages isolated from PI3Kp110δ deficient mice have increased IL-12p40 production and reduced IL-10 production in response to heat-killed *E. coli* and GM-CSF-derived bone marrow macrophages from these mice have increased pro-inflammatory responses to toll-like receptor ligands.[190] As such, macrophages have been implicated in driving inflammation in this model but a direct role for $PI3Kp110\delta^{D910A/D910A}$

macrophages in colonic inflammation has not been demonstrated. PI3Kp110δ$^{D910A/D910A}$ mice in our animal facility do not show clinical or histological signs of disease at 8-12 weeks of age but histological sections did show increased staining for nitrotyrosine suggesting that there was more pro-inflammatory nitric oxide produced in the colons of these mice. As such, PI3Kp110δ$^{D910A/D910A}$ mice were highly sensitive to DSS-induced colitis. Mice had significantly higher clinical disease activity and histological damage scores compared to their wild type littermates. Importantly, we depleted phagocytes (macrophage and dendritic cells) from these mice with clodronate-containing liposomes and this reduced disease severity. Furthermore, adoptive transfer of M2-skewed macrophages from wild type mice, but not those from PI3Kp110δ deficient mice, rescued mice from intestinal inflammation. Taken together, these results suggest that a lack of M2 macrophages and increased pro-inflammatory macrophage activity exacerbates DSS-induced colitis in PI3Kp110δ deficient mice.

I, and others, have reported that the PI3K pathway is required for IL-4 induced gene transcription of specific M2 macrophage products[172, 195] and, using pharmacological inhibitors, I found that ArgI induction was sensitive to PI3Kp110δ inhibition. Data presented here confirm this finding by demonstrating that macrophages that do not have PI3Kp110δ activity are impaired in their ability to induce ArgI, TREM2, and FIZZ1 in response to IL-4 treatment. This defect in PI3Kp110δ$^{D910A/D910A}$ macrophages ability to induce M2 macrophage markers in response to IL-4 correlates with their inability to protect mice from DSS-induced intestinal inflammation and may underlie, or contribute to, the development of spontaneous colitis in PI3Kp110δ deficient mice.

Arginase acts as an endogenous inhibitor of nitric oxide production by competing with nitric oxide synthase for their common substrate, L-arginine, and leads to production of polyamines that can contribute to tissue restitution. Herein, I have shown that the protective effect of M2 macrophages in DSS-induced colitis requires arginase activity. While *in vitro* inhibition of arginase activity by BEC or ABH in M2 macrophages resulted in an increase in arginase activity, *in vivo* studies showed a decrease in activity in whole colon homogenates. This may be due to a negative feedback loop in tissue where inhibiting activity reduced mRNA expression. In summary, my results identify a critical role for ArgI in the anti-inflammatory functions of M2 macrophages in mice.

5 Chapter: Conclusion

Current research, including the data presented here, has shown that targeting macrophages is a promising therapeutic strategy for the treatment of IBD. Disease presentation has been associated with an influx of monocytes that play a central role in disease pathogenesis.[156-159] Consistent with this, I see a 4.6-fold increase in F4/80^{+} cells in mouse colons during DSS-treatment. Depletion of pro-inflammatory macrophages reduces disease severity in IL-10 deficient mice that have hyper-inflammatory macrophages.[57] However, in wild type mice, depleting colonic macrophages exacerbates DSS-induced intestinal inflammation suggesting that macrophages can play a role in protecting the colon during intestinal inflammation.[143] Alternatively activated macrophages contribute to normal physiological processes and have been implicated in pathologies but also have the potential to be protective in inflammatory diseases due to their anti-inflammatory and healing phenotype. With an increasing population of patients that are refractory to current therapies[42], manipulating macrophage phenotype to dampen inflammation and promote tissue healing has the potential to reduce inflammation and disease symptoms in patients with IBD.

Several lines of evidence suggest that M2 macrophages can confer protection against intestinal inflammation. Human studies have shown that the parasitic worms, *Trichuris suis* or *Necator americanus*, reduce clinical disease activity indices in people with Crohn's Disease and it is believed that these infections dampen down inflammation by skewing macrophages to an M2 phenotype.[200-202] During the course of this work, a complementary pre-clinical study demonstrated that *Hymenolepis diminuta* protected mice from DNBS-induced colitis, protection was macrophage-mediated, and protection correlated with the induction of the M2 macrophage markers, ArgI and FIZZ1.[147] Moreover, adoptive transfer

