THE ROLE OF SHIP1 IN IL-10 SIGNALLING PATHWAY IN PRIMARY MACROPHAGES

by

Yee Wah So

B.Sc. (Hons), The University of British Columbia, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January 2014

© Yee Wah So 2014

Abstract

The inflammatory response is an important physiological mechanism for hosts' defense against pathogens and post-assault tissue repair. However, excessive inflammation leads to tissue damage and pathology. Therefore, inflammation is tightly regulated by anti-inflammatory factors such as interleukin-10 (IL-10) in order to maintain homeostasis. IL-10 inhibits macrophages' activation by suppressing macrophages' antigen presenting ability and production of proinflammatory cytokines such as tumor necrosis factor α (TNF α). The signal transducer and activator of transcription 3 (STAT3) pathway has been regarded as the only downstream pathway of IL-10 for decades. However, our lab has previously shown that IL-10's early antiinflammatory action uses the lipid phosphatase SH2 domain containing inositol 5 phosphatase (SHIP1) but not STAT3. Previous results in our lab suggested that IL-10 activates SHIP1 to inhibit the phosphoinositide 3-kinase (PI3K) pathway; and this accounts for IL-10's early antiinflammatory effects. Since the previous results were mostly obtained using cell lines, we sought to verify the results in the more physiological relevant mouse primary cells. We first investigated whether IL-10 activates SHIP1 by assessing the physical interaction of IL-10 receptor (IL-10R) and SHIP1, the localization of SHIP1, and the phosphorylation state of SHIP1 upon IL-10 stimulation. We could not observe any effect of IL-10 on altering the activation state of SHIP1. We next investigated IL-10's effect on the activation of Akt, a downstream molecule of PI3K, in lipopolysaccharide (LPS) activated macrophages. We demonstrated that IL-10 inhibited phosphorylation of Akt in macrophages from C57BL/6 mice but not macrophages from Balb/C mice. Lastly, we investigated the roles of SHIP1 and STAT3 in IL-10's inhibition of TNFα protein. We found that the TNFα production profile in SHIP1^{-/-} and STAT3^{-/-} cells were extremely similar. Closer examination showed that SHIP1messenger RNA (mRNA) expression

was significantly reduced in STAT3 knock out (-/-) macrophages. Although this work failed to demonstrate some of the observations obtained in cell lines, it shows the significance of genetic background of the cells used in experiments. It also suggests that STAT3-/- macrophages' unresponsiveness to IL-10 may due to the lower SHIP1 level in these cells, indicating a potentially important role of SHIP1 in IL-10's anti-inflammatory properties.

Preface

Design of all experiments and analysis of all research data were completed under the supervision of Dr. Alice Mui.

All experiments were performed by the author with the following exceptions:

Primary cells harvesting from mice was performed with the assistance of Dr. Alice Mui and Sylvia Cheung.

Immunoblotting was performed with the assistance of Alix Thomson (Figures 6-9).

Continuous Flow Cell culture experiments were performed by Joanna Chen (Figure 12).

Quantitative PCR analysis of SHIP1 mRNA expression in STAT3^{+/+} and STAT3^{-/-} bone marrow derived macrophages (BMDM) were performed with the assistance of Sylvia Cheung.

Appendices:

Data and Figures in Appendix A were provided courtesy of Dr. Ali Ghanipour.

Data and Figures in Appendix B were provided courtesy of Drs. Andrew Ming-Lum and Alice Mui.

Publication:

The data presented in the thesis are part of the following manuscript that is currently in preparation:

Cheung ST*Ming-Lum A*, **So EY**, McCarrell E, Chan CS, Chang T, Ghanipour A, Qasimi P, Sly LM, Chung SW, Anderson RJ, Ong CJ, Krystal G, Mui ALF. Interleukin 10 triggers rapid anti-inflammatory effects through SHIP1.

(* co-first authors)

Ethics Approval:

All animal experiments performed under the following protocols as approved by the UBC Animal Care Committee:

SHIP1^{-/-} and STAT3^{-/-} Mouse Colonies: A06-0336

Table of Contents

Abstract	ii
Preface	iv
Table of Contents	vi
List of Figures	ix
List of Abbreviations	x
1 Introduction	1
1.1 Inflammation	1
1.2 Macrophages	2
1.2.1 Activation of macrophages	3
1.3 Interleukin-10	4
1.3.1 IL-10 signalling pathway	5
1.3.2 IL-10 regulation of macrophages	6
1.3.3 IL-10 in diseases	6
1.4 LPS/TLR4 signalling pathway	7
1.4.1 MyD88 dependent and independent pathways	8
1.5 TNFα	10
1.5.1 TNFα signalling pathway	11
1.6 PI3K pathway	11
1.6.1 Protein kinase B/Akt	14
1.6.2 Targets of Akt	14
1.7 SH2-containing inositol-5'-phosphatase 1 (SHIP1)	15
1.7.1 Regulation of SHIP1 expression	17
1.7.2 SHIP1 in disease	18
1.8 Linking IL-10, STAT3 and SHIP1: Previous results	18
1.8.1 IL-10 regulation of PI3K pathway	19
1.8.2 Relative contributions of SHIP1 and STAT3 in IL-10 signalling pathway	20
1.9 Hypothesis and objectives	23

2. Materials and methods	24
2.1 Mouse colonies	24
2.2 Generation of bone marrow derived macrophage and peritoneal macrophages	24
2.3 Antibodies and drugs	25
2.4 SHIP1 and IL-10R immunoprecipitation	25
2.5 IL-10R peptide pull down assay	26
2.6 Immunofluorescence	27
2.7 Immunoblotting and analysis	28
2.8 RNA extraction, DNase treatment, cDNA synthesis and quantitative PCR	28
2.9 Continuous flow cell stimulation	30
2.10 TNFα ELISA	31
2.11 Statistical analyse	32
3. Results	33
3.1 IL-10R interaction with SHIP1	33
3.2 IL-10 induction of translocation of SHIP1	35
3.3 IL-10 induction of phosphorylation of SHIP1	35
3.4 IL-10 effects on phosphorylation of Akt	38
3.4.1 Inhibition of pAkt: time course	38
3.4.2 Inhibition of pAkt: LPS concentration	38
3.4.3 Inhibition of pAkt: IL-10 concentration	40
3.4.4 Inhibition of pAkt: effect of pre-adding IL-10	40
3.4.5 Inhibition of pAkt: downstream molecules of PI3K pathway	43
3.4.6 Inhibition of phospho-Akt: cell type (perimac vs BMDM)	46
3.4.7 Inhibition of pAkt: genetic background of primary cells	46
3.5 TNFα production in static culture	49
3.6 TNFα production in continuous flow culture	51
3.7 SHIP1 expression in STAT3 ^{-/-} primary cells	54
4. Discussion	56
4.1 STAT3 independent IL-10 signalling pathway	56
4.2 SHIP1 in the IL-10 signalling pathway	57
4.3 Conclusion	64

References	65
Appendices	73

List of Figures

Figure 1 LPS signalling pathway	9
Figure 2 TNFα signalling pathway	2
Figure 3 The PI3K signalling pathway	3
Figure 4 Interaction of SHIP1 and IL-10R	4
Figure 5 Localization of SHIP1 upon IL-10 stimulation	6
Figure 6 IL-10's effect on phosphorylation of SHIP1	7
Figure 7 IL-10's effect on LPS induced phospho-Akt: optimization of stimulation condition 39	9
Figure 8 IL-10's effect on LPS induced phosphorylation of proteins in the PI3K pathway 45	5
Figure 9 IL-10's effect on LPS induced pAkt: effect of cell identity	8
Figure 10 SHIP1 and STAT3 mRNA and protein expression in SHIP1 ^{-/-} and STAT3 ^{-/-} peritoneal	
macrophages	О
Figure 11 IL-10 inhibition of TNF α in SHIP1 $^{+/+}$ / SHIP1 $^{-/-}$ and STAT3 $^{+/+}$ / STAT3 $^{-/-}$ BMDM in	ì
static culture	2
Figure 12 Both SHIP1 and STAT3 are required for IL-10 inhibition on the first peak of TNF α	
protein production in the continuous flow system	3
Figure 13 SHIP1 expression in STAT3 +/+ and STAT3 -/- primary cells	5

List of Abbreviations

ALL Acute lymphoblastic leukemia

AML Acute myeloid leukemia

AP-1 Activator protein 1

ATF-2 Activating transcription factor 2

ARE Adenylate-uridylate -rich elements

BCL-3 B-cell lymphoma 3-encoded protein

BMDM Bone marrow derived macrophages

BSA Bovine serum albumin

CD Cluster of differentiation

C/EBP CCAAT/enhancer binding protein

ChIP Chromatin immunoprecipitation

CNBr Cyanogen bromide

CSF Colony stimulating factor

DAMP Danger associated molecular pattern

DEPC Diethyl pyrocarbonate

DMEM Dulbecco's modified eagle medium

Dok Docking protein

Dox Doxycycline

ETV3 Ets variant 3

FADD Fas-associated death domain

FC γ R FC γ receptor

FCS Fetal calf serum

FOXO Forkhead box transcription factor

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GFP Green fluorescent protein

Grb2 Growth factor receptor-bound protein 2

GSK3 Glycogen synthase kinase 3

IBD Inflammatory bowel disease

ICAM Intercellular adhesion molecule 1

IFNγ Interferony

IKK Inhibitor of κB kinase

IL-1RA IL-1 receptor agonist

IL-10 Interleukin-10

IL-10R Interleukin-10 receptor

IMDM Iscove's modified Dulbecco's medium

IRAK4 IL-1 receptor associated kinase 4

ITAM Immunoreceptor tyrosine based activation motif

ITIM Immunoreceptor tyrosine based inhibitory motif

Jak1 Janus kinase 1

JNK c-Jun N-terminal kinase

KD Knock down

KO Knockout

KSRP KH-type splicing regulatory protein

LBP Lipopolysaccharide binding protein

LPS Lipopolysaccharide

MAP3K MAP kinase kinase kinase

MAPK Mitogen activated protein kinase

Mal MyD88-adaptor-like

MCP-1 Monocyte chemotactic protein 1

M-CSF-1 Macrophage colony stimulating factor

MHC Major histocompatibility complex

MIP-1 Macrophage inflammatory protein 1

miRNA Micro RNA

MNK-1 MAP kinase signal integrating kinase-1

mTor Mammalian target of rapamycin

MyD88 Myeloid differentiation primary response gene (88)

NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells

NLR Nod-like receptor

PAMP Pathogen associated molecular pattern

PBS Phosphate buffered saline

PDK1 3 phosphoinositide-dependent kinase-1

PH Pleckstrin homology

PI Phosphatidylinositol

PI3K Phosphoinositide 3'-kinase

PI(3,4)P₂ Phosphatidylinositol 3,4-biphosphate

PI(4,5)P₂ Phosphatidylinositol 4,5-biphosphate

PIP₃ Phosphatidylinositol 3,4,5-triphosphate

PKC Protein kinase C

perimac Peritoneal macrophages

PRR Proline rich region

PTEN Phosphatase and tensin homology

qPCR Quantitative polymerase chain reaction

RHEB Ras homolog enriched in brain

RIP1 receptor interacting protein 1

RNS Reactive nitrogen species

ROS Reactive oxygen species

SARM Selective androgen receptor modulators

s-SHIP1 Stem cell SHIP1

SBNO2 Strawberry notch homolog 2

SDS Sodium dodecyl sulphate

SGK1 serum-and glucocorticoid-induced kinase 1

SH2 Src homology 2

Shc Src homology 2 containing protein

SHIP1 SH2 domain containing inositol 5 photphatase

siRNA Small interfering RNA

SMAD Sma and Mad related protein

SOCS3 Suppressor of cytokine signalling 3

Sp1/3 Specificity protein 1/3

STAT Signal transducer and activator of transcription

TACE TNF α converting enzyme

TAB 1, 2, and 3 TAK-1 binding proteins 1, 2 and 3

TAK1 TGFβ-activated protein kinase 1

TBK1 TANK-binding kinase 1

TBS Tris-buffered saline

TRAF TNF receptor associated factor

TGF β Transforming growth factor β

TIA-1 T cell antigen-1

TIAR TIA-1-related protein

TIR Toll/IL-1R

TIRAP Toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TLR Toll-like receptor

TNF α Tumor necrosis factor α

TNFR TNF α receptor

TRADD TNFR associated death domain

TRAF TNFR associated factor

TRAM TRIF related adaptor molecule

TRIF TIR-domain-containing adaptor inducing interferon β

TSC2 Tuberous sclerosis complex 2

TTP Tristertrapolin

Tyk2 Tyrosine kinase 2

UTR Untranslated region

WGA Wheat-germ-agglutinin

1 Introduction

1.1 Inflammation

Inflammation was first described to be the combined symptoms of redness, swelling, heat and pain in the first century [1]. In the 19th century, a fifth symptom – disturbance of function-was added to the description of inflammation [2]. We now appreciate inflammation as a complex physiological response of the body to damaging stimuli such as pathogens, chemical irritants and tissue damage. The main function of inflammation is to combat and remove the damaging stimuli in order to protect the host and initiate the restoration of the damaged tissue [3].

Therefore inflammation is an important self-defence mechanism of the host and is generally a beneficial process. However, excessive and uncontrolled inflammation leads to tissue damage and might result in a number of pathologies including inflammatory bowel disease (IBD), atherosclerosis, and rheumatoid arthritis [3-6].

Stimuli that can trigger inflammation include allergens, pathogen associated molecular patterns (PAMPs) that are expressed on the surface of microbes [7, 8] and damage associated molecular patterns (DAMPs) that are molecules released by damaged/dying cells to the extracellular environment [9, 10]. When mast cells and macrophages residing in the tissues encounter these stimuli, they become activated and release a number of soluble proinflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), macrophage inflammatory protein1 (MIP1), histamine, prostaglandins, and reactive oxygen and nitrogen species (ROS, RNS) [1, 11]. The release of these mediators causes vasodilation and increases permeability of the local blood vessels, which results in increased blood flow to the affected local region causing redness, swelling, and heat. These molecules also increase the expression of adhesion molecules on the surface of the endothelial cells lining the

blood vessels. As a result, circulating neutrophils that come in contact with the endothelial cells are slowed down and migrate through the endothelial wall to the affected site [12, 13].

Leukocytes migrate according to the chemotactic gradient such that they move toward the region with the highest chemokine concentration [13]. The recruited neutrophils are then activated by the pro-inflammatory mediators at the affected site and in turn produce more pro-inflammatory mediators and cytotoxic reagents in order to eliminate the invading pathogens [1]. If the neutrophils cannot clear the pathogens and the inflammatory responses persist, then other leukocytes such as macrophages and T-cells are recruited to the affected site to aid in removing the invading pathogens. After the pro-inflammatory stimuli have been eliminated, the inflammatory response is switched to the repairing mode, which initiates the clearance of damaged cells/debris and the repairing of damaged tissue [3].

Although inflammation is generally a beneficial process that aims to remove dangerous stimuli and protect the host, a number of the products that are released to destroy pathogens are also damaging to host's tissue and cells. Therefore inflammation has to be tightly regulated and quickly terminated to prevent unwanted side-effects. One of the main mechanisms to regulate inflammation is through the release of anti-inflammatory molecules such as interleukin-10 (IL-10), transforming growth factor- β (TGF- β), and glucocorticoids [14, 15]. These anti-inflammatory molecules down-regulate inflammation by reducing the production of pro-inflammatory mediators, deactivating the recruited leukocytes, and switching the target cells (e.g. macrophages) from a pro-inflammatory state to an anti-inflammatory state.

1.2 Macrophages

Macrophages were first described as cells that engulf large particles. Engulfment of large particles, or phagocytosis, is one of the main roles of macrophages: phagocytosis of apoptotic

cells and old red blood cells maintain homeostasis in the body, while phagocytosis of invading microbes and pathogens is a central part of host defense and pathogen elimination [16].

Macrophages belong to the innate immune system and act as the bridge between the innate and adaptive immune system. After the phagocytosis of pathogens, macrophages present parts of the pathogens to activate cells in the adaptive immune system. Macrophages are derived from circulating monocytes, which are in turn derived from a common myeloid progenitor cell that can also differentiate into neutrophils [16, 17]. Residual macrophages are present in virtually all types of tissue in the body and form the first line of defense in those local areas. Macrophages are extremely heterogeneous and the phenotype of tissue-specific resident macrophages is influenced by the micro-environment in which they reside.

1.2.1 Activation of macrophages

Under normal circumstances, macrophages are in a "resting" state such that they do not produce pro-inflammatory cytokines but are still capable of phagocytosis. Resting macrophages are "classically" activated by PAMPs from pathogens and DAMPs. The toll-like receptors (TLRs) expressed on the surface of macrophages and Nod-like receptors (NLRs) expressed within macrophages are responsible for the detection of PAMPs and the activation of downstream signalling pathways [16, 18]. Classically activated macrophages, also commonly known as M1 macrophages, are pro-inflammatory and produce high levels of reactive oxygen and nitrogen species and pro-inflammatory cytokines such as $TNF\alpha$, IL-1, and IL-6. Also, these macrophages have increased expression of major histocompatibility complex II (MHC II) and co-stimulatory molecules CD80/CD86 and hence have enhanced antigen presenting ability [18-20]. Macrophages can also be "alternatively" activated by stimuli such as IL-4, IL-3, and TGF β . These alternatively activated macrophages are classified as the "healer" or M2 macrophages and

they are associated with the tissue healing/repairing process in inflammation. Unlike the M1 macrophages, M2 macrophages do not express high level of pro-inflammatory cytokines and ROS, and they do not have up-regulation of antigen presenting molecules. In contrast, M2 macrophages have enhanced endocytic activity and enhanced expression of the anti-inflammatory cytokine IL-10 [18-22].

1.3 Interleukin-10

Interleukin-10 was first discovered in 1989 as a factor produced by Th2 cells to inhibit Th1 cells' cytokine production [23]. Later, it was recognized that IL-10 could affect many immune cells other than the T cells and that it was, in fact, the main negative regulator of the immune system [24]. IL-10 is a glycosylated, 17 kDa protein with a structure that is composed of six α-helices. The two carboxyl-terminal helices are important for interacting with another IL-10 protein to form the active form of IL-10, a soluble, non-covalently linked homodimer.

Structurally, IL-10 belongs to the class 2 α-helical cytokine family [25, 26]. IL-10 is expressed by a number of immune cells and it can in turn affect these immune cells. Although IL-10 has a wide range of target cells, it is believed that the main target cells of IL-10 are the activated macrophages and dendritic cells since these cells express the highest amount of IL-10 and IL-10 receptor (IL-10R) upon stimulation [25-27]. IL-10 expression is mediated by transcription factors including specificity protein-1 (Sp-1) and 3 (Sp-3), CCAAT/enhancer binding protein (C/EBP) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [28-30].

IL-10 is a soluble factor that binds to a receptor complex composed of a ligand-binding subunit (IL-10R1, also known as IL-10R α in mice) [31] and a signalling subunit (IL-10R2, also known as IL-10R β in mice) [32]. IL-10R1 is expressed constitutively at low level in most hematopoietic cells and its expression is up-regulated by many inflammatory-stimuli, such as

lipopolysaccharide (LPS) stimulation [25, 33]. IL-10R2 is also expressed in many immune cell types but unlike IL-10R1, its expression is not affected by inflammatory stimuli [25, 32]. The broad range of cells that express the IL-10R complex support the idea that many immune cells can respond to IL-10, although the responsiveness differs depending on the level of IL-10R expression. IL-10R1 has a much higher binding affinity for IL-10 than IL-10R2, so IL-10R1 is responsible for most of the interaction between the receptor and IL-10 [25, 34], while IL-10R2 is necessary for the downstream IL-10 signalling pathway.

