The role of SHIP2 in suppressing inflammatory signaling induced by LPS in Immortalized Murine Macrophage Cell Line

by

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Abstract

Inflammation is an important step in the body's defense against pathogen infection. However, it must be tightly regulated and appropriately terminated to prevent pathological consequences. Interleukin-10 (IL10) is one of the body's most important anti-inflammatory cytokine that can inhibit many molecular events necessary for promoting inflammation including production of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα). Our laboratory has recently shown that SH2-domain containing Inositol 5′ phosphatase (SHIP1) is involved in IL10 signaling in macrophages, and although the mechanism of how this occurs is not well studied, our laboratory have obtained data suggesting SHIP1 mediates IL10 signalling through its phosphatase activity or interaction with other signalling proteins. SHIP2 is the only other known homologue of SHIP1 with approximately 38% amino acid sequence identity, yet they possess several similar functions including mediating FcγIIB signaling and phagocytosis. Because of their similarities and SHIP1's involvement in IL10 signaling, we sought to investigate whether SHIP2 is also involved in inhibiting inflammatory response in macrophage by knocking it out using CRISPR/Cas9-mediated genome editing. Overall, we were unable to determine whether SHIP2 plays a role in macrophage anti-inflammatory response due to the large variation in cell sensitivity to IL10 and we also observed that transduction of macrophages with CRISPR/Cas9 virus alters the cellular response to IL10 which confounded our investigation of SHIP2 function.

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 assistance from Sylvia Cheung and Lisa Lee on CRISPR/Cas9 constructs design and cloning.

Biosafety Approval

The author has completed Biological Safety Training Course conducted by Risk Management Services of UBC.

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List of Abbreviations

AP1	Activator protein 1
Akt	Protein kinase B (PKB)
Arap3	Ankyrin repeat and PH domain 3
ARE	AU-rich element
Bcl3	B-cell lymphoma 3-encoded protein
BCAP	B-cell adaptor protein
BCR	B-cell receptor
cPPT	Central polypurine tract
CD	Cluster of differentiation
CRISPR	Clustered, regularly interspaced, short palindromic repeat
CRISPR/Cas9	CRISPR-associated protein-9 nuclease
crRNA	CRISPR-RNA
DAMPs	Danger Associated Molecular Patterns
DD	Death domain
DMEM	Dulbecco's Modified Eagle Medium
DSB	Double stranded break
dsRNA	Double-stranded RNA
EphA2	Ephrin type A receptor 2
EFP	Elongation factor-1α promoter
FCS	Fetal calf serum
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
MHC	Histocompatability Complex
HDR	Homology directed repair
HGFR	Met/hepatocyte growth factor receptor

iNOS	inducible nitric oxide synthase
KSRP	KH-type splicing regulatory protein
IL-6	Interleukin-6
IL10	Interleukin-10
IL10R	Interleukin-10 Receptor
IMDM	Iscove's modified Dulbecco's medium
IRAK1	Interleukin-1 Receptor Associated Kinase
IRF3	Interferon regulatory factor 3
IRF5	Interferon regulatory factor 5
ITIM motif	Immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal Kinase
J2M WW cell	J2M Wild-type cell
J2M KW cells (ΔSHIP1)	J2M KW cells (ΔSHIP1)
J2M KK_3 cells	J2M KK_3 cells (ΔSHIP1, ΔSHIP2)
LPS	Lipopolysaccharide
LBP	LPS-binding protein
M-CSF	Macrophage colony stimulating factor
MAPKK6	MAP Kinase Kinase 6
MD2	Lymphocyte antigen 96
MyD88	Myeloid differentiation primary response gene 88
Mal	MyD88-adaptor like
NHEJ	Non-homologous end joining
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated molecule pattern
PDGFR	Platelet-derived growth factor receptor

PI3K	Phosphatidylinositol-3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
p38	p38 mitogen-activated protein kinase
pTyr	Phosphorylated tyrosine
PTEN	Phosphatase and Tensin Homolog
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PTB	Phospho-tyrosine binding
PH-R	Pleckstrin homology-related
PRR	Proline-rich region
RIP1	Receptor interacting protein 1
RRE	Rev response element
SARM	Sterile α- and armadillo-motif containing protein
SH2	Src Homology 2
SHIP1	SH2-domain containing Inositol 5′ phosphatase 1
SHIP2	SH2-domain containing inositol 5´phosphatase 2
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
SAM	Sterile α motif
TAB2	TAK1-associated binding protein 2
TACE	Tumor necrosis factor α converting Enzyme
TBS	Tris buffered saline
TBS-T	Tris-buffered saline supplemented with 0.1% Tween 20
TAK1	TGF-β activated kinase 1

