

ROLE OF SHIP1 IN THE INTERLEUKIN 10 SIGNALING PATHWAY

by

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Role of SHIP1 in the interleukin 10 signaling pathway

submitted by Soroush Shakibakho in partial fulfillment of the requirements for

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## **Abstract**

**Background:** Inflammation is a major immune response that protects the host from infections. Upon pathogen encounter, immune cells are activated and produce pro-inflammatory mediators. Although inflammation is a critical step in both innate and acquired immune response, excessive inflammation can damage tissues and lead to inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. Interleukin 10 (IL10) is a key anti-inflammatory cytokine that deactivates various immune cells including macrophages. IL10 signaling has classically been described to include ligand stimulated phosphorylation of the IL10 receptor (IL10R), recruitment of the signal transducer and activator of transcription 3 (STAT3) and the subsequent phosphorylation, dimerization and translocation of STAT3 to the nucleus, where it stimulates transcription of anti-inflammatory genes. Our studies have shown that in addition to the canonical STAT3 signaling, IL10 mediates its anti-inflammatory effect through SHIP1 inositol phosphatase. We hypothesized that IL10 signaling leads to the recruitment and activation of a SHIP1-STAT3 complex that then mediates the anti-inflammatory effects of IL10.

Our SHIP1 pulldown studies have revealed that SHIP1 and STAT3 form a complex in response to IL10. In addition, we observed the AMP-activated protein kinase (AMPK), which has been previously reported to play a role in IL10 signaling, to be part of this complex. The pro-inflammatory counterpart of IL10, IL6 is unable to initiate the formation of such complex although both IL10 and IL6 induce tyrosine phosphorylation of STAT3. In addition to IL10, our small molecule SHIP1 agonist, AQX-151, was also found to induce the formation of the SHIP1-STAT3-AMPK complex. Our experiments in STAT3 knock-out bone marrow derived macrophages suggested that STAT3 mediates the association of SHIP1 and AMPK. We also mutated a number of phospho-Tyr residues in SHIP1 and assessed IL10 signaling. Tyr190 was identified to play an important role in both complex assembly and cellular response to IL10 (evaluated by inhibition of TNF $\alpha$  secretion).

These studies showed for the first time the importance of SHIP1, STAT3 and AMPK complex in IL10 signaling. Understanding the mechanisms by which IL10 down regulates immune cell function will give insight into development of novel therapeutics that mimic the beneficial effects of IL10.

## **Lay Summary**

Inflammation is a major immune response that protects us from infections. After encountering a microbe, our immune cells are activated and produce pro-inflammatory mediators. Although inflammation is a critical step in both innate and acquired immune response, excessive inflammation can damage tissues and lead to inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

Interleukin 10 (IL10) is a key anti-inflammatory cytokine that deactivates various immune cells. IL10 signaling has classically been described to use the signal transducer and activator of transcription 3 (STAT3) pathway to turn-on anti-inflammatory genes. However, our studies have shown that in addition to the canonical STAT3 signaling, IL10 mediates its anti-inflammatory effect through SHIP1 inositol phosphatase. Understanding the mechanisms by which IL10 down regulates immune cells will give insight into development of novel drugs that can mimic the beneficial effects of IL10.

## **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:

- Site directed mutagenesis of SHIP1 was performed by Dr. Sylvia Cheung
- Data presented in Appendix A is courtesy of Dr. Andrew Ming-Lum
- Data presented in Appendix B is courtesy of Dr. Sylvia Cheung

## **Ethics Approval**

All animal experiments were performed in accordance with the UBC Animal Care Committee guidelines under the following protocols:

- SHIP1 KO mouse colony: A13-0203
- Harvest of tissues and cells: A11-0218

## Table of Contents

Abstract.....	iii
Lay Summary.....	iv
Preface.....	v
Table of Contents.....	vi
List of Tables.....	viii
List of Figures.....	ix
List of Abbreviations.....	x
<b>1 Introduction.....</b>	<b>1</b>
1.1 Inflammation.....	1
1.2 Innate immunity and macrophages.....	2
1.3 LPS/TLR4 Signaling pathway.....	3
1.4 Tumor necrosis factor $\alpha$ .....	6
1.5 PI3K pathway.....	8
1.6 SH2-containing inositol-5'-phosphatase 1 (SHIP1).....	9
1.6.1 SHIP1's Biological activity.....	9
1.6.2 Non-Catalytic functions of SHIP1.....	10
1.6.3 SHIP1 in disease.....	10
1.7 Interleukin 10.....	10
1.7.1 IL10 Signaling.....	11
1.7.2 IL10 and SHIP1.....	12
1.8 Interleukin 6.....	12
1.8.1 IL6 Signaling.....	12
1.9 AMP-activated protein kinase (AMPK) .....	13
1.9.1 AMPK inhibition of inflammation.....	13
1.9.2 AMPK and IL10 signaling.....	14
1.10 Linking IL10, STAT3 and SHIP1.....	14
1.11 Hypothesis and objectives.....	15

<b>2 Materials and methods</b> .....	15
2.1 Mouse colonies.....	15
2.2 Generation of bone marrow derived macrophage and peritoneal macrophages.....	15
2.3 Cell lines.....	16
2.4 Antibodies and drugs.....	17
2.5 SHIP1 pull down assay.....	17
2.6 Immunoblotting and analysis.....	17
2.7 TNF $\alpha$ ELISA.....	18
2.8 Statistical analysis.....	18
<b>3 Results</b> .....	19
3.1 SHIP1 interacts with STAT3 and AMPK to mediate IL10 signaling.....	19
3.2 Complex formation is independent of STAT3 phosphorylation.....	24
3.3 STAT3 mediates the interaction between SHIP1 and AMPK.....	25
3.4 A catalytically inactive mutant of SHIP1 is responsive to IL10.....	27
3.5 Identification of domains and residues required for complex formation.....	30
3.5.1 SHIP1's PH and C2 domains are important for complex formation.....	30
3.6 Phosphorylation of Tyr-190 is required for complex formation.....	33
3.7 Tyr-190 mutants are less responsive to IL10.....	36
<b>4 Discussion</b> .....	37
4.1 STAT3 independent IL10 signaling pathway.....	37
4.2 SHIP1 in IL10 signaling.....	37
<b>5 References</b> .....	41
<b>6 Appendices</b> .....	48

**List of Tables**

**Table 1 Cell lines used in this study.....16**

**Table 2 Tyrosine point mutants..... 33**

## List of Figures

<b>Figure 1 LPS/TLR signaling pathway</b> .....	5
<b>Figure 2 Tumor necrosis factor <math>\alpha</math> signaling pathway</b> .....	7
<b>Figure 3 Canonical IL10 signaling pathway via STAT3</b> .....	11
<b>Figure 4 SHIP1 binds STAT3 in response to IL10</b> .....	20
<b>Figure 5 SHIP1 physically interacts with STAT3 and AMPK</b> .....	23
<b>Figure 6 STAT3 phosphorylation and complex formation are independent events</b> .....	24
<b>Figure 7 STAT3 mediates the interaction between SHIP1 and AMPK</b> .....	26
<b>Figure 8 SHIP1 phosphatase dead mutant is fully responsive to IL10</b> .....	28
<b>Figure 9 Schematic diagram of the domains in WT SHIP1 and our SHIP1 mutants</b> .....	29
<b>Figure 10 SHIP1 <math>\Delta</math>PH mutant does not form a complex</b> .....	31
<b>Figure 11 SHIP1 <math>\Delta</math>C2 mutant does not form a complex in response to AQX-151</b> .....	32
<b>Figure 12 Schematic diagram of STAT3 and its domains</b> .....	34
<b>Figure 13 Phosphorylation of Tyr190 plays a role in complex formation</b> .....	35
<b>Figure 14 Tyr190 is crucial for IL10 signaling</b> .....	36
<b>Figure 15 Proposed model for IL10 signaling</b> .....	40

## List of Abbreviations

AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
ATF-1	Activating transcription factor 1
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
CSF	Colony stimulating factor
DAMP	Danger associated molecular pattern
DMEM	Dulbecco's modified eagle medium
Dok	Docking protein
ETV3	Ets variant 3
FADD	Fas-associated death domain
FC $\gamma$ R	FC $\gamma$ receptor
FCS	Fetal calf serum
Grb2	Growth factor receptor-bound protein 2
IBD	Inflammatory bowel disease
IFN $\gamma$	Interferon $\gamma$
IKK	Inhibitor of $\kappa$ B kinase
IL10	Interleukin 10
IL10R	Interleukin 10 receptor
IMDM	Iscove's modified Dulbecco's medium
IRAK4	IL1 receptor associated kinase 4
Jak1	Janus kinase 1
JNK	c-Jun N-terminal kinase
KD	Knock down

KO	Knockout
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAP3K	MAP kinase kinase kinase
MAPK	Mitogen activated protein kinase
MCSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
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
RIP1	Receptor interacting protein 1
SDS	Sodium dodecyl sulphate
SH2	Src homology 2 containing protein
SHIP1	SH2 domain containing inositol 5' photphatase
SMAD	Sma and Mad related protein
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription

TAB 1, 2, 3	TAK-1 binding proteins 1, 2, 3
TAK1	TGF $\beta$ -activated protein kinase 1
TBS	Tris-buffered saline
TGF $\beta$	Transforming growth factor $\beta$
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 $\alpha$	Tumor necrosis factor $\alpha$
TNFR	TNF $\alpha$ receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing interferon $\beta$
TTP	Tristetrapolin
Tyk2	Tyrosine kinase 2
UTR	Untranslated region

## 1. Introduction

### 1.1 Inflammation

Inflammation is classically described as the occurrence of redness, swelling, heat, pain and disturbance of function of the affected tissue<sup>[1]</sup>. We now appreciate inflammation as a crucial step in the activation of the immune response to pathogens and chemical irritants. The primary goal of inflammation is to protect the host and initiate tissue repair and healing<sup>[2, 3]</sup>. However, like any other physiologic response, regulation and balance is necessary. In fact, excessive and uncontrolled inflammation may cause tissue damage and a wide array of pathologies including inflammatory bowel disease (IBD) and rheumatoid arthritis<sup>[3-6]</sup>.

Inflammation can be triggered by a number of pathogen associated molecular patterns (PAMPs) present on the cell membrane of various microbes, and damage associated molecular patterns (DAMPs) released by damaged or expiring cells<sup>[7, 8]</sup>. When tissue resident macrophages and mast cells encounter these signals, they are activated and release a number of pro-inflammatory mediators including histamine, prostaglandins, interleukin 1 (IL1), interleukin 6 (IL6), interleukin 8 (IL8) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ )<sup>[9]</sup>. These pro-inflammatory mediators cause vasodilation and increase permeability of blood vessels supplying the affected tissue, leading to cardinal signs of inflammation: swelling, redness and heat. In addition, these pro-inflammatory molecules enhance the expression of different adhesion molecules by the endothelial cells that line the blood vessels. This leads to the recruitment, attachment and migration of neutrophils to the site of infection<sup>[10]</sup>. The recruited neutrophils are activated by the pro-inflammatory mediators and subsequently release more of these factors and other cytotoxic molecules to combat the invading pathogen. Clearance of the pathogens triggers the resolution phase and initiates regeneration and repair of the damaged tissue. On the other hand, inability of neutrophils to clear the microbes, and persistence of the inflammatory response results in the recruitment of macrophages and lymphocytes<sup>[3]</sup>.

Although inflammation is necessary to generate an appropriate response against the microbes, continuous inflammation results in insidious release of cytotoxic mediators and tissue damage. As a result, inflammation needs to be tightly regulated via anti-inflammatory molecules such as interleukin 10

(IL10) and transforming growth factor  $\beta$  (TGF $\beta$ )<sup>[11, 12]</sup>. In fact, there are many cases where loss of function mutations of these anti-inflammatory products has led to chronic inflammation and devastating pathologies<sup>[4, 6, 13]</sup>. These anti-inflammatory mediators act by repressing the leukocytes and inhibiting synthesis and release of pro-inflammatory molecules, switching the cells from an inflammatory state to an anti-inflammatory state<sup>[14]</sup>.

## 1.2 Innate immunity and macrophages

Inflammation is primarily mediated by cells of the innate immune system such as the phagocytes, granulocytes, antigen-presenting cells and natural killer cells<sup>[7]</sup>. Phagocytes engulf and kill the invading pathogen and include macrophages, neutrophils and dendritic cells. In addition to eradicating the microbe, they can process and present antigens via Major Histocompatibility Complex II (MHC II) to activate cells of adaptive immunity<sup>[7]</sup>. Granulocytes contain cytotoxic granules packed with reactive oxygen and nitrogen species and include basophils, eosinophils and mast cells. Natural killer cells are specialised to kill tumor cells and virally infected cells.

Macrophages are special among the innate immune cells as they play a key role in both the initiation and resolution of inflammation. In addition, macrophages act as a bridge between innate and adaptive immunity by presenting parts of the engulfed microbe to lymphocytes<sup>[15]</sup>. Macrophages are equipped with a family of immune receptors known as the Toll-like receptors (TLRs) which are able to recognise PAMPs expressed by the invading pathogen. This will trigger the activation of macrophages and result in the production of various pro-inflammatory mediators<sup>[15]</sup>. Activated macrophages, often referred to as M1 macrophages, potentiate inflammation by producing reactive oxygen and nitrogen species and pro-inflammatory cytokines including IL1, IL6 and TNF $\alpha$ . Furthermore, these macrophages have higher expression of MHC II and co-stimulatory molecules CD80/CD86 and therefore have enhanced antigen presenting ability<sup>[15-17]</sup>. On the other hand, macrophages may get activated by other stimuli such as IL3, IL4, and TGF $\beta$ . These alternatively activated macrophages are classified as M2 macrophages and are associated with healing and resolution of the inflammatory response. Unlike the M1 macrophages, M2 macrophages do not express pro-inflammatory cytokines and they do not have

increased synthesis of antigen presenting molecules. In fact, M2 or “healer” macrophages have enhanced endocytic activity and produce anti-inflammatory cytokine IL10<sup>[15-19]</sup>.

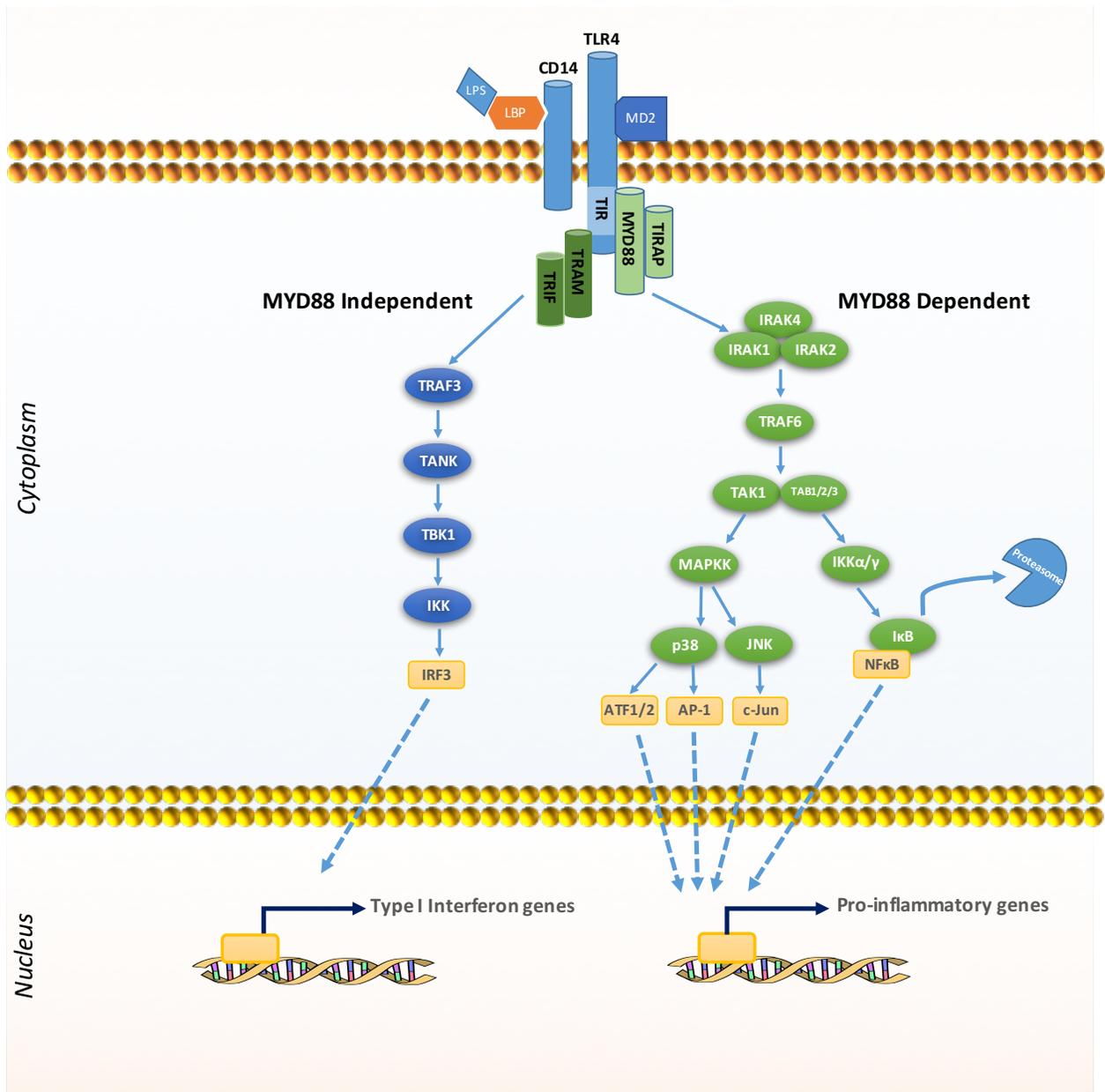
### 1.3 LPS/TLR4 Signaling pathway

Macrophages detect PAMPs via the TLRs expressed on their cell membrane. TLRs are type I transmembrane proteins and carry a conserved Toll/IL1R (TIR) domain in their cytoplasmic side capable of interacting with adaptor proteins<sup>[20]</sup>. To date, thirteen TLRs have been identified in mouse, and ten of these are also found in human. TLR1, 2, and 6 recognize bacterial peptidoglycans, TLR3 recognizes double stranded RNA in viruses, TLR4 recognizes lipopolysaccharide (LPS), TLR5 recognizes bacterial flagellin, TLR7 and 8 recognize single stranded RNA in viruses, TLR9 recognizes unmethylated CpG sequence on DNA, and TLR11 recognizes flagellin and profilin present on certain microbes<sup>[21]</sup>. LPS is part of the cell wall of Gram-negative bacteria and it is recognised by TLR4. LPS is bound by LPS binding protein (LBP) which couples LPS to CD14, a GPI-anchored membrane protein on the surface of macrophages. This facilitates the recognition of LPS by TLR4-MD2 complex and initiates TLR4 dimerization and signaling<sup>[22]</sup>.