1.3.1 IL-10 signalling pathway

The Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) pathway has been regarded as the only signalling pathway that IL-10 acts through, and the activation of this pathway can account for all of IL-10's anti-inflammatory effects [35]. Upon IL-10 binding to the IL-10R, the Janus associated kinase Jak1 and Tyk2, which are constitutively associated with IL-10R1 and IL-10R2 respectively, become trans-phosphorylated and thus activated [36]. After the activation of these kinases, they phosphorylate residue Tyr446 and Tyr496 on the human IL-10R (Tyr427 and Tyr477 on the mouse IL-10R). These two phosphorylated tyrosine residues then can be bound by the Src homology 2 (SH2) domain of the transcription factor STAT3 [37], followed by the phosphorylation of STAT3 by the receptor associated kinases at residues Tyr705 and Ser727. Phosphorylation of Tyr705 facilitates the dimerization of STAT3 through the SH2 domain and phosphorylation of Ser727 enhances the transcriptional activity of STAT3 [38]. The dimerized STAT3 molecules dissociate from the IL-10R and translocate into the nucleus to bind to the STAT3 binding elements within the promoters of IL-10 responsive genes. These genes include anti-inflammatory genes such as Suppressor of cytokine signalling 3 (SOCS3), Strawberry notch homolog 2 (SBNO2) and Ets variant 3 (ETV3) [36, 39-42].

1.3.2 IL-10 regulation of macrophages

Since activated macrophages express a high level of IL-10R1, it is commonly believed that they are the immune cells that are most responsive to IL-10 and are the main target of IL-10. In activated macrophages, IL-10 represses the production of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12, TNFα, macrophage colony stimulating factor (M-CSF)) [43], chemokines (ie. monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1)) [44, 45] and the production of nitric oxide [46]. In addition, IL-10 depresses the expression of surface molecules on the macrophages including the MHC II and the co-stimulatory molecules CD80 and CD86, thereby reducing the antigen presenting ability of the activated macrophages [47]. IL-10 also alters activated macrophages' adhesion ability by down-regulating intercellular adhesion molecule 1 (ICAM-1), which is necessary for macrophages to establish cell to cell contact with endothelial cells [48]. Lastly, IL-10 up-regulates the production of a number of anti-inflammatory molecules such as the IL-1 receptor antagonist (IL-1RA) and soluble TNF receptor which bind to the respective pro-inflammatory cytokines and restricting their effect on other immune cells [49, 50].

1.3.3 IL-10 in diseases

Due to IL-10's importance in regulating inflammation and the immune system, defects that affect IL-10's normal functions are associated with inflammatory diseases. For example, both IL- $10^{-/-}$ and IL- $10R^{-/-}$ mice developed colitis spontaneously [51] and genome-wide studies had identified mutations in the IL-10 and IL-10R genes that correlate with increased susceptibility to IBD in human [52, 53]. IL-10 was shown to be protective in mouse endotoxemia experiments and had beneficial effects in treating rheumatoid arthritis and allergy [33]. Also, IL-10 was reported to inhibit allograft rejection after organ transplantation [54, 55]. These studies highlight

the importance of IL-10 in negatively regulating inflammation and autoimmune diseases. On the other hand, many pathogens have evolved to inhibit the host's immune system by utilizing IL-10. For example, *M. tuberculosis* can manipulate macrophages and dendritic cells to increase IL-10 production, which protect the pathogen from being eliminated by the immune system [56]. It has been reported that certain types of cancer are also associated with increased IL-10 production, which represses the immune system and enhances cancer cells' survival. Therefore it is important for IL-10 to maintain proper homeostasis since enhanced and defective IL-10 level both result in pathologies.

1.4 LPS/TLR4 signalling pathway

Macrophages detect PAMPs through TLRs expressed on the surface of the cells. To date, there are 11 TLRs identified in mammals that have different ligand specificity: TLR1, 2 and 6 recognize lipopeptides, TLR3 recognizes double stranded RNA, TLR4 recognizes LPS, TLR5 recognizes bacterial flagellin, TLR7 and 8 recognize GU rich single stranded RNA, and TLR9 recognizes unmethylated CpG DNA [57-59]. TLRs are classified as type-1 membrane proteins and possess a conserved toll/IL-1R (TIR) domain at the intracellular portion of the receptor. The TIR domain is responsible for protein-protein interaction between the TLRs and TIR domain containing adaptor proteins, such as Myeloid differentiation primary response gene (88) (MyD88), TIR-domain-containing adaptor inducing interferon β (TRIF), TRIF related adaptor molecule (TRAM), MyD88-adaptor-like (Mal) and Selective androgen receptor modulators (SARM). The TLRs interact with various combinations of these adaptor proteins to activate different downstream signalling pathways [57-59].

LPS is a component of the cell wall of Gram-negative bacteria and it is the ligand of TLR4. TLR4 requires additional proteins to facilitate the ligand binding. LPS binding protein (LBP) is a

serum lipid transferase that transfers LPS from the bacteria cell wall to CD14, a GPI-linked membrane protein on the surface of the macrophages. CD14 then facilitates the transfer of LPS to TLR4, which is associated with another membrane protein that aids in LPS presentation called MD2. The binding of LPS-LPB complex to the TLR-MD2 complex causes the dimerization of the TLR4 [60].

1.4.1 MyD88 dependent and independent pathways

There are two known pathways downstream of LPS/TLR4 binding: the MyD88 dependent and independent pathways (Figure 1). In the MyD88 dependent pathway, LPS binding induces the recruitment of the adaptor proteins TIR-domain containing adaptor protein (TIRAP) and MyD88 to TLR4. MyD88 contains a death domain, which is responsible for protein-protein interaction with another death domain containing protein called IL-1 receptor associated kinase 4 (IRAK4). IRAK4 is phosphorylated and activated once recruited to the membrane. Once activated, IRAK4 recruits and phosphorylates IRAK1 and 2. The activated IRAKs dissociate from the receptor and form a complex with tumor-necrosis-factor receptor associated factor 6 (TRAF6), which then activates the TGFβ-activated protein kinase 1 (TAK1) and TAK-1 binding proteins 1, 2 and 3 (TAB 1, 2 and 3). TAK1 is a MAP kinase kinase kinase (MAP3K) that activates kinases upstream of pathways such as NFκB, p38 and c-Jun N-terminal kinase (JNK). For example, TAK1 activates the nuclear factor κB kinase complex (IKK) α/γ , which then phosphorylates IkB thus promoting its degradation. The degradation of IkB would release NFkB and allows it to translocate to the nucleus to induce the transcription of pro-inflammatory genes [58, 59].

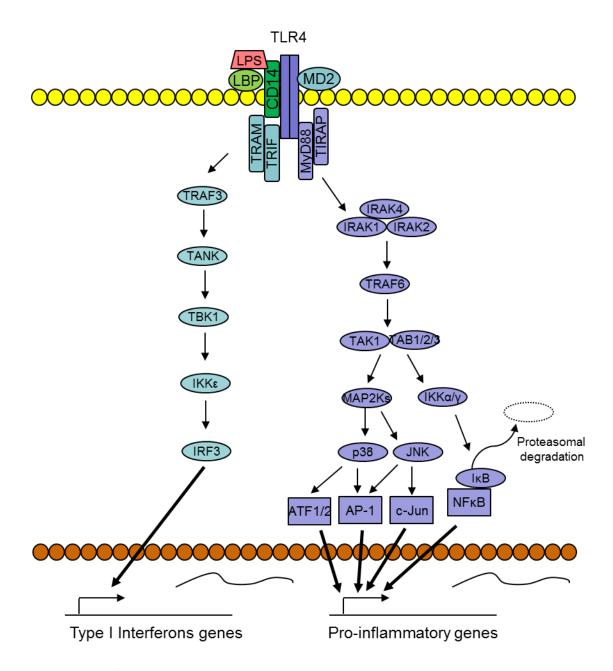


Figure 1 LPS signalling pathway

LPS-LBP complex is transferred by CD14 to the TLR4-MD2 complex. LPS binding initiates the MyD88 dependent and independent pathways. In the MyD88 dependent pathway, MyD88 and TIRAP are recruited to the receptor complex and activate the downstream signalling cascades resulting in the activation of p38, JNK and NFκB pathways and production of pro-inflammatory cytokines. In the MyD88 independent pathway, TRIF and TRAM are recruited to the TLR4 and initiate downstream signalling cascades leading to the activation of the IRF transcription factors and the production of type I interferons.

In the MyD88 independent pathway, LPS binding induces the association of TLR4 and TIRdomain containing adaptor inducing interferon β (TRIF) via the TRIF related adaptor molecule (TRAM). TRIF then activates receptor interacting protein 1 (RIP1), which in turn activates the IKK complex to drive NFκB. TRIF can also bind to TRAF3, which serves as an adaptor protein between TRIF, TANK, TANK-binding kinase 1 (TBK1) and IKKε. TANK, TBK1 and IKKε are kinases that phosphorylate the transcription factor interferon regulatory factor 3 (IRF3), which is responsible for the transcription of type I interferon genes [58, 59].

1.5 TNFα

TNF α was first discovered as a soluble factor in the serum of mice treated by LPS and was an inducer of hemorrhagic necrosis of tumors [61]. TNF α is one of the earliest genes that gets expressed after LPS stimulation of macrophages. Its mRNA can be detected within 30 minutes of stimulation and the protein can be detected in abundance within one hour of stimulation. Since its discovery, it has been a hallmark of inflammation [62, 63].

TNFα protein is initially synthesized as a 27 kDa membrane anchored protein. It is then cleaved by TNFα-converting enzyme (TACE) to produce the soluble 17 kDa mature protein [64]. The main producer cell of TNFα after LPS stimulation is the macrophage. LPS stimulation activates the TLR4 pathway, which leads to the activation of transcription factors such as NFκB and Activator protein 1 (AP-1) [65, 66]. These transcription factors then bind to the promoter region of the TNFα gene to initiate transcription. TNFα mRNA is also post-transcriptionally regulated by RNA binding proteins, such as tristertrapolin (TTP), KH-type splicing regulatory protein (KSRP) that interacts with the 5 ′ and 3 ′ untranslated region (UTR) of the mRNA, affecting mRNA stability and translation efficiency [67-70].

1.5.1 TNFα signalling pathway

TNF α signals through two receptors: TNF receptor 1 and 2 (TNFR1 and 2) (Figure 2). TNFR1 is expressed in almost all cell types and TNFR2 is mainly expressed on particular immune cells such as the macrophages [71]. TNFR1 contains a cytoplasmic death domain that can interact with TNFR1 associated death domain (TRADD) to initiate apoptosis [72]. Alternatively, TNF α binding to TNFR1 and TNFR2 recruits TNF receptor associated factors (TRAFs) to activate the NF κ B and MAPKs pathways, which promote cell survival and the production of pro-inflammatory cytokines, including TNF α itself [73]. The second wave of TNF α acts in an autocrine manner to stimulate the second wave of pro-inflammatory cytokines production.

1.6 PI3K pathway

The PI3 kinases (PI3K) are a family of kinases that phosphorylate the 3´-hydroxyl position of phosphatidylinositol (PI) lipids. The resulting products are secondary messengers of a number of signalling pathways that affect many cellular functions including cell proliferation, growth, motility, and activation (Figure 3). Dysregulation of the PI3K pathway has been implicated in pathologies such as cancer and inflammatory diseases [74].

To date there are 8 PI3K isoforms identified and they are divided into 3 classes according to their structures and substrate specificities. The class I PI3Ks are all heterodimers composed of the p110 catalytic subunits and the p85 or p101 regulatory subunit. The class I PI3Ks are subcategorized into classes IA and IB, in which class IA is activated by receptors with tyrosine kinase activity and class IB is activated by G-protein coupled receptors. Class IA includes p110 α , p110 β and p110 δ and they all share a common regulatory subunit p85. Class IB only has one member, p110 γ , and the catalytic subunit is associated with the regulatory subunit p101 [74-78].

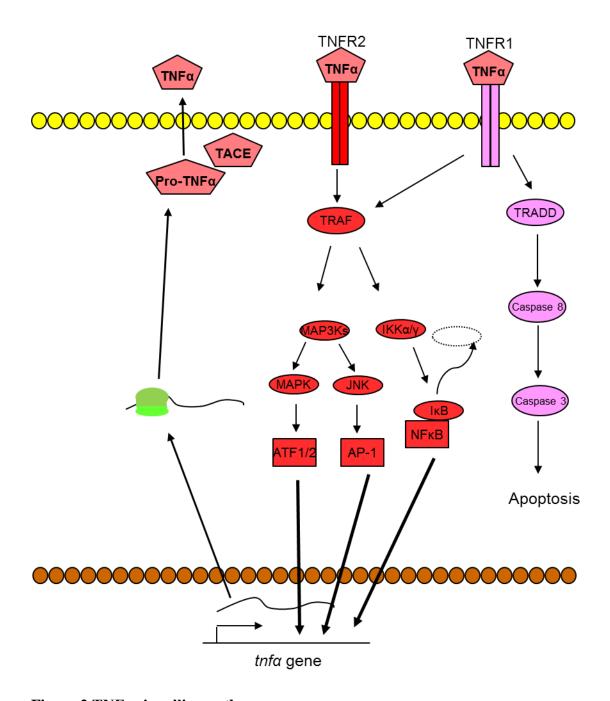


Figure 2 TNFα signalling pathway

Stimulus such as LPS binding to TLR4 results in the production of pro-TNF α , which is cleaved into the mature TNF α by TACE. TNF α binding to TNFR1 leads to the recruitment of TRADD and results in the activation of the caspase pathway and apoptosis. TNF α binding to TNFR2 recruits TRAF2 which leads to the activation of the MAPK and NF κ B pathways. Activation of these pathways results in the production of pro-inflammatory cytokines, including TNF α itself, and promotes cell survival.

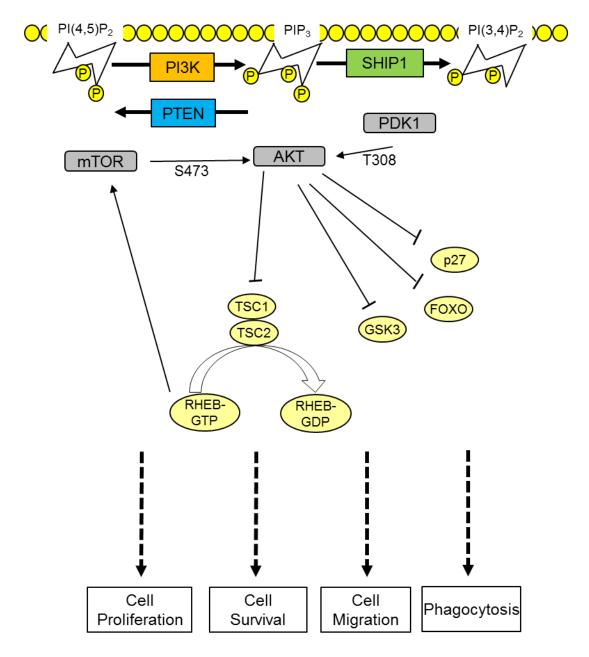


Figure 3 The PI3K signalling pathway

PI(4,5)P₂ is phosphorylated by PI3K at the membrane to generate PIP₃, which recruits PH domain containing protein such as Akt. Akt is phosphorylated and activated by PDK1 and mTOR, leading to the downstream signalling cascades that ultimately activate in a number of cellular processes including cell proliferation, survival, migration and increased phagocytosis. PIP₃ is dephorphorylated by PTEN to form PI(4,5)P₂ or by SHIP1 to form PI(3,4)P₂, which antagonize the PI3K pathway.

1.6.1 Protein kinase B/Akt

Although PI3K can phosphorylate the 3 'position of different inositol lipids, the preferred substrate of class IA PI3Ks is PI(4,5)P₂. The product, PIP₃, then recruits proteins containing domains that interact with lipid, such as the pleckstrin homology (PH) domain. PH domains are found in more than 150 proteins to date and they bind phospholipids with high affinity. Some PH domains bind to PIP₃ more strongly than to other PIs. One example of a PH domain containing protein that binds to PIP₃ with higher affinity than other PIs is the Akt kinase, which also binds to PI(3,4)P₂ with high affinity [79-81]. Akt is a 57 kDa serine/threonine kinase and is commonly referred as protein kinase B (PKB) because it was first identified as a kinase that has high homology with protein kinases A and C [82]. Akt has three isoforms $(\alpha, \beta, \text{ and } \gamma)$ in mammalian cells. In mice, the a isoform mediates signalling pathways that promote cell survival and proliferation, the β isoform is associated with insulin-related metabolism, and the γ isoform is involved with controlling cell size [83]. All isoforms consist of a N-terminal PH domain, a central kinase domain and a C-terminal regulatory domain. Akt is essentially cytoplasmic in resting cells and it translocates to the cell membrane upon stimulation. At the cell membrane, Akt is first phosphorylated at residue Thr308 located in the active site by 3'phosphoinositidedependent kinase-1 (PDK1), a Ser/Thr kinase that also binds to PIP₃. Then Ser473 in the Cterminal regulatory domain of Akt is phosphorylated by several possible kinases, one of which is mammalian target of rapamycin (mTOR) when mTORC2 is associated with Rictor [84]. Phosphorylation of both sites is required for full activation of Akt [85].

1.6.2 Targets of Akt

Akt can phosphorylate a number of proteins, leading to their activation. The activation of these proteins ultimately results in enhanced cell survival/proliferation. These proteins include

p27, the forkhead box transcription factor (FOXO), tuberous sclerosis complex 2 (TSC2), glycogen synthase kinase 3 (GSK3), and serum-and glucocorticoid-induced kinase 1 (SGK1)[86, 87], p27 is a cell-cycle inhibitor and phosphorylation of p27 leads to its inactivation and promotion of cell cycle entry. FOXO is a transcription factor that is responsible for the transcription of p27, as well as another cell-cycle inhibitor cyclin G2 and the pro-apoptotic molecule BIM. When FOXO is phosphorylated by Akt, it translocates from the nucleus to the cytoplasm thus it cannot regulate transcription of the cell-cycle inhibitor and pro-apoptotic genes. Unphosphorylated TSC2 heterodimerizes with TSC1 and the dimerized complex acts as a Ras homolog enriched in brain (RHEB) -GTPase activating protein. GTP-bound RHEB activates the kinase TOR, which is a nutrient sensor and the target of rapamycin. Akt phosphorylates TSC2, which inactivates the TSC2-TSC1 complex and thus increases the amount of RHEB-GTP and the subsequent activation of TOR [86, 87]. GSK3 is a serine/threonine kinase that has more than 50 substrates, allowing it to regulate many cellular processes. GSK3 exists as two isoforms, GSK3α and GSK3β. Both isoforms can be inhibited by phosphorylation (Ser21 of GSK3α and Ser9 of GSK3β) by Akt [88].