TBK1	TRAF family member-associated NFκB activator (TANK)-binding kinase 1
TIR	Toll/IL1R
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
TracrRNA	transactivating CRISPR-RNA
TRAF	TNF receptor associated factor
TRIF	TIR-domain containing adaptor protein inducing interferon-β
TRAM	TRIF related adaptor molecule
TRAF6	Tumor necrosis factor receptor-associated factor 6
TTP	Tristetraprolin
UIM	ubiquitin interacting motif

1 Introduction

1.1 Inflammation

In a world that is replete with microorganisms and viruses, an immune system is crucial for vertebrate survival. Inflammation is the key step in activating the immune system to fight against these pathogens and is also the key control point in the body's attempt to modulate the degree of immunological activity[1]. Inflammation is classically defined as the occurrence of redness, swelling, heat, pain[1,2], and disturbance of function of the tissue affected by inflammation. Because of these damaging characteristics, inflammation poses a threat to the host's health, and inappropriate regulation of inflammatory response can potentially lead to inflammatory diseases[3–8].

Inflammation's main function is the rapid elimination of pathogen and damaged cells and restoration of immune homeostasis. It is initiated and orchestrated by immune cells when they come into contact with signals of infection or tissue damage[2,9–11]. These signals can be in the form of cellular debris or components of pathogens, which upon recognition by immune cells, cause a behavioral change in the immune cells to combat the presented threat by activating or repressing specific intracellular signaling pathways that regulate antimicrobial functions such as engulfment of pathogens (phaghocytosis) and release of cytokines, which can recruit effector immune cells such as neutropils and monocytes[12,13]. The recruitment of some of these immune cells will further exacerbate the cytotoxic environment at the site of inflammation.

Cytokines can either promote or inhibit the inflammatory state of immune cells. For example, pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) and Interleukin-1 (IL-1) are some of the earliest cytokines induced by pathogen signals, and they act to further enhance their own production, promote the production of molecules that induce fever, vasodilation, and destruction of pathogens, and recruit immunocompetent cells to the site of infection[14–17]. Anti-inflammatory cytokines such as IL-10 and transforming growth factor-β repress the recruitment of immune cells to the site of infection or damage, inhibit the release of pro-inflammatory cytokines and reprogram the immune cells' behavior to an anti-inflammatory state[18–22]. Therefore, cytokines are crucial regulators of inflammation.

1.2 Innate immunity

The inflammatory response involves cells of innate immunity that perform anti-microbial functions immediately upon recognition of pathogens[23]. The major innate immune cells are phagocytes, antigen-presenting cells, granulocytes, and natural killer cells[24]. Phagocytes are capable of engulfing pathogens, and they include macrophages[25], neutrophils[26], and dendritic cells[27]. Macrophages and dendritic cells can also present antigen by redirecting the components of the phagocytosed pathogen to the cell surface together with Major Histocompatability Complex (MHC), which leads to the activation of adaptive immunity[28,29]. Granulocytes, as its name suggests, contain granules that pack cytotoxic proteins and reactive nitrogen and oxygen species that kill and inhibit growth of bacteria and fungi[30–32]. Neutrophils, basophils, eosinophils and mast cells are the most abundant granulocytes. Lastly, the natural killer cells secrete cytotoxic substances in the

presence of cells that have abnormally low surface expression of MHC, which are typically tumor cells or cells infected by virus[33].

1.2.1 Macrophages

Macrophage is involved in both the initiation and resolution of inflammation. During initiation, macrophages recognize molecules secreted by or expressed on the surface of pathogenic organisms called pathogen-associated molecule patterns (PAMP) using a family of immune receptors called Toll-like receptors. When these receptors are activated macrophages secret inflammatory cytokines to recruit and activate effector cells such as neutrophils and monocytes to eliminate pathogens[2]. Macrophages also reduce inflammation by phagocytosing apoptotic inflammatory cells[34,35] and secreting anti-inflammatory cytokines to inhibit the inflammatory activity of other immune cells'.