LPS/TLR4 signaling is known to happen via two distinct pathways: the MyD88 dependent and MyD88 independent pathways. As shown in **Figure 1**, in the MyD88 dependent pathway, LPS binding induces the recruitment of TIR-domain containing adaptor protein (TIRAP) and MyD88 to TLR4. MyD88 will subsequently bind IL1 receptor associated kinase 4 (IRAK4) via its death domain. Once at the membrane, IRAK4 is phosphorylated and activated. Active IRAK4 will recruit and phosphorylate IRAK1 and 2 and the activated IRAKs form a complex with tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 is a ubiquitin E3 ligase, and together with the ubiquitin conjugating enzyme Ubc13 and Ubc-like protein Uev1a, it catalyzes the addition of polyubiquitin chains on itself and other proteins<sup>[23]</sup>. Active TRAF6 will recruit and activate TGF $\beta$ -activated protein kinase 1 (TAK1) and TAK-1 binding proteins 1, 2 and 3 (TAB 1, 2 and 3)<sup>[24, 25]</sup>. TAK1 is a MAP kinase kinase kinase (MAP3K) and activates important signaling pathways such as NF $\kappa$ B, p38 and c-Jun N-terminal kinase (JNK)<sup>[24, 25]</sup>. For instance, TAK1 activates nuclear factor  $\kappa$ B kinase complex (IKK)  $\alpha/\gamma$ , which subsequently phosphorylates I $\kappa$ B thus

promoting its degradation <sup>[24, 25]</sup>. The degradation of I $\kappa$ B releases NF $\kappa$ B and allows it to translocate to the nucleus to induce the transcription of pro-inflammatory mediators <sup>[24, 25]</sup>.

In the MyD88 independent pathway, TLR4 associates with TIR-domain containing adaptor inducing interferon  $\beta$  (TRIF) via TRIF related adaptor molecule (TRAM) <sup>[24, 25]</sup>. TRIF will activate receptor interacting protein 1 (RIP1), which in turn activates the IKK complex <sup>[24, 25]</sup>. Similar to the MyD88 dependent pathway, IKK activation initiates NF $\kappa$ B signaling <sup>[24, 25]</sup>. TRIF may also regulate the activation of transcription factor interferon regulatory factor 3 (IRF3), which is responsible for the transcription of type I interferon gene products (**Figure 1**).



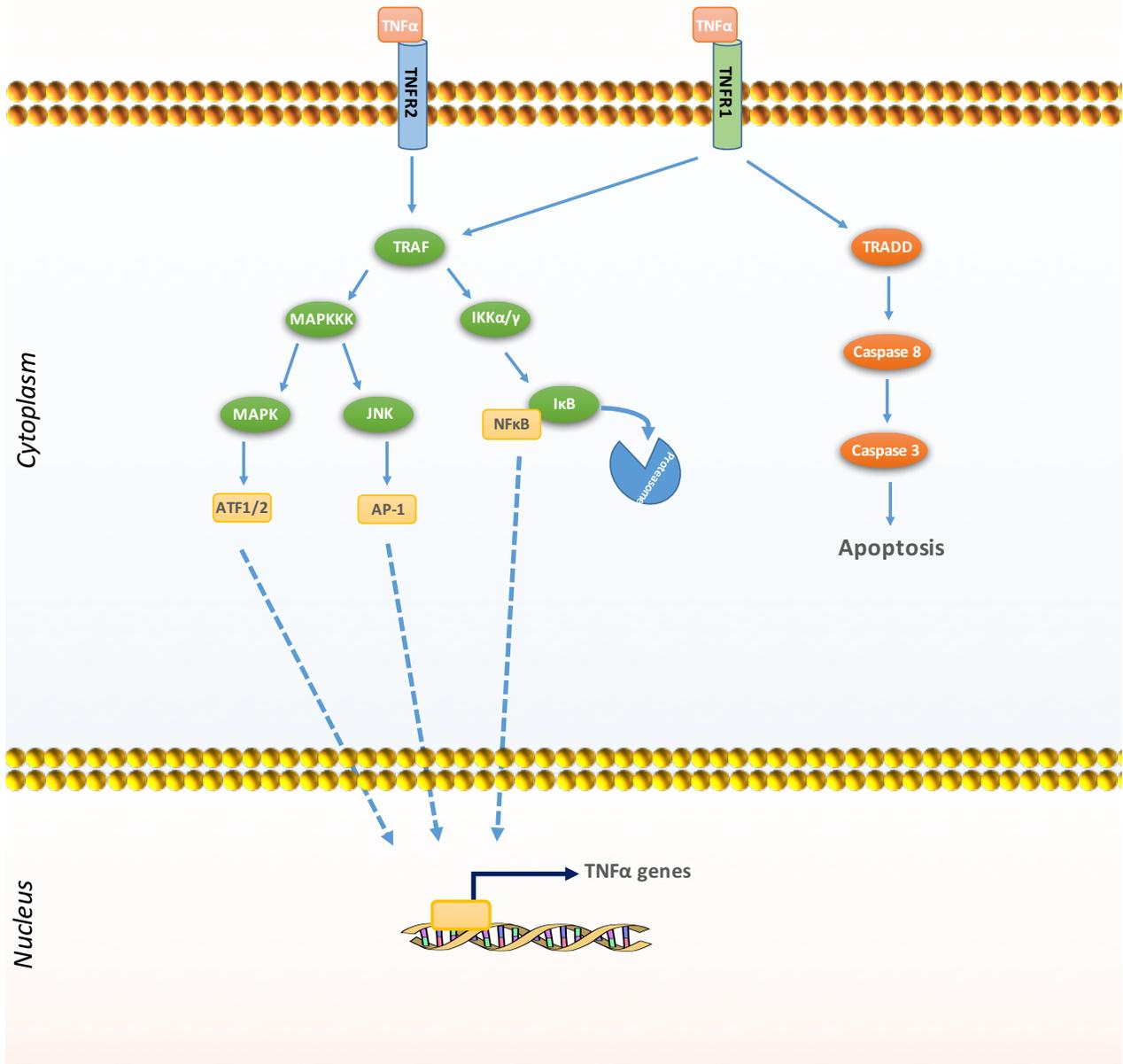
**Figure 1 LPS/TLR signaling pathway**

LPS binding to the TLR4-MD2 complex initiates the MyD88 dependent and MyD88 independent pathways. In the MYD88 dependent pathway, TIRAP is recruited to the receptor complex and ultimately activates pro-inflammatory pathways including ATF1/2, AP-1, c-Jun and NF $\kappa$ B. In the MyD88 independent pathway TRIF and TRAM are recruited to TLR4 and initiate a signalling cascades leading to the activation of IRF3 transcription factor. This ultimately leads to the production of type I interferons and other genes involved in the inflammatory response.

## 1.4 Tumor necrosis factor $\alpha$

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) was first described as an inducer of hemorrhagic necrosis of tumors, hence its name<sup>[26]</sup>. It was later found that TNF $\alpha$  plays a crucial role in endotoxin stimulated septic shock and is a hallmark of inflammation<sup>[27, 28]</sup>. In fact, TNF $\alpha$  is one of the earliest molecules expressed by macrophages following LPS exposure and it can be detected in abundance within one hour of stimulation. TNF $\alpha$  protein is initially synthesized as a 27 kDa membrane attached protein<sup>[28, 29]</sup>. It is later cleaved to produce the soluble 17 kDa mature protein<sup>[28, 29]</sup>. LPS stimulation activates the TLR4 pathway, which in turn promotes transcription of TNF $\alpha$  via NF $\kappa$ B and Activator protein 1 (AP-1)<sup>[30, 31]</sup>. In addition, RNA binding proteins, such as tristetrapolin (TTP), post-transcriptionally regulate TNF $\alpha$  synthesis by interacting with the 5' and 3' untranslated region (UTR) of the mRNA<sup>[32-35]</sup>.

TNF $\alpha$  acts through two receptors: TNF receptor 1 and 2 (TNFR1 and 2). TNFR1 is ubiquitously expressed in almost all cell types and TNFR2 is only present on immune cells including the macrophages<sup>[36]</sup>. Binding of TNF $\alpha$  to TNFR1 leads to its association with TNFR1 associated death domain (TRADD) and initiates apoptosis (**Figure 2**)<sup>[37]</sup>. In addition, TNF $\alpha$  binding to TNFR1 and TNFR2 recruits TNF receptor associated factors (TRAFs) that activate the NF $\kappa$ B and MAPKs pathways, resulting in production of pro-inflammatory cytokines, including TNF $\alpha$  itself (**Figure 2**)<sup>[38]</sup>.



**Figure 2 Tumor necrosis factor α signaling pathway**

TNFα binding to TNFR1 leads to the recruitment of TRADD and results in the activation of the caspases and ultimately apoptosis. TNFα binding to TNFR2 recruits TRAF which leads to the activation of the MAPK and NFκB signaling pathways. This results in the production of pro-inflammatory gene products, such as TNFα itself.

## 1.5 Phosphatidylinositol-3 kinase (PI3K) pathway

The phosphatidylinositol-3 kinases (PI3K) are a family of kinases that phosphorylate the 3' hydroxyl position of phosphatidylinositol lipids. The PI3K products are important secondary messengers for a wide range of signaling pathways that regulate many cellular functions including growth, motility, and proliferation. There are eight PI3K isoforms identified and they are divided into three classes according to their structures and substrate specificities<sup>[39-45]</sup>. Class IA PI3Ks phosphorylate PI(4,5)P<sub>2</sub> and make PI(3,4,5)P<sub>3</sub>, which can recruit proteins containing pleckstrin homology (PH) domain. One example of a PH domain containing protein that binds to PIP<sub>3</sub> is protein kinase B (PKB)<sup>[43, 45, 46]</sup>. PKB, also known as Akt, is a serine/threonine kinase that mediates various signaling pathways important for growth and proliferation<sup>[47-49]</sup>. PI3K signaling is also known to be involved in the LPS/TLR4 pathway. PI3K is reported to interact with the TLR4 signaling complex via its p85 subunit<sup>[50, 51]</sup>. Although, there are conflicting findings regarding the role of PI3K in LPS/TLR4 pathway, overall PI3K appears to enhance LPS-stimulated production of pro-inflammatory mediators such as TNF $\alpha$ <sup>[52-56]</sup>.

Dysregulation of PI3K is commonly found in cancer and inflammatory diseases<sup>[57]</sup>. As a result, regulating the activity of PI3K is crucial for normal cellular behavior and function. PI3K activity is antagonized by inositol phosphatases. The most studied inositol phosphatase is phosphatase and tensin homolog (PTEN), which converts phosphatidylinositol (3,4,5) trisphosphate (PIP<sub>3</sub>) to phosphatidylinositol (4,5) bisphosphate (PIP<sub>2</sub>)<sup>[58]</sup>. The SH2-domain containing inositol 5'-phosphatase 1 and 2 (SHIP1 and SHIP2) are another one of these phosphatases that dephosphorylates PIP<sub>3</sub> at the 5' position, converting PIP<sub>3</sub> into phosphatidylinositol (3,4) bisphosphate<sup>[59, 60]</sup>. PTEN and SHIP2 are expressed in a variety of tissues, whereas SHIP1 is predominantly present in hematopoietic cells<sup>[60]</sup>. Considering SHIP1's unique expression pattern, it is a strong therapeutic candidate to treat hematopoietic disorders involving PIP signaling<sup>[61, 62]</sup>.

## 1.6 SH2-containing inositol-5'-phosphatase 1 (SHIP1)

SHIP1 is a 145 kDa protein with a N-terminal SH2 domain, a PH-related domain (PH-R), a centrally located phosphatase domain, a C2 domain, followed by a proline-rich region (PRR) at the C-terminus<sup>[63, 64]</sup>. The SH2 domain is important for SHIP1's association with tyrosine-phosphorylated proteins<sup>[65-67]</sup>. PH-R domain is required for the recruitment of SHIP1 to the plasma membrane to mediate phagocytosis<sup>[64]</sup>. C2 domain is involved in binding of SHIP1's product, PI (3,4) P<sub>2</sub>, and it is also the allosteric binding site responsible for enhancing SHIP1's activity<sup>[61, 62]</sup>. Furthermore, SHIP1 can bind several SH3 domain containing proteins including growth factor receptor-bound protein 2 (Grb2) via its PRR<sup>[68, 69]</sup>. In addition, the PRR domain has two NPXY motifs which are prone to phosphorylation. When phosphorylated, SHIP1 can bind to proteins containing phospho-tyrosine binding domains (PTB), such as Shc and docking protein (Dok 1 and 2)<sup>[70]</sup>.

### 1.6.1 SHIP1's Biological activity

Knowing SHIP1's ability to inhibit PI3K signaling, it was not surprising to discover its role as a negative regulator of immune activity. In fact, several studies have shown that knocking out SHIP1 leads to enhanced production of pro-inflammatory cytokines in macrophages and higher levels of Akt phosphorylation and activation<sup>[71]</sup>. In addition, as PI3K signaling induces proliferation and survival, it has been suggested that SHIP1 plays an important role in regulating macrophage activation<sup>[45, 72]</sup>. Several groups have shown that SHIP1 can inhibit transcription factors such as NFκB by downregulating PI3K and lowering PIP<sub>3</sub> levels<sup>[73, 74]</sup>. As the phosphatase activity of SHIP1 remains constant upon cytokine stimulation, it is believed that SHIP1's activity is dictated by its physical location within the cell and that it performs its role by translocating to sites containing PIP<sub>3</sub><sup>[75, 76]</sup>.

Regulation of SHIP1's expression further demonstrates its anti-inflammatory role. At the transcriptional level, SHIP1's expression is regulated by the Sma and Mad related protein (SMAD) transcription factors<sup>[77]</sup>. TGFβ, a negative regulators of immune cell proliferation, strongly enhances the expression of SHIP1 mRNA in a SMAD dependent manner<sup>[77]</sup>. At the post-transcriptional level, SHIP1 is regulated by the pro-inflammatory micro-RNA-155 (miR-155)<sup>[78]</sup>. The anti-inflammatory cytokine IL10,

inhibits miR-155 expression and this inhibition is found to be dependent on SHIP1 and STAT3<sup>[79, 80]</sup>.

These findings suggest that SHIP1 and miR-155 regulate each other in a mutual manner, creating a fine balance between pro- or anti-inflammatory response of the cells<sup>[80]</sup>.

### **1.6.2 Non-Catalytic functions of SHIP1**

Several reports have shown that SHIP1 does not function exclusively by antagonizing PI3K. SHIP1 contains a Src-homology 2 (SH2) domain, a PH related domain and a proline rich region that all carry the potential to participate in protein-protein interactions. Furthermore, SHIP1 carries several tyrosine residues that can be phosphorylated and interact with Shc, inhibiting Grb2-mediated Ras/Erk signaling pathway<sup>[75, 81-83]</sup>. Ultimately preventing the translocation of TNF $\alpha$  mRNA to the cytoplasm and inhibiting the production of pro-inflammatory IL6 and IL1 $\beta$ <sup>[84]</sup>. Studies that reconstituted cell lines with non-catalytic mutants of SHIP1 reported that regions other than the phosphatase domain are necessary for SHIP1's function in TLR4, PDGFR, and HGFR signaling<sup>[85, 86]</sup>.

### **1.6.3 SHIP1 in disease**

Most of our appreciation of SHIP1's role in inflammation and immune cells comes from studying SHIP1<sup>-/-</sup> mice. Splenomegaly, a Paget's like osteoporosis, shortened life span, and an asthma like hypersensitivity are examples of pathologies seen in these studies<sup>[87, 88]</sup>. Furthermore, SHIP1<sup>-/-</sup> mice are more susceptible to endotoxin shock and their macrophages produce higher levels of pro-inflammatory cytokines following LPS stimulation. In humans, heterozygosity at chromosome 2q36 (chromosomal location of SHIP1) is associated with familial Paget-like osteoporosis<sup>[89]</sup>. Moreover, mutations in SHIP1 have been found to play a role in the pathogenesis of acute myelogenous leukemia and acute lymphoblastic leukemia<sup>[90, 91]</sup>.