1.7 SH2-containing inositol-5'-phosphatase 1 (SHIP1)

Since the PI3K pathway has very broad and profound effects on cells, it must be tightly regulated and terminated shortly after its activation. A major mechanism to antagonize the pathway is through the degradation of PIP₃. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is the phosphatase that directly opposes PI3K activity by removing the 3 phosphate from PIP3 to generate PI(4,5)P₂, the original substrate of PI3K. On the other hand, SH2-domain containing inositol 5 phosphatase 1 and 2 (SHIP1 and SHIP2) convert PIP₃ into PI(3,4)P₂. PTEN and SHIP2 are expressed ubiquitously but SHIP1 is expressed predominantly in

hematopoietic cells. The restricted expression of SHIP1 makes it an attractive therapeutic target since altering its activity would not have a global effect in the body [89].

SHIP1 is a 145 kDa protein with a N-terminal SH2 domain, a PH-related domain (PH-R), a centrally located 5 phosphatase followed by a C2 domain, and a proline-rich region (PRR) containing two NPXY motifs at the C-terminus [90, 91]. The SH2 domain is important for SHIP1's association with tyrosine-phosphorylated proteins such as FcyRIIa and FcyRIIb through the immune receptor tyrosine based-activating motif (ITAM) and the immune receptor tyrosine based-inhibiting motif (ITIM) respectively [92-94]. The PRR is also responsible for SHIP1's interaction with a subset of SH3 domain containing proteins. Src homology 2 containing protein (Shc), doking protein (Doks) 1 and 2, growth factor receptor-bound protein 2 (Grb2) and p85α subunit of PI3K are proteins that are capable to bind to SHIP1's PRR when the NPXY motifs are phosphorylated [95, 96]. The PH-R domain is important in SHIP1's ability to interact with lipid and it has been shown that mutation in the PH-R domain reduced SHIP1's translocation to the cell membrane upon FcyR-dependent phagocytosis[91]. The C2 domain of SHIP1 binds to PI(3-4)P₂, the product of SHIP1, and such binding allosterically activates SHIP1's enzymatic activity, which suggests the C2 domain is important for the regulation and activation of the phosphatase [91].

Beside the 145 kDa full length protein, SHIP1 has several isoforms that are generated from alternate splicing [97]. Studies have shown a 135 kDa SHIP1 isoform that is co-expressed with the full length SHIP1. Similar to the 145 kDa SHIP1, this 135 kDa isoform is tyrosine phosphorylated by M-CSF-1 stimulation and it associates with Shc and Grb2. However, since the spliced isoform lacks the binding site for the PI3K subunit p85, it has weaker interaction with p85 than the full length SHIP1 [98]. In embryo, full length SHIP1 is not expressed but a 104 kDa

stem-cell specific SHIP1 (s-SHIP1) is encoded from an internal promoter between exon 5 and 6. It is believed that s-SHIP1 plays an important role in the development and survival of pluripotent stem cells [99].

1.7.1 Regulation of SHIP1 expression

SHIP1's expression is controlled at several levels. At the transcriptional level, SHIP1 has been described to be regulated by the Sma and Mad related protein (SMAD) transcription factors [100]. TGFβ and activin, which are negative regulators of immune cells proliferation, strongly induce the expression of SHIP1 mRNA and protein [100]. At the post-transcriptional level, SHIP1 is a known target of the pro-inflammatory micro-RNA-155 (miR-155), which controls the stability of the SHIP1 mRNA [101]. It is worth noting that IL-10 inhibits miR-155 expression and this inhibition is SHIP1 and STAT3 dependent [102, 103]. This suggests that SHIP1 and miR-155 regulate each other bi-directionally and creates a fine balance between pro- or anti-inflammatory status in the host. On the protein level, the turn-over rate of the SHIP1 protein is relatively slow (more than 24 hours) but the level of SHIP1 protein can be controlled by proteasomal degradation [104].

Since the PI3K pathway induces cells proliferation and survival, it has been suggested that the PI3K negative regulator SHIP1 plays an important role in regulating macrophages proliferation [80, 105]. Studies have shown that SHIP1 can inhibit the activation of Akt as well as transcription factors such as NFκB by decreasing the level of PIP₃ [106, 107]. The phosphatase activity of SHIP1 remains constant upon cytokine stimulation as well as the phosphorylation of the tyrosine residues at the NPXY motifs [108, 109]. Therefore it is commonly believed that SHIP1's activity is dictated by its physical location and that it exerts its phosphatase function by translocating to sites containing PIP₃ [105, 109].

1.7.2 SHIP1 in disease

Much of the understanding of SHIP1's role in immune cells comes from studying the SHIP1-/- mice [110, 111]. The phenotypes of SHIP1-/- mice include splenomegaly, a Paget's like osteroporosis, shortened life-span, and an asthma-like symptom. Furthermore, SHIP1-/- mice are hypersensitive to immune stimulation. For example, they are significantly more susceptible to endotoxin shock and their macrophages produce a much higher level of many pro-inflammatory cytokines during LPS challenge. It has also be found that SHIP1-/- macrophages are skewed [112] toward the alternatively activated M2 phenotype.

In humans, heterozygosity at the chromosomal location of SHIP1 (chromosome 2q36) is associated with a subset of patients with familial Paget-like osteoporosis [113]. Moreover, mutation in SHIP1 has been reported to be in the blast cells of acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) patients [114, 115]. These studies in leukemic patients suggest that SHIP1 functions as a tumour suppressor, likely via the inhibition of the PI3K pathway.

1.8 Linking IL-10, STAT3 and SHIP1: Previous results

IL-10 is the major anti-inflammatory cytokine and one of its mechanisms on regulating inflammation is through inhibiting the production of pro-inflammatory cytokines. Studies have shown that IL-10 reduces TNFα production transcriptionally as well as post-transcriptionally [70, 116]. IL-10 binds to IL-10R to activate STAT3 and results in the production of STAT3 regulated gene products such as SOCS3, Bcl-3, ETV3 and SBNO2 [36, 39-42]. SOCS3 has been reported to inhibit the TLR4 signalling pathway which directly suppresses LPS induced TNFα production [41]. On the other hand, Bcl3, ETV3 and SBNO2 are repressors of transcription and are capable of interfering with TNFα transcription [39, 117]. Post-transcriptionally, TNFα mRNA contains

AU-rich elements (ARE) at the 3 UTR, which target the mRNA for degradation [118]. IL-10 reduces the stability of TNF α mRNA by inhibiting the stabilizing effect of ARE-binding proteins such as T cell antigen-1 (TIA-1), TIA-1-related protein (TIAR), human antigen R (HuR) [119] through targeting the p38 MAPK pathway. Also, IL-10 induces the expression of TTP, which binds to the ARE of TNF α mRNA to promote the mRNA degradation, in a STAT3 dependent manner [120]. Recently, it has been shown that IL-10 decreases TNF α translation by shifting the mRNA from associating with poly-ribosomes to mono-ribosome. This shifting mechanism appears to be SHIP1 dependent and involves the inhibition of MAP kinase signal integrating kinase-1 (MNK-1) [121].

It is well established that the PI3K pathway has important implications in the immune system since it can affect a large number of cellular pathways. However, there is contradicting evidence on PI3K involvement in the cellular pathways, which lead to the debate of whether the PI3K pathway is pro-inflammatory or anti-inflammatory [122-125].

1.8.1 IL-10 regulation of PI3K pathway

Our lab is interested in investigating the role of IL-10 in the LPS-activated PI3K pathway. It has been shown previously in our lab that in macrophages that were stimulated with LPS and IL-10, the level of PIP₃ was dramatically decreased compared with cells that were stimulated with LPS alone (Appendix A). Also, the level of PI(3,4)P₂ was increased in the cells that were stimulated with LPS and IL-10 compared to the cells that were stimulated with LPS alone (Appendix A). This initial result indicated that LPS induced the production of PIP₃ while IL-10 caused PIP₃ dephosphorylation to generate PI(3,4)P₂, and suggested that a 5 ´ inositol phosphatase was responsible for this dephosphorylation (Appendix A). Therefore we speculated that IL-10 might activate SHIP1, a 5 ´phosphatase that acts as a negative regulator of PI3K in

macrophages. We found that in a macrophages cell line, IL-10 treatment led to the phosphorylation of SHIP1 on a tyrosine residue and this phosphorylation was dependent on the Tyr446 and 496 residues on IL-10R (Appendix A). SHIP1 only interacted with the phosphorylated form of these two tyrosine residues, but not the unphosphorylated form (Appendix A). These previous results indicated that IL-10 can activate SHIP1, and that activated SHIP1 is recruited to active IL-10R in cell lines.

Our lab continued to examine whether IL-10 activation of SHIP1 may affect the downstream signalling events of the PI3K pathway. The phosphorylation state of Akt on Ser473 residue was measured in RAW 264.7 cells after LPS +/- IL-10 stimulation. LPS stimulation induced phosphorylation of Akt and the addition of IL-10 reduced the phosphorylation. Next, the production of TNF α protein was used as an indicator of the PI3K pathway activity. RAW 264.7 macrophages were treated with LPS +/- IL-10 and supernatant was collected for TNF α protein measurement. Similar to the observation seen in phosphorylation of Akt, LPS induced the production of TNF α protein whereas IL-10 inhibited its production. These results suggested that IL-10 can negatively regulate the PI3K pathway (Appendix B).

1.8.2 Relative contributions of SHIP1 and STAT3 in IL-10 signalling pathway

The transcription factor STAT3 has been suggested to be the only signalling pathway that IL-10 utilizes. But the results from our lab suggest the possibility that SHIP1 might be the key molecule in a novel, STAT3-independent arm of the IL-10 signaling pathway [121]. To determine the relative contribution of STAT3 and SHIP1 in IL-10's inhibition on LPS induced TNFα protein production, we generated RAW264.7 cell lines in which STAT3 and SHIP1 protein were knocked down by RNA interference. siRNA sequences that target SHIP1, STAT3 or a scrambled sequence were cloned into a lentiviral vector in which the siRNA were expressed

under the control of a doxycycline (Dox)-dependent promoter. The addition of Dox for 48 hours resulted in >95% of SHIP1 knock down and around 50% of STAT3 knock down. It was not possible to achieve a larger reduction of the STAT3 protein probably because STAT3 is necessary for the survival of these cells. After the establishment of the stably siRNA transduced cells, they were treated with Dox for 48 hours. After the removal of Dox, the cells were stimulated with LPS +/- IL-10 and supernatant was collected at 1, 2 and 24 hours after stimulation. The TNFα protein levels in the LPS+IL-10 culture were calculated relative to the TNFα protein level in the parallel LPS alone cultures and were expressed as percentage. We found that IL-10 inhibited the LPS-induced TNFα protein production in parental and Scrambled RAW cells at a similar degree at all time points. STAT3 knock down (STAT3 KD) cells responded to IL-10 similarly to parental and Scrambled cells at 1 hour, but they were resistant to IL-10 at 2 hour and 24 hours. The SHIP1 knock down (SHIP1 KD) cells responded to LPS+IL-10 stimulation differently from the STAT3 KD cells, which were resistant to IL-10 at 1 and 2 hours, but responded similarly to parental cells and Scrambled cells at 24 hours. These data suggest that SHIP1 and STAT3 are responsible for different phases of the IL-10 signaling pathway: SHIP1 is required for the early inhibitory effect of IL-10 (such as at 1 hour) whereas STAT3 is required for the later time point (such as 24 hours) (Appendix B).

The major disadvantage of utilizing a system in which the same stimulation solution remains with the cells for the entire duration of the experiment is that the stimulation results in the production of cytokine, and the accumulation of cytokines might cause secondary responses in the cells and confound the results. Therefore, we developed the continuous-flow culture to overcome the intrinsic confounding variable of the static culture. In the continuous-flow culture, the stimulating media are pumped into the wells while the supernatant from the wells is removed

at the same time. This allows the collection of the supernatant over a continuous period of time and mimics the actual physiological environment more closely than the static culture system.

In the continuous flow cell system, LPS stimulation induced two peaks of TNFα production in all of parental, Scrambled, SHIP1 KD and STAT3 KD cells. The first peak appeared at about 50 minutes and the second peak appeared at about 110 minutes. IL-10 effectively inhibited both peaks in parental and Scrambled cells but failed to inhibit the first peak in SHIP1 knock down cells. For STAT3 KD cells, the first peak was inhibited by IL-10 in a similar way as the parental and Scrambled cells; but unlike the parental and Scrambled cells, the second peak of STAT3 KD cells was not completely inhibited by IL-10. These data suggested that LPS induced TNFα protein in a bi-phasic manner. Consistent with the static culture data, SHIP1 is responsible for the first phase (early time point) of IL-10's inhibitory effect. The effect of STAT3 deficiency on the two phases was not as clear as the SHIP1 knock down. This is most likely due to the fact that STAT3 KD was not complete and the remaining ~50% of STAT3 protein was enough to compensate for the IL-10 dependent STAT3 action in these cells (Appendix B).

Taken together, previous studies in our lab that used RAW 264.7 macrophages suggest that IL-10 stimulation activates and recruits SHIP1. LPS induced activation of PI3K is reduced by IL-10 which might be dependent on the IL-10 activation of SHIP1. Also, the early anti-inflammatory effect of IL-10, such as the inhibition of TNF α protein expression, is SHIP1 dependent, and the late anti-inflammatory effect of IL-10 is STAT3 dependent.

1.9 Hypothesis and objectives

The overall objective of the thesis is to investigate the role of SHIP1 in the IL-10 signalling pathway in LPS activated primary macrophages and the molecular mechanisms that regulate SHIP1 activity. The conclusions that were drawn regarding the relationship between IL-10 and SHIP1 were based on experiments done in the RAW 264.7 macrophage cell line. In order to investigate these conclusions in a more physiologically relevant setting, my main goal was to validate the observations in murine primary cells (either peritoneal macrophages [perimac] or bone marrow derived macrophages [BMDM]). We hypothesized that results similar to those in cell lines would be obtained in primary cells, in which 1) IL-10 activates SHIP1 by recruiting the enzyme to the IL-10R; 2) SHIP1 is the key signalling molecule in one arm of the IL-10 signalling pathway that is STAT3 independent; 3) SHIP1's anti-inflammatory activities are in part through the inhibition of the PI3K pathway and SHIP1's pathway is earlier than the STAT3 dependent pathway.

2. Materials and methods

2.1 Mouse colonies

SHIP1^{-/-} mice backcrossed 3 times to Balb/C mice were kindly provided by Dr. Gerald Krystal (BC Cancer Research Centre, Vancouver, B.C.), and further backcrossed to the F6 generation. C57Bl/6 STAT3^{flox/flox} mice were purchased from Dr. S. Akira (Hyogo College of Medicine, Nishinomiya, Japan) and C57BL/6 LysMCre mice were purchased from Jackson Laboratory. STAT3^{flox/flox} mice were crossed with the LysMCre mice to generate offspring that were heterozygous on both alleles. The doubly heterozygous mice were then crossed with homozygous STAT3^{flox/flox} mice. The resultant offspring, which had the genotype of STAT3 flox/flox /LysMCre^{+/-}, were referred as the STAT3^{-/-} mice. The STAT3^{-/-} mice were crossed with the homozygous STAT3^{flox/flox} mice so that half of the progeny were STAT3^{-/-}, while the other half were homozygous STAT3^{flox/flox} mice. These F3 progenies were used for the experiments described in the study. All mice were maintained in accordance to the ethic protocols approved by the University of British Columbia Animal Care Committee.

2.2 Generation of bone marrow derived macrophage and peritoneal macrophages

Femurs and tibias were collected from mice and the bone marrow was flushed out by passing Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher Scientific, Nepean, ON), supplemented with 10% fetal calf serum (FCS, Fisher Scientific, Ottawa, ON), which had been heat inactivated by incubation at 56° C for 90 minutes, $10~\mu$ M β -mercaptoethanol (Sigma Aldrich, Oakville ON), $150~\mu$ M monothioglycolate, and 1 mM L-glutamine through a 26G needle (BD Scientific, Mississauga, ON). Extracted cells were cultured on a 10-cm tissue culture dish (Fisher Scientific, Ottawa, ON) for 2 hours. Non-adherent cells were collected and seeded at 9×10^6 cells per 10-cm tissue culture dish. Cells were left undisturbed for seven days before use in the

supplemented IMDM media with 5 ng/ml Colony Stimulating Factor-1 (CSF-1) (Stem Cell Technologies, Vancouver, BC). All cells were maintained at 37°C, 5% CO₂ and 95% humidity. Peritoneal macrophages from male and female mice, age 6-12 weeks, were extracted by peritoneal lavage using 5 ml of sterile phosphate buffered saline (PBS, Fisher Scientific, Ottawa, ON) and IMDM mixture (3:1 ratio mixture). Cells were then transferred to supplemented IMDM and 2.5×10^5 cells were plated per well in tissue culture treated 24-well plates. Cells were allowed to adhere for 4 hours at 37°C, 5% CO₂ and 95% humidity and non-adhered cells were removed prior to stimulation.

2.3 Antibodies and drugs

Primary antibodies used in the experiments include α-SHIP1 (P1C1) and α-IL-10R purchased from Santa Cruz (Dallas, Tx), α-STAT3 purchased from Upstate Biotechnology (Lake Placid, NY), α-phospho-Akt (pAkt) S473 (D9E), α-pPDK1, α-pIkBα, α-pGSK3 (37F11), α-p85 (19H8) purchased from Cell Signaling Technologies (Pickering, ON), α-actin (AC15) and α-vinculin (hVIN1)purchased from Sigma (Oakville ON). The secondary antibodies, including Alexa Fluor® 488 Goat Anti-Mouse IgG, Alexa Fluor® 680 Goat Anti-Rabbit IgG, and Alexa Fluor® 680 Goat Anti-Mouse IgG, were all purchased from Life Technology (Burlington, ON) unless otherwise stated. LY294002 was purchased from Cayman Technologies (Pickerington, OH).

2.4 SHIP1 and IL-10R immunoprecipitation

Seven to ten day old bone marrow derived macrophages (BMDM) cultured in 10-cm dish were stimulated by 100 ng/ml IL-10 (recombinant murine IL-10 expressed in 293T cells and purified to >95% purity). Cells were washed twice with 20 ml of PBS and lysed in 1 ml of Nonidet P-40 (NP-40) (Sigma Aldrich, Oakville ON) lysis buffer (50 mM HEPES, 2 mM EDTA,

1 mM NaVO4, 100 mM NaF, 50 mM NaPPi, 1% NP-40), supplemented with Complete Protease Inhibitor Cocktail (PIC, Roche Diagnostics, Laval, QC). Lysate was rocked at 4°C for 45 minutes and clarified by centrifugation at 12,000×g for 20 minutes. For SHIP1 immunoprecipitation, clarified lysates were incubated with 1 μg of α-SHIP1 (P1C1) (Santa Cruz, Dallas, Tx) antibody at 4°C for 2 hours. The lysate/antibody was then incubated with protein G agarose beads (Sigma Aldrich, Oakville ON) at 4°C for 2 hours. For IL-10R immunoprecipitation, the clarified lysate was incubated with 1 μg of α-IL-10R (37A5) (DNAX, Palo Alto, CA), which was conjugated to Cyanogen bromide (CNBr) activated agarose beads, at 4°C for 2 hours. The beads were then washed 3 times with 0.1% NP-40 in lysis buffer and resuspended in 100 μl of 2× Laemmli's buffer for both types of immunoprecipitation.