Because macrophage can be either pro- or anti-inflammatory depending on the type of stimuli it receives, nomenclatures for describing macrophage activation state were developed. Classically, macrophage has been divided into M1 and M2 activation states[36], denoting that the macrophage is pro- and anti-inflammatory, respectively. But a nomenclature of macrophage only possessing two polar opposite activation states leads to a problem of over-grouping macrophages with different molecular signatures. As a result of this issue, a new nomenclature was recently proposed where the two broad activation states are replaced with a spectrum of macrophage phenotypes defined by the stimulus they receive[37].

Over the years, many macrophage cell lines have been developed by immortalizing macrophages obtained directly from mouse[38]. One such cell line called J2M, derived by

transducing mice bone marrow stem cells with J2 recombinant v-raf/v-myc retrovirus while culturing in macrophage colony stimulating factor (M-CSF), has been used to look at the effect of anti-inflammatory cytokine on macrophage activity. In addition, another J2M cell line was generated from bone marrow stem cells that were extracted from mice lacking expression of SH2-domain containing inositol 5´phosphatase 1 (SHIP1), and this cell line has been used by our laboratory to study the function of SHIP1 in macrophages.

1.3 TLR/LPS signaling pathway

Activating macrophages and recruiting them to the site of infection requires pathogen recognition[9,10]. Two activating signals that macrophages can detect are Pathogen Associated Molecular Patterns (PAMPs) and Danger Associated Molecular Patterns (DAMPs). PAMPs are molecules that are associated with pathogens such as Lipopolysaccharide (LPS) found on cell wall of bacteria and double-stranded RNA found in viruses, whereas DAMPs are debris or substances secreted by damaged cells such as the normally intracellular protein HMGB1. These molecules can bind to a family of receptors expressed on the surface of macrophages called Toll-like receptors (TLR) and trigger an inflammatory response.

TLRs are type I transmembrane proteins, possessing Toll/IL1R (TIR) domain in its cytoplasmic side capable of interacting with other TIR domains[39]. TLRs can form dimer with other TLRs, which allows them to bind to a variety of ligands[40]. So far, thirteen TLRs have been identified in mouse, and ten of them are also found in human. TLR1, 2, and 6 recognize peptidoglycan, TLR3 recognizes double stranded DNA in viruses, TLR4 recognizes lipopolysaccharide (LPS), TLR5 recognizes flagellin, TLR7 and 8 recognize

single stranded DNA in RNA viruses, TLR9 recognizes unmethylated CpG sequence on DNA, and TLR11 recognizes flagellin and profilin[9].

LPS, the prototypical endotoxin and one of the most well-studied PAMPs, binds to TLR4 expressed on the surface of macrophages[41]. The interaction of LPS with TLR4 is initiated by LPS-binding protein (LBP)[42,43], a serum lipid transferase that transfers the LPS on bacterial cell wall to a macrophage surface protein called CD14[44,45]. CD14 then facilitates the transfer of LPS to TLR4 with the help of MD2[46,47], which leads to the dimerzation of TLR4 and activation of TLR4's downstream signaling cascade[40] (Figure 1).

The dimerized TIR-TIR interface on TLR4 acts as an adaptor for downstream signaling molecules[48] including Myeloid differentiation primary response gene 88 (MyD88)[49], MyD88-adaptor like (Mal)[50], TIR-domain containing adaptor protein inducing interferon-β (TRIF)[51], TRIF-related adaptor molecule (TRAM)[52], and Sterile α- and armadillo-motif containing protein (SARM)[53]. Mal and TRAM act as adaptors for MyD88 and TRIF, respectively, which allows TLR4 to facilitate a MyD88-dependent and a MyD88-independent (TRIF-dependent) signaling pathway.

In the MyD88-dependent pathway, the recruitment of MyD88 to TLR4 receptor leads to further recruitment of downstream signaling molecules through its N-terminal death domain (DD), including Interleukin-1 Receptor Associated Kinase 1 and 4 (IRAK1 and IRAK4)[54,55]. When MyD88 and IRAK4 interact, IRAK4 becomes activated and phosphorylates IRAK1, which in turn activates Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6)[55]. TRAF6 is an ubiquitin E3 ligase that catalyzes the addition of polyubiquitin chain on itself and other proteins along with ubiquitin

conjugating enzyme Ubc13 and Ubc-like protein Uev1a[56]. Upon ubiquitination, TRAF6 recruits and activates TAK1-associated binding protein 2 (TAB2), an activator of TGF-β activated kinase 1 (TAK1)[57–59]. MyD88 also recruits the δ and γ isoforms[60] of Phosphatidylinositol-3-kinase (PI3K) to the plasma membrane upon activation by LPS[61–63] and positively regulates the production of TNFα.