of *ex vivo* derived, IL-4-skewed M2 macrophages recapitulated the protective effect.[147] I have also demonstrated that M2 macrophages can protect mice from acute intestinal inflammation. Mice deficient in the lipid phosphatase, SHIP, have increased numbers of M2 macrophages in the colon and are protected during DSS-induced colitis. Protection is macrophage-mediated and could be conferred to a susceptible, wild type host by adoptive transfer of *ex vivo* derived, M2 macrophages. In addition, I have shown that mice with a point mutation disrupting the active site in PI3Kp110δ are defective in their ability to skew macrophages to an M2 phenotype and have exacerbated disease during induced intestinal inflammation. When these mice are reconstituted with *ex vivo* derived M2 macrophages, they are also protected from DSS-induced colitis. In both wild type and PI3Kp110$\delta^{D910A/D910A}$ mice, a significant reduction in clinical disease symptoms and histological damage is apparent after treatment with M2 macrophages. These results suggest that M2 macrophages are able to reduce inflammation and tissue damage during an induced inflammatory response.

In addition to IBD, M2 macrophages have been identified as a potential target in other inflammatory conditions. Studies of Type 1 diabetes have shown that nonobese diabetic mice infected with *Heligmosomoides polygyrus* had a reduced incidence of disease correlating with an increase in pancreatic M2 macrophages.[203] Similar results were seen with nonobese diabetic mice infected with *Schistosoma mansoni* or treated with helminth or soluble worm extract where a decreased incidence of Type 1 diabetes is associated with increased numbers of M2 macrophages.[204, 205] Importantly, adoptive transfer of *in vitro* IL-4 treated macrophages was shown to reduce hyperglycemia and insulitis in the pancreas of diabetic mice.[206] Type 2 diabetes has also been associated with dysregulated macrophage phenotype where adipose tissue macrophages from mice treated with a high fat diet had a

shift from an M2 phenotype to an inflammatory M1 phenotype, which correlated with the onset of disease.[207] Additionally, a relapsing experimental autoimmune encephalomyelitis rat model of multiple sclerosis showed that an increase in M1 macrophages was associated with disease relapse, whereas administration of *ex vivo* activated M2 macrophages suppressed disease.[208] Finally, M1 macrophages lyse muscle cells via an iNOS-dependent mechanism in the dystrophin-deficient mdx murine model of muscular dystrophy. M2 macrophages inhibited this effect via ArgI outcompeting iNOS for their common substrate, L-arginine.[209] Taken together, these studies show that alternatively activated macrophages are critical for protection from chronic inflammation and manipulating macrophages to skew to an M2 phenotype may be an effective therapeutic option for multiple inflammatory diseases.

SHIP, the hematopoietic-specific negative regulator of the PI3K pathway, is a potential therapeutic target for IBD. *In vivo* differentiated SHIP-/- macrophages are profoundly M2 skewed [139] and this has been cited as important in their response to enteric pathogens.[161] Here, I have shown that alternative activation of murine macrophages is dependent on the degradation of SHIP and that more SHIP-/- colonic macrophages express the M2 macrophage markers, ArgI and Ym1, relative to SHIP+/+ littermate controls. Importantly, I demonstrate that SHIP-/- mice are protected during DSS-induced colitis with respect to the clinical presentation of disease and the histological damage observed. SHIP-/- mice do develop disease in response to DSS but disease onset is delayed and severity of disease is reduced at the time points examined. Macrophage depletion experiments demonstrated that protection was mediated by SHIP-/- macrophages. Since liposome-encapsulated PBS also caused macrophage depletion in our model, as has been described previously,[143] macrophage□depletion experiments were compared to a PBS injection control,

wherein disease activity and histological damage was comparable to DSS-treated mice without injection. Bone marrow derived macrophages from SHIP-/- mice can be skewed to an M2 phenotype by differentiation with GM-CSF [140] and importantly, when SHIP-/- macrophages are skewed to an M2 phenotype during differentiation, they do not revert to an M1 phenotype.[139, 140] We differentiated SHIP-/- bone marrow macrophages in the presence of MCSF, to generate M1 macrophages, or GM-CSF, to generate M2 macrophages, and injected them via tail vein into SHIP+/+ mice during the development of colitis. GM-CSF-derived SHIP-/- M2 macrophages, but not MCSF-derived (M1 control), conferred protection to wild type mice (SHIP+/+) during DSS-induced colitis. This demonstrates that SHIP deficiency in macrophages is not sufficient to protect mice from intestinal inflammation, but rather, the M2 macrophage phenotype is responsible for protection. Thus manipulating SHIP levels, together with skewing macrophages to an M2 phenotype, may provide an ideal strategy to treat IBD.