2.5 IL-10R peptide pull down assay

Streptavidin coupled Dynabeads® (0.2 mg) were blocked with 0.5% BSA at 23°C for 15 minutes. One of the two IL-10R biotinylated peptides (50 nM, peptide that contained either the Tyr427 or Tyr477 residue, and the peptides were either in the unphosprylated or phosphorylated form) were incubated with the blocked Dynabeads® in a final volume of 500 µl at room temperature for 1 hour (final peptide concentration: 100 µM, Tyr427 peptide sequence: TFQGYQKQTRWK, Tyr477 peptide sequence: LAAGYLKQESQG) (GenScript, Piscataway, NJ). The beads/peptide mixture was washed twice by 0.5% BSA in PBS and incubated with IL-10 stimulated BMDM cell lysate, which was prepared as described in the immunoprecipitation section, at 4°C for 4 hours. The beads were then washed 3 times with 0.1% NP-40 in lysis buffer and resuspended in 50 µl of 2× Laemmli's buffer.

2.6 Immunofluorescence

Coverslips (Fisher Scientific, Ottawa, ON) were sterilized by autoclaving. Sterile coverslips were placed in 24-well plates and coated with poly-L-lysine (Sigma Aldrich, Oakville ON) at 37°C overnight. The poly-L-lysine was removed and the coverslips were washed three times with PBS. 2.5×10⁵ BMDM per well were plated onto the dried coverslips and allowed to adhere at 37°C overnight, 5% CO₂, and 95% humidity. The cells were stimulated by 100 ng/ml IL-10 and were then washed twice by ice-cold PBS and incubated with 2 μg/ml of wheat-germagglutinin (WGA) conjugated to Alexa Fluor 550® (Life Technology, Burlington, ON) at 4°C for 10 minutes. Cells were washed three times with PBS, cross-linked with 500 µl of 4% paraformaldehyde at 37°C for 15 minutes, permeabilized by 500 µl of 0.5% saponin (Sigma Aldrich, Oakville ON) at 23°C for 10 minutes and blocked with 1% bovine serum albumin (BSA) (Sigma Aldrich, Burlington, ON) in Tris-buffered saline (TBS) at 23°C for 1 hour. Three PBS washes, with 1 ml of PBS per well for each wash, were included between each step. After blocking, the cells were probed with 5 μg/ml α-SHIP1 antibody at room temperature overnight. Primary antibody was removed by three PBS washes and the cells were incubated with α-MS Alexa Fluor 488® secondary antibody (10 µg/ml diluted in PBS) (Life Technology, Burlington, ON) for 1 hour at room temperature. The coverslips were washed three times with PBS and mounted on glass slide (Fisher Scientific, Ottawa, ON) using ProLong-Gold antifade-reagent® (Life Technology, Burlington, ON). The edge of the coverslip was sealed with clear nail polish after overnight curing. Images were taken using a Zeiss LSM78 confocal microscope equipped with a 63× oil immersion lens (Carl Zeiss, Oberkochen, Germany).

2.7 Immunoblotting and analysis

After cell lysate samples were prepared in 2× Laemmli's buffer [126], they were boiled for 3 minutes and loaded onto 7.5% or 10% SDS-polyacrylamide gels at constant current. Resolved proteins were transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Etobicoke ON, Canada) using a wet transfer apparatus, blocked with 3% BSA in Tris buffered saline (TBS) for 45 minutes at room temperature, and probed with primary antibodies that recognize specific protein or phospho-proteins overnight at room temperature. Membranes were washed three times with Tris-buffered saline supplemented with 0.1% Tween (TBS-T) for 5 minutes per wash and incubated with Alexa-Fluor 680® secondary antibodies (Life Technology, Burlington, ON) diluted 1:10,000 in TBS-T for 60 minutes. Membranes were then washed three times with TBS-T for 5 minutes per wash and imaged using a Li-Cor Odyssey Infrared Imager (LI-COR biosceince, Lincoln NB, USA). Densitometru analysis was performed using the Image Studio software (LI-COR bioscience, Lincoln NB, USA) by measuring the integrated signal of each protein band and normalized to the integrated signal of an appropriate endogenous control protein band, which has a constant protein expression that is not affected by the treatment.

2.8 RNA extraction, DNase treatment, cDNA synthesis and quantitative PCR

Macrophages (2.5×10⁵ per well) were plated in 24 well plates and were allowed to adhere for 4 hours at 37°C, 5% CO₂, and 95% humidity prior to lysis using 200 μl of TRIZOL® Reagent (Life Technology, Burlington, ON). TRIZOL® dissolved cell lysate was collected into a 1.5 ml Eppendorf tube and 80 μl of chloroform was added. The two solutions were mixed vigorously for 15 seconds and underwent centrifugation for 15 minutes at 12000 rpm and 4°C. After centrifugation, 100 μl of the top aqueous solution was collected and transferred to a new

1.5 ml Eppendorf tube. Back extraction was then performed by added 250 μl of diethyl pyrocarbonate (DEPC) water to the TRIZOL® /chloroform containing tube. The tube underwent centrifugation for 15 minutes at 12000 rpm and 4°C. After the centrifugation, 250 μl of the top aqueous solution was combined with the 100 μl of clear aqueous solution in the new Eppendorf tube. Glycogen and sodium acetate were added to the tube to a final concentration of 100 μg/ml and 0.3 M respectively. 350 μl of isopropanol was then added to the tube and the tube was incubated at 23°C for 10 minutes, followed by a centrifugation for 10 minutes at 12000 rpm and 4°C. The supernatant was removed by aspiration and a RNA pellet was visible. 500 μl of 75% ethanol was added to the tube followed by a centrifugation for 5 minutes at 12000 rpm and 4°C. After aspirating the ethanol from the tube, the RNA pellet was allowed to dry at 23°C for 5 minutes and resuspended in 30 μl of DEPC treated water.

RNA (500 ng) was incubated with 10 units of DNaseI (Roche Diagnostics, Mississauga ON) in a total volume of 25 µl at 30°C for 30 minutes, followed by the addition of 2 µl of 0.1 M EDTA, and incubation at 75°C for 10 minutes to terminate the reaction. DNaseI treated RNA (120 ng) was used as template, and the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mississauga ON) was used for cDNA generation. Briefly, 50 pmol of oligo-dT primers was mixed with 6 ul of DPEC water and incubated at 65°C for 5 minutes. The primer was then mixed with the Triscriptor RT reaction buffer (5× concentrated), dNTP at a final concentration of 1 mM, 20 units of Protector RNase Inhibitor, 10 units of Transcriptor Reverse Transcriptase, and the DNaseI treated RNA. The mixture was incubated for 1 hour at 50°C, followed by 5 minutes incubation at 85°C.

The cDNA samples were diluted $5\times$ with DEPC water and 4 μ l was mixed with $1\times$ FastStart SYBR Green reagent (Roche Diagnostics, Mississauga ON), 30 μ M of forward and

reverse primers, and DEPC water in 15 μL total volume. Each sample was run in technical triplicates on a 96-well optical plate (Life Technology, Burlington, ON) using a StepOnePlusTM Real Time PCR apparatus (Life Technology, Burlington, ON). The primers used in the studies were: mGAPDH (Forward: AATGTGTCCGTCGTGGATCT, Reverse: GCTTCACCACCTTCTTGATGT), SHIP1 (Forward: GCCCCTGCATGGGAA ATCAA, Reverse: TGGGTAGCTGGTCATAACTCC) and STAT3 (Forward: CCTGAAGACCAAGTTC ATCTGTGTGAC, Reverse: AGACGATATGGGGTTCGGCT). Ct values of each sample were obtained using the StepOnePlusTM Real Time PCR software and the comparative Ct method was used for quantification.

2.9 Continuous flow cell stimulation

Peritoneal macrophages (perimac) or BMDM were plated at a density of 3×10^5 cells per well in 24 well plates that had been coated with poly-L-lysine for overnight at $37\,^{\circ}$ C. After 4 hours or overnight resting, culture media was removed and cells were equilibrated in Leibovitz's L-15 media (Life Technology, Burlington, ON) supplemented with 9% FCS, $10\,\mu\text{M}$ β -mercaptoethanol and 150 μ M monothioglycolate for 1 hour at $37\,^{\circ}$ C. After equilibration, cells were placed in a continuous flow cell apparatus in which the cells were covered by modified rubber stoppers with inlet and outlet lines. Stimulation media were passed through the inlet line over the cells by a syringe pump (New Era Syringe Pumps Inc., Farmingdale, NY) at a constant flow rate set at $150\,\mu\text{l/min}$. At the same time, an equal volume of supernatant in the well was removed at the same flow rate through the outlet line. The effluent was collected by a fraction collector at 5-minute intervals for 4 hours.

2.10 TNFa ELISA

2.5X10⁵ macrophages per well were plated in 24 well plates for either 4 hours or overnight. For stimulation, the cells were stimulated with 1 ng/ml LPS +/- IL-10 (ranged from 0.1 to 100 ng/ml IL-10) in a total volume of 500 μl. 100 μl of supernatant was removed from each well per hour up to four hours. Alternatively, supernatant from stimulated cells were collected using the flow cell apparatus as described above for up to 4 hours of stimulation. TNF α protein concentration was assayed by enzyme-linked immunosorbant assay (ELISA) using BD OptEIATM Mouse TNF ELISA Set II kit (BD Scientific, Mississauga, ON. 50 µl of the supernatant was loaded on 96 well plate (Sigma Aldrich, Oakville ON) that was precoated with the capture antibody (α -TNF α antibody, diluted 1:250× in 0.05 M carbonate/ bicarbonate buffer, pH 9.6) and the supernatant was incubated for overnight at 4°C. On the next day, the supernatant was removed and the plate was blocked with assay diluent (10% FCS in PBS) for one hour at 23°C. The blocking solution was washed away with 5× washes using the wash buffer (0.05% Tween in PBS). The plate was then incubated with the 50 µl of detection antibody (biotinylated α -TNF α antibody, diluted 1:250× in assay diluent) for 1 hour at 23°C. The detection antibody was removed by 5× washes using the wash buffer. 50 µl of streptavidin-HRP solution (diluted 1:250× in assay diluent) was then added to the plate and was incubated for 30 minutes at 23°C, followed by 7× washes using the wash buffer. The assay was developed by adding 50 μl of the 3,3', 5,5' tetramethyl benzidine solution (TMB, 0.005% TMB, 0.006% H₂O₂ in 0.01M Acetate Buffer and 0.05% Sodium Nitroferricyanide) and the reaction was stopped by added 25 µl of 2 M HCl. The plate was then read by the Epoch® Microplate Spectrophotometer at an absorbance of 450 nm.

2.11 Statistical analyse

All ANOVA and student-t test analysis were performed with GraphPad Prism 6 software.

3. Results

3.1 IL-10R interaction with SHIP1

IL-10R possesses tyrosine residues that can be phosphorylated upon IL-10 stimulation and STAT3 can interact with these residues via the SH2 domain. Therefore we decided to investigate the potential physical interaction between IL-10R and SHIP1, which also contains a SH2 domain. We first performed immunoprecipitations to determine the direct physical interaction of SHIP1 and IL-10R upon IL-10 stimulation in BMDMs. BMDMs were cultured in CSF-1 containing media for seven days and then they were stimulated with 100 ng/ml IL-10 for 10 or 20 minutes. α-SHIP1 antibody was used for the immunoprecipitation and the immunoblot was probed with α -IL-10R α antibody. The same blot was also probed with α -SHIP1 antibody to ensure that there was equal protein immunoprecipitated across the different samples. As shown in Figure 4A, there seems to be no detectable increased association between IL-10R with SHIP1 upon IL-10 stimulation. Alternatively, we tried to perform the immunopreipitation using α -IL- $10R\alpha$ antibody and probed the immunoblot with α -SHIP1 antibody. However, we failed to observe any association between IL-10R and SHIP1 with or without IL-10 stimulation (Figure 4B). We obtained different immunoprecipitation patterns of the SHIP1 and IL-10R protein from the two types of immunoprecipitations. Therefore, we utilized synthetic human IL-10R peptides that include the tyrosines 427 and 477 residues to perform a peptide pull down assay. Our laboratory had previously shown that the phosphorylated form but not the unphosphorylated form of these IL-10R peptides can interact with STAT3. We also observed similar results when we used the peptides to pull down SHIP1 in the RAW 264.7 cell line. However, the peptides pull

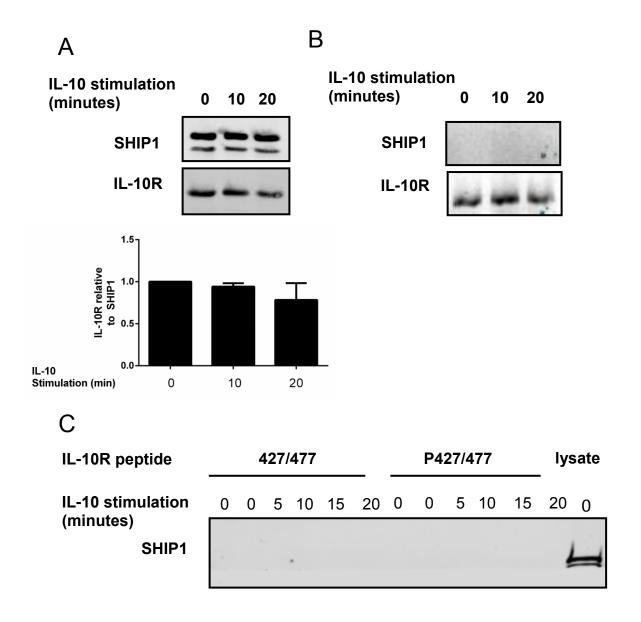


Figure 4 Interaction of SHIP1 and IL-10R

(A) Immunoblot analysis of SHIP1 immunoprecipitation of SHIP1 WT BMDM stimulated with IL-10 (100 ng/ml) for the indicated times, probed with anti-IL-10R α and SHIP1 (loading control). (B) Immunoblot analysis of IL-10R α immunoprecipitation of SHIP1 WT BMDM treated by IL-10 (100 ng/ml) for the indicated times, probed with anti-SHIP1 and IL-10R (loading control). (C) Immunoblot analysis of IL-10R α peptide pull down of SHIP1 WT BMDM stimulated with IL-10 (100 ng/ml) for the indicated times, probed with anti-SHIP1 antibody.

Down using cell lysates from IL-10 stimulated BMDM showed that both the phosphorylated and unphosphorylated peptides could not pull down detectable SHIP1 (Figure 4C).

3.2 IL-10 induction of translocation of SHIP1

It is widely accepted that SHIP1 translocates to the plasma membrane after its activation since its substrate PIP3 is located at the membrane [105, 109]. We reasoned that if IL-10 activates SHIP1, SHIP1 would translocate to either the IL-10R or the membrane. The localization of SHIP1 after IL-10 stimulation was determined by immunofluorescence. In Figure 5, plasma membrane was represented by Alexa Fluor® 550 conjugated wheat germ agglutinin (WGA) and the co-localization of SHIP1 and WGA was shown. The degree of co-localization was quantified and there was no IL-10 dependent SHIP1/WGA co-localization.

3.3 IL-10 induction of phosphorylation of SHIP1

The tyrosine phosphorylation of SHIP1 has commonly been attributed to its activation, although this notion remains unproven. Since we could not establish an increase in physical interaction between SHIP1 and IL-10R upon IL-10 treatment, we next investigated the effect of IL-10 on the tyrosine phosphorylation state of SHIP1. BMDMs were stimulated by 100 ng/ml IL-10 for the indicated time and the level of pSHIP (Tyr1020) was determined by immunoblotting. As shown in Figure 6, basal pTyr1020 SHIP1 was detected even in unstimulated BMDM. The level of the phosphorylation of this residue remained unchanged upon IL-10 stimulation.

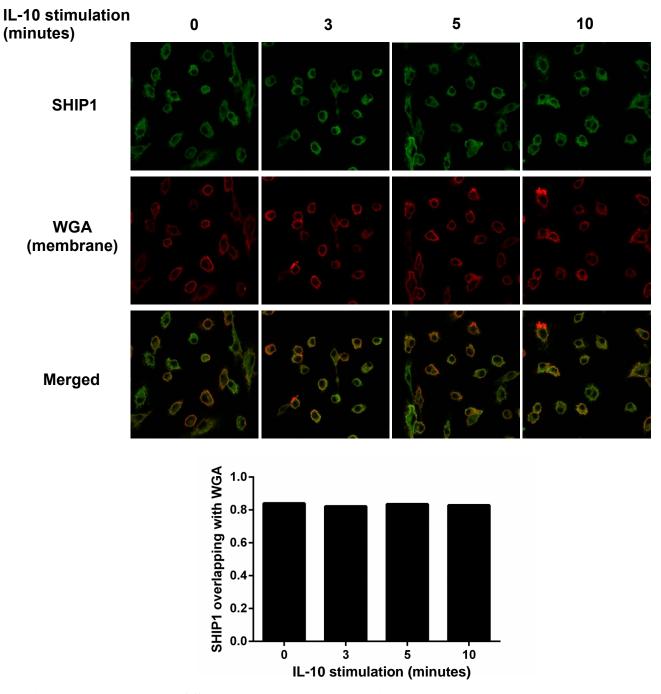


Figure 5 Localization of SHIP1 upon IL-10 stimulation

SHIP1 WT BMDM were treated with IL-10 (100 ng/ml) for the indicated times. Cells were incubated with Alexa Fluor® 550 conjugated WGA for 10 minutes (Red, middle panels), fixed, permeabilized, and stained with α -SHIP1 (P1C1) primary antibody followed by FITC labelled secondary antibody (Green, top panels). Merged images of the green and red signals are represented in the bottom panels.

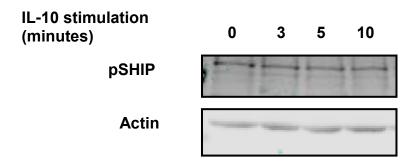


Figure 6 IL-10's effect on phosphorylation of SHIP1

Immunoblot analysis of SHIP1 WT BMDM treated by IL-10 (100 ng/ml) for the indicted times, probed with α -pSHIP1 (Tyr 1020) and actin (loading control)

3.4 IL-10 effects on phosphorylation of Akt

It was previously shown in our lab that addition of IL-10 could inhibit the phosphorylation of Akt in LPS activated RAW 264.7 macrophages. This suggests that IL-10 can inhibit the PI3K pathway. We also showed that IL-10 induces the production of PI-3,4-P₂ and activates SHIP1. Taken together, these data suggest that IL-10 activates SHIP1 to dephosphorylate PIP₃ which inhibits the PI3K pathway. My goal was to test for IL-10 inhibition of phospho-Akt in primary cells and to show the role of SHIP1 in the IL-10 signalling pathway by using SHIP1-/- primary cells.

3.4.1 Inhibition of pAkt: time course

We first wanted to determine IL-10's inhibition on pAkt in wild type BMDM using the same stimulation conditions as the ones used for stimulating RAW 264.7 macrophages. BMDM were stimulated with 1 ng/ml LPS +/- 100 ng/ml IL-10 for the indicated times and the immunoblot was probed with α-pAkt-Ser473 antibody. The same blot was also probed with vinculin as loading control. As shown in Figure 7A, phosphorylation of Akt could be detected as early as 10 minutes after LPS stimulation, peaked at around 30 minutes. This kinetic profile of the BMDM is different from the one of the RAW 264.7 cells. In the cell line, phosphorylated Akt was only detectable after 20 minutes, the signal was the strongest at 25 minutes and the amount of pAkt started to decline at 30 minutes. Quantification result shows that addition of IL-10 did not alter the phosphorylation intensity of Akt at all time points.