Activation of TRAF6 leads to the up-regulation of pro-inflammatory cytokines via three transcription factors. One of the transcription factors is interferon regulatory factor 5 (IRF5) which translocates to the nucleus to regulate gene expression after it is activated by MyD88 and TRAF6[64]. The other two transcription factors, Activator Protein 1 (AP1) and Nuclear Factor κ-light-chain-enhancer of Activated B Cells (NFκB), are regulated by TAK1. AP1 is activated by kinases downstream of TAK1 including MAP Kinase Kinase 6 (MAPKK6), c-Jun N-terminal Kinase (JNK) and p38 mitogen-activated protein kinase (p38)[58]. NFκB translocates to the nucleus to activate transcription after its inhibitor is targeted for proteasomal degradation by the activated TAK1[58,65].

In the MyD88-independent pathway, TRAM recruits TRIF to the plasma membrane[52,66,67], leading to the activation of Receptor Interacting Protein 1 (RIP1)[68] that targets the inhibitor of NFκB for proteasomal degradation so that NFκB can translocate to the nucleus to activate transcription of pro-inflammatory genes in a similar fashion as MyD88-dependent pathway. TRIF can also regulate transcriptional activity of IRF3, the transcription factor of Interferon-γ (IFNγ), by activating TRAF family member-associated NFκB activator (TANK)-binding kinase 1 (TBK1) that phosphorylates IRF3[67,69].

1.4 Tumor Necrosis Factor-α

Tumor necrosis factor α (TNFα) was initially identified as a cytotoxic molecule secreted by immune cells in response to endotoxin stimulation that had tumor-killing capabilities[70]. It was later found that TNFα is the driver of endotoxin stimulated septic shock[71–73] and its presence in the body became a hallmark of inflammation[74,75]. TNFα sustains inflammation by binding to its cell surface receptor that enhances expression and activates pro-inflammatory signaling molecules such as inducible Nitric Oxide Synthase (iNOS)[76,77] and NFκB[78].

Because it is a hallmark of inflammation, the production of TNFα in macrophages is a