A critical caveat when considering using M2 macrophages to dampen down inflammation is that macrophages are plastic, meaning that their phenotype is not fixed. Rather, they are polarized or skewed by their local microenvironment. This limits the use of M2 macrophages for treatment of chronic inflammatory diseases, like IBD, because when IL-4 is removed or when an M2 macrophage encounters an inflammatory environment, its protective M2 phenotype will be lost. It may acquire properties of inflammatory macrophages that would be predicted to exacerbate inflammation.[79, 139] SHIP has been shown to be degraded by the proteasome following tyrosine phosphorylation and therefore, the induction of SHIP phosphorylation could be used to deplete endogenous protein levels.[170] I have demonstrated that SHIP protein levels can be reduced by siRNA and SHIP deficient

macrophages are more sensitive to skewing to an M2 phenotype. Recent studies have reported that oral delivery of siRNA targeting TNFα and Map4k4 successfully reduced intestinal inflammation.[210, 211] This technology, together with our ability to lock macrophages into an M2 phenotype by SHIP depletion, may provide a novel approach to permit the generation of 'fixed' M2 macrophages that can be used therapeutically to dampen down intestinal inflammation in IBD patients.

Another potential target for treatment of IBD is class I PI3K, which has been implicated as a key signalling pathway in IBD.[212] However, PI3K has been reported to play disparate roles in murine colitis models, likely due to the unique roles that specific catalytic subunits play in inflammation.[213-215] Pertinent to a role for PI3K in macrophage phenotype in intestinal inflammation, mice deficient for class IA PI3Kp110δ catalytic subunit activity develop spontaneous colonic inflammation,[216] which has recently been attributed to macrophage dysfunction and includes elevated levels of M1 macrophage-associated IL-12 and IL-23 production.[215] These PI3Kp110δ deficient mice lack giant cells in the spleen, gut, mesenteric lymph nodes, and Peyer's Patches suggesting that they may be defective in their ability to skew to an M2 phenotype, which is required for macrophage fusion and giant cell formation.[216] Indeed, I have recently found that PI3Kp110δ is required for IL-4-induced alternative activation of macrophages and so I hypothesized that this may contribute to their spontaneous colonic inflammation.[172, 217, 218] Consistent with this, IL-4 and STAT6, which are required for M2 macrophage skewing, are protective in allergic and DSS-induced murine models of intestinal inflammation, respectively.[219, 220] Though PI3Kp110γ has been reported to contribute to intestinal inflammation, interestingly, pharmacological inhibition of PI3Kp110γ causes a significant increase in tissue IL-4 levels. This may contribute to

protection reported in PI3Kp110γ deficient mice during DSS-induced colitis.[221] Indeed, IL-4 responses in monocytes and macrophages are impaired in IBD patients and this may contribute to disease pathology.[222]

PI3Kp110δ deficient mice are a knock-in mouse model, in which inactivation of the p110δ catalytic subunit of class I PI3-kinase leads to the development of colitis.[189] Colonic inflammation has been reported in these mice as early as 8 weeks of age and inflammation increases with age.[190] Colonic macrophages isolated from PI3Kp110δ deficient mice have increased IL-12p40 production and reduced IL-10 production in response to heat-killed *E. coli* and GM-CSF-derived bone marrow macrophages from these mice have increased pro-inflammatory responses to toll-like receptor ligands.[190] As such, macrophages have been implicated in driving inflammation in this model but a direct role for PI3Kp110$\delta^{D910A/D910A}$ macrophages in colonic inflammation has not been demonstrated. PI3Kp110$\delta^{D910A/D910A}$ mice in our animal facility do not show clinical or histological signs of disease at 8-12 weeks of age, but histological sections did show increased staining for nitrotyrosine suggesting that there was more pro-inflammatory nitric oxide produced in the colons of these mice. PI3Kp110$\delta^{D910A/D910A}$ mice were highly sensitive to DSS-induced colitis. Mice had significantly higher clinical disease activity and histological damage scores compared to their wild type littermates. Importantly, I depleted phagocytes (macrophage and dendritic cells) from these mice with clodronate-containing liposomes and this reduced disease severity demonstrating for the first time, a direct role for phagocytes in intestinal inflammation in PI3Kp110$\delta^{D910A/D910A}$ mice. Furthermore, adoptive transfer of M2-skewed macrophages from wild type mice, but not those from PI3Kp110δ deficient mice, rescued mice from intestinal inflammation. Taken together, these results suggest that a lack of M2 macrophages

and increased pro-inflammatory macrophage activity exacerbates DSS-induced colitis in PI3Kp110δ deficient mice.