3.4.2 Inhibition of pAkt: LPS concentration

The LPS concentration used to stimulate RAW 264.7 cells might not be suitable for treating primary cells. Excess LPS might induce an over-excited pro-inflammatory response,

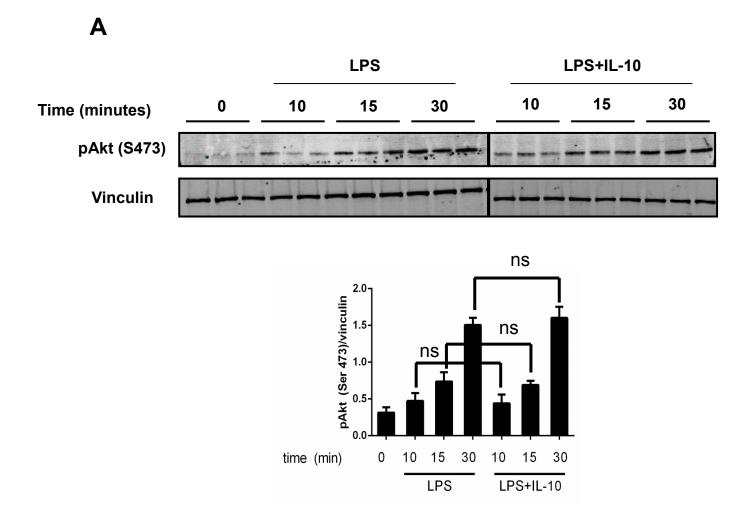


Figure 7 IL-10's effect on LPS induced phospho-Akt: optimization of stimulation condition

(A) Immunoblot analysis of SHIP1 WT BMDM stimulated with LPS (1 ng/ml) with or without IL-10 (100 ng/ml) for the indicated times, probed with α -pAkt (Ser 473) and vinculin (loading control). Cell lysate samples were run in triplicates on the blot. Bottom left panel is the quantification of each individual sample on the immunoblot after normalizing the pAkt signal to the vinculin signal. Bottom right panel is the combined representation of the triplicates' densitometry quantification. ns=not significantly different (One-way ANOVA).

which could not be inhibited by IL-10. Therefore, we next tried to determine the LPS concentration that was sufficient to stimulate the phosphorylation of Akt but low enough so that the pAkt could be inhibited by IL-10. We stimulated BMDM with LPS concentrations in a range from 0.1 to 1 ng/ml while the IL-10 concentration was kept constant at 100 ng/ml. 0.1 ng/ml LPS induced weak phosphorylation of Akt while the phosphorylation reached its plateau at around 0.5 ng/ml of LPS (Figure 7B). Quantification of the pAkt and the loading control, p85, showed that addition of IL-10 did not reduce the pAkt signal significantly at all LPS concentrations.

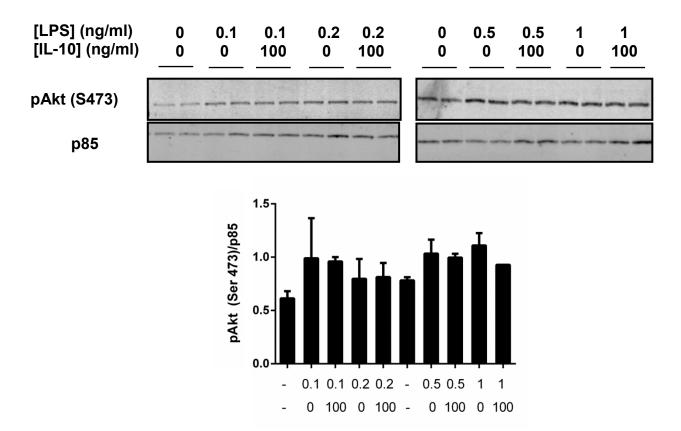
3.4.3 Inhibition of pAkt: IL-10 concentration

100 ng/ml IL-10 was used in the previous studies but it was possible that the IL-10 concentration is too low hence it could not inhibit LPS-induced pAkt. Therefore, we performed an IL-10 titration from 1 ng/ml up to 1000 ng/ml to determine the IL-10 concentration that would yield the best pAkt inhibition (Figure 7C). A sample was also treated with LY294002 as a positive control for inhibition of pAkt. After quantification against the loading control, actin, it showed that cells treated with various IL-10 concentrations exhibited similar pAkt intensity as the LPS alone sample.

3.4.4 Inhibition of pAkt: effect of pre-adding IL-10

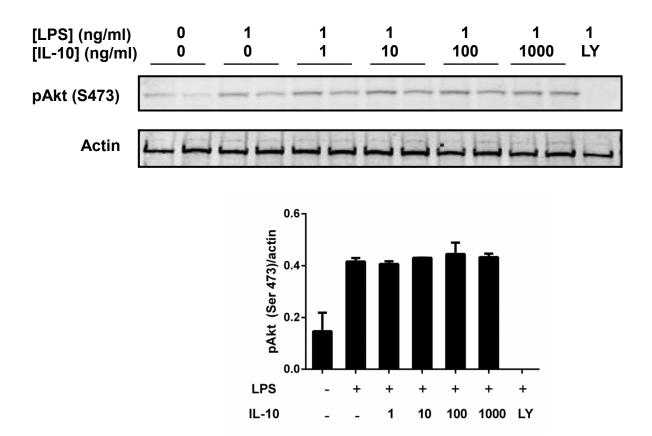
The expression of IL-10R is relatively less abundant than TLR4. Therefore the time taken for IL-10 to act might not be as rapid as the LPS stimulation. Since the stimulation time points that we chose were short, the potential response time between LPS and IL-10 might mask the IL-10 inhibitory effect within the experimental time points. Therefore some investigators prefer to

В



(B) Immunoblot analysis of SHIP1 WT BMDM stimulated with various LPS concentrations as indicated with or without IL-10 (100 ng/ml) for 15 minutes, probed with anti-phospho-Akt (Ser 473) and p85 (loading control). Cell lysate samples were run in duplicates on the blot. Bottom left panel is the quantification of each individual sample on the immunoblot after normalizing the phospho-Akt signal to the p85 signal. Bottom right panel is the combined representation of the duplicates' densitometry quantification.

C



(C) Immunoblot analysis of SHIP1 WT BMDM stimulated with LPS (1 ng/ml) with various concentrations of IL-10 as indicated for 15 minutes, LY294002 (LY, 25 μ M) was added 30 minutes prior to LPS stimulation. Immunoblot was probed with anti-phospho-Akt (Ser 473) and actin (loading control). Cell lysate samples were run in duplicates on the blot. Bottom left panel is the quantification of each individual sample on the immunoblot after normalizing the phospho-Akt signal to the actin signal. Bottom right panel is the combined representation of the duplicates' densitometry quantification.

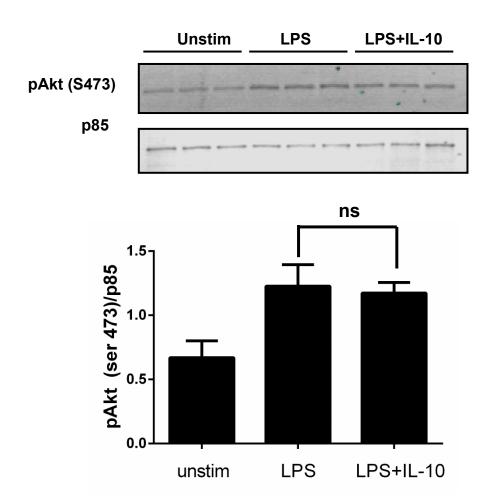
pre-add IL-10 before the other stimulation [42]. We tried to pre-add IL-10 to the cells 5 minutes before the addition of LPS (Figure 7D). After quantification using pAkt and p85, it showed that pre-adding IL-10 did not result in IL-10 inhibition of pAkt.

3.4.5 Inhibition of pAkt: downstream molecules of PI3K pathway

We wished to validate the role of IL-10 in terminating the PI3K pathway via SHIP1 and pAkt was chosen as the experimental target because Akt is the key kinase directly downstream of the production of PIP3. However, we failed to observe any IL-10-dependent reduction of pAkt in our experiments. One possible reason is that phosphorylation of Akt might not be the best indicator of the effect of IL-10 on LPS activated macrophages. Therefore we looked at the possibility of using the phosphorylation state of other proteins that are in the PI3K pathway and downstream of Akt as markers of PI3K pathway activity.

BMDMs that were treated by LPS +/- IL-10 for 15, 30, or 45 minutes and the cell lysate was run on immunoblot. The blot was then probed with either pPDK1, pIkBα or pGSK antibody, and vinculin was used as the loading control. PDK1 is basally phosphorylated and the phosphorylation level did not change when LPS or LPS+IL-10 were added to the cell culture. IkBα was also basally phosphorylated and the addition of LPS further intensified the phosphorylation; addition of IL-10 did not alter the phosphorylation level induced by LPS. For GSK3, the basal phosphorylation level was weak and LPS strongly induced the phosphorylation. Similar to the observations seen for PDK1 and IkBα, the addition of IL-10 did not have any significant change on the GSK3 phosphorylation level in comparison to the LPS alone stimulated samples (Figure 8).

D



(**D**) Immunoblot analysis of SHIP1 WT BMDM stimulated with LPS (0.2 ng/ml) with or without IL-10 (100 ng/ml) for 15 minutes with IL-10 added 5 minutes prior to LPS. Immunoblot was probed with α -pAkt (Ser 473) and p85 (loading control). Cell lysate samples were run in triplicates on the blot. Bottom left panel is the quantification of each individual sample on the immunoblot after normalizing the pAkt signal to the p85 signal. Bottom right panel is the combined representation of the triplicates' densitometry quantification.

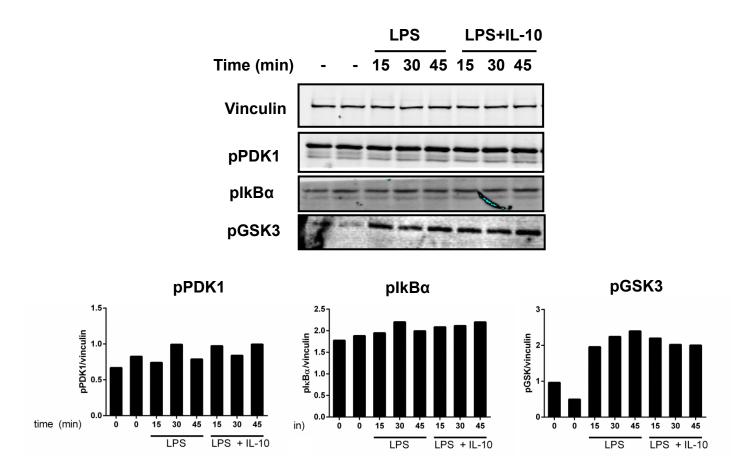


Figure 8 IL-10's effect on LPS induced phosphorylation of proteins in the PI3K pathway

Immunoblot analysis of SHIP1 $^{+/+}$ BMDM stimulated with LPS (0.2 ng/ml) with or without IL-10 (100 ng/ml) for the indicated times. Immunoblot was probed with α -pPDK1, pIkB α , pGSK3, and vinculin (loading control). Bottom panels are the densitometry quantification of each individual sample on the immunoblot after normalizing the phospho-protein signal to the vinculin signal.

3.4.6 Inhibition of phospho-Akt: cell type (perimac vs BMDM)

The previous experiments were done in BMDM that were cultured in CSF-1 containing media for 7 days after extraction. We decided to test out the stimulation in freshly derived peritoneal macrophages (perimac) since perimacs are more mature than BMDM [127]. BMDM has less defined macrophage markers and might not respond to LPS and IL-10 in the same way. Therefore, we stimulated both BMDMs and perimacs with 0.2 ng/ml LPS +/- 100 ng/ml IL-10 and measured pAkt and actin by immunoblotting (Figure 9A). The quantification shows that although perimacs seemed to be more responsive to LPS, as suggested by the higher level of pAkt, IL-10 could not inhibit the phosphorylation in either cell types.

3.4.7 Inhibition of pAkt: genetic background of primary cells

We wished to first reproduce the IL-10 inhibition on pAkt in LPS activated macrophages in wild type primary cells and then moved on to determine the role of SHIP1 by using SHIP1^{-/-} primary cells. Our SHIP1^{-/-} mice colony is on a Balb/C background therefore the primary cells used in the above experiments were all derived from mice with Balb/C background. After the above experiments failed to reproduce the IL-10 inhibition observed in cell lines, we investigated the possibility that the strain background of the primary cells might affect their responsiveness to LPS and/or IL-10 treatments. We derived BMDM from mice on a C57BL/6 background and stimulated them with 0.2 ng/ml LPS +/- 100 ng/ml IL-10. As shown in Figure 9B, the level of phosphorylation of Akt in the LPS+IL-10 samples are significantly lower than the ones of the LPS alone samples.

A

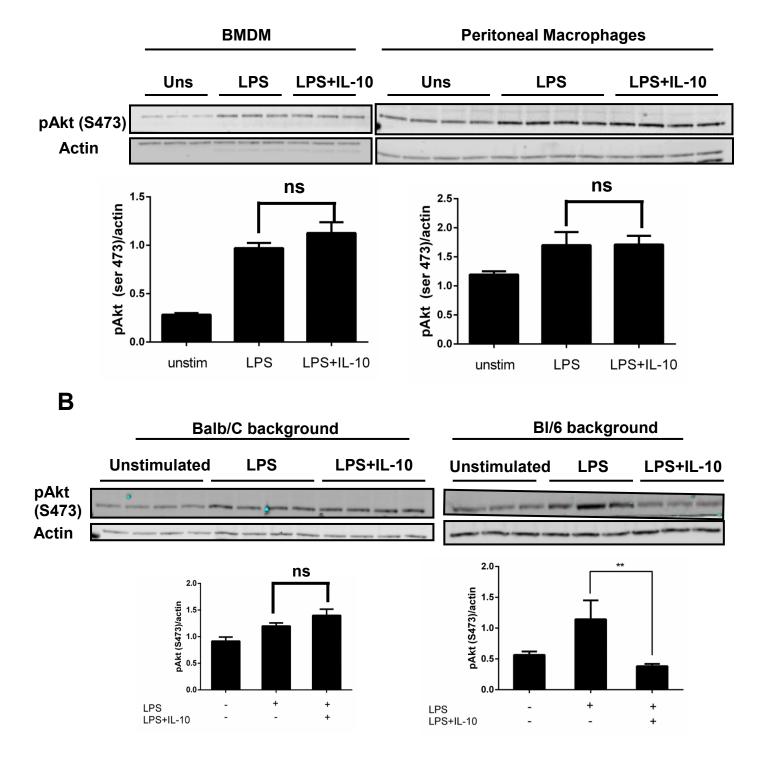


Figure 9 IL-10's effect on LPS induced pAkt: effect of cell identity

(A) Immunoblot analysis of SHIP1^{+/+} BMDM or peritoneal macrophages stimulated with LPS (0.2 ng/ml) with or without IL-10 (100 ng/ml) for 15 minutes. Immunoblots were probed with α -pAkt (Ser 473) and actin (loading control). Cell lysates were run in triplicate for BMDM and quadruplicates for peritoneal macrophages on the blot. Middle panels are the quantification of each individual sample on the immunoblots after normalizing the pAkt signal to the actin signal. Bottom panels are the combined representation of the triplicates' and quadruplicates' densitometry quantification. (B) Immunoblot analysis of Balb/C background BMDM or Bl/6 background BMDM stimulated with LPS (0.2 ng/ml) with or without IL-10 (100 ng/ml) for 15 minutes. Immunoblots were probed with α -pAkt (Ser 473) and actin (loading control). Cell lysate samples were run in triplicate. Middle panels are the quantification of each individual sample on the immunoblots after normalizing the pAkt signal to the actin signal. Bottom panels are the combined representation of the triplicates' densitometry quantification. ns=not significantly different, **p<0.01 (One-way ANOVA).

3.5 TNFα production in static culture

We previously generated RAW 264.7 cells that were transduced with siRNA constructs that selectively knocked down SHIP1 or STAT3 in order to understand the relative contribution of these proteins to the IL-10 signalling pathway. By stimulating these transduced cells with LPS +/- IL-10 for various time points, we concluded that SHIP1 is involved in the early anti-inflammatory effect of IL-10 and STAT3 is responsible for the effect at later time points.

One of the biggest problems of drawing conclusions from experiments using the STAT3 KD cells was that we only successfully knocked down about 50% of the STAT3 at the protein level. Incomplete knock down may cofound the conclusions we drew about STAT3's role in IL-10 inhibition of TNFα protein production. We overcame this problem by crossing STAT3^{flox/flox} mice with LysMcre mice [128] to obtain STAT3^{flox/flox} LysMcre mice which had STAT3 knocked out only in the monocytes. The STAT3^{-/-} peritoneal macrophages had over 90% of STAT3 mRNA reduction and about 80% of STAT3 protein levels reduction in comparison to the wild type counterpart (Figure 10B; right). SHIP1^{+/+} and -/- primary cells were also utilized so that the relative contributions of SHIP1 and STAT3 could be compared. For SHIP1^{-/-} peritoneal macrophages, the knock out was close to completion for both SHIP1 mRNA and protein expression (Figure 10A).

SHIP1^{+/+} and ^{-/-} BMDM, and STAT3^{+/+} and ^{-/-} BMDM were stimulated with 1 ng/ml LPS +/- 1 or 10 ng/ml IL-10 for 1 to 4 hours. Supernatant was collected every hour and the TNF α protein level was determined by ELISA. SHIP1^{+/+} and ^{-/-} BMDM responded to IL-10 in a dose dependent manner similarly at all time points. Also, there was a gradual increase in the percentage of TNF α protein reduction as time passed by. At 1 hour, the reduction was about 10-30% and there was 70-80% reduction at 4 hours. In contrast to what was observed in parental

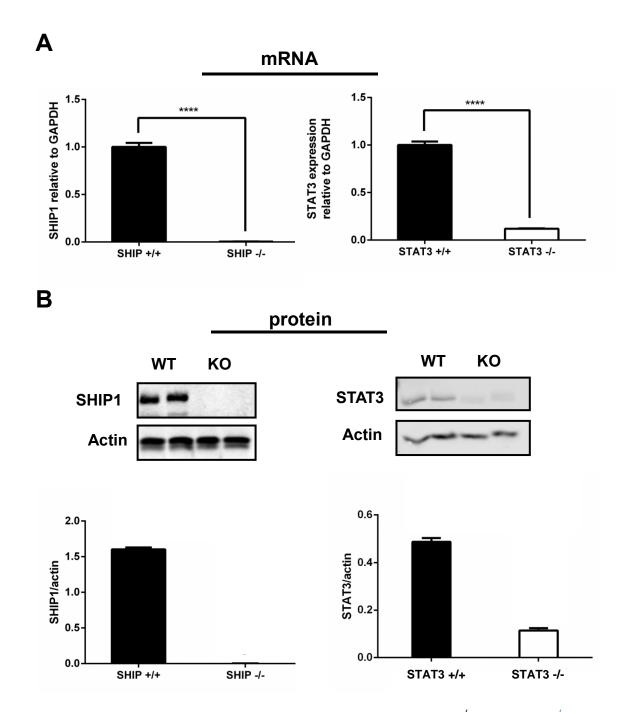


Figure 10 SHIP1 and STAT3 mRNA and protein expression in SHIP1 $^{-/-}$ and STAT3 $^{-/-}$ peritoneal macrophages

(A) RNA was extracted from SHIP1 */+/-/- or STAT3*/-/- peritoneal macrophages and was subjected to qPCR analysis. Data represent the mean SHIP1 (left panel) STAT3 (right panel) mRNA expression relative to GAPDH mRNA expression as determined by qPCR. (B) Immunoblot analysis of lysate from SHIP1*/-/- (left panel) or STAT3*/-/- (right panel) peritoneal macrophages, probed with anti-SHIP1 or anti-STAT3 and actin (loading control). Each sample was run in duplicate. Bottom graph represents the densitometry quantification of SHIP1 and STAT3 signal normalized to actin signal.