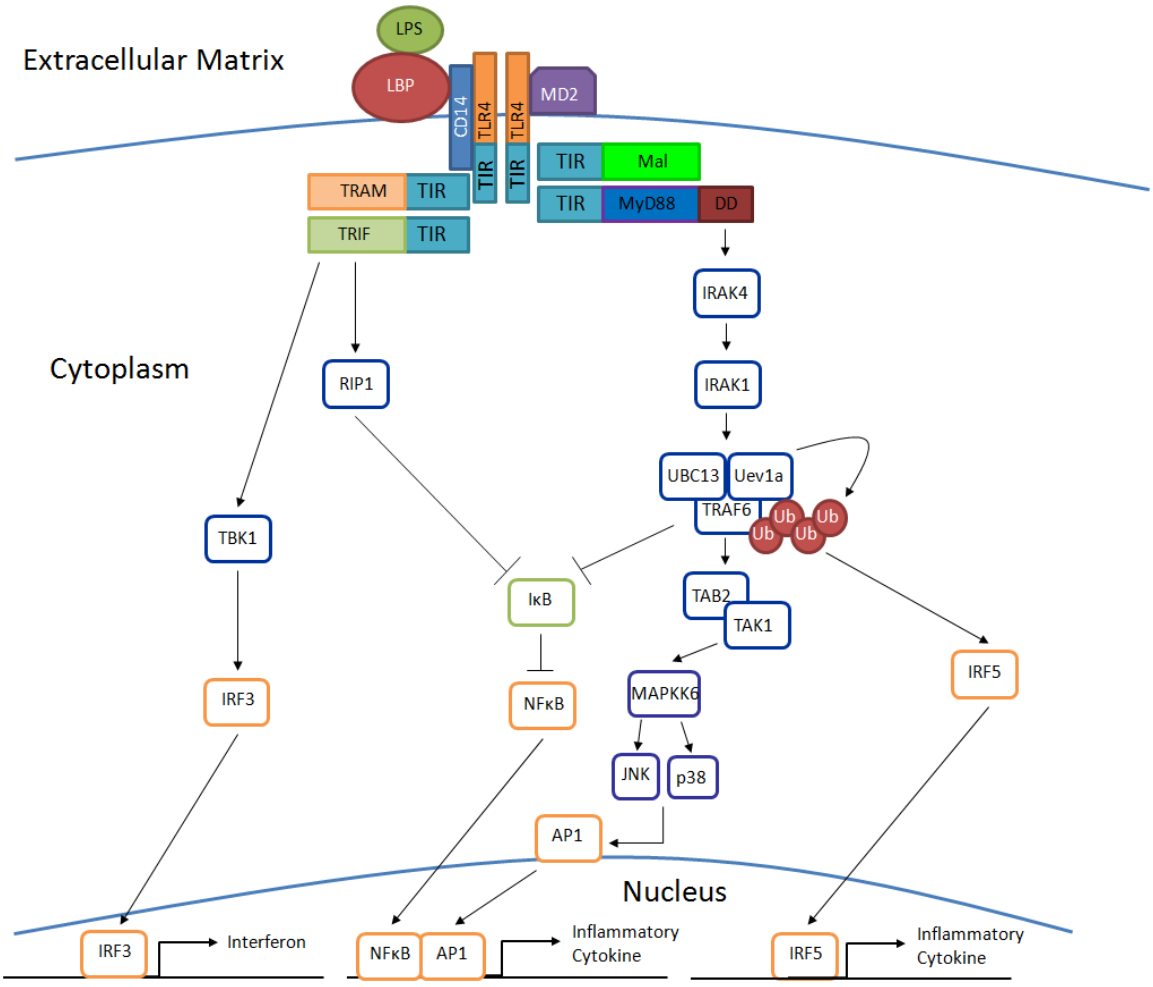

Illustration 1. TLR4 signaling pathway

well studied phenomenon, with numerous regulatory events characterized. LPS stimulated TLR4 signaling pathway can activate transcription of TNFα by utilizing transcription factor NFκB[79] and AP1[80]. TNFα production can also be regulated post-transcriptionally by proteins that recognize and bind to the AU-rich element (ARE) in the 3′UTR of TNFα mRNA. LPS activates the TPL2/Erk signaling pathway that promotes the translocation of TNFα mRNA to the cytoplasm, a process that is enhanced when the ARE is removed[81]. There are also several stabilizing and destabilizing factors that control the level of TNFα mRNA by binding to its ARE, such as KSRP[82], TTP[83], and HuR[84]. TNFα translation is enhanced by LPS-stimulated p38 activation[85], but TIA1[86] and hnRNP A1[87] are capable of inhibiting TNFα mRNA translation. Lastly, TNFα can be regulated at the level of its secretion by Tumor Necrosis Factor α Converting Enzyme (TACE)[88,89].

1.5 Interleukin-10

IL10 was first discovered in 1989 as a factor secreted by Th2 cells to inhibit Th1 cytokine production[18]. Ever since then, studies have shown that Interleukin-10 (IL10) is the body's most important anti-inflammatory cytokine that inhibits a broad spectrum of activated macrophage functions[19], including production of cytokines such as TNF-α, IL-1β, IL-6, IL-8, G-CSF, GM-CSF[90–92], and IL-12[93]. This powerful attribute of IL10 is further highlighted in studies showing that genetic insufficiencies in IL10 or its receptor leads to inflammation in mouse models[94,95] and human diseases[96–98]. Current research is aiming to harness the anti-inflammatory effect of IL10 as a way of treating diseases of chronic inflammation[99–105]. However, early clinical trials of IL10 showed mixed results, with some trials showing improvement with IL10 treatment[106,107], while some showing IL10 having little to no effect[108,109]. These inconsistent results were speculated to be caused by

the non-anti-inflammatory effects of IL10, the activation of B-cell, $CD4^+$, $CD8^+$, and natural killer cells, and also due to the dose of IL10 administered in the trials being too low to have its intended effect on the target site of treatment. To circumvent these problems, researchers are investigating alternative methods of delivering IL10 to the site of inflammation to enhance the specificity of the therapy and to reduce the dose of IL10 that is necessary to elicit a beneficial anti-inflammatory effect[110–113].