The role of arginase activity in intestinal inflammation and DSS-induced colitis is controversial. Arginase acts as an endogenous inhibitor of nitric oxide production by competing with nitric oxide synthase for L-arginine, and leads to production of polyamines that can contribute to tissue restitution. Arginase has been reported to be protective in the *C. rodentium* model of infectious intestinal inflammation[198] and ArgI produced by macrophages inhibits intestinal inflammation in mice infected with the Th2-inducing pathogen, *Schistosoma mansoni*.[223] L-arginine supplementation also improves disease outcome during *C. rodentium* infection and DSS-induced colitis by increasing arginase-dependent polyamine production and wound healing.[198, 224] In contrast, a recent study demonstrated that DSS-induced colitis was reduced in CH3 mice by treatment with an arginase inhibitor, nor-NOHA.[225] This was associated with increased production of NO, which may reduce pathology by contributing to inflammation and inducing epithelial cell proliferation and tissue restitution.[225-227] My data is consistent with the former studies and demonstrate that the protective effect of M2 macrophages in DSS-induced colitis requires arginase activity. I have shown a critical role for ArgI in M2 macrophage-mediated protection from intestinal inflammation. In the absence of PI3Kp110δ, mice had modestly, but significantly, reduced numbers of colonic macrophages and significantly reduced numbers of ArgI-expressing M2 macrophages. This led to increased pro-inflammatory nitric oxide production, increased clinical disease activity, and increased histological damage during DSS-induced colitis. PI3Kp110δ deficient macrophages treated with IL-4 were impaired in their ability to induce the M2 macrophage marker, ArgI, and failed to protect mice during DSS-induced intestinal

inflammation. Blockade of arginase activity in mice also prevented wild type $ArgI^{+}$ M2 macrophages from protecting mice during DSS-induced colitis. This highlights a critical role for arginase activity in the anti-inflammatory properties of M2 macrophages during intestinal inflammation in mice.

The work presented in this thesis contributes significantly to the current understanding of how macrophage phenotype can diminish, or contribute to, intestinal inflammation. While M2 macrophages have previously been shown to protect mice in models of intestinal inflammation, macrophage skewing has been induced by infection with parasitic worms.[147] The results that I have presented here using the SHIP-/- mice are the first demonstrating this M2 macrophage mediated protection in a genetic model. The advantage of this approach is that I was able to identify SHIP as a potential target for treatment of IBD. I was also able to further our understanding of SHIP's involvement in the alternative activation of macrophages by determining that its degradation is required for M2 macrophage skewing. While conducting these studies, I was also able to show that both STAT6 and PI3Kp110δ are required for the degradation of SHIP protein and loss of SHIP activity, creating an environment that potentiates expression of STAT6-driven alternative macrophage activation. Expanding our understanding of IL-4 signalling and macrophage skewing allowed me to identify PI3Kp110δ as an additional therapeutic target. Subsequently, I was able to show that the deficiencies of $PI3Kp110\delta^{D910A/D910A}$ macrophages to completely skew to an M2 phenotype, exacerbates DSS-induced colitis. By comparing IL-4 treated $PI3Kp110\delta^{D910A/D910A}$ bone marrow derived macrophages to controls, I was able to identify differential expression of specific factors that may be potential targets for manipulation to promote M2 macrophage skewing and treatment of IBD. I further showed that, in fact,

inhibition of arginase activity prevented protection by M2 macrophages during induced intestinal inflammation.

Our genetic approach to investigate the role of macrophage phenotype in intestinal inflammation also highlights the potency of anti-inflammatory M2 macrophages in maintaining intestinal homeostasis. I was surprised to find that a seemingly modest increase in colonic M2 macrophage numbers, 2.3-fold increased numbers in SHIP-/- mice, was sufficient to confer protection against DSS-induced colitis. Consistent with this, PI3Kp110δ deficient mice had a seemingly modest 2.2-fold reduction in $ArgI^+$ M2 colonic macrophages. A recent report by Jenkins, S. *et al.* (2011), demonstrated that IL-4 causes proliferation of resident tissue macrophages in the pleural cavity, peritoneal cavity, and liver that were formerly believed to be terminally differentiated and incapable of further proliferation.[228] Our results are consistent with a role for IL-4 signalling establishing homeostatic, intestinal macrophage populations. Specifically, these data suggest that small changes in the number of resident M2 macrophages are required to maintain, and are sufficient to disrupt, intestinal homeostasis.