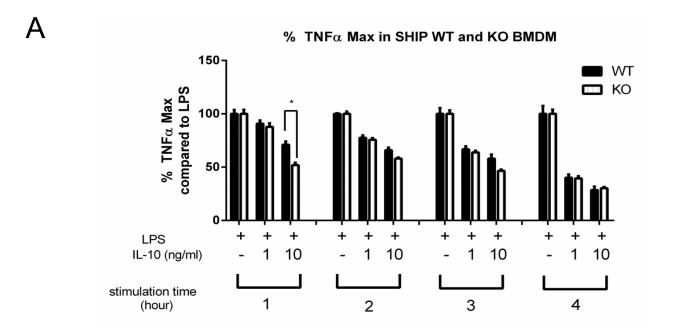
and SHIP1 KD RAW 264.7 cells, the SHIP1^{-/-} cells were not more resistant to IL-10 treatment than SHIP1^{+/+} cells (Figure 11A). The degree of IL-10 inhibition on TNF α protein was similar in SHIP1^{+/+} and ^{-/-} cells at almost all the conditions and this observation was consistent in multiple replications of the experiments (Appendix C).

STAT3^{+/+} and ^{-/-} BMDM also responded to IL-10 in a dose-dependent manner. Unlike the SHIP1^{-/-} BMDM, IL-10 inhibition on TNFα protein was impaired in the STAT3^{-/-} BMDM at all time points. While a higher degree of inhibition was achieved at longer time points in STAT3^{+/+} BMDM and TNFα protein production was almost completely inhibited at 4 hours, the maximum IL-10 inhibition was reached at 2 hours and remained relatively constant up to 4 hours in STAT3^{-/-} BMDM (Figure 11B and Appendix C).

3.6 TNFa production in continuous flow culture

In SHIP1 and STAT3 KD RAW 264.7 cells stimulated by LPS+/-IL-10 under a continuous flow system, we observed that the TNF α protein production differed in the two cell types. The TNF α protein profile suggested that SHIP1 and STAT3 may contribute to different phases of IL-10 inhibition on LPS induced TNF α production in the RAW 264.7 cell lines. We repeated the stimulation by using SHIP1^{+/+} and -/-, and STAT3^{+/+} and -/- BMDM. In SHIP1 +/+ and -/- cells, LPS induced a large TNF α protein peak at around 55 minutes and a smaller one at around 110 minutes (Figure 12A). Addition of IL-10 reduced the first peak by over 50% in SHIP1^{+/+} cells but failed to inhibit the peak in SHIP1^{-/-} cells. IL-10 could inhibit the second peak of TNF α protein at a similar degree in both +/+ and -/- cell types.

In STAT3^{+/+} and ^{-/-} BMDM, there were also two TNF α peaks induced by LPS, and the amount of TNF α protein produced in each peak was similar in both cell types (Figure 12B). In STAT3^{+/+} cells, IL-10 inhibited more than 50% of the first peak and almost completely inhibited



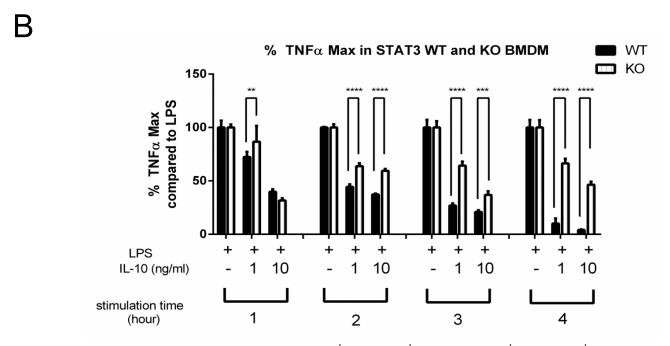


Figure 11 IL-10 inhibition of TNF α in SHIP1 $^{+/+}$ / SHIP1 $^{-/-}$ and STAT3 $^{+/+}$ / STAT3 $^{-/-}$ BMDM in static culture

(A) TNF α ELISA of supernatant of SHIP1^{+/+}/-^{-/-} and STAT3^{+/+}/-^{-/-} (B) BMDM that were treated by LPS (1 ng/ml) with or without IL-10 (1 or 10 ng/ml) for the indicated times. Data represent the percentage of TNF α relative to LPS alone treated samples \pm S.D. (n=3). TNF α level of LPS alone stimulated samples at 1, 2, 3, and 4 hours respectively were: 126 \pm 3, 587 \pm 2, 6.0 \pm 0.2×10², 9.5 \pm 0.5×10² for SHIP1^{+/+} BMDM; 168 \pm 4, 6.5 \pm 0.1×10², 6.0 \pm 0.1×10² and 10.5 \pm 0.3×10² for SHIP1^{-/-}BMDM; 50 \pm 2, 5.8 \pm 0.3×10², 12.0 \pm 0.6×10² and 1404 \pm 5 for STAT3^{+/+} BMDM; 38 \pm 1, 6.2 \pm 0.3×10², 10.9 \pm 0.2×10² and 11.4 \pm 0.5×10² for STAT3^{-/-}BMDM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Two-way ANOVA)

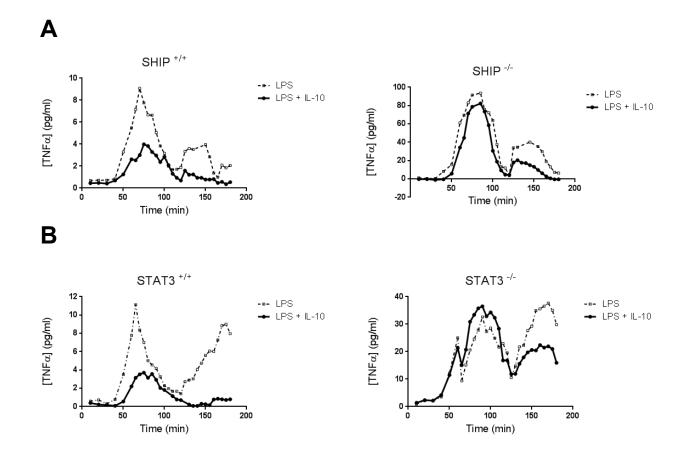


Figure 12 Both SHIP1 and STAT3 are required for IL-10 inhibition on the first peak of TNF α protein production in the continuous flow system

(A)TNF α ELISA of continuous flow cell system from SHIP1^{+/+}/ SHIP1^{-/-} and (B) STAT3^{+/+}/ STAT3^{-/-}BMDM stimulated by LPS (1 ng/ml) with or without IL-10 (10 ng/ml). Data represent the TNF α concentration of each fraction (5 minutes per fraction) collected over the course of 3 hours.

the second peak. In STAT3^{-/-} cells, IL-10 failed to inhibit the first peak while it could still inhibit the second peak by about 50%.

3.7 SHIP1 expression in STAT3 -/- primary cells

We observed that the TNF α protein production profiles in the SHIP1 and STAT3^{-/-} cells are almost identical: IL-10 failed to inhibit the first TNF α peak that occurred at around 55 minutes and IL-10 reduced the second TNF α peak, which occurred at around 110 minutes, by about 50%. This result was surprising since it is different from what we determined in the RAW 264.7 cell lines. Also, we did not expect the effect of STAT3 deficiency would impact IL-10 inhibition of TNF α within the first hour (first peak at 55 minutes) since all of STAT3 functions require *de novo* protein synthesis.

Since STAT3 is a transcription factor, we investigated on the possibility that STAT3 deficiency was not directly affecting IL-10's inhibition of TNFα but indirectly through changing the level of other signalling molecules that play a role in IL-10's early inhibitory effect. The first candidate was SHIP1 because we have shown that the lack of SHIP1 resulted in IL-10's inability to inhibit the first TNFα peak after LPS stimulation. We extracted RNA from peritoneal macrophages and BMDM from STAT3^{+/+} and -/- mice with Bl/6 background. The mRNA was reverse transcribed into cDNA and the level of SHIP1 mRNA was determined by qPCR.

GAPDH was used as the normalization control. As shown in Figure 13, both STAT3^{-/-} peritoneal macrophages and BMDM expressed significantly less SHIP1 mRNA than the STAT3^{+/+} cells.

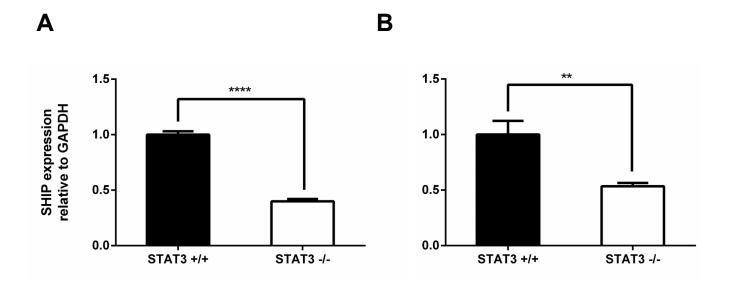


Figure 13 SHIP1 expression in STAT3 $^{+/+}$ and STAT3 $^{-/-}$ primary cells

(A) RNA was extracted from STAT3 ^{+/+} / STAT3 ^{-/-} peritoneal macrophages and **(B)** BMDM and was subjected to qPCR analysis. Data represent the mean SHIP1 mRNA expression relative to GAPDH mRNA expression as determined by qPCR.

4. Discussion

4.1 STAT3 independent IL-10 signalling pathway

IL-10 has been studied for decades as the main negative regulator of activated immune cells and is an important anti-inflammatory cytokine. It is widely accepted in the literatures that all of IL-10 function depends on the STAT3 pathway. However, there is evidence in recent literatures that suggests IL-10 utilizes a quick pathway that does not depend on novel transcription or translation. In a study published by O'Farrell et al, a J774.1 macrophage cell line that expressed a dominant negative STAT3 was stimulated by LPS +/- IL-10 [129]. The study showed that IL-10 could still inhibit TNFα protein production in these cells [129]. In another study, Williams et al demonstrated that IL-10 inhibition of TNFα mRNA did not depend on de novo protein synthesis at early time points such as one hour [36]. Furthermore, IL-10 inhibition of TNFα protein appeared to be STAT3-independent at early time points and the inhibition became more dependent on STAT3 at longer time points [36]. Similar observations that suggest IL-10 can act in a STAT3-independent way were also seen in *in vivo* models. Berg *et al* showed that IL-10 KO mice were highly susceptible to LPS treatment in which the mortality and serum TNF α level were much higher in the IL-10^{-/-} mice than in the wild type mice after LPS injection [130]. This suggested that endogenous IL-10 is important for the survival of the mice and the inhibition of TNFα in LPS-induced endotoxemia model. In a study conducted by Takeda et al, myeloid cell specific STAT3^{-/-}mice were generated and LPS was injected to induce endotoxemia. In contrast to the IL- $10^{-/-}$ mice in which the serum TNF α level remained high after 6 hours of LPS injection, TNFα level in the STAT3^{-/-} mice began to decline after 1.5 hours of LPS stimulation and returned to baseline level after 3 hours [131]. The decline of TNF α at 1.5 hours coincided with the increased production of endogenous IL-10. Lastly, our lab has previously

shown that IL-10 represses TNFα production by shifting TNFα mRNA from associating with polysomes to monosomes via a SHIP1 dependent mechanism [121]. Collectively, these *in vitro* and *in vivo* experiments suggest that IL-10 acts through a STAT3 independent pathway that does not require de novo protein synthesis and takes action at early timepoints.

4.2 SHIP1 in the IL-10 signalling pathway

Previous studies from our lab suggested that SHIP1 might be the central molecule in the STAT3 independent IL-10 signalling pathway. We showed that IL-10 activates and recruits SHIP1 to the IL-10R in the J774.1 macrophage cell line, and that SHIP1 deficiency in macrophages resulted in IL-10 resistance in these cells at early time points. There are a few important implications of SHIP1 being an important signalling molecule in the IL-10 signalling pathway. Firstly, the STAT3 dependent IL-10 signalling pathway has been studied extensively. The appearance of a novel IL-10 signalling pathway that is STAT3 independent will result in a better understanding of IL-10 and the mechanisms by which it regulates the immune system. Secondly, we showed that SHIP1 was required for IL-10 to inhibit TNFα protein production and this indicates that SHIP1 has an anti-inflammatory role. Since SHIP1 is a negative regulator of the PI3K pathway, the anti-inflammatory property of SHIP1 suggests that PI3K is a positive regulator of TNFα production and is pro-inflammatory under the experimental conditions that were tested. This adds an extra piece of evidence of PI3K being pro-inflammatory to the much debated topic of whether PI3K pathway is pro- or anti-inflammatory. Lastly, direct activation of SHIP1 might be a potential therapeutic that aims to mimic the beneficial anti-inflammatory effect of IL-10 and this direct activation is especially important in diseases that are caused by defects in IL-10 or IL-10R expression. Also, this therapeutic acts as a potential alternative in treating PI3K related diseases in immune cells.

Due to the important physiological roles that PI3K pathway plays in cells, it has been a heavily investigated pathway for the development of potential therapeutics for diseases such as cancer. The PI3K inhibitors, wortmannin and LY-292004, have been widely used experimentally and are important tools for understanding the pathway. However, they fail to be applicable in clinical settings since they inhibit all members of the PI3K family thus lack the specificity required for effective therapeutics. More recently, the development of isoform-specific PI3K inhibitors has suggested a promising avenue in controlling PI3K pathway related diseases. For example, PI3K isoforms p110 δ and p110 γ are enriched in immune cells and their specific inhibitors are being studies as therapeutics in treating inflammatory diseases [132].

Inhibition of PI3K is one way to directly terminate the activity of this pathway. As an alternative, activation of phosphatases that are negative regulators of PI3K should also inhibit the pathway. Our lab had developed a small molecule SHIP1 agonist, AQX-MN100 that has been shown to be beneficial in reducing inflammatory symptoms in mouse models of inflammatory diseases [133]. SHIP1 is an ideal target for immune cell-related diseases since its expression is mainly restricted to hematopoietic cells.

Since cell lines behave differently from primary cells, we wanted to confirm the RAW 264.7 cell finding in murine primary macrophages such as perimac and BMDM. We first investigated the physical interaction of SHIP1 and IL-10R upon IL-10 stimulation. SHIP1 possesses a SH2 domain that interacts with phosphorylated tyrosines, and there are two tyrosine residues on the IL-10R that become phosphorylated upon IL-10 binding. Therefore, we first tried to determine whether SHIP1 is recruited to the IL-10R when IL-10 binds to and activates its receptor. However, we failed to observe IL-10 dependent association between SHIP1 and IL-10R. Also, SHIP1 could not be pulled down by both the phosphorylated and unphosphorylated IL-10R

peptide. We speculated that since SHIP1 also interacts with other protein via its SH2 domain, it is possible that SHIP1 and IL-10R do not interact directly but associate through bridging protein(s). The protein complex might have dissociated during the IL-10R immunoprecipitation and the IL-10R peptide pull down, thus SHIP1 could not be observed in the immunoblots. Further studies should be done to determine the proteins that become associated with the IL-10R and/or SHIP1 upon IL-10 stimulation. Analysis of these two populations of proteins may identify proteins that become associated with both IL-10R and SHIP1 and serve as an adaptor protein between them. If such an adaptor protein cannot be found, determining the associating protein complex will still shed light on the possible novel signalling molecules that become activated by IL-10. This will result in a better understanding of the IL-10 pathway and the possible mechanism(s) by which SHIP1 operates within the IL-10 pathway.

The commonly accepted belief in the literature is that SHIP1 activity is dictated by its location. Therefore we investigated the effect of IL-10 on SHIP1 activity by determining SHIP1's spatial location upon IL-10 stimulation using immunofluorescence. Although the study that utilized α-SHIP1 antibody and WGA did not show SHIP1 translocating to the membrane, more optimization and experiments should be done. SHIP1 protein is relatively abundant and it is possible that only a certain percentage of the protein translocates to the cell membrane upon IL-10 stimulation. Therefore the SHIP1 level might only have a subtle change at the membrane and it is important to have a robust membrane marker. Different membrane markers, such as CD11b and cadherin, can be used in place of WGA. Another possible way to determine SHIP1's localization upon IL-10 stimulation is to fractionate the cell lysate into membrane and cytosolic fractions and detect SHIP1. Lastly, immunofluorescence has a potential technical flaw in short stimulations since the cellular activities are not terminated instantaneously which might result in

the stimulation time being longer than expected. In order to observe SHIP1's localization, a more suitable alternative is to construct a GFP-tagged SHIP1 protein and track its movement when the transfected cells are stimulated by IL-10. An advantage of live cell imaging is that the full record of SHIP1's movement can be observed whereas immunofluorescence is limited by the time points chosen and important information may be neglected.

We then assessed SHIP1's activation state by its phosphorylation state. We found that SHIP1 is basally phosphorylated in BMDM and the addition of IL-10 did not induce significant changes in the phosphorylation level. In fact, there seems to be a slight decrease in the phosphorylation signal after 10 minutes of IL-10 stimulation. It is interesting to note that LPS stimulation promotes SHIP1 phosphorylation and tyrosine-phosphorylation of SHIP1 is related to the proteasome degradation of SHIP1. Therefore it would be interesting to determine the effect of IL-10 on phosphorylation state of SHIP1 in LPS activated macrophages.

A large proportion of the thesis was dedicated to determining IL-10's inhibition on LPS induced phosphorylation of Akt. Despite varying many different parameters such as, LPS and IL-10 concentration, stimulation time, pre-adding IL-10 before LPS, cell type and looking at other members in the PI3K pathway, we could not observe IL-10 inhibition of LPS-induced phospho-Akt in cells obtained from Balb/C mice. However, significant IL-10 inhibition of phospho-Akt signal could be seen when we used BMDM derived from Bl/6 mice. Future experiments should be performed in both BMDM and perimac to determine if the IL-10 inhibition can be reproduced in other macrophage cell types with Bl/6 background. In order to determine the role of SHIP1 in IL-10's inhibition of pAkt, the same LPS +/- IL-10 stimulation should be set up using primary cells derived from SHIP1^{-/-} mice with Bl/6 background. The

SHIP1^{+/+} and ^{-/-} primary cells with Bl/6 background. Also, it is interesting to determine the molecular mechanistic differences in the IL-10 signalling pathway of macrophages from the two mice strains that result in difference IL-10 responsiveness in these cells. For example, the expression levels of proteins in the IL-10 signalling pathway, such as IL-10R and SHIP1, might be higher in cells with Bl/6 background and cause an enhanced IL-10 sensitivity in these cells.