1.5.1 Biology of IL10

Cytokines are small proteins between the sizes of 5 – 20 kDa that affect the intracellular signaling cascade of their target cells by binding to their cognate receptors. They have been known to influence a wide range of biological processes[114], but they are especially important in regulating inflammation, as mentioned in a previous section. Interleukin is a category of cytokine that was first observed to be expressed by lymphocytes[115], and today, it is known that the function of the immune system depends on a large part to the activities of the various different types of Interleukins.

IL10 is an Interleukin made up of six helices that are folded in a barrel shape that becomes fully active when it homodimerzes via its C-terminus helix. IL10 is expressed by almost all leukocytes, but most important sources of IL10 *in vivo* appear to be T-helper cells and macrophages[116–118]. IL10 targets T-helper cells and macrophages in addition to other immune cell types such as mast cells, B-cells and natural killer cells (Table 1). In macrophages, IL10 inhibits the production of pro-inflammatory cytokines, enhances production of IL-1 receptor antagonist[119], downregulate the expression of class II Major Histocompatibility Complex[120], promotes the expression of anti-inflammatory

genes through its downstream signaling pathway[121], and drive differentiation of monocytes into macrophages[122].

Cell Type	Biological Effect
Macrophage and monocyte	Inhibits secretion of TNF-α, IL-1β, IL-6[92] Inhibits secretion of TNF-α, IL-1α, IL-1β, IL-6 and GM-CSF induced by LPS and IFN-γ[91] Inhibits secretion of TNF-α and hydrogen peroxide[90] induced by LPS
	Enhances IL-1 receptor antagonist and soluble TNF-α receptor[119]
	Downregulates expression of Major Histocompatibility Complex II molecules induced by IFN-γ[120]
	Inhibits synthesis of IL-12[93]
	Drives differentiation of monocyte into macrophage[122]
T – cells	Inhibits synthesis of IL-2 and IFN-γ by Th1 and IL-4/5 by Th2[123]
Neutrophilic Granulocytes	Inhibits production of TNF-α and IL-1β that are induced by LPS and phagocytosis of bacteria[124] Inhibits production of prostaglandin E2[125]
Eosinophilic Granulocytes	Inhibits production of TNF-α, GM-CSF, and IL-8[126]
Mast Cells	Inhibits production of TNF-α and GM-CSF[127]
	Inhibits expression of IgE receptor, Syk, Fyn, Akt, and Stat5[128]
B – cells	Promotes survival[129] and differentiation into plasma cell[130]
Natural Killer Cells	Enhances cytotoxicity[131]

Table 1. Biological effect of IL10 on different immune cells

IL10 signals through IL10-receptor (IL10R), which is composed of two subunits, IL10R1 and IL10R2[132–134] (Figure 2). Upon binding of IL10, the receptor subunits dimerize, leading to the recruitment of tyrosine kinases Jak1 and Tyk2 to the cytoplasmic side of IL10R1 and IL10R2, respectively[135]. The recruitment and activation of these kinases leads to the phosphorylation of two residues on IL10R1. Signal Transducer and Activator of Transcription 3 (STAT3) contains a Src Homology 2 (SH2) domain that recognizes and binds to the phosphorylated Tyrosine residue on IL10R1[136]. The recruited STAT3 becomes phosphorylated, which can then dimerize with STAT1[132], STAT3, or STAT5 molecule[137]. The dimerized STAT3 translocates to the nucleus to activate transcription of genes that promote anti-inflammatory response. Examples include Bcl3[121], which inhibits the transcriptional activation function of LPS-induced NFκB by binding to its subunits[138,139], and Etv3 and SBNO2[140], which also inhibits NFκB transcriptional activity by acting as transcriptional co-repressors[141–143]. By an unknown mechanism, IL10 also

inhibits activation of PI3K/Akt signaling pathway to enhance the activation of IκB Kinase activity[144], leading to enhanced stability of NFκB inhibitor IκB.