## **1.7 Interleukin 10**

Interleukin 10 (IL10) is a major anti-inflammatory cytokine that inhibits the production of various pro-inflammatory mediators. Current research aims to utilize the anti-inflammatory effect of IL10 to treat diseases of chronic inflammation<sup>[92-98]</sup>. IL10 is a class II  $\alpha$ -helical cytokine consisting of six helices that weighs 21 kDa. IL10 is expressed by almost all leukocytes, yet the major sources of IL10 *in vivo* are the

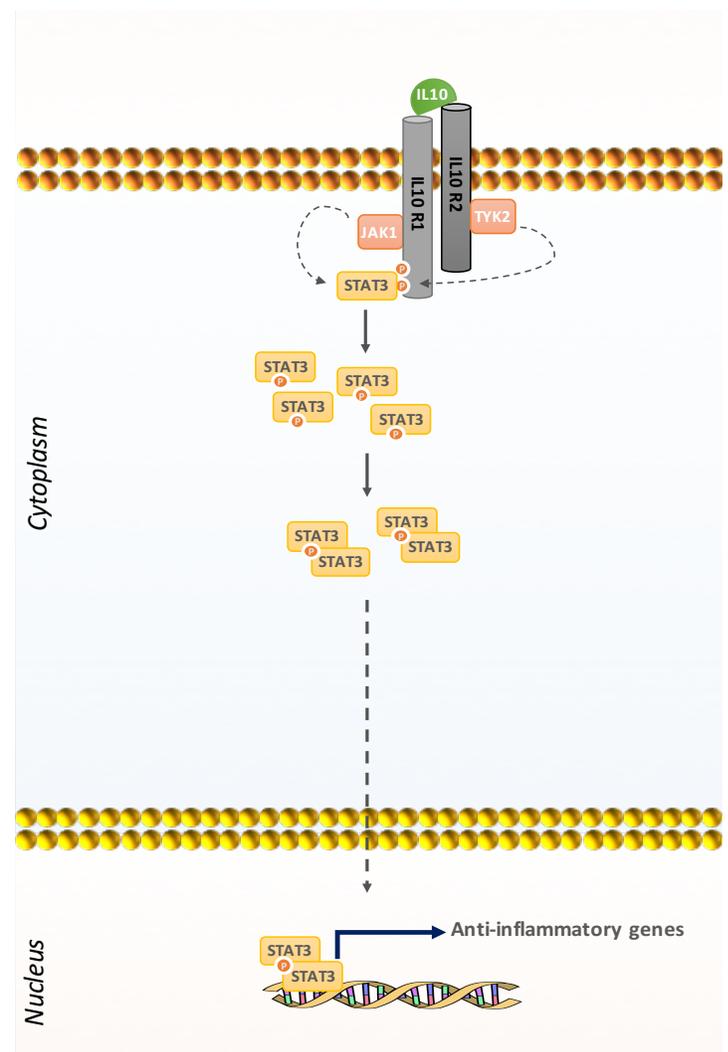
macrophages and T-helper cells [99-101]. In macrophages, IL10 inhibits the production of pro-inflammatory cytokines including IL1 $\beta$ , IL6, IL8, IL12 and TNF $\alpha$  [102, 103]. It also upregulates production of IL1 receptor antagonist and downregulates the expression of MHC II [104, 105].

### 1.7.1 IL10 signaling through STAT3

Binding of IL10 to its receptor (IL10R) leads to receptor dimerization, causing the recruitment of tyrosine kinases JAK1 and TYK2 [106]. This leads to the phosphorylation of two tyrosine residues on IL10R which serve as the docking site for the Signal transducer and activator of transcription 3 (STAT3)

transcription factor (**Figure 3**) [106]. STAT3 has a Src-homology 2 (SH2) domain which recognises these phosphorylated tyrosine residues. Next, the recruited STAT3 is itself phosphorylated and can form a dimer with another STAT3 molecule. The dimerized STAT3 will translocate to the nucleus where it promotes the transcription of anti-inflammatory mediators. Examples of these gene include the Bcl3, ETV3 and SBNO2 which inhibit TLR4 and NF $\kappa$ B signaling [107-109].

In addition, TNF $\alpha$  mRNA contains AU-rich elements (ARE) at its 3' untranslated region, which target the mRNA for degradation. IL10 is found to reduce the stability of TNF $\alpha$  mRNA by inhibiting ARE-binding proteins, for example T cell antigen-1 (TIA-1) and TIA-1-related protein (TIAR) [110, 111]. Furthermore, it has been shown that IL10 lowers TNF $\alpha$  translation by shifting the mRNA from interacting with poly-ribosomes to mono-



**Figure 3 Canonical IL10 signaling pathway via STAT3**

Binding of IL10 to IL10R leads to receptor dimerization, causing the activation of tyrosine kinases JAK1 and TYK2. This leads to the phosphorylation of IL10R, recruitment of STAT3, STAT3 phosphorylation and dimerisation. The dimerized STAT3 will translocate to the nucleus where it promotes the transcription of anti-inflammatory mediators.

ribosome. This shift in translation efficiency was found to be SHIP1 dependent and involves the inhibition of MAP kinase signal integrating kinase <sup>[112]</sup>.

### **1.7.2 IL10 and SHIP1**

It has been previously shown in our lab that macrophages stimulated with LPS and IL10 have a significantly lower level of PIP<sub>3</sub> and higher levels of PI(3,4)P<sub>2</sub> than the ones stimulated with LPS alone (Appendix A). This observation indicates that the initial LPS treatment increased PIP<sub>3</sub> production and IL10 caused PIP<sub>3</sub> dephosphorylation into PI(3,4)P<sub>2</sub>. Accordingly, we hypothesised that IL10 may activate SHIP1, a 5'-phosphatase that acts as a negative regulator of PI3K in macrophages. We further examined the effect of IL10 activation of SHIP1 on the downstream signaling events of PI3K pathway and IL10 was found to lower LPS induced phosphorylation and activation of protein kinase B.

## **1.8 Interleukin 6**

Interleukin 6 (IL6) is a critical cytokine with a diverse repertoire of physiological functions including immune cell proliferation and differentiation. IL6 is a 184 amino acid glycosylated protein, which is synthesized and secreted by monocytes, T-cells and endothelial cells <sup>[113, 114]</sup>. IL6 has inflammatory action on macrophages <sup>[113]</sup>. Dysregulation of IL6 pathway is associated with various inflammatory disorders including rheumatoid arthritis and castleman disease <sup>[115, 116]</sup>. In addition, monoclonal antibodies against IL6 or IL6 receptor have been developed for the treatment of autoimmune pathologies such as rheumatoid arthritis <sup>[113, 115]</sup>.

### **1.8.1 IL6 Signaling**

IL6 dependent activation of the JAK-STAT pathway, the MAPK cascade, and the PI3K pathway have been studied extensively <sup>[114]</sup>. However, the communication between these signaling mechanisms still needs to be explained in more detail. IL6 binds to its specific receptor (IL6R), an 80 kDa type I transmembrane protein <sup>[113, 117]</sup>. IL6 binding to its receptor leads to the recruitment of a second transmembrane protein, gp130, which serves as a signal transducer of IL6. The first enzymatic step in IL6 signal transduction is the activation of JAK <sup>[117]</sup>. JAKs are constitutively interacting with the cytoplasmic tail of gp130 <sup>[113, 118]</sup>. In response to IL6 binding to the receptor, JAK kinases are activated by auto-

phosphorylation at a tyrosine motif of the activation loop within the C-terminal JH1-domain <sup>[113, 114]</sup>. Subsequently, the active JAKs will phosphorylate tyrosine motifs within the cytoplasmic part of gp130 <sup>[118]</sup>. These phosphorylated tyrosine residues act as recruitment sites for signaling molecules containing SH2 domains <sup>[118, 119]</sup>. The most important protein recruited to phosphorylated gp130 is STAT3 <sup>[120, 121]</sup>. Similar to the IL10 pathway, STAT3 is subsequently phosphorylated, dimerize and translocate to the nucleus <sup>[114]</sup>. In the nucleus, STAT3 dimers bind to enhancer elements of promoters of IL6-inducible pro-inflammatory genes <sup>[113, 114]</sup>. Considering that both IL6 (a pro-inflammatory cytokine) and IL10 (an anti-inflammatory cytokine) use the JAK-STAT3 signaling, our goal was to assess whether SHIP1 plays a role in their difference.

## **1.9 AMP-activated protein kinase (AMPK)**

The AMP-activated protein kinase (AMPK) is a conserved serine/threonine protein kinase that acts as a sensor of energy status and regulates cellular energy balance <sup>[122, 123]</sup>. AMPK is activated by falling energy status and it activates catabolic pathways that generate ATP, while inhibiting anabolic pathways that consume energy <sup>[122, 123]</sup>. AMPK activation will inactivate aerobic glycolysis seen in rapidly dividing tumor cells and cells involved in inflammation, and promotes oxidative metabolism. AMPK has dozens of physiological targets and it is now regarded as a critical regulator of cellular energy <sup>[122, 123]</sup>. Given the crucial nature of cellular energy balance in cell function, it is not surprising that AMPK has a role in a wide array of pathologies including diabetes, cancer, inflammatory disorders and viral infections <sup>[122, 124-129]</sup>. Furthermore, AMPK activation is found to be associated with an anti-inflammatory response <sup>[130, 131]</sup>. Salicylate (the major in vivo metabolite of aspirin) has been reported to mediate its anti-inflammatory effect by activating AMPK <sup>[130, 131]</sup>.

### **1.9.1 AMPK inhibition of inflammation**

There is growing evidence that AMPK has an anti-inflammatory effect which may be mediated by its metabolic actions. Inactive neutrophils or macrophages use oxidative metabolism (including fatty acid oxidation) to generate ATP while they are at rest <sup>[122, 128, 132]</sup>. Once activated, such as in an inflammatory state, they often switch to aerobic glycolysis. In fact, classically activated pro-inflammatory M1

macrophages utilise aerobic glycolysis while alternatively activated anti-inflammatory M2 macrophages tend to use oxidative metabolism<sup>[128, 132]</sup>. This switch has been found to be associated with reduced AMPK activation and is promoted by AMPK inhibition<sup>[128, 132]</sup>. Moreover, studies where AMPK has been down-regulated in macrophages show enhanced production of pro-inflammatory mediators<sup>[128, 132]</sup>. These experiments illustrated reduced acetyl-CoA carboxylase and reduced rates of fatty acid oxidation which promoted accumulation of pro-inflammatory diacylglycerols<sup>[128, 132]</sup>. This elicited M1 skewing in macrophages derived from either bone marrow or adipose tissue<sup>[128, 129, 132, 133]</sup>. The role of AMPK in macrophages was also evaluated using mice with myeloid specific knockouts of AMPK- $\alpha$ 1, the isoform expressed in macrophages<sup>[129, 133]</sup>. Regeneration of muscle tissue in response to injury was defective in these mice, and this was due to reduced skewing towards M2 macrophages<sup>[133]</sup>.

### **1.9.2 AMPK and IL10 signaling**

Considering AMPK's anti-inflammatory effect on macrophages it was not surprising to discover its involvement in IL10 signaling<sup>[134]</sup>. IL10 induced phosphorylation of tyrosine residues in STAT3 has been reported to be AMPK $\alpha$ 1 dependent<sup>[134]</sup>. These phosphorylation events were blocked by inhibition of CaMKK $\beta$ , an upstream activator of AMPK<sup>[134]</sup>. Furthermore, the impaired IL10-induced STAT3 phosphorylation in AMPK $\alpha$ 1 deficient macrophages was accompanied by reduced SOCS3 expression and an inability of IL10 to suppress LPS-induced pro-inflammatory cytokine production<sup>[134]</sup>.

### **1.10 Linking IL10, STAT3 and SHIP1**

Although it is commonly suggested that IL10 signaling only occurs through STAT3 activation, results from our lab propose a critical role for SHIP1<sup>[112]</sup>. Our experiments in SHIP1 knock down cells illustrate the importance of SHIP1 in the inhibitory effect of IL10 (Appendix B). Using a unique continuous supernatant collection system, we found LPS to induce TNF $\alpha$  production in two waves, one at 50 minutes and another at 110 minutes. Both of these peaks are inhibited by IL10 in parental cells. However, the first peak is not inhibited by IL10 in STAT3 KD cells. Similarly, the first peak is not inhibited in SHIP1 KD, indicating the importance of both STAT3 and SHIP1 in IL10 signaling. Furthermore, our previous experiments suggest that IL10 stimulation recruits SHIP1 to the membrane and activates it, subsequently

SHIP1 can dephosphorylate PIP<sub>3</sub> and counteract LPS induced PI3K signaling. However, the molecular mechanism through which IL10 signaling leads to and coordinates SHIP1 and STAT3 activation still remains unclear.

### **1.11 Hypothesis and objectives**

The overall aim of this thesis project was to evaluate the crosstalk between SHIP1 and STAT3 in IL10 signaling. As discussed earlier both IL6 and IL10 use the JAK-STAT3 signaling, however, they initiate a pro- and an anti-inflammatory response respectively. Considering SHIP1's involvement in IL10 signaling and downregulating PIP<sub>3</sub> levels, we hypothesized that SHIP1 could indeed make this difference. To evaluate this, I used a variety of SHIP1 WT/KO or STAT3 WT/KO cell lines and primary cells including bone marrow derived macrophages (BMDM) and peritoneal macrophages (perimacs).

## **2. Materials and methods**

### **2.1 Mouse colonies**

Balb/C SHIP1<sup>+/+</sup> and SHIP1<sup>-/-</sup> mice were kindly provided by Dr. Gerald Krystal (BC Cancer Research Centre, Vancouver, British Columbia). C57BL/6 STAT3<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice were purchased from Dr. S. Akira (Hyogo College of Medicine, Nishinomiya, Japan) and C57BL/6 LysMCre mice were purchased from Jackson Laboratory. All mice were housed and 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**

Tibias and femurs were harvested from mice and the marrow was extracted by flushing Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher Scientific, Nepean, ON), supplemented with 10% fetal calf serum (FCS, Fisher Scientific, Ottawa, ON), 10 μM β-mercaptoethanol (Sigma Aldrich, Oakville ON), 150 μM monothioglycolate, and 1 mM L-glutamine using a 26G needle (BD Scientific, Mississauga, ON). Cells were cultured on a 10-cm tissue culture plate (Fisher Scientific, Ottawa, ON) for 2 hours. Non-adherent cells were collected and seeded at 9×10<sup>6</sup> cells per 10-cm tissue culture plate. Cells were

incubated for seven days in the IMDM media supplemented with 5 ng/ml Colony Stimulating Factor-1 (CSF-1) (Stem Cell Technologies, Vancouver, BC). Peritoneal macrophages were extracted by peritoneal lavage using 5 ml of sterile phosphate buffered saline (PBS, Fisher Scientific, Ottawa, ON) and IMDM mixture (3:1 ratio). Subsequently, cells were plated at  $2.5 \times 10^5$  per well in 24-well tissue culture plates in IMDM. Cells were allowed to adhere for 4 hours and non-adhered cells were removed prior to stimulation. All cells were maintained at 37°C, 5% CO<sub>2</sub> and 95% humidity.

## 2.3 Cell lines

SHIP1 wild-type and knockout J2M cell line were kindly provided by Dr. Gerald Krystal (BC Cancer Research Centre, Vancouver, British Columbia) and maintained in IMDM media, supplemented with 10% (v/v) FCS, 10  $\mu$ M  $\beta$ -mercaptoethanol, 150  $\mu$ M monothioglycolate, and 1 mM L-glutamine. These cells were reconstituted with the indicated lentiviral vectors as described in table 1<sup>[64, 80]</sup>. The mammalian (lentiviral) expression plasmids of SHIP1 in FUGWBW were generated using Gateway LR reactions from pENTR1A (Invitrogen, Burlington, ON) constructs<sup>[135]</sup>. All cell lines were maintained at 37°C, 5% CO<sub>2</sub> and 95% humidity.

**Table 1 Cell lines used in this study**

Cell line name	Parental Cell	Vector	Property
<b>J2M WT</b>	N/A	N/A	SHIP1 wildtype J2M macrophage cell lines
<b>J2M KO</b>	N/A	N/A	SHIP1 knockout J2M macrophage cell lines
<b>J2M BW</b>	J2M KO	FUGWBW lentiviral vector	SHIP1 KO J2M macrophages reconstituted with empty lentiviral vector
<b>J2M His-SHIP1</b>	J2M KO	His-SHIP1 in FUGWBW	SHIP1 KO J2M macrophages reconstituted with His-Tagged SHIP1
<b>J2M <math>\Delta</math> PH</b>	J2M KO	$\Delta$ PH-SHIP1 in FUGWBW	SHIP1 lacking PH domain
<b>J2M <math>\Delta</math> C2</b>	J2M KO	$\Delta$ C2-SHIP1 in FUGWBW	SHIP1 lacking C2 domain
<b>J2M KAKA</b>	J2M KO	KAKA-SHIP1 in FUGWBW	Two point mutations in the PH domain, making it functionally inactive
<b>J2M PD (3pt)</b>	J2M KO	PD-SHIP1 in FUGWBW	Three point mutations in the catalytic domain, making phosphatase dead SHIP1 (PD)
<b>J2M Y190F J2M Y657F J2M Y659F J2M Y799F</b>	J2M KO	Point mutants of SHIP1 in FUGWBW	Individual point mutants of different Tyr residues that had a high phosphorylation prediction score and a consensus sequence similar to the p-Tyr recognised by STAT3's SH2 domain

## **2.4 Antibodies and reagents**

Primary antibodies used in the experiments include anti-SHIP1 (P1C1) purchased from Santa Cruz (Dallas, Tx), anti-STAT3 (9D8) from Thermo Fisher Scientific (Nepean, ON), anti-Phosphotyrosine (4G10) from EMD Millipore (Nepean, ON), anti-Phospho-STAT3 pTyr705 (G.374.10) from Thermo Fisher Scientific (Nepean, ON), anti-AMPK alpha 1 from Abcam (Cambridge, MA), anti-actin from Sigma (Oakville, ON). The secondary antibodies, 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). Human Recombinant IL6 used in the experiments was purchased from Stemcell Technologies (Vancouver, BC) and AICAR (5-Aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranoside) was purchased from Sigma Aldrich (Oakville, ON).