Next we wanted to verify the role of SHIP1 and STAT3 in IL-10's ability to inhibit TNFα production in LPS activated macrophages. The TNFα protein production profile in the SHIP1^{+/+} and ^{-/-} BMDM static culture was different from the static culture done in parental RAW 264.7 and SHIP1 KD cells. In the cells lines, SHIP1 KD cells were resistant to IL-10's inhibition on LPS induced TNFα production. In contrast, SHIP1^{-/-} BMDM was as responsive to IL-10 as the SHIP1 +/+ cells. This might be due to the intrinsic differences of cell line and BMDM in response to IL-10. It has been shown that high concentration of IL-10 (>100 ng/ml) would inhibit TNFα production at a similar level in both the parental and SHIP1 KD RAW 264.7 cell lines. The IL-10 concentrations used to stimulate the BMDM were chosen according to the ones used in the cell lines. However, BMDM might be more sensitive to IL-10 and even lower IL-10 concentration is needed to show a differential IL-10 effect on SHIP1^{+/+} and ^{-/-} BMDM. We showed that IL-10 inhibited TNFα protein production in both STAT3^{+/+} and ^{-/-} BMDM. However, the percentage of inhibition was significantly less in STAT3^{-/-} cells than in WT cells, indicating that STAT3^{-/-} cells were more resistance to the effect of IL-10. These observations strengthened the idea that STAT3 is not the only signalling molecule by which IL-10 acts upon, since STAT3^{-/-} BMDM could still partially respond to IL-10 treatment.

In the continuous flow cell culture, it was surprising that the TNF α protein production profiles were almost identical in SHIP1^{-/-} and STAT3^{-/-}BMDM. The first peak of LPS-induced

TNFα production, which occurred at around 55 minutes, was resistant to IL-10 in both SHIP1^{-/-} and STAT3^{-/-} cell types as compared to the respective wild type cells. It should be noted that at the 1 hour time point of the static culture, both SHIP1^{-/-} and STAT3^{-/-} BMDM were not resistant to IL-10. This indicated that the continuous flow system and the static culture do have dissimilar environments which lead to different results. We speculated that the observations seen in the continuous flow cells culture give us better insights into the TNFα protein profile since it resembles the physiological environment more than the static cultures. LPS +/- IL-10 stimulations of STAT3 ^{+/+}/₋ and SHIP1 ^{+/+}/₋ BMDM using the continuous flow cell system were limited to a maximum of 4 hours in the experiments presented in the thesis. Longer stimulation, such as up to 24 hours, might allow us to gain more insight into the relative contribution of SHIP1 and STAT3 in IL-10 inhibition of TNFα protein.

From the continuous flow cell system, we observed that the TNFα production profiles for the LPS +/- IL-10 stimulated SHIP1^{-/-} and STAT3^{-/-} BMDM were extremely similar. One possible explanation is that SHIP1 level is reduced in STAT3^{-/-} cells, and the TNFα profile observed is due to SHIP1 deficiency in both SHIP1 and STAT3^{-/-} cells. We found that SHIP1 mRNA expression is significantly reduced in STAT3^{-/-} perimac in comparison to the STAT3^{+/+} peritoneal macrophages. This suggested that resistance to IL-10 in the STAT3^{-/-} BMDM during the first TNFα peak might be due to the lack of SHIP1. The next step will be to show the level of SHIP1 protein in STAT3^{-/-} primary cells in comparison to STAT3^{+/+} primary cells. In order to verify that deficiency of SHIP1 is the cause of STAT3^{-/-} cells being resistant to IL-10 at early time points, SHIP1 protein can be reconstituted into STAT3^{-/-} cells. Then the STAT3^{+/+}, STAT3^{-/-} and SHIP1 reconstituted STAT3^{-/-} cells will be treated by LPS +/- IL-10 and the TNFα protein levels will be measured. If SHIP1 deficiency in STAT3^{-/-} cells was the cause of IL-10

resistance, then we expect the reconstitution of SHIP1 will regain IL-10 inhibition on the first peak of TNF α protein production.

Further studies should be performed to determine the mechanisms by which STAT3 controls SHIP1 mRNA or protein expression. Since STAT3 is a transcription factor, SHIP1's promoter region should be examined for a STAT3 binding sequence in order to determine if STAT3 can directly affect SHIP1 mRNA expression. If such a sequence is found through bioinformatic analysis, it can be verified by performing ChIP experiment. Since STAT3 might transcriptionally control SHIP1 expression indirectly via regulating SHIP1's transcription factors, the promoter region of the known transcription factors of SHIP1, such as SMAD, can also be investigated using the genetic approach and ChIP experiment described above.

Deficiency of STAT3 can affect SHIP1 expression and cellular response to LPS and IL-10 stimulation in two ways. Firstly, lack of STAT3 would change the development and growth of the cells thus creating a cellular environment that is different from the STAT3^{+/+} cells. It has been shown that STAT3 is essential in embryonic stem cells development and global STAT3^{-/-} is embryonic lethal [134]. Although the LysMCre system can limit the knock out in monocytes, these cells might potentially develop abnormally. The second possibility is that STAT3's effect is limited to the known targets of STAT3 and SHIP1, and does not affect the overall cellular environment significantly. Utilization of the STAT3 KD RAW 264.7 cells might allow us to gain insight into which possibility is the correct one. Since inducing STAT3 KD would bring short term effect on the cells and would not affect the developmental stage of the cells.

4.3 Conclusion

The data presented in the research project demonstrated the reoccurring concern of utilizing cell lines and further our understanding in the IL-10 signalling pathway. We failed to replicate some of the results observed in cell lines including the interaction between IL-10R and SHIP1. Under the experimental conditions presented, we showed that IL-10 inhibited PI3K pathway only in BMDM derived from mice with Bl/6 background but not Balb/C background. This indicated the genetic background of the experimental animals played an important role in the IL-10 signalling pathway. It also emphasizes on the potential flaws in applying the knowledge obtained from animals to the human-based system. Lastly, we demonstrated that the TNFα protein profile of SHIP1-^{7/2} and STAT3-^{7/2} BMDM were similar and STAT3-^{7/2} primary cells have reduced SHIP1 mRNA expression. This finding suggests the phenotypes of STAT3-^{7/2} animals or cells, such as being resistant to IL-10 treatment, might due to the deficiency of SHIP1. This highlights the significance of SHIP1 being an essential molecule in the IL-10 signalling pathway.

References

- 1. Nathan, C., *Points of control in inflammation*. Nature, 2002. **420**(6917): p. 846-852.
- 2. Rather, L.J., Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. Bull N Y Acad Med, 1971. **47**(3): p. 303-322.
- 3. Medzhitov, R., *Origin and physiological roles of inflammation*. Nature, 2008. **454**(7203): p. 428-435.
- 4. Itzkowitz, S.H. and X. Yio, *Inflammation and Cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation*. American Journal of Physiology Gastrointestinal and Liver Physiology, 2004. **287**(1): p. G7-G17.
- 5. Koltsova, E.K., C.C. Hedrick, and K. Ley, *Myeloid cells in atherosclerosis: a delicate balance of anti-inflammatory and proinflammatory mechanisms*. Current Opinion in Lipidology, 2013. **24**(5): p. 371-380 10.1097/MOL.0b013e328363d298.
- 6. Moelants, E.A.V., et al., *Regulation of TNF-[alpha] with a focus on rheumatoid arthritis.* Immunol Cell Biol, 2013. **91**(6): p. 393-401.
- 7. Janeway, C.A. and R. Medzhitov, *INNATE IMMUNE RECOGNITION*. Annual Review of Immunology, 2002. **20**(1): p. 197-216.
- 8. Medzhitov, R. and C.A. Janeway Jr, *Innate immune recognition and control of adaptive immune responses*. Seminars in Immunology, 1998. **10**(5): p. 351-353.
- 9. Nace G, E.J., Eid R, Tsung A., *Dendritic cells and damage-associated molecular patterns: endogenous danger signals linking innate and adaptive immunity.* J Innate Immun, 2012. **4**(1): p. 6-15.
- 10. Geetha Srikrishna, H.H.F., Endogenous Damage-Associated Molecular Pattern Molecules at the Crossroads of Inflammation and Cancer. Neoplasia, 2009. **11**(7): p. 615-628.
- 11. Medzhitov, R., *Inflammation 2010: New Adventures of an Old Flame.* Cell, 2010. **140**(6): p. 771-776.
- 12. Rose, D.M., R. Alon, and M.H. Ginsberg, *Integrin modulation and signaling in leukocyte adhesion and migration*. Immunological Reviews, 2007. **218**(1): p. 126-134.
- 13. Imhof, B.A. and M. Aurrand-Lions, *Adhesion mechanisms regulating the migration of monocytes*. Nat Rev Immunol, 2004. **4**(6): p. 432-444.
- 14. Serhan, C.N. and J. Savill, *Resolution of inflammation: the beginning programs the end.* Nat Immunol, 2005. **6**(12): p. 1191-1197.
- 15. Freire, M.O. and T.E. Van Dyke, *Natural resolution of inflammation*. Periodontology 2000, 2013. **63**(1): p. 149-164.
- 16. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nat Rev Immunol, 2008. **8**(12): p. 958-969.
- 17. Gordon, S., *The macrophage: Past, present and future*. European Journal of Immunology, 2007. **37**(S1): p. S9-S17.
- 18. Mantovani, A., A. Sica, and M. Locati, *New vistas on macrophage differentiation and activation*. European Journal of Immunology, 2007. **37**(1): p. 14-16.
- 19. Martinez, F.O., *Regulators of macrophage activation*. European Journal of Immunology, 2011. **41**(6): p. 1531-1534.

- 20. Goerdt, S., et al., *Alternative versus Classical Activation of Macrophages*. Pathobiology, 1999. **67**: p. 222-226.
- 21. Byers, D.E. and M.J. Holtzman, *ALternatively activated macrophages and airway disease*. CHEST Journal, 2011. **140**(3): p. 768-774.
- 22. Tomioka, H., et al., Characteristics of Suppressor Macrophages Induced by Mycobacterial and Protozoal Infections in relation to Alternatively Activated M2 Macrophages. Clinical and Developmental Immunology, 2012. **2012**: p. 19.
- 23. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones.* The Journal of Experimental Medicine, 1989. **170**(6): p. 2081-2095.
- 24. Fiorentino, D.F., et al., *IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells*. The Journal of Immunology, 1991. **146**(10): p. 3444-51.
- 25. Moore, K.W., et al., *Interleukin-10 and the Interleukin-10 Receptor*. Annual Review of Immunology, 2001. **19**(1): p. 683-765.
- 26. Pestka, S., et al., *Interleukin-10 and Related Cytokines and Receptors*. Annual Review of Immunology, 2004. **22**(1): p. 929-979.
- 27. Ouyang, W., et al., *Regulation and Functions of the IL-10 Family of Cytokines in Inflammation and Disease*. Annual Review of Immunology, 2011. **29**(1): p. 71-109.
- 28. Tone, M., et al., *IL-10 Gene Expression Is Controlled by the Transcription Factors Sp1 and Sp3*. The Journal of Immunology, 2000. **165**(1): p. 286-291.
- 29. Liu, Y.-W., et al., Functional Cooperation of Simian Virus 40 Promoter Factor 1 and CCAAT/Enhancer-Binding Protein β and δ in Lipopolysaccharide-Induced Gene Activation of IL-10 in Mouse Macrophages. The Journal of Immunology, 2003. **171**(2): p. 821-828.
- 30. Liu, Y.-W., et al., Lipopolysaccharide-induced transcriptional activation of interleukin-10 is mediated by MAPK- and NF-κB-induced CCAAT/enhancer-binding protein δ in mouse macrophages. Cellular Signalling, 2006. **18**(9): p. 1492-1500.
- 31. Liu, Y., et al., *Expression cloning and characterization of a human IL-10 receptor*. The Journal of Immunology, 1994. **152**(4): p. 1821-9.
- 32. Kotenko, S.V., et al., *Identification and functional characterization of a second chain of the interleukin-10 receptor complex.* EMBO J, 1997. **16**(19): p. 5894-5903.
- 33. Mosser, D.M. and X. Zhang, *Interleukin-10: new perspectives on an old cytokine*. Immunological Reviews, 2008. **226**(1): p. 205-218.
- 34. Raymond P. Donnelly, H.D., and David S. Finbloom, *The Interleukin-10 Signal Transduction Pathway and Regulation of Gene Expression in Mononuclear Phagocytes* Journal of Interferon & Cytokine Research, 1999. **19**(6): p. 563-573.
- 35. Murray, P.J., *Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response*. Current Opinion in Pharmacology, 2006. **6**(4): p. 379-386.
- 36. Williams, L., et al., Signal Transducer and Activator of Transcription 3 Is the Dominant Mediator of the Anti-Inflammatory Effects of IL-10 in Human Macrophages. The Journal of Immunology, 2004. **172**(1): p. 567-576.
- 37. Lai, C.-F., et al., Receptors for Interleukin (IL)-10 and IL-6-type Cytokines Use Similar Signaling Mechanisms for Inducing Transcription through IL-6 Response Elements. Journal of Biological Chemistry, 1996. **271**(24): p. 13968-13975.

- 38. Riley, J.K., et al., *Interleukin-10 Receptor Signaling through the JAK-STAT Pathway:* Requirement for Two Distinct Receptor-Derived Signals for Anti-Inflammatory Action. Journal of Biological Chemistry, 1999. **274**(23): p. 16513-16521.
- 39. Kuwata, H., et al., *IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-α production in macrophages.* Blood, 2003. **102**(12): p. 4123-4129.
- 40. Cassatella, M.A., et al., Interleukin-10 (IL-10) Selectively Enhances CIS3/SOCS3 mRNA Expression in Human Neutrophils: Evidence for an IL-10–Induced Pathway That Is Independent of STAT Protein Activation. Blood, 1999. **94**(8): p. 2880-2889.
- 41. Berlato, C., et al., *Involvement of Suppressor of Cytokine Signaling-3 as a Mediator of the Inhibitory Effects of IL-10 on Lipopolysaccharide-Induced Macrophage Activation.* The Journal of Immunology, 2002. **168**(12): p. 6404-6411.
- 42. Lang, R., et al., Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10. The Journal of Immunology, 2002. **169**(5): p. 2253-2263.
- 43. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* The Journal of Experimental Medicine, 1991. **174**(5): p. 1209-1220.
- 44. Berkman, N., et al., *Inhibition of macrophage inflammatory protein-1 alpha expression by IL-10. Differential sensitivities in human blood monocytes and alveolar macrophages.* The Journal of Immunology, 1995. **155**(9): p. 4412-8.
- 45. Rossi, D.L., et al., *Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3alpha and MIP-3beta*. The Journal of Immunology, 1997. **158**(3): p. 1033-6.
- 46. Bogdan, C., Y. Vodovotz, and C. Nathan, *Macrophage deactivation by interleukin 10*. The Journal of Experimental Medicine, 1991. **174**(6): p. 1549-1555.
- 47. Ding, L., et al., *IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression*. The Journal of Immunology, 1993. **151**(3): p. 1224-34.
- 48. Willems, F., et al., *Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes*. European Journal of Immunology, 1994. **24**(4): p. 1007-1009.
- 49. Jenkins JK, M.M., Arend WP, *The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils.* Lymphokine Cytokine Res, 1994. **13**(1): p. 47-54.
- 50. Dickensheets, H.L., et al., *Interleukin-10 Upregulates Tumor Necrosis Factor Receptor Type-II (p75) Gene Expression in Endotoxin-Stimulated Human Monocytes.* Blood, 1997. **90**(10): p. 4162-4171.
- 51. Kühn, R., et al., *Interleukin-10-deficient mice develop chronic enterocolitis*. Cell, 1993. **75**(2): p. 263-274.
- 52. Franke, A., et al., Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat Genet, 2008. **40**(11): p. 1319-1323.
- 53. Glocker, E.-O., et al., *Inflammatory Bowel Disease and Mutations Affecting the Interleukin-10 Receptor*. New England Journal of Medicine, 2009. **361**(21): p. 2033-2045.
- 54. Zeller, J.C., et al., *Induction of CD4+ T Cell Alloantigen-Specific Hyporesponsiveness by IL-10 and TGF-β*. The Journal of Immunology, 1999. **163**(7): p. 3684-3691.

- 55. Li, W., et al., Recipient pretreatment with mammalian IL-10 prolongs mouse cardiac allograft survival by inhibition of anti-donor T cell responses. Transplantation Proceedings, 1999. **31**(1–2): p. 115.
- 56. Redford, P.S., P.J. Murray, and A. O'Garra, *The role of IL-10 in immune regulation during M. tuberculosis infection.* Mucosal Immunol, 2011. **4**(3): p. 261-270.
- 57. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen Recognition and Innate Immunity*. Cell, 2006. **124**(4): p. 783-801.
- 58. Akira, S. and K. Takeda, *Toll-like receptor signalling*. Nat Rev Immunol, 2004. **4**(7): p. 499-511.
- 59. Kawai, T. and S. Akira, *TLR signaling*. Seminars in Immunology, 2007. **19**(1): p. 24-32.
- 60. Jerala, R., *Structural biology of the LPS recognition*. International Journal of Medical Microbiology, 2007. **297**(5): p. 353-363.
- 61. E A Carswell, L.J.O., R L Kassel, S Green, N Fiore, and B Williamson, *An endotoxin-induced serum factor that causes necrosis of tumors*. Proc Natl Acad Sci U S A, 1975. **72**(9): p. 3666-3670.
- 62. Patial, N.P.a.S., *Tumor Necrosis Factor-α Signaling in Macrophages*. Crit Rev Eukaryot Gene Expr, 2010. **20**(2): p. 87-103.
- 63. Zelová, H. and J. Hošek, *TNF-α signalling and inflammation: interactions between old acquaintances*. Inflammation Research, 2013. **62**(7): p. 641-651.
- 64. Wenda Shurety, A.M.-T., Darren Brown, David A. Hume, and Jennifer L. Stow, Localization and Post-Golgi Trafficking of Tumor Necrosis Factor-alpha in Macrophages Journal of Interferon & Cytokine Research, 2000. **20**(4): p. 427-438.
- 65. Collart, M.A., P. Baeuerle, and P. Vassalli, *Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B.* Molecular and Cellular Biology, 1990. **10**(4): p. 1498-1506.
- 66. Rhoades, K.L., S.H. Golub, and J.S. Economou, *The regulation of the human tumor necrosis factor alpha promoter region in macrophage, T cell, and B cell lines.* Journal of Biological Chemistry, 1992. **267**(31): p. 22102-7.
- 67. Lai, W.S., et al., Evidence that Tristetraprolin Binds to AU-Rich Elements and Promotes the Deadenylation and Destabilization of Tumor Necrosis Factor Alpha mRNA.

 Molecular and Cellular Biology, 1999. 19(6): p. 4311-4323.
- 68. Buxadé, M., et al., *The Mnks Are Novel Components in the Control of TNFα Biosynthesis and Phosphorylate and Regulate hnRNP A1.* Immunity, 2005. **23**(2): p. 177-189.
- 69. Piecyk, M., et al., *TIA-1 is a translational silencer that selectively regulates the expression of TNF-[alpha]*. EMBO J, 2000. **19**(15): p. 4154-4163.
- 70. Kontoyiannis, D., et al., *Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology*. EMBO J, 2001. **20**(14): p. 3760-3770.
- 71. Tartaglia, L.A., et al., *The two different receptors for tumor necrosis factor mediate distinct cellular responses*. Proceedings of the National Academy of Sciences, 1991. **88**(20): p. 9292-9296.
- 72. Hsu, H., J. Xiong, and D.V. Goeddel, *The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation*. Cell, 1995. **81**(4): p. 495-504.