The concept of inducing macrophage skewing or providing *ex vivo* derived alternatively activated macrophages to patients suffering from IBD is a promising therapeutic option. However, in future studies it will be critical to be cognizant of three important caveats to this approach. First, M1 macrophages are critical in our bodies' recognition and response to infectious organisms.[89] M2 macrophage-based immunotherapy could increase susceptibility to infection. This is also an important consideration for current therapies used to treat IBD, steroids and biological therapies. In that regard, I would not predict that macrophage-based immunotherapy would pose a greater risk of infection in patients than

current therapies that globally reduce inflammatory responses. Second, M1 macrophages are important for tumor surveillance. Theoretically, if a neoplastic event occurred in the intestine, a paucity of M1 macrophages could compromise tumor surveillance, increasing the risk of developing inflammation associated cancer.[9] Moreover, M2 macrophages are phenotypically similar to tumor associated macrophages so providing patients with M2 macrophages to treat intestinal inflammation may provide a niche for cancer development or growth.[229] It has been demonstrated that tumors grow faster when transplanted onto SHIP-/- mice compared to wild type littermates and this may be due, in part, to the increased numbers of M2 macrophages in SHIP-/- mice.[139] Finally, M2 macrophages have been linked to pathological wound healing or fibrosis in animal models, though their role remains controversial.[224, 230] I have contributed to additional studies from our laboratory demonstrating that SHIP-/- mice develop spontaneous inflammation in the ileum accompanied by arginase-dependent fibrosis.[192] While I believe that skewing macrophages to an M2 phenotype to dampen down inflammation and promote restitution remains a promising therapeutic strategy to treat IBD, we must rigorously assess these potential consequences prior to implementing this strategy in patients.

Future studies will focus on three main strategies to reduce intestinal inflammation. First, depleting macrophages may be an effective treatment for inflammatory diseases. Results presented here show that depleting macrophages from the inflammatory environment reduced the severity of intestinal inflammation. In fact, recent studies in IBD patients have shown that leukocyte apheresis, which removes macrophages and granulocytes, is a novel, safe, and effective strategy to reduce intestinal inflammation,[231-233] including disease that is refractory to current therapies.[234] A second strategy that may be useful to treat intestinal

inflammation is increasing the number of protective anti-inflammatory macrophages at sites of inflammation by polarizing macrophages *in situ*. Further studies are needed to determine if targeting SHIP or PI3Kp110δ would be a useful therapeutic approach. In addition, other factors involved in M2 skewing should be identified and their therapeutic potential determined. For example, studies have shown that mice with reduced IL-10 signalling or deficiencies in STAT6 have impaired M2 macrophage skewing and are more susceptible to DSS-induced colitis.[150, 220] ArgI is expressed in M2 macrophages from mice but is not in human M2 macrophages so protective effects of ArgI in murine M2 macrophages will not be translatable to people with IBD. However, neutrophils are a source of ArgI in people[89] and are among the most abundant leukocytes present at sites of intestinal inflammation in people with IBD.[235] It seems paradoxical, then, that nitric oxide production is also elevated in inflamed tissues from people with IBD.[236] In future studies, it would be interesting to determine whether neutrophil ArgI activity was compromised in people with IBD or insufficient to limit NO production at sites of intestinal inflammation. Investigating anti-inflammatory properties of human M2 macrophages may provide insight into additional mechanisms of action and identify novel immunotherapeutic strategies that can be used to reduce intestinal inflammation and treat people with IBD. Here, I have identified TREM2 as a potential target as studies with PI3Kp110δ deficient macrophages have shown that, in contrast to wild type macrophages, TREM2 is not induced in response to IL-4. TREM2 is a cell surface receptor that attenuates macrophage response to microbial products,[237] and as such, it may also contribute to the anti-inflammatory properties of M2 macrophages. A third strategy that may be useful to treat intestinal inflammation is increasing the number of anti-inflammatory macrophages by adoptive transfer of *ex vivo* polarized macrophages. To avoid

some of the limitations associated with M2 macrophages discussed earlier, regulatory macrophages may also be useful in reducing intestinal inflammation. These macrophages down-regulate IL-12 production but produce high levels of the immunosuppressive cytokine IL-10 in response to inflammatory stimuli, like LPS. Their primary role may be to stop the inflammatory response and minimize tissue damage.[238, 239] Importantly, unlike M2 macrophages, they do not contribute to the production of the extracellular matrix.[239] Current research, including the results presented here, show that M2 macrophages are protective during induced intestinal inflammation. Future studies are necessary to further support the thesis that manipulating intestinal macrophages can dampen down inflammation and promote tissue healing in IBD to provide relief to patients.

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