## **2.5 SHIP1 pull down assay**

Following the appropriate stimulations, cells were washed twice with PBS and lysed in 0.5 ml of 1% Octyl-beta-Glucoside lysis buffer (50 mM HEPES, 4 mM EDTA, 2 mM NaVO<sub>4</sub>, 100 mM NaF, 10 mM NaPPi, 10 mM imidazole) supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Laval, QC). Lysates were rocked at 4°C for 30 minutes and clarified by centrifugation at 10,000 rpm for 20 minutes. The lysates were then incubated with cOmplete™ His-Tag Purification Resin (Sigma Aldrich, Oakville ON) at 4°C for 2 hours. The beads were subsequently washed three times with 0.5% Octyl-beta-Glucoside in lysis buffer and resuspended in 100  $\mu$ l of 2 $\times$  Laemmli's buffer.

## **2.6 Immunoblotting and analysis**

After the addition of 2  $\times$  Laemmli's buffer, samples were boiled for 5 minutes and loaded onto 10% SDS polyacrylamide gels at constant 100 V for 90 minutes. Resolved proteins were then transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Etobicoke ON, Canada) using a wet transfer apparatus. Membranes were 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 0.05% Tween 20 (TBST) for 5 minutes per wash and incubated with Alexa Fluor®488

or Alexa Fluor®680 secondary antibodies diluted 1:10,000 in TBST for 60 minutes. Following secondary antibody incubation, membranes were washed three times with TBST and imaged using a LI-Cor Odyssey Infrared Imager (LI-COR bioscience, Lincoln NB, USA). Densitometry analysis was performed using the Image Studio software (LI-COR bioscience, Lincoln NB, USA) by measuring the signal of each protein band and normalizing it to the signal of an appropriate endogenous control protein with a constant protein expression that is not affected by the treatment.

## **2.7 TNF $\alpha$ ELISA**

J2M cells were plated at  $5 \times 10^4$  per well and allowed to adhere overnight. Cells were stimulated with LPS (1 ng/ml) and increasing concentrations of IL10 (ranging from 0 – 20 ng/ml). Supernatant was removed from each well for TNF $\alpha$  concentration measurement. TNF $\alpha$  protein concentration was assayed by enzyme-linked immunosorbent assay (ELISA) using BD OptEIA™ Mouse TNF ELISA Set II kit (BD Scientific, Mississauga, ON). Assay plate (Sigma Aldrich, Oakville ON) was precoated with capture antibody (anti-TNF $\alpha$  antibody, diluted 1:250 in 0.05 M carbonate/ bicarbonate buffer, pH 9.6) overnight at 4°C. The following day, plate was blocked with assay diluent (10% FCS in PBS) for at least one hour at 23°C. The blocking solution was removed by washing the plate three times with wash buffer (0.05% Tween 20 in PBS). Samples were loaded on 96 well plates, and incubated at 23°C for two hours. The plates were then incubated with detection antibody (biotinylated anti-TNF $\alpha$  antibody, diluted 1:250 in assay diluent) for 1 hour at 23°C. The detection antibody solution was removed by washing the plates three times. Plates were then incubated with streptavidin-HRP solution (diluted 1:250 in assay diluent) for 30 minutes at 23°C, followed by removal of the solution by washing seven times using wash buffer. The assay was developed by adding 3,3', 5,5' tetramethyl benzidine solution (0.005% TMB, 0.006% H<sub>2</sub>O<sub>2</sub> in 0.01 M Acetate Buffer and 0.05% Sodium Nitroferricyanide) and the reaction was stopped by adding 25  $\mu$ l of 2 M HCl. The plates were read by the Epoch® Microplate Spectrophotometer at an absorbance wavelength of 450 nm.

## **2.8 Statistical analysis**

All ANOVA analysis were performed using the GraphPad Prism 6 software.

### 3. Results

#### 3.1 SHIP1 interacts with STAT3 and AMPK to mediate IL10 signaling

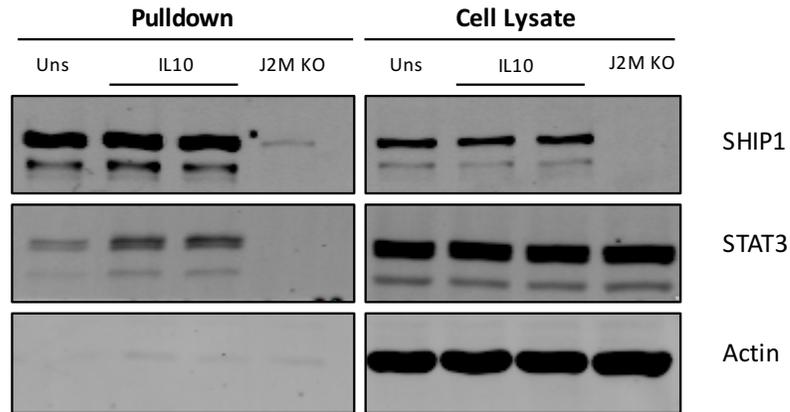
Considering the resistance of SHIP1 KO cells to IL10 mediated TNF $\alpha$  suppression (Appendix B) and the increased levels of PI(3,4)P<sub>2</sub> following IL10 stimulation (Appendix A) we hypothesized SHIP1 to play an important role in IL10 signaling. We first assessed whether SHIP1 physically interacts with STAT3 in response to IL10 in J2M cell lines. The J2M cell lines were established by the G. Krystal lab by infecting SHIP WT or SHIP KO bone marrow derived macrophages with v-raf virus<sup>[64]</sup>. J2M KO cells reconstituted with His-SHIP1 (as described in materials and methods), were stimulated with 10 ng/ml IL10 for 10 minutes and SHIP1 protein was pulled down using cOmplete™ His-Tag Purification Resin (Ni-beads) (**Figure 4A**). As shown in Figure 4A, we found that incubating cell lysates with Ni-beads enriched SHIP1 protein levels compared to the starting cell lysate. We also observed STAT3 protein in the pulldowns, but importantly more on IL10 stimulated cells. In addition, STAT3 appears to be present in the pulldowns through their interaction with SHIP1 since there is no STAT3 in the SHIP1 KO pulldown sample (**Figure 4A**).

We then examined whether IL6, another cytokine which leads to tyrosine phosphorylation of STAT3<sup>[113]</sup> would induce association of SHIP1 and STAT3. **Figure 4B** shows that although the level of phospho-STAT3 increases in both IL6 and IL10 stimulated cell lysates (lanes 3,4 vs. 5,6), only IL10 treatment increased the amount of STAT3 found in pulldowns of His-tagged SHIP1 (pulldown lanes 5,6). Interestingly, stimulation of cells with the small molecule SHIP1 agonist AQX-151 developed by our lab also induced STAT3 association with SHIP1 (pulldown lanes 11,12), even though it did not result in tyrosine phosphorylation of STAT3 (lysate lanes 11,12).

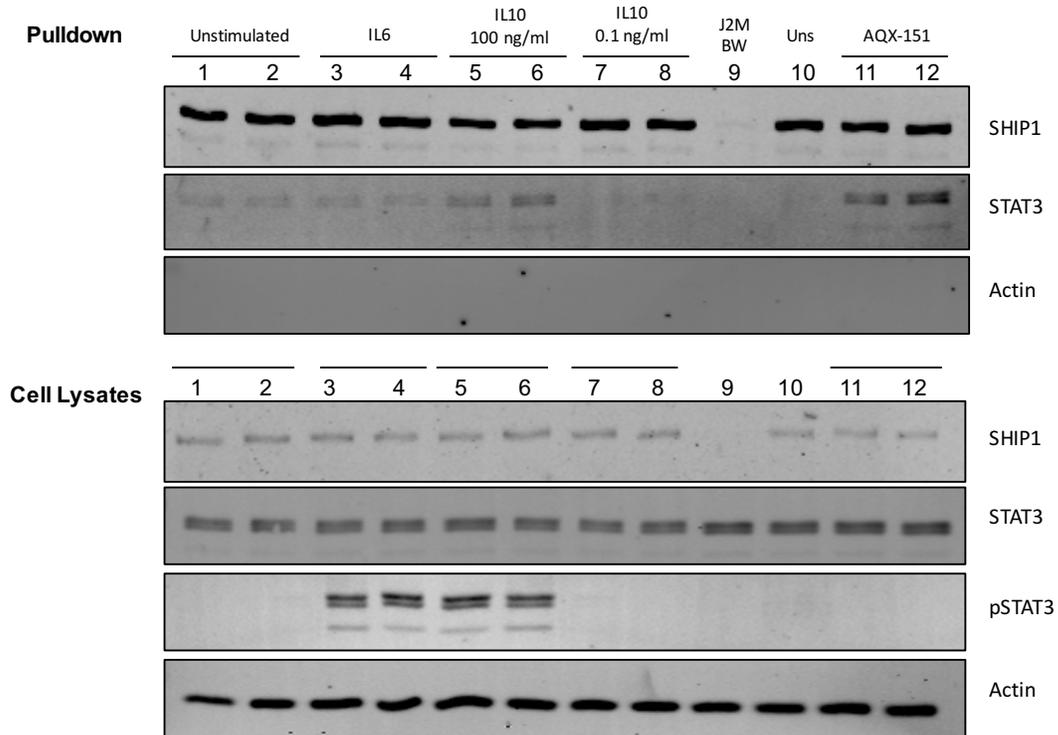
To extend our studies to primary cells which do not express His-tagged SHIP1, we looked at the possibility that nickel beads might pull down endogenous, wild-type SHIP1. Although a stretch of 6 histidines is the commonly used recombinant tag for Ni-bead chromatography, stretches of fewer than 6 histidines can coordinate with nickel and other cations<sup>[135]</sup>. Wild-type SHIP1 has stretch of three histidine residues (his<sup>1169</sup>, his<sup>1170</sup>, his<sup>1173</sup>) at its C-terminus which may mediate binding to Ni-beads (**Figure 12C**). To examine this possibility, we tested whether Ni-beads can pull down endogenous SHIP1 as well as it

pulls down His-SHIP1 (**Figure 4C**). J2M cells expressing His-SHIP1 (J2M His-SHIP1), wild-type SHIP1 (J2M WT) or no SHIP1 (J2M KO, J2M BW) were stimulated with IL10 or IL6 and the lysates were incubated with Ni-beads. As shown in Figure 4C, SHIP1 protein was enriched in the Ni-bead pulldowns of both His-SHIP1 (lanes 1-3) and wild-type SHIP1 (lane 6) as compared to the levels in their respective starting cell lysates (**Figure 4C**). As seen earlier, the level of STAT3 in the SHIP1 pulldown was increased in IL10 (lane 3) but not IL6 (lane 2) treated cells. No STAT3 was seen in the Ni-bead pulldowns from J2M KO and J2M BW cells. These data indicate that Ni-beads can pulldown endogenous, wild-type SHIP1.

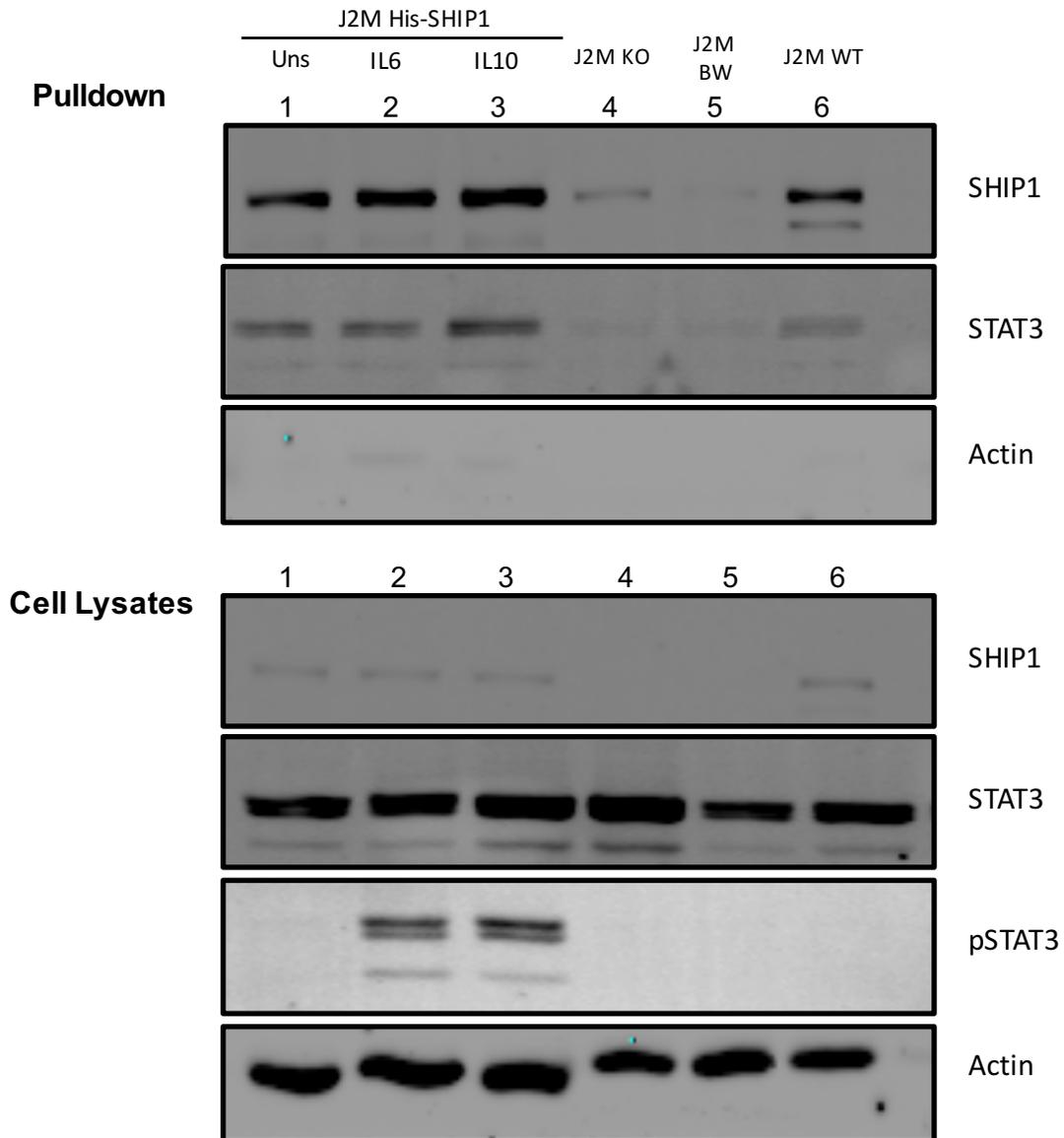
4A



4B



4C

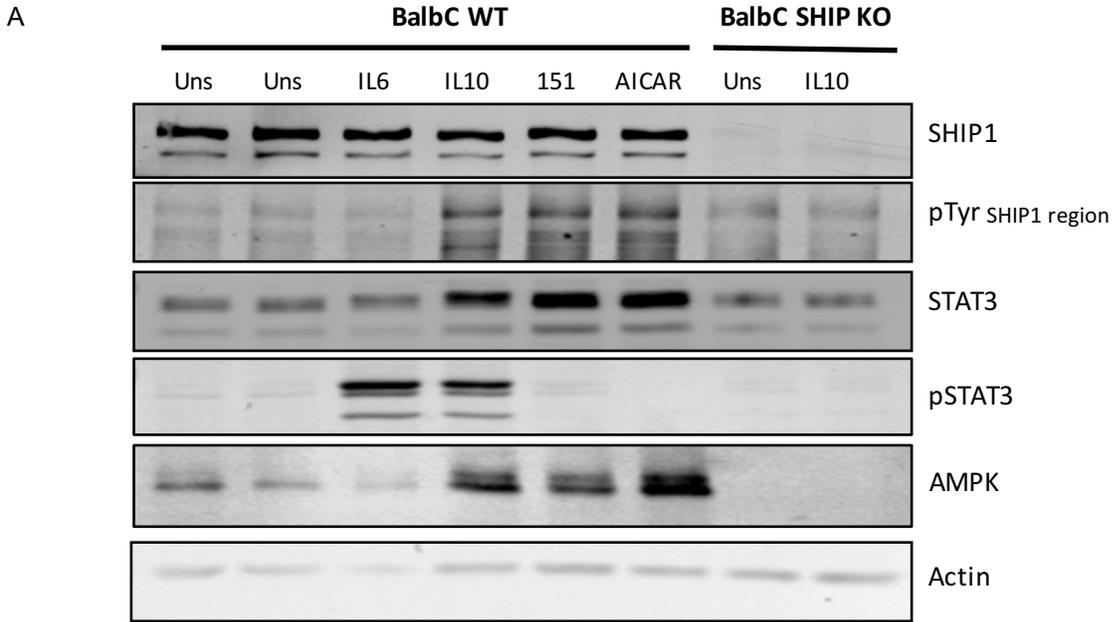


**Figure 4 SHIP1 binds STAT3 in response to IL10**

**(A)** Immunoblot analysis of SHIP1 pull-down and lysates in J2M KO cells reconstituted with or not with His-SHIP1, stimulated with 10 ng/ml IL10 for 10 minutes **(B)** Cells as indicated were stimulated with 100 ng/ml IL6, 0.1 ng/ml IL10, 100 ng/ml IL10 and 20  $\mu$ M AQX-151 for 5 minutes. Cell lysates (bottom panels) were subjected to Ni-bead pull-down (top panels). Lane numbers in pull-down and cell lysate panels indicate the same cell type and stimulation. **(C)** Cells as indicated were stimulated with 100 ng/ml IL6 and 100 ng/ml IL10 for 5 minutes. Lane numbers in pull-down and cell lysate panels indicate the same cell type and stimulation. Samples were probed with anti-SHIP1 protein, Phospho-STAT3 (Tyr 705), STAT3 Protein and Actin (Loading control).