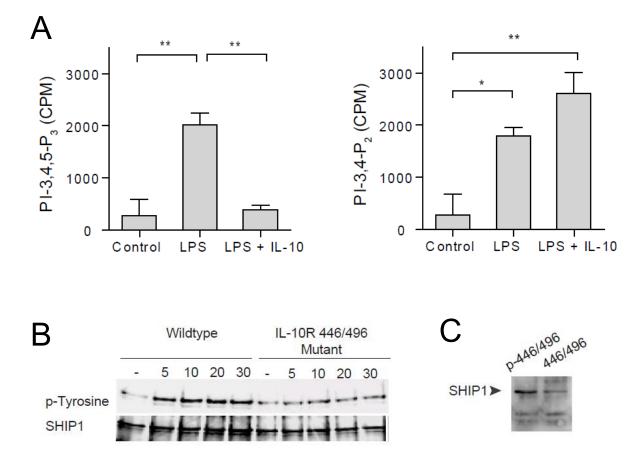
- 73. Rothe M, S.V., Dixit VM, Goeddel DV., *TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD4*. Science, 1995. **269**(5229): p. 1424-7.
- 74. Katso, R., et al., Cellular Function of Phosphoinositide 3-Kinases:: Implications for Development, Immunity, Homeostasis, and Cancer. Annual Review of Cell and Developmental Biology, 2001. **17**(1): p. 615-675.
- 75. Koyasu, S., *The role of PI3K in immune cells*. Nat Immunol, 2003. **4**(4): p. 313-319.
- 76. Wymann, M.P. and L. Pirola, *Structure and function of phosphoinositide 3-kinases*. Biochimica et Biophysica Acta (BBA) Molecular and Cell Biology of Lipids, 1998. **1436**(1–2): p. 127-150.
- 77. Fruman, D.A., R.E. Meyers, and L.C. Cantley, *Phosphoinositide Kinases*. Annual Review of Biochemistry, 1998. **67**(1): p. 481-507.
- 78. Vanhaesebroeck, B., et al., *Synthesis and Function of 3-Phosphorylated Inositol Lipids*. Annual Review of Biochemistry, 2001. **70**(1): p. 535-602.
- 79. Vanhaesebroeck, B. and D.R. Alessi, *The PI3K-PDK1 connection: more than just a road to PKB*. Biochem. J., 2000. **346**(3): p. 561-576.
- 80. Rameh, L.E. and L.C. Cantley, *The Role of Phosphoinositide 3-Kinase Lipid Products in Cell Function.* Journal of Biological Chemistry, 1999. **274**(13): p. 8347-8350.
- 81. Fruman, D.A., L.E. Rameh, and L.C. Cantley, *Phosphoinositide Binding Domains: Embracing 3-Phosphate*. Cell, 1999. **97**(7): p. 817-820.
- 82. Jones, P.F., et al., *Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily*. Proceedings of the National Academy of Sciences, 1991. **88**(10): p. 4171-4175.
- 83. Zhang, Y., et al., *Kinase AKT controls innate immune cell development and function*. Immunology, 2013: p.
- 84. Sarbassov, D.D., et al., *Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex.* Science, 2005. **307**(5712): p. 1098-1101.
- 85. D R Alessi, M.A., B Caudwell, P Cron, N Morrice, P Cohen, and B A Hemmings, *Mechanism of activation of protein kinase B by insulin and IGF-1*. EMBO J, 1996. **15**(23): p. 6541-6551.
- 86. Chang, F., et al., *Involvement of PI3K//Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy.* Leukemia, 2003. **17**(3): p. 590-603.
- 87. Liu, P., et al., *Targeting the phosphoinositide 3-kinase pathway in cancer*. Nat Rev Drug Discov, 2009. **8**(8): p. 627-644.
- 88. Jope, R.S. and G.V.W. Johnson, *The glamour and gloom of glycogen synthase kinase-3*. Trends in Biochemical Sciences, 2004. **29**(2): p. 95-102.
- 89. Krystal, G., *Lipid phosphatases in the immune system*. Seminars in Immunology, 2000. **12**(4): p. 397-403.
- 90. Rohrschneider, L.R., et al., *Structure, function, and biology of SHIP proteins*. Genes & Development, 2000. **14**(5): p. 505-520.
- 91. Ming-Lum, A., et al., *A pleckstrin homology-related domain in SHIP1 mediates membrane localization during Fcy receptor-induced phagocytosis.* The FASEB Journal, 2012. **26**(8): p. 3163-3177.

- 92. Huang, Z.-Y., et al., *The effect of phosphatases SHP-1 and SHIP-1 on signaling by the ITIM- and ITAM-containing Fcy receptors FcyRIIB and FcyRIIA*. Journal of Leukocyte Biology, 2003. **73**(6): p. 823-829.
- 93. Maresco, D.L., et al., *The SH2-Containing 5'-Inositol Phosphatase (SHIP) Is Tyrosine Phosphorylated after Fcy Receptor Clustering in Monocytes.* The Journal of Immunology, 1999. **162**(11): p. 6458-6465.
- 94. Pearse, R.N., et al., *SHIP Recruitment Attenuates FcyRIIB-Induced B Cell Apoptosis*. Immunity, 1999. **10**(6): p. 753-760.
- 95. Lamkin, T.D., et al., Shc Interaction with Src Homology 2 Domain Containing Inositol Phosphatase (SHIP) in Vivo Requires the Shc-Phosphotyrosine Binding Domain and Two Specific Phosphotyrosines on SHIP. Journal of Biological Chemistry, 1997. **272**(16): p. 10396-10401.
- 96. Harmer, S.L. and A.L. DeFranco, *The Src Homology Domain 2-Containing Inositol Phosphatase SHIP Forms a Ternary Complex with Shc and Grb2 in Antigen Receptor-stimulated B Lymphocytes*. Journal of Biological Chemistry, 1999. **274**(17): p. 12183-12191.
- 97. Geier, S.J., et al., *The Human SHIP Gene Is Differentially Expressed in Cell Lineages of the Bone Marrow and Blood*. Blood, 1997. **89**(6): p. 1876-1885.
- 98. Lucas, D.M. and L.R. Rohrschneider, *A Novel Spliced Form of SH2-Containing Inositol Phosphatase Is Expressed During Myeloid Development*. Blood, 1999. **93**(6): p. 1922-1933.
- 99. Bai, L. and L.R. Rohrschneider, *s-SHIP promoter expression marks activated stem cells in developing mouse mammary tissue*. Genes & Development, 2010. **24**(17): p. 1882-1892.
- 100. Valderrama-Carvajal, H., et al., *Activin/TGF-[beta] induce apoptosis through Smaddependent expression of the lipid phosphatase SHIP*. Nat Cell Biol, 2002. **4**(12): p. 963-969.
- 101. O'Connell, R.M., et al., *Inositol phosphatase SHIP1 is a primary target of miR-155*. Proceedings of the National Academy of Sciences, 2009. **106**(17): p. 7113-7118.
- 102. McCoy, C.E., et al., *IL-10 Inhibits miR-155 Induction by Toll-like Receptors*. Journal of Biological Chemistry, 2010. **285**(27): p. 20492-20498.
- 103. Sylvia T. Cheung, E.Y.S., David Chang, Andrew Ming-Lum, Alice L-F. Mui, *Interleukin-10 Inhibits Lipopolysaccharide Induced miR-155 Precursor Stability and Maturation* PLoS One, 2013. **8**(8).
- 104. Ruschmann, J., et al., *Tyrosine phosphorylation of SHIP promotes its proteasomal degradation*. Experimental hematology, 2010. **38**(5): p. 392-402.e1.
- 105. Sly, L.M., et al., *SHIP, SHIP2, and PTEN activities are regulated in vivo by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide.* Experimental hematology, 2003. **31**(12): p. 1170-1181.
- 106. Tridandapani, S., et al., *Src Homology 2 Domain-Containing Inositol Polyphosphate Phosphatase Regulates NF-κB-Mediated Gene Transcription by Phagocytic FcγRs in Human Myeloid Cells*. The Journal of Immunology, 2002. **169**(8): p. 4370-4378.
- 107. Aman, M.J., et al., *The Inositol Phosphatase SHIP Inhibits Akt/PKB Activation in B Cells*. Journal of Biological Chemistry, 1998. **273**(51): p. 33922-33928.

- 108. Damen, J.E., et al., *The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase.* Proceedings of the National Academy of Sciences, 1996. **93**(4): p. 1689-1693.
- 109. Phee, H., A. Jacob, and K.M. Coggeshall, *Enzymatic Activity of the Src Homology 2 Domain-containing Inositol Phosphatase Is Regulated by a Plasma Membrane Location*. Journal of Biological Chemistry, 2000. **275**(25): p. 19090-19097.
- 110. Helgason, C.D., et al., *Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span.* Genes & Development, 1998. **12**(11): p. 1610-1620.
- 111. Takeshita, S., et al., *SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts*. Nat Med, 2002. **8**(9): p. 943-949.
- 112. Sly LM, H.V., Antignano F, Ruschmann J, Hamilton M, Lam V, Rauh MJ, Krystal G., *The role of SHIP in macrophages*. Front Biosci, 2007. **12**: p. 2836-48.
- 113. Hocking, L.J., et al., Genomewide Search in Familial Paget Disease of Bone Shows Evidence of Genetic Heterogeneity with Candidate Loci on Chromosomes 2q36, 10p13, and 5q35. The American Journal of Human Genetics, 2001. **69**(5): p. 1055-1061.
- 114. Luo, J.M., et al., *Possible dominant-negative mutation of the SHIP gene in acute myeloid leukemia*. Leukemia, 2003. **17**(1): p. 1-8.
- 115. Luo JM, L.Z., Hao HL, Wang FX, Dong ZR, Ohno R., *Mutation analysis of SHIP gene in acute leukemia*. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 2004. **12**(4).
- 116. Murray, P.J., *The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(24): p. 8686-8691.
- 117. El Kasmi, K.C., et al., *Cutting Edge: A Transcriptional Repressor and Corepressor Induced by the STAT3-Regulated Anti-Inflammatory Signaling Pathway*. The Journal of Immunology, 2007. **179**(11): p. 7215-7219.
- 118. Chen, C.-Y.A. and A.-B. Shyu, *AU-rich elements: characterization and importance in mRNA degradation*. Trends in Biochemical Sciences, 1995. **20**(11): p. 465-470.
- 119. Khera, T.K., A.D. Dick, and L.B. Nicholson, *Mechanisms of TNFα regulation in uveitis: Focus on RNA-binding proteins.* Progress in Retinal and Eye Research, 2010. **29**(6): p. 610-621.
- 120. Schaljo, B., et al., *Tristetraprolin Is Required for Full Anti-Inflammatory Response of Murine Macrophages to IL-10.* The Journal of Immunology, 2009. **183**(2): p. 1197-1206.
- 121. Chan, C.S., et al., *Interleukin-10 Inhibits Lipopolysaccharide-induced Tumor Necrosis Factor-α Translation through a SHIP1-dependent Pathway*. Journal of Biological Chemistry, 2012. **287**(45): p. 38020-38027.
- 122. Vanhaesebroeck, B., et al., *Signalling by PI3K isoforms: insights from gene-targeted mice*. Trends in Biochemical Sciences, 2005. **30**(4): p. 194-204.
- 123. Utsugi, M., et al., *PI3K p110β Positively Regulates Lipopolysaccharide-Induced IL-12 Production in Human Macrophages and Dendritic Cells and JNK1 Plays a Novel Role.* The Journal of Immunology, 2009. **182**(9): p. 5225-5231.
- 124. Díaz-Guerra, M.J.M., et al., *Negative Regulation by Phosphatidylinositol 3-Kinase of Inducible Nitric Oxide Synthase Expression in Macrophages*. The Journal of Immunology, 1999. **162**(10): p. 6184-6190.

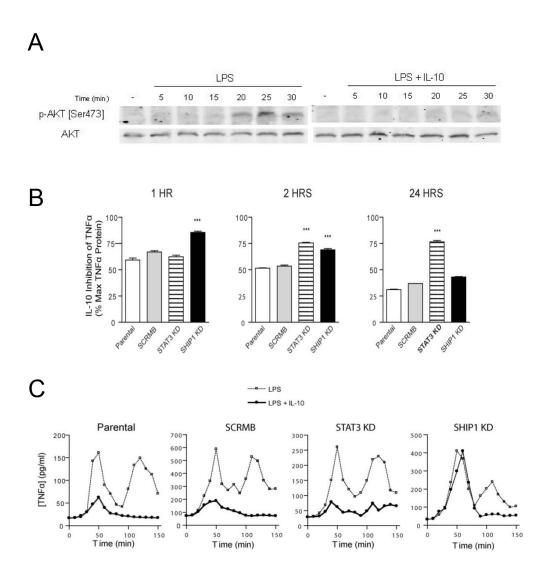
- 125. Guha, M. and N. Mackman, *The Phosphatidylinositol 3-Kinase-Akt Pathway Limits Lipopolysaccharide Activation of Signaling Pathways and Expression of Inflammatory Mediators in Human Monocytic Cells.* Journal of Biological Chemistry, 2002. **277**(35): p. 32124-32132.
- 126. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. nature, 1970. **227**(5259): p. 680-685.
- 127. Wang, C., et al., *Characterization of murine macrophages from bone marrow, spleen and peritoneum.* BMC immunology, 2013. **14**(1): p. 6.
- 128. Clausen, B., et al., Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic research, 1999. **8**(4): p. 265-277.
- 129. O'Farrell, A.-M., et al., *IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways.* EMBO J, 1998. **17**(4): p. 1006-1018.
- 130. Berg, D.J., et al., *Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance*. The Journal of Clinical Investigation, 1995. **96**(5): p. 2339-2347.
- 131. Takeda, K., et al., Enhanced Th1 Activity and Development of Chronic Enterocolitis in Mice Devoid of Stat3 in Macrophages and Neutrophils. Immunity, 1999. **10**(1): p. 39-49.
- 132. Blunt, M.D. and S.G. Ward, *Targeting PI3K isoforms and SHIP in the immune system:* new therapeutics for inflammation and leukemia. Current Opinion in Pharmacology, 2012. **12**(4): p. 444-451.
- 133. Ong, C.J., et al., *Small-molecule agonists of SHIP1 inhibit the phosphoinositide 3-kinase pathway in hematopoietic cells.* Blood, 2007. **110**(6): p. 1942-1949.
- 134. Takeda, K., et al., *Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality*. Proceedings of the National Academy of Sciences, 1997. **94**(8): p. 3801-3804.

Appendices



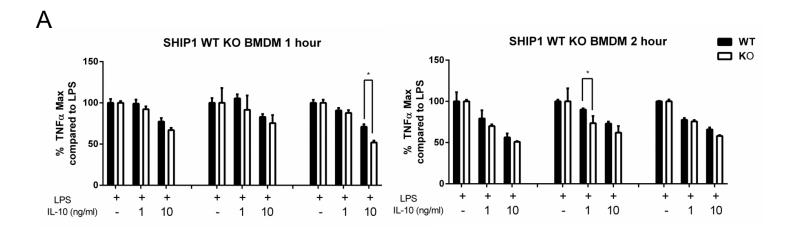
Appendix A Previous results demonstrating IL-10 activates SHIP in macrophage

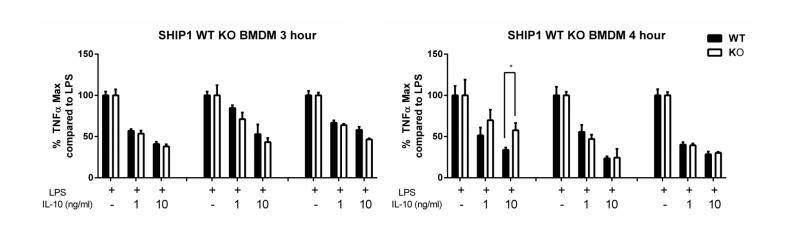
(A) Orthophosphate labeled wild type BMDM were treated with LPS (50 ng/ml) +/- IL-10 (100 ng/ml) for 15 minutes and underwent HPLC inositol phospholipid analysis. Data represent mean CPM \pm SD (n=3). *p<0.05, **p<0.01 (one-way ANOVA). (B) Immunoblot analysis of J774.1 macrophages that were transduced to express wild type hIL-10R or Tyr446/496^{FF} hIL-10R, stimulated by hIL-10 (50 ng/ml) in the presence of anti-mIL-10R antibody (IBI.2, 10 μ g/ml) for the indicated times. Blots were probed with anti-phospho tyrosine antibody (4G10) and protein SHIP1 as loading control. (C) Immunoblot analysis of phosphorylated or unphosphorylated hIL-10R peptides pull down of J774.1 macrophages probed with anti-SHIP1 antibody.

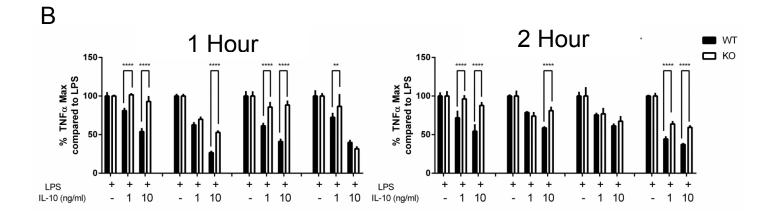


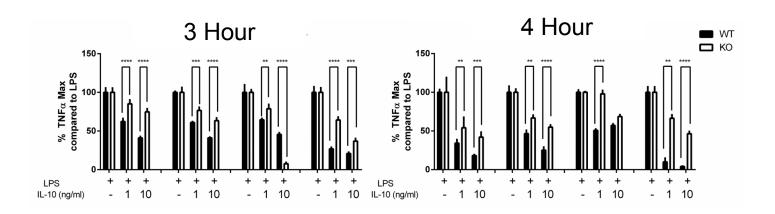
Appendix B Previous results of IL-10's role in the PI3K pathway and involvement of SHIP1 and STAT3 in IL-10's inhibitory effect on TNFα protein production

(A) Immunoblot analysis of RAW 264.7 cells stimulated with LPS (10 ng/ml) +/- IL-10 (100 ng/ml) for the indicated times, probed with anti-phospho-Akt (Ser 473) and protein Akt (loading control). (B) TNF α ELISA of supernatant of parental RAW264.7 cells, Scrambled, SHIP1 or STAT3 siRNA transduced RAW 264.7 cells that were treated by Doxycycline (2 µg/ml) for 48 hours prior to stimulation then were stimulated by LPS (10 ng/ml) with or without IL-10 (100 ng/ml) for the indicated times. Data represent the percentage of TNF α level relative to LPS alone treated samples \pm S.D. (n=3). (C) TNF α ELISA of continuous flow cell system from parental RAW264.7 cells, Scrambled, SHIP1 or STAT3 siRNA transduced RAW 264.7 cells that were treated by Doxycycline (2 µg/ml) for 48 hours prior to stimulation then were stimulated by LPS (10 ng/ml) with or without IL-10 (10 ng/ml). Data represent the TNF α concentration of each fraction (5 minutes per fraction) collected over the course of 3 hours









Appendix C Replicate experiments of TNF α protein production in SHIP1 and STAT3 $^{+\!/\!+}$ and $^{-\!/\!-}$ BMDM

(A) TNF α ELISA of supernatant of SHIP1 */+ /-/- (n=3) and (B) STAT3 */+ /-/- BMDM (n=4) that were treated by LPS (1 ng/ml) with or without IL-10 (1 or 10 ng/ml) for the indicated times. Data represent the percentage of TNF α level relative to LPS alone treated samples \pm S.D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001 (Two-way ANOVA)