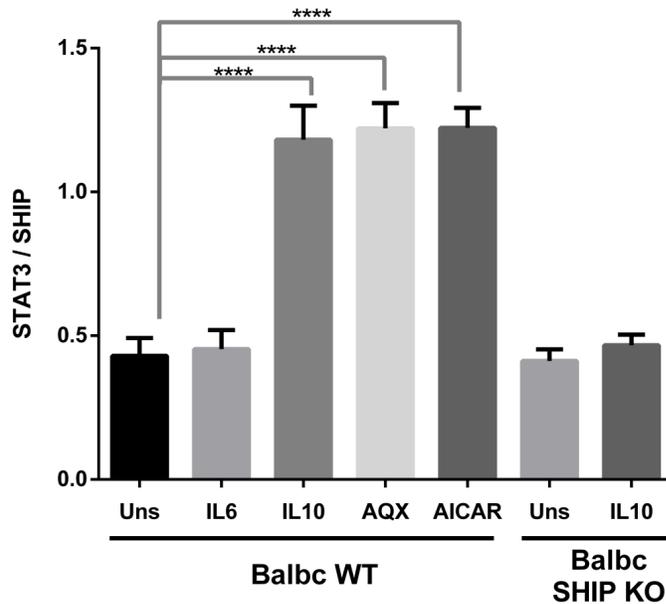
We next assessed SHIP1, STAT3 and AMPK association in peritoneal macrophages. Peritoneal macrophages (perimacs) from Balb/C SHIP1<sup>+/+</sup> were stimulated with 100 ng/ml of IL6 for 5 minutes, 100 ng/ml of IL10 for 5 minutes, 20 uM of AQX-151 for 5 minutes, and 0.5 mM of AICAR (an analog of adenosine monophosphate that stimulates AMPK<sup>[122]</sup>) for 10 minutes. As detailed earlier, SHIP1 was pulled-down and immunoblots were probed with anti-SHIP1 antibody to ensure that there were equal amounts of SHIP1 protein pulled down across the different samples. The same blots were probed with anti-phosphotyrosine (SHIP1 region), anti-STAT3 and anti-AMPK antibodies (**Figure 5A**). It is interesting that although stimulation with both IL6 and IL10 cause tyrosine phosphorylation of STAT3 as observed in cell lysates (data not shown) the levels of STAT3 and AMPK proteins in SHIP1 pulldowns only increase after stimulation with IL10 (**Figure 5A**).

Furthermore, both SHIP1 agonist (AQX-151) and AMPK activator (AICAR) are found to induce the interaction between SHIP1, STAT3 and AMPK proteins. **Figure 5B** further supports this by illustrating the densitometric analysis of the levels of STAT3 protein normalised against SHIP1 Protein (from Figure 5A, panel 3). Overall these findings illustrate that IL10 stimulation triggers the physical interaction between SHIP1, STAT3 and AMPK, whereas IL6 is not able to cause the formation of such a complex. In fact, this may be causing the difference between the outcomes of these two signaling pathways. In addition, the amount of pSTAT3 pulled down with SHIP1 is the same following both IL6 and IL10 stimulation, which suggests that contrary to common belief, STAT3 phosphorylation may not be the sole pathway for IL10 signaling.



**B**

**STAT3 associated with SHIP  
in SHIP WT and KO Perimacs**

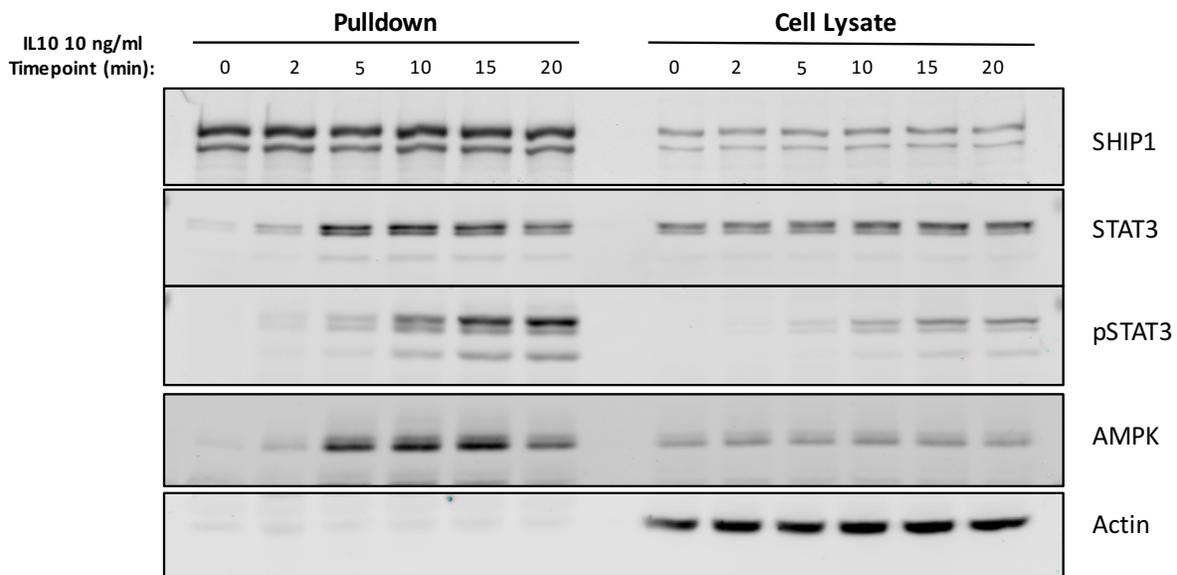


**Figure 5 SHIP1 physically interacts with STAT3 and AMPK**

**(A)** Immunoblot analysis of SHIP1 pull-down in WT BalbC and SHIP1 KO Perimacs stimulated with 100 ng/ml IL10 for 5 minutes, 100 ng/ml IL6 for 5 minutes, 20  $\mu$ M AQX-151 for 5 minutes and 0.5 mM AICAR for 10 minutes. Samples were probed with anti-SHIP1 protein, Phospho-Tyrosine, Phospho-STAT3 (Tyr 705), STAT3 Protein, AMPK Protein and Actin (Loading control). **(B)** Densitometric analysis of the levels of STAT3 protein normalised against SHIP1 protein. The experiment was repeated to obtain three biological replicates for each of the stimulation conditions. Data from all of the replicates were combined, the bar graphs represent the mean and error bars illustrate the SD.  $p < 0.0001$  One-way ANOVA with Tukey's multiple comparisons test.

### 3.2 Complex formation is independent of STAT3 phosphorylation

To further investigate the interaction between phosphorylated-STAT3 (pSTAT3) and SHIP1, as well as SHIP1-STAT3-AMPK complex, the following time course experiment was designed and performed. J2M KO macrophages reconstituted with His-SHIP1, were stimulated with 10 ng/ml IL10 for 2, 5, 10, 15 and 20 minutes. His-SHIP1 protein was pulled down using Ni-beads and the immunoblot was probed with anti-STAT3, anti-pSTAT3 (pTyr705) and anti-AMPK antibodies (**Figure 6**). The same blot was also probed with anti-SHIP1 antibody to ensure that there were equal amounts of SHIP1 protein pulled down across the different samples. As shown in **Figure 6**, STAT3 and AMPK levels increase in the pull-down samples at 2 minutes, peak at 10 minutes and it goes down 20 minutes after IL10 stimulation. On the other hand, pSTAT3 levels start increasing at 5 minutes, peaks at 15 minutes and is still high at 20 minutes. This observation further supports the idea that STAT3 phosphorylation and SHIP1-STAT3-AMPK complex formation are separate events that happen in the cell following IL10 stimulation.



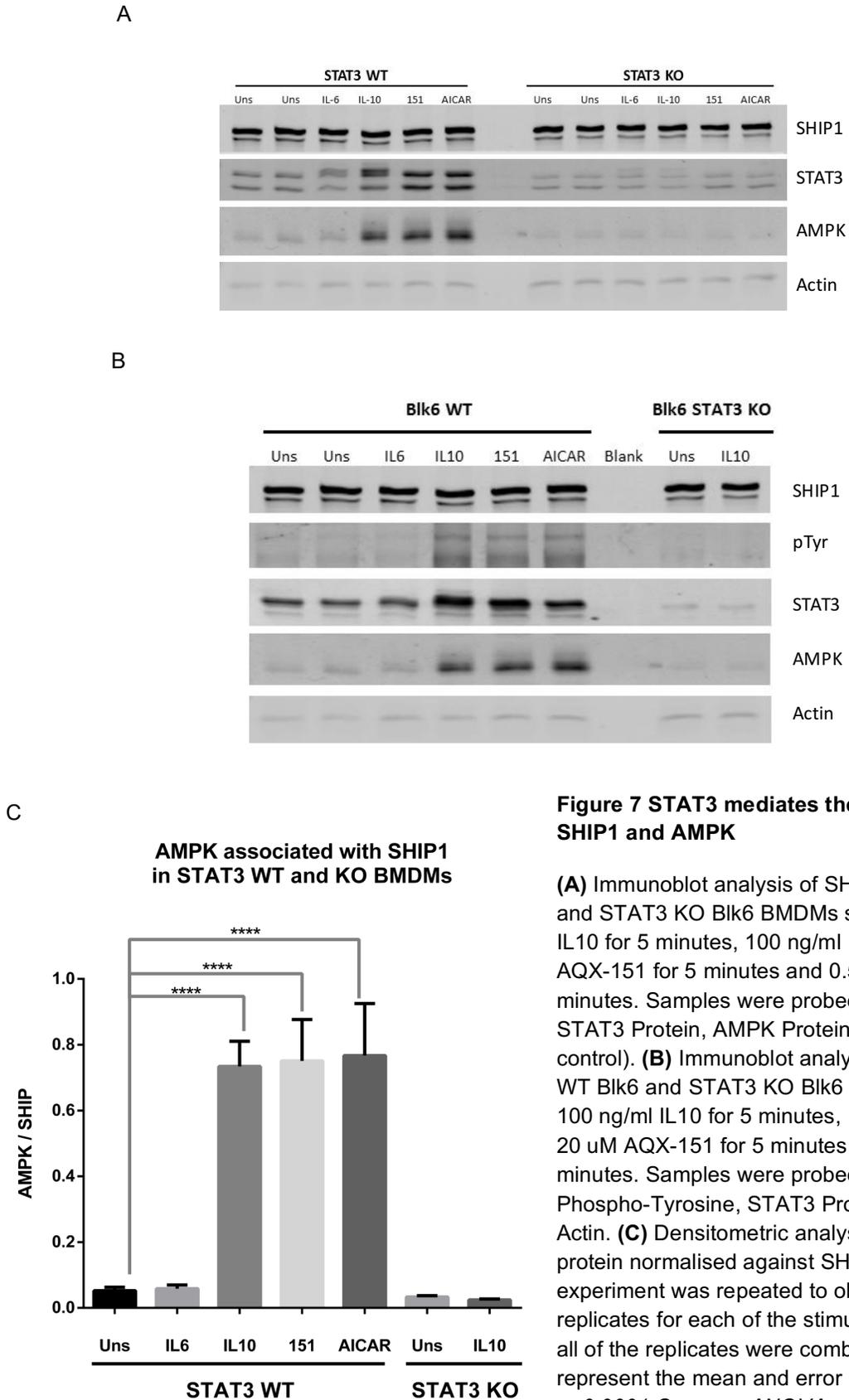
**Figure 6 STAT3 phosphorylation and complex formation are independent events**

Immunoblot analysis of SHIP1 pull-down in J2M KO cells reconstituted with His-SHIP1, stimulated with 10 ng/ml IL10 for the indicated times. Samples were probed with anti-SHIP1 protein, Phospho-STAT3 (Tyr 705), STAT3 Protein, AMPK Protein and Actin (Loading control). The experiment was performed twice.

### 3.3 STAT3 mediates the interaction between SHIP1 and AMPK

To evaluate the binding order in the SHIP1-STAT3-AMPK complex, we next performed the pulldown experiments in STAT3 KO bone marrow derived macrophages (**Figure 7A**) and perimacs (**Figure 7B**). Bone marrow derived macrophages (BMDMs) from C57BL/6 STAT3 KO mice were cultured in CSF-1 containing media for seven days and then they were stimulated with 100 ng/ml IL10 for 5 minutes, 100 ng/ml IL6 for 5 minutes, 20  $\mu$ M AQX-151 for 5 minutes and 0.5 mM AICAR for 10 minutes. Again SHIP1 protein was pulled down using His-Tag Purification Resin and the immunoblot was probed with anti-STAT3 and anti-AMPK antibodies. The same blot was also probed with anti-SHIP1 antibody to ensure that there were equal amounts of SHIP1 protein pulled down in the different samples. As shown in **Figure 7A**, IL10, AQX-151 and AICAR all trigger complex formation and cause higher levels of STAT3 and AMPK to be present in pulldown samples. However, there are no AMPK proteins present in the pulldown samples of STAT3 KO cells after stimulation. This observation suggests that STAT3 mediates the interaction between SHIP1 and AMPK and is crucial for the formation of SHIP1-STAT3-AMPK complex.

In addition, as shown in **Figure 7B** perimacs from WT C57BL/6 and STAT3 KO cells were stimulated with 100 ng/ml IL10 for 5 minutes, 100 ng/ml IL6 for 5 minutes, 20  $\mu$ M AQX-151 for 5 minutes and 0.5 mM AICAR for 10 minutes. Again, although IL10, AQX-151 and AICAR all trigger complex formation, there are no AMPK proteins present in the pulldown samples of STAT3 KO cells following our stimulation. This is further supported by densitometric analysis (**Figure 7C**) of the levels of AMPK protein normalised against SHIP1 Protein. These observations collectively support the importance of STAT3 in the formation of SHIP1-STAT3-AMPK complex in response to IL10.



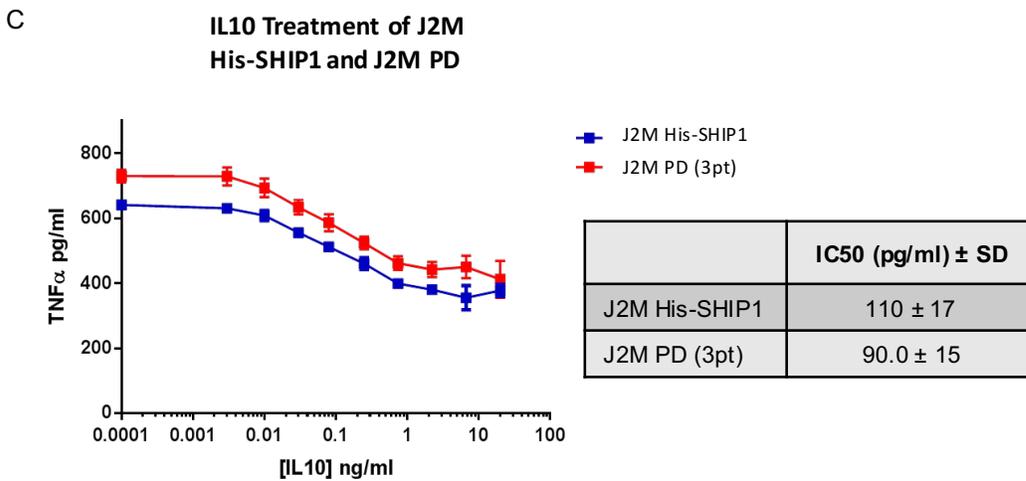
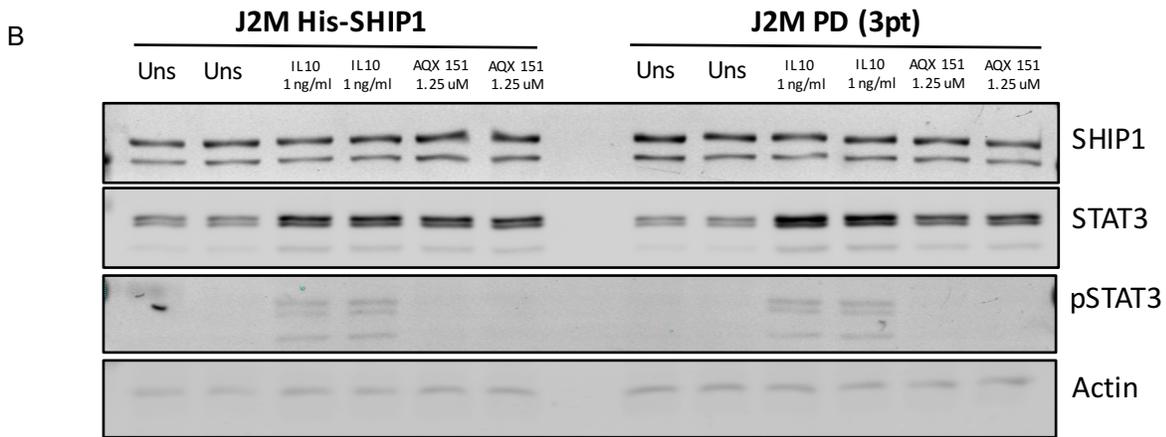
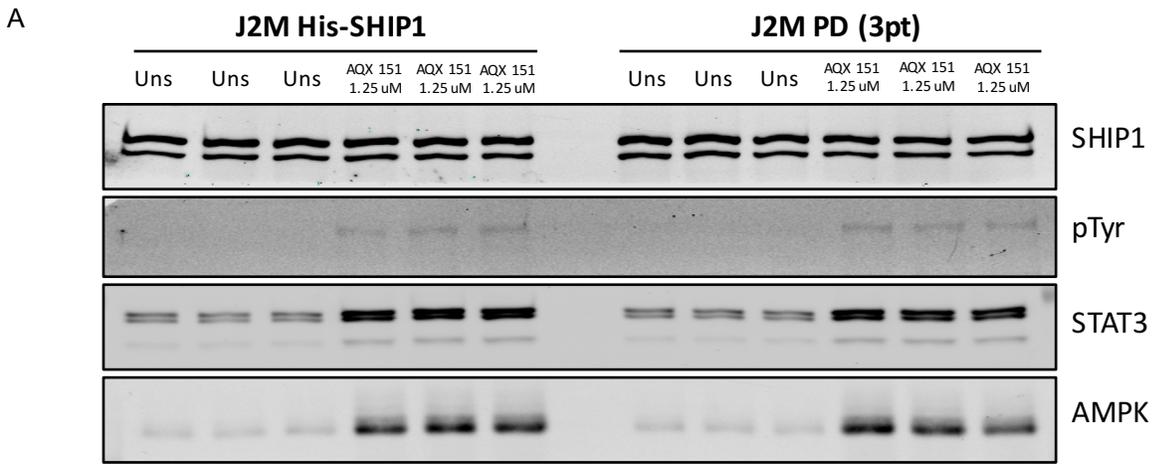
**Figure 7 STAT3 mediates the interaction between SHIP1 and AMPK**

(A) Immunoblot analysis of SHIP1 pull-down in WT Blk6 and STAT3 KO Blk6 BMDMs stimulated with 100 ng/ml IL10 for 5 minutes, 100 ng/ml IL6 for 5 minutes, 20  $\mu$ M AQX-151 for 5 minutes and 0.5 mM AICAR for 10 minutes. Samples were probed with anti-SHIP1 protein, STAT3 Protein, AMPK Protein and Actin (Loading control). (B) Immunoblot analysis of SHIP1 pull-down in WT Blk6 and STAT3 KO Blk6 perimacs stimulated with 100 ng/ml IL10 for 5 minutes, 100 ng/ml IL6 for 5 minutes, 20  $\mu$ M AQX-151 for 5 minutes and 0.5 mM AICAR for 10 minutes. Samples were probed with anti-SHIP1 protein, Phospho-Tyrosine, STAT3 Protein, AMPK Protein and Actin. (C) Densitometric analysis of the levels of AMPK protein normalised against SHIP1 protein in BMDMs. The experiment was repeated to obtain three biological replicates for each of the stimulation conditions. Data from all of the replicates were combined, the bar graphs represent the mean and error bars illustrate the SD.  $p < 0.0001$  One-way ANOVA with Tukey's multiple comparisons test.

### 3.4 A catalytically inactive mutant of SHIP1 is responsive to IL10

As discussed earlier, it has been previously shown in our lab that macrophages stimulated with LPS and IL10 have a significantly lower level of PIP<sub>3</sub> and higher levels of PI(3,4)P<sub>2</sub> than the ones stimulated with LPS alone (Appendix A). This observation indicated that the initial LPS treatment increased PIP<sub>3</sub> production and IL10 caused PIP<sub>3</sub> dephosphorylation into PI(3,4)P<sub>2</sub>. Accordingly, we hypothesised that IL10 may activate SHIP1, a 5'-phosphatase that acts as a negative regulator of PI3K in macrophages. To test this hypothesis, we next assessed whether a catalytically inactive mutant of SHIP1 (a phosphatase dead mutant) can still form the SHIP1-STAT3-AMPK complex. J2M KO macrophages reconstituted with His-SHIP1 and 3PT phosphatase dead SHIP1 were stimulated with **(8A)** 1.25 uM AQX-151 for 10 minutes and **(8B)** 1 ng/ml IL10 and 1.25 uM AQX-151 for 10 minutes. SHIP1 protein was pulled down using Ni-beads and the immunoblots were probed with anti-SHIP1 protein, anti-phospho-tyrosine, anti-STAT3 protein, anti-pSTAT3 (pTyr705) and anti-AMPK antibodies. As shown in **Figure 8A** and **8B**, it is interesting to note that the phosphatase dead mutant SHIP1 can still form the complex with STAT3 and AMPK in response to IL10 and AQX-151.

Furthermore, we also evaluated whether this mutant is able to respond to IL10 by measuring TNF $\alpha$  response to LPS and IL10. J2M KO macrophages reconstituted with His-SHIP1 and mutant SHIP1 were plated at  $5 \times 10^4$  per well in a 96 well plate and allowed to adhere overnight. Cells were stimulated with LPS (1 ng/ml) and increasing concentrations of IL10 (ranging from 0 – 20 ng/ml). Supernatant was removed from each well for TNF $\alpha$  concentration measurement using ELISA. As shown in **Figure 8C** the levels of TNF $\alpha$  decrease with increasing IL10 concentration in both His-SHIP1 and mutant cells. Furthermore, the half maximal inhibitory concentration (IC<sub>50</sub>) was calculated for the two curves and they were found to be 110 pg/ml for WT and 90 pg/ml for the phosphatase dead mutant, indicating that both cell types were responsive to IL10. This finding further supports, that SHIP1's involvement in IL10 pathway is independent of its role as a 5'-phosphatase.

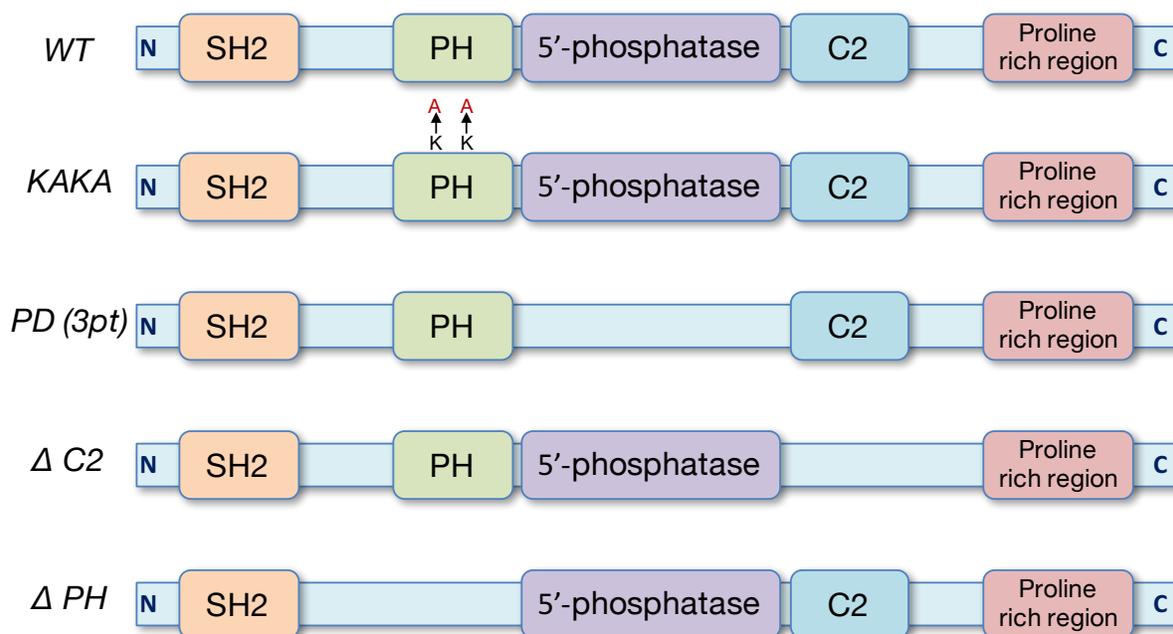


**Figure 8 SHIP1 phosphatase dead mutant is fully responsive to IL10**

Immunoblot analysis of SHIP1 pulldown in J2M KO cells reconstituted with His-SHIP1 and 3PT mutant phosphatase dead (PD). Cells were stimulated with **(A)** 1.25 uM AQX-151 for 10 minutes and **(B)** 1 ng/ml IL10 and 1.25 uM AQX-151 for 10 minutes. Samples were probed with STAT3 Protein, AMPK Protein, Phospho-Tyrosine and SHIP1 Protein (Loading control). **(C)** TNF $\alpha$  ELISA of cell supernatants from J2M KO reconstituted with His-SHIP1 and PD (3pt). Cells were stimulated with LPS (1 ng/ml) and increasing concentrations of IL10 (ranging from 0 – 20 ng/ml). Data represent mean TNF $\alpha$  levels at 1 hour time point. Each cell line was tested in three independent experiments. The mean TNF $\alpha$  level is plotted with error bars illustrating the SD. The half maximal inhibitory concentration (IC50) was accordingly calculated using GraphPad Prism 6.

### 3.5 Identification of domains and residues required for complex formation

In order to identify the SHIP1 domains that play a role in the formation of the SHIP1-STAT3-AMPK complex, we performed the pulldown experiments with various SHIP1 mutants previously made in our lab. SHIP1 is a 145 kDa protein with a N-terminal SH2 domain, a PH-related domain (PH-R), a centrally located phosphatase domain, a C2 domain, followed by a proline-rich region (PRR) at the C-terminus<sup>[63, 64]</sup>. As described earlier, each of these domains has been identified to play an important role in SHIP1's function and interaction with other proteins. **Figure 9** illustrates the critical domains of WT SHIP1 and our SHIP1 mutants. We had previously found that SHIP1's PH-related (PH-R) domain is required for its recruitment to the plasma membrane upon cell stimulation via direct interactions with PIP<sub>3</sub>. The KAKA mutant is the result of mutating K370 and K397 (found in the PH domain) to alanines. These lysine residues were found to be important for SHIP1's ability to bind PIP<sub>3</sub> and PI(3,4)P<sub>2</sub>. The C2 domain of SHIP1 binds to PI(3,4)P<sub>2</sub>, the product of SHIP1, and such binding allosterically activates SHIP1's enzymatic activity, suggesting C2 domain's importance for the regulation and activation of the phosphatase (**Figure 9**).



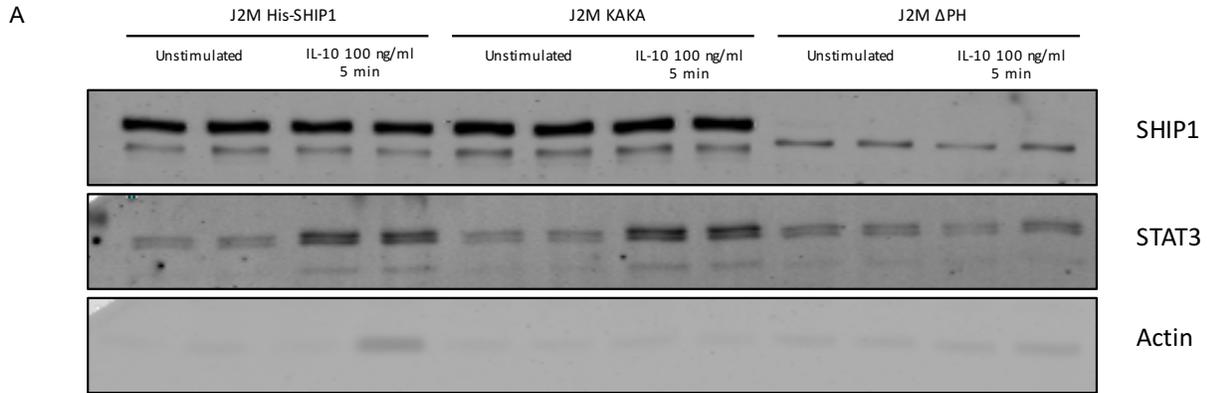
**Figure 9 Schematic diagram of the domains in WT SHIP1 and our SHIP1 mutants**

SHIP1 carries a SH2 domain, a PH-R domain, a phosphatase domain, a C2 domain, followed by a proline-rich region (PRR). Various SHIP1 mutants (names on the left) were reconstituted into J2M KO cells to evaluate the importance of these domains in SHIP1-STAT3 complex formation.

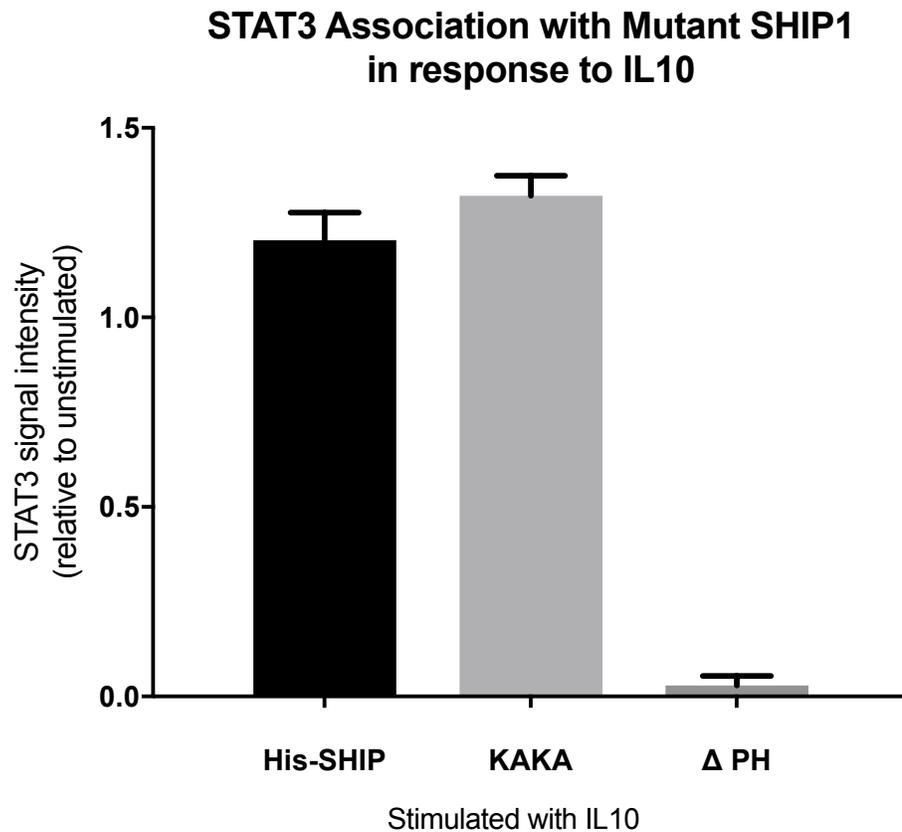
### 3.5.1 SHIP1's PH and C2 domains are important for complex formation

J2M KO macrophages reconstituted with His-SHIP1,  $\Delta$ PH,  $\Delta$ C2 and KAKA mutants were stimulated with 100 ng/ml IL10 for 5 minutes (**Figure 10**) and 20  $\mu$ M AQX-151 for 5 minutes (**Figure 11**). SHIP1 protein was pulled down using Ni-beads and the immunoblots were probed with anti-SHIP1 protein and anti-STAT3 protein. As shown in both the immunoblot (**Figure 10A**) and the densitometric analysis (**Figure 10B**) there is no enhancement in STAT3 levels following IL10 stimulation in the  $\Delta$ PH samples. This suggests that SHIP1's PH domain is required for the formation of SHIP1-STAT3 complex in response to IL10. Knowing PH domain's role in recruiting SHIP1 to the plasma membrane, it is reasonable to hypothesise that the SHIP1-STAT3-AMPK complex forms at the plasma membrane. However, it is interesting to note that the STAT3 levels rise following IL10 stimulation in the KAKA mutant (functionally inactive PH domain). This indicates that the absence of STAT3 in the pulldown samples of  $\Delta$ PH cells is not simply due to SHIP1's inability to bind PIP<sub>3</sub> or recruitment to plasma membrane, and may have other explanations.

Another interesting finding was the inability of SHIP1 lacking C2 Domain to form a complex in response to AQX-151. As shown in **Figure 11A**, there is no enhancement in STAT3 levels following AQX-151 stimulation in the  $\Delta$ C2 and the  $\Delta$ PH mutants. This is also supported by the densitometric analysis of the levels of STAT3 protein normalised against SHIP1 protein (**Figure 11B**). This again suggests the importance of PH and C2 domain for SHIP1-STAT3 complex formation. In addition, it may indicate that AQX-151 binds to the C2 domain to cause a conformational change, activate SHIP1, and trigger SHIP1's interaction with STAT3.



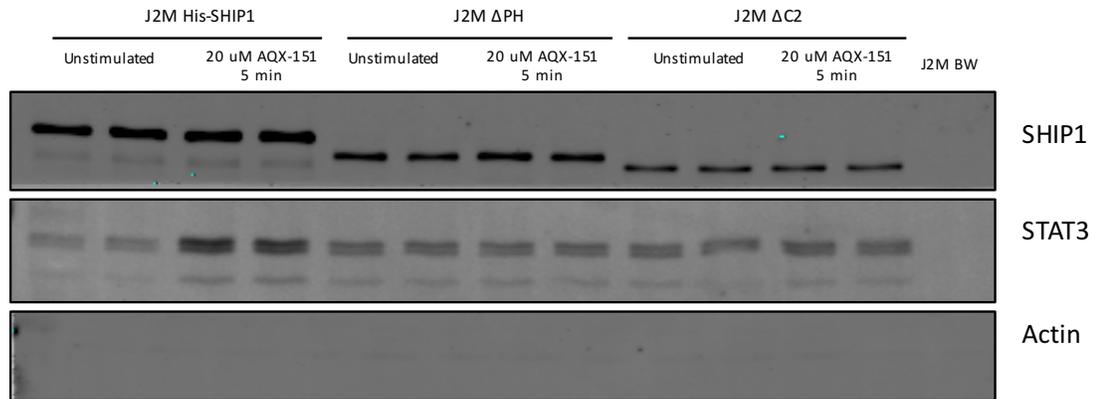
**B**



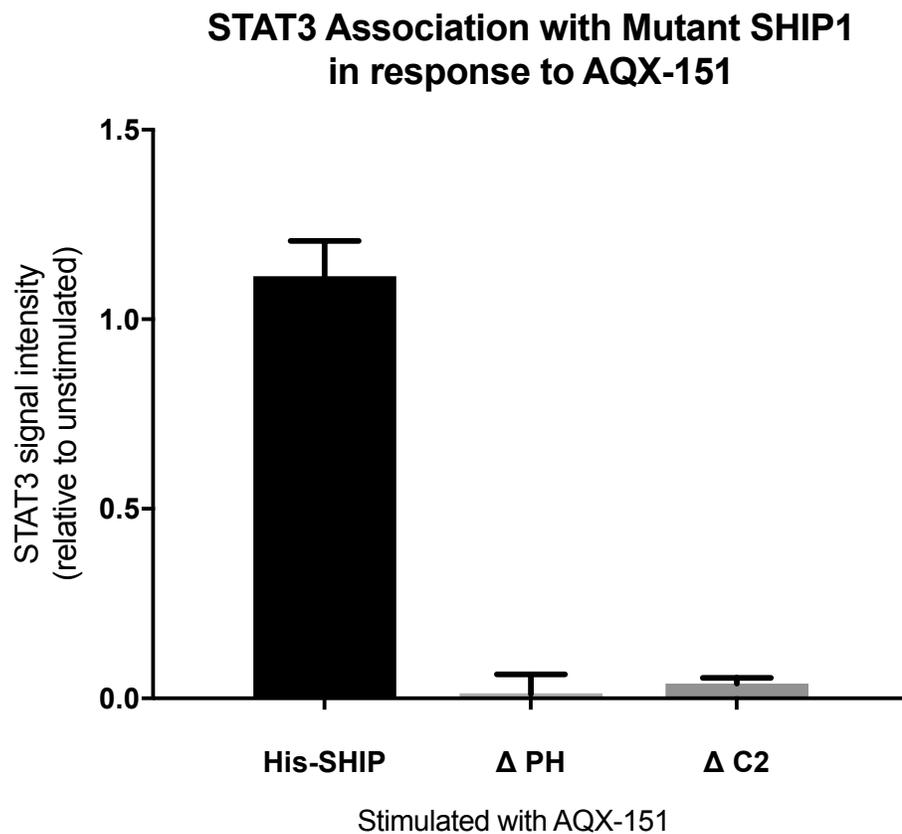
**Figure 10 SHIP1  $\Delta$ PH mutant does not form a complex**

**(A)** Immunoblot analysis of SHIP1 pulldown in J2M KO cells reconstituted with His-SHIP1, KAKA and  $\Delta$ PH mutants. Cells were stimulated with 100 ng/ml IL10 for 5 minutes and probed with anti-SHIP1 protein, STAT3 Protein, and Actin. **(B)** Densitometric analysis of the levels of STAT3 protein following IL10 stimulation relative to unstimulated samples. This experiment was performed with two biological replicates, the average value of the replicates is presented and error bars illustrate the range.

A



B



**Figure 11 SHIP1 ΔC2 mutant does not form a complex in response to AQX-151**

**(A)** Immunoblot analysis of SHIP1 pull-down in J2M KO cells reconstituted with His-SHIP1, ΔPH and ΔC2 mutants. Cells were stimulated with 20 μM AQX-151 for 5 minutes and probed with anti-SHIP1 protein, STAT3 Protein, and Actin. **(B)** Densitometric analysis of the levels of STAT3 protein following AQX-151 stimulation relative to unstimulated samples. This experiment was performed twice with two biological replicates, the average value of the replicates is presented and error bars illustrate the range.

### 3.6 Phosphorylation of Tyr-190 is required for complex formation

As discussed earlier, STAT3 is recruited to IL10R via its SH2 domain, which binds to phosphorylated tyrosine residues. The SH2 domain of STAT3 recognises phosphorylated Tyrosines on IL10R in specific consensus sequences YXXQ and YXXC (**Figure 12A**). In addition, once phosphorylated, two STAT3 molecules will dimerize via their SH2 domains. The consensus sequence recognised by the SH2 domains for STAT3 dimerization is reported to be YLKT (**Figure 12A**). As a result, we hypothesised that the same principle may apply to SHIP1 and STAT3's interaction. To test this hypothesis, the NetPhos 2.0 Server was used to identify potential SHIP1 p-Tyrosines that could be targeted by STAT3's SH2 domain (**Figure 12B**). Four potential tyrosine residues with high phosphorylation prediction scores and a consensus sequence similar to the p-Tyr recognised by STAT3's SH2 domain were found in SHIP1's sequence (highlighted in yellow **Figure 12B**). In fact, these tyrosines could be responsible for SHIP1-STAT3 complex formation.

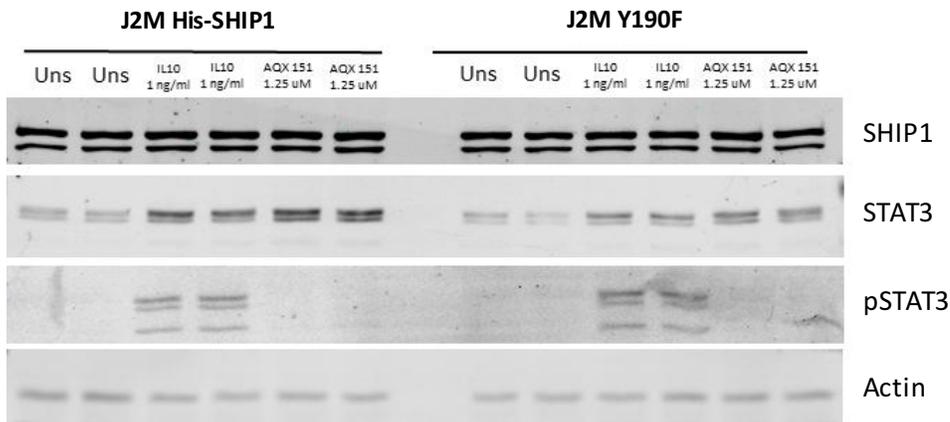
Accordingly, we chose several targets and mutated them in SHIP1 sequence (**Table 2**). Again, J2M KO macrophages reconstituted with His-SHIP1 and the mutant versions of SHIP1 were stimulated with 1 ng/ml IL10 for 5 minutes and 1.25  $\mu$ M AQX-151 for 10 minutes. Out of four different mutants, Tyr190 was found to play a role in the SHIP1-STAT3-AMPK complex formation (**Figure 13A**). As seen in the densitometric analysis of the levels of STAT3 protein normalised against SHIP1 protein, the amount of STAT3 present in Y190F mutant is less than WT following both IL10 and AQX-151 stimulation (**Figure 13B**). These results collectively indicate that phosphorylation of Tyr-190 is required for SHIP1 and STAT3's interaction possibly through STAT3's SH2 domain. In fact, in the absence of Tyr-190 SHIP1-STAT3-AMPK complex does not form.

**Table 2 Tyrosine point mutants**

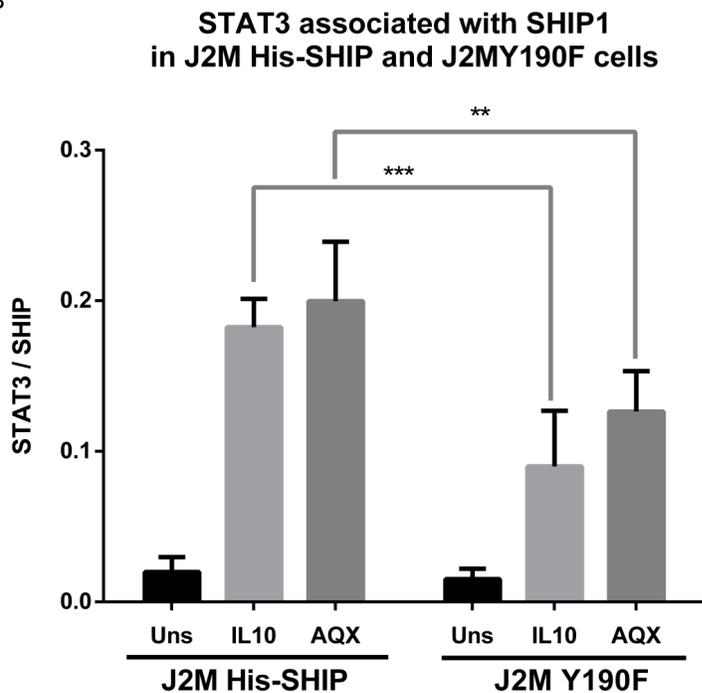
Mutant cells	Phosphorylation Prediction Score <i>NetPhos 2.0</i>	Amino acid residues surrounding the relevant Y
J2M Y190F	0.239	QDY <del>Y</del> LSTQ
J2M Y657F	0.908	DKYAYTK
J2M Y659F	0.316	YAYTKQK
J2M Y799F	0.888	PEYLLDQ



A



B

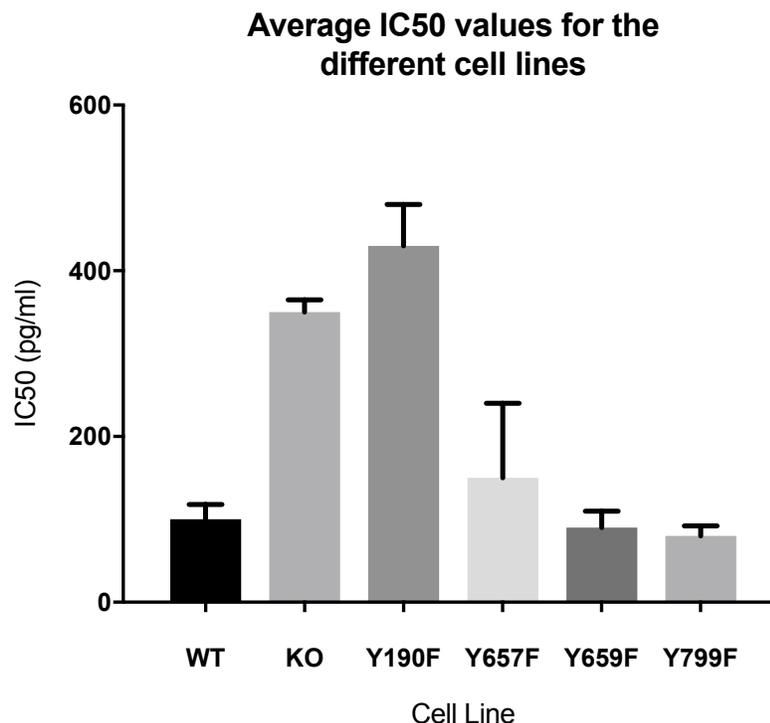


**Figure 13 Phosphorylation of Tyr190 plays a role in complex formation**

Immunoblot analysis of SHIP1 pulldown in J2M KO cells reconstituted with His-SHIP1 and Y190F mutant SHIP1. **(A)** Cells were stimulated with 1 ng/ml IL10 for 5 minutes and 1.25 uM AQX-151 for 10 minutes and probed with STAT3 Protein, pSTAT3 (Y705), AMPK Protein, SHIP1 Protein and Actin **(B)** Densitometric analysis of the levels of STAT3 protein normalised against SHIP1 Protein shown in part A. The experiment was repeated to obtain four biological replicates for each of the stimulation conditions. Data from all of the replicates were combined, the bar graphs represent the mean and error bars illustrate the SD. \*\* p < 0.01, \*\*\* p < 0.001, Two-way ANOVA with Sidak's multiple comparisons test.

### 3.7 Tyr-190 mutants are less responsive to IL10

We next evaluated the biological response of these cells to IL10 using a TNF $\alpha$  assay. J2M KO macrophages, J2M His-SHIP1, J2M Y190F, J2M Y657F, J2M Y659F and J2M Y799F were plated at  $5 \times 10^4$  per well in a 96 well plate and allowed to adhere overnight. Cells were stimulated with LPS (1 ng/ml) and increasing concentrations of IL10 (ranging from 0 – 20 ng/ml) and supernatants were collected for TNF $\alpha$  measurement at 1 hour. As expected, we observed a decrease in TNF $\alpha$  levels with increasing IL10 concentration (TNF $\alpha$  suppression curves are presented in appendix C). In order to further discern between the response of the WT and mutant cells, the half maximal inhibitory concentration (IC<sub>50</sub>) was calculated (**Figure 14**). As seen in Figure 14, the IC<sub>50</sub> value in J2M Tyr-190 mutant (430 pg/ml) is higher than rest of the cells indicating that the cells are unresponsive to IL10. As we saw previously out of all the different point mutants, Y190F was also unable to form the SHIP1-STAT3-AMPK complex. This indicates that phosphorylation at Tyr-190 is required for complex formation which is necessary for IL10 signaling.



**Figure 14 Tyr190 is crucial for IL10 signaling**

The half maximal inhibitory concentration (IC<sub>50</sub>) for different cell lines was calculated and graphed. Each cell line was tested in three independent experiments. The graphs show the mean IC<sub>50</sub> and error bars indicate SD of the replicate experiments.

## 4. Discussion

### 4.1 STAT3 independent IL10 signaling pathway

Being a crucial anti-inflammatory cytokine, IL10 signaling pathway has been extensively studied over the past few decades. It is commonly accepted that IL10 mediates its effect via the JAK-STAT3 signaling pathway. However, more recent studies have shown IL10 to use another faster mechanism which does not require gene transcription. In fact, studies have shown that macrophage cell line that expressed a dominant negative STAT3 was still responsive to IL10 inhibition of TNF $\alpha$  <sup>[136]</sup>. In addition, studies in *in vivo* models have also suggested that IL10 can act in a STAT3-independent manner <sup>[137, 138]</sup>. Takeda et al have shown that in myeloid cell specific STAT3<sup>-/-</sup> mice, TNF $\alpha$  levels began to decline 1.5 hours after LPS stimulation and returned to baseline level after 3 hours <sup>[138]</sup>. Lastly, our lab has shown that IL10 represses TNF $\alpha$  production by shifting TNF $\alpha$  mRNA from associating with poly-ribosomes to mono-ribosome via a SHIP1 dependent mechanism <sup>[112]</sup>. Together these results suggest that IL10 utilises a STAT3-independent pathway to mediate its anti-inflammatory effect.

### 4.2 SHIP1 in IL10 signaling

Previous results from our lab suggested that SHIP1 may be the central molecule in mediating STAT3-independent IL10 signaling. We have previously shown that IL10 activates and recruits SHIP1 to the IL10R and that SHIP1 deficiency results in IL10 resistance in macrophage cell lines. Furthermore, our lab has developed a small molecule SHIP1 agonist, AQX-151, which is found to be beneficial in reducing inflammatory symptoms in mouse models of inflammatory bowel disease <sup>[62]</sup>. As SHIP1's expression is mainly restricted to hematopoietic cells, it is an ideal target for immune cell related diseases.

To further investigate SHIP1's role in IL10 signaling, we first assessed whether SHIP1 physically interacts with STAT3 and possibly AMPK in response to IL10. Using primary peritoneal macrophages (perimacs) from Balb/C SHIP1<sup>+/+</sup> mice, we showed that while IL10 stimulation triggers the physical interaction between SHIP1, STAT3 and AMPK. On the other hand, IL6 is not able to cause the formation of such a complex. In fact, the involvement of SHIP1 and the formation of SHIP1-STAT3-AMPK complex may be the determining factor for the difference between the outcome of these two signaling pathways.

Furthermore, our small molecule SHIP1 agonist (AQX-151) and AMPK activator (AICAR) also trigger complex formation. It was interesting to note that the amount of pSTAT3 pulled down with SHIP1 was the same following both IL6 and IL10 stimulation, which suggests that complex formation is independent of STAT3 phosphorylation. This was further confirmed by analysing the kinetics of SHIP1-STAT3-AMPK complex formation. As we saw, complex was formed 2 minutes after IL10 stimulation, while pSTAT3 levels start increasing at 5 minutes. This suggests that STAT3 phosphorylation and SHIP1-STAT3-AMPK complex formation are separate events that occur following IL10 stimulation.

We next evaluated the binding order in the SHIP1-STAT3-AMPK complex, and performed the pulldown experiments in STAT3 KO bone marrow derived macrophages and perimacs. Again we saw that IL10, AQX-151 and AICAR all triggered complex formation and caused higher levels of STAT3 and AMPK to be present in pulldown samples of both BMDMs and perimacs. However, there were no AMPK proteins present in the pulldown samples of STAT3 KO cells after stimulation. This suggests that STAT3 is crucial for complex formation and mediates the interaction between SHIP1 and AMPK protein. It has been previously shown in our lab that macrophages stimulated with LPS and IL10 have lower levels of PIP<sub>3</sub> and higher levels of PI(3,4)P<sub>2</sub> than the ones stimulated with LPS alone (Appendix A). Based on this observation we hypothesised that IL10 may activate SHIP1, a 5'-phosphatase that converts PI(3,4,5)P<sub>3</sub> to PI(3,4)P<sub>2</sub>. However, it was interesting to find that the phosphatase dead mutant SHIP1 is still responsive to IL10. In fact, TNF $\alpha$  levels decreased with increasing IL10 concentration in both J2M His-SHIP and mutant cells. In addition, we observed that the phosphatase dead can still form the complex with STAT3 and AMPK in response to IL10 and AQX-151. These results showed that SHIP1's involvement in IL10 signaling pathway is independent of its role as a 5'phosphatase.

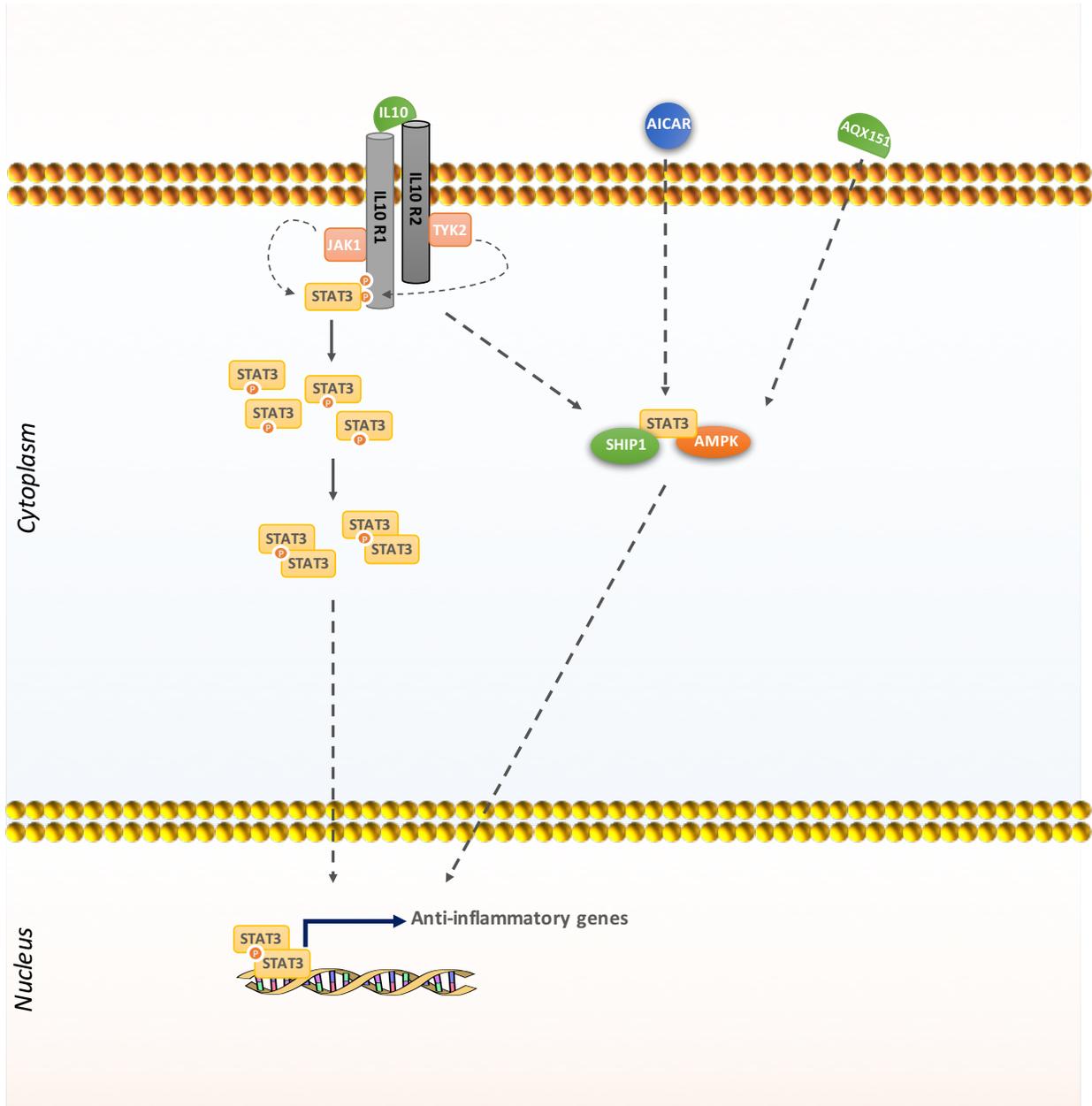
After recognising the importance of complex formation as a non-canonical IL10 signaling pathway, we aimed to identify the SHIP1 domains that play a role in the formation of the SHIP1-STAT3-AMPK complex. We performed the pulldown experiments with various SHIP1 mutants and the PH domain was found to be required for SHIP1-STAT3 complex formation in response to IL10. However, it was interesting to note that complex formation was normal in the KAKA mutant (functionally inactive PH domain). This observation indicates that  $\Delta$ PH SHIP1's inability to bind STAT3 may not be related to its

recruitment to the cell membrane and its association with PIP<sub>3</sub>. In fact, it indicates that the formation of SHIP1-STAT3 complex is independent of SHIP1's role as a 5'-phosphatase. In addition, SHIP1 lacking C2 Domain failed to form a complex in response to AQX-151. This suggests that AQX-151 binds to the C2 domain to cause a conformational change, activate SHIP1, and trigger SHIP1's interaction with STAT3. It has been commonly accepted that STAT3 associates with pTyr residues via its SH2 domain [109] This applies to both STAT3's recruitment to the IL10R and STAT3 dimerization when they are phosphorylated. Accordingly, we next aimed to identify potential SHIP1 p-Tyrosines that could be targeted by STAT3's SH2 domain. Four different potential targets in SHIP1's sequence were identified and mutated. Out of these mutants, Tyr190 was found to be critical for SHIP1-STAT3-AMPK complex formation. Furthermore, Y190F mutants were remarkably resistant to IL10 mediated inhibition of LPS-stimulated TNF $\alpha$  secretion. These finding confirmed the importance of SHIP1-STAT3-AMPK complex as a STAT3-independent pathway for IL10 signaling.

Although we have seen that either IL10, AQX-151 and AICAR can trigger complex assembly (**Figure 15**) the mechanism by which these intercellular proteins mediate the anti-inflammatory response requires further investigation. We hypothesise that the binding of AQX-151 to SHIP1's C2 domain causes a conformational change in SHIP1, allowing its phosphorylation at Tyr190 and subsequently its interaction with STAT3 and AMPK. Similarly, binding of AICAR to AMPK can induce a conformation change in the protein and allow its association with STAT3 and SHIP1. Furthermore, we postulate that the downstream targets of STAT3 change once it becomes part of the complex and interacts with SHIP1 and AMPK. Indeed, phosphorylated STAT3 induced by IL6 could induce the expression of pro-inflammatory genes while non-phosphorylated STAT3 in the SHIP1-AMPK complex leads to expression of anti-inflammatory genes.

To conclude, the data presented in this research project enhanced our understanding of IL10 signaling. Considering IL10's potent anti-inflammatory function, we can stimulate this pathway to combat various inflammatory diseases such as inflammatory bowel disease or rheumatoid arthritis. Here we showed that IL10 initiates the formation of SHIP1-STAT3-AMPK complex independent of JAK-STAT3 pathway. In fact, formation of this complex was found to be necessary for IL10 signaling. Future studies

can further reveal the details of the protein-protein interactions required for such assembly. Overall, recognition of this unique mechanism will give insight into development of novel therapeutics that mimic the beneficial effects of IL10.



**Figure 15 Proposed model for IL10 signaling**

We have shown that IL10, AQX-151 and AICAR can induce complex assembly and mediate IL10 signaling independent of the canonical JAK-STAT3 pathway.

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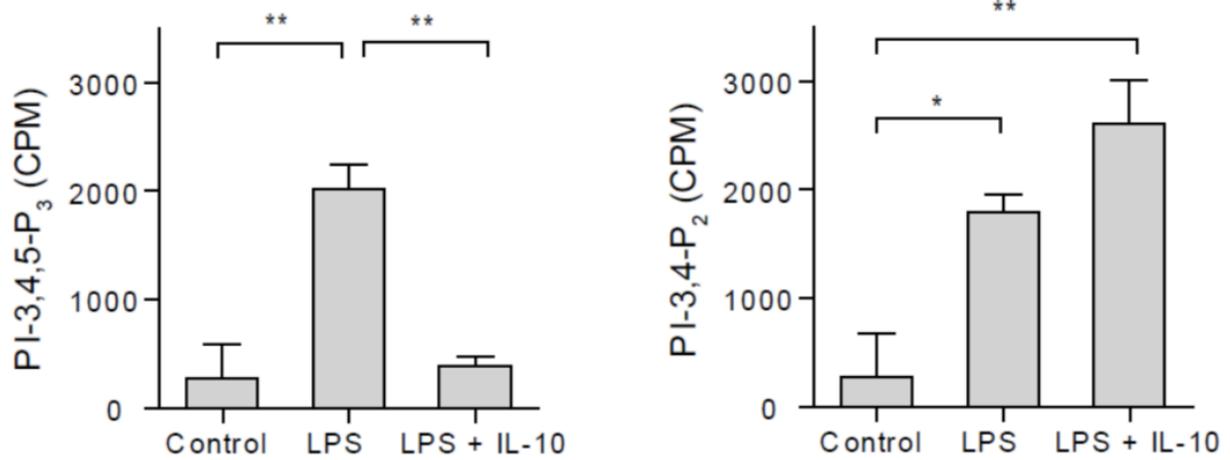
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## 6. Appendices

### Appendix A

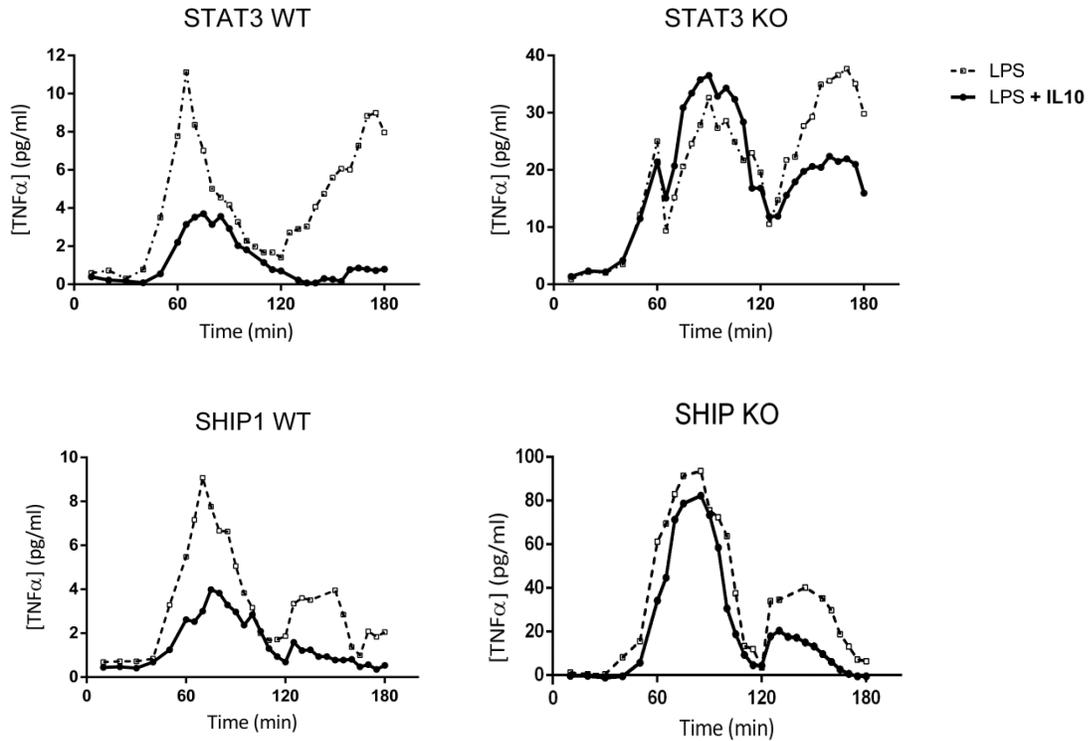


#### **Appendix A Previous results illustrating that IL10 activates SHIP1**

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<0.01 (one-way ANOVA)

*Courtesy of Dr. Andrew Ming-Lum*

## Appendix B



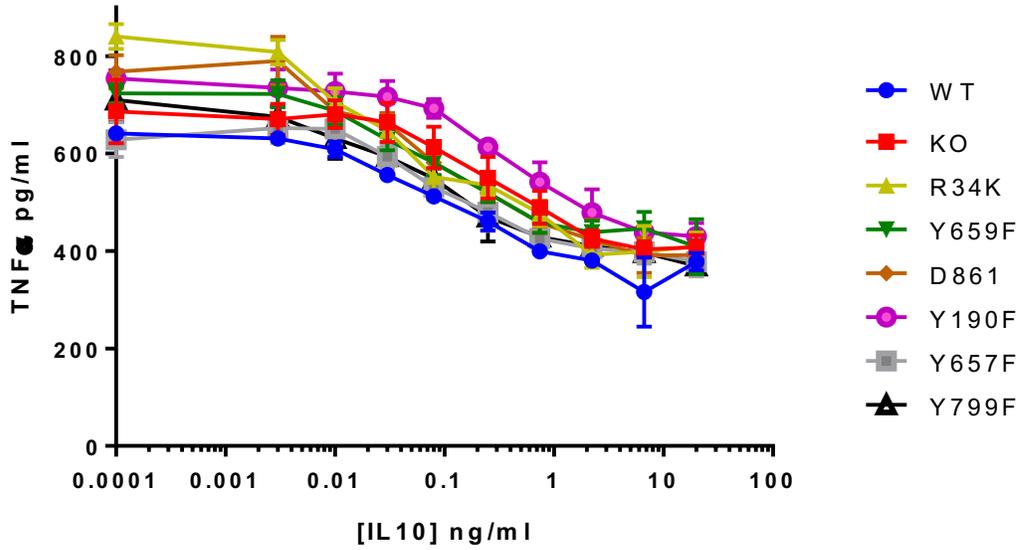
### Appendix B Previous results illustrating the importance of SHIP1 in IL10 signaling

STAT3<sup>+/+</sup>, STAT3<sup>-/-</sup>, SHIP1<sup>+/+</sup> and SHIP1<sup>-/-</sup> BMDMs were stimulated with LPS (dotted line) or LPS + IL10 (solid line) over the course of 200 minutes in a continuous-flow apparatus. Fractions were collected every 5 minutes for measurement of TNFα levels using ELISA.

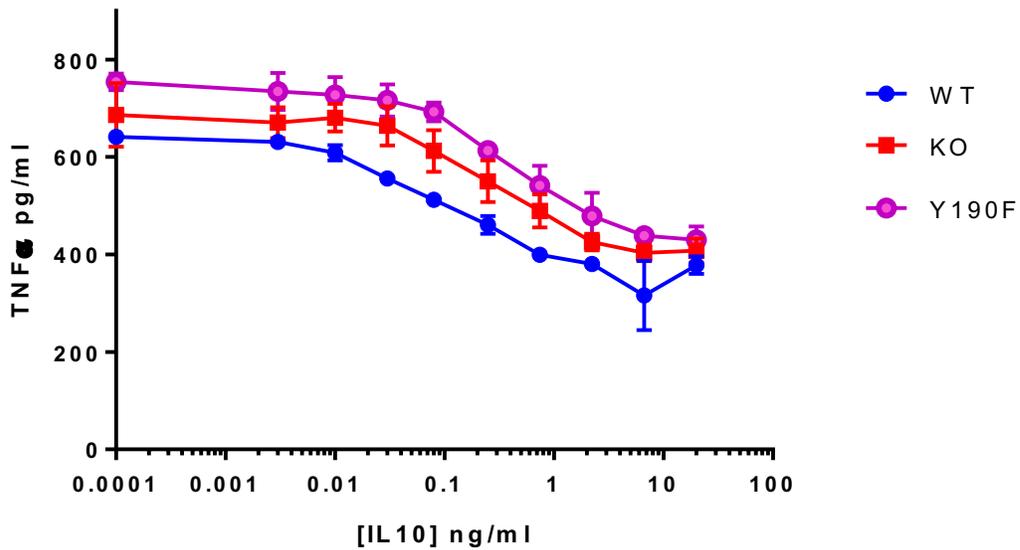
*Courtesy of Dr. Tsz Ying Sylvia Cheung*

**Appendix C**

**IL10 Treatment of J2M Mutants 1 Hr (10 ng/ml LPS)**



**IL10 Treatment of J2M Mutants 1 Hr (10 ng/ml LPS)**



**Appendix C TNFα suppression curves for different cell lines**

TNFα ELISA of cell supernatants from J2M KO reconstituted with WT, R34K, Y659F, D861, Y657F, Y799F and Y190F SHIP1. Cells were stimulated with LPS (1 ng/ml) and increasing concentrations of IL10 (ranging from 0 – 20 ng/ml). Data represent TNFα levels at 1 hour time point. Each cell line was tested in three independent experiments and the mean TNFα level is plotted with error bars illustrating the SD.