1.5.2 IL10 inhibition of TNFα production

One of IL10's most well-known functions is the inhibition of TNFα production. At the level of transcription, IL10 inhibits the translocation of NFκB to the nucleus by inhibiting the MyD88-dependent TLR4 signaling pathway, which leads to a concomitant reduction in TNFα production[145–147]. IL10 also reduces TNFα mRNA stability in a tristetraprolin (TTP)-dependent manner by inhibiting its upstream inhibitor p38 MAPK[83]. p38 MAPK also promotes translation of TNFα, and thus IL10 inhibition of p38 pathway inhibits TNFα translation[85,148]. Lastly, IL10 can inhibit secretion of TNFα by enhancing the expression of TIMP3[88], an inhibitor of TACE.

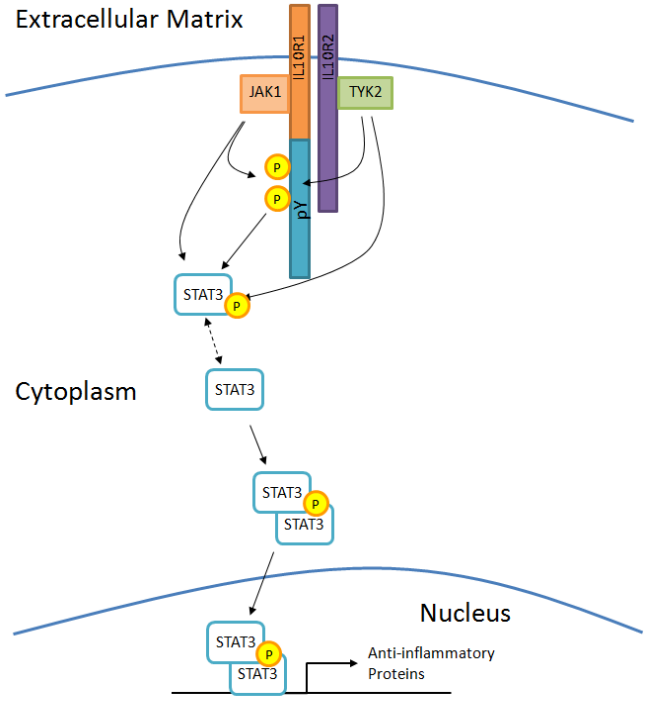

Illustration 2. IL10 signaling pathway

1.6 PI3K pathway

Phosphotinositide-3-kinase (PI3K) is a class of enzymes that catalyze the addition of phosphate to the 3′ hydroxyl group on the inositol ring of phosphatidylinositol that are enriched on the cytoplasmic surface of plasma membrane[149]. Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), the product generated by PI3K, is an important second messenger

signaling molecule that mediates a variety of cellular functions such as differentiation, growth, proliferation, and survival.

PI3K is divided into classes of I_A, I_B, II, and III. The class I enzymes, consisting of I_A and I_B, are heterodimers of a regulatory subunit and a catalytic subunit[150]. The catalytic subunits consists of p110α, p110β, p110δ, and p110γ isoforms. p110α and p110β are expressed ubiquitously in many tissues and organs, whereas p110δ and p110γ are primarily expressed in leukocytes. The regulatory subunits include p85α, p55α, p50α, p85β, and p55γ, p85α being the most expressed. p85α and p85β possess SH2 domains that allow them to interact with phosphorylated tyrosine (pTyr). Upon external stimulus, receptor tyrosine kinases become phosphorylated in their tyrosine residues, which lead to the recruitment of p85 subunits to the plasma membrane. Because p85 possesses an "inter-SH2" domain that interacts constitutively with the p110 subunit, recruitment of p85 also leads to the recruitment of p110 to the plasma membrane.

1.6.1 Function of PI3K in TLR4 pathway

It is well known that PI3K becomes activated from stimulation of TLR4 by LPS. However, the molecular mechanism underlying this activation is not well studied. Several reports suggest that PI3K is recruited to the TLR4 signaling complex by the physical interaction between p85 subunit of PI3K with either MyD88[61,62] or B-cell adaptor protein (BCAP)[151–153], another TLR4 adaptor protein. Also, whether PI3K activates or inhibits TLR4-mediated LPS activation of macrophages has been controversial, with some labs reporting that PI3K activates this pathway[154–166] and other labs reporting that PI3K inhibits this pathway[151,152,167–174]. However, most of these studies investigated the function of PI3K in this pathway using pan-PI3K inhibitors without taking into account

the specific function played by individual PI3K isoforms. Indeed, part of the controversy can be addressed using isoform specific inhibitors of PI3K, and one paper has shown that p110δ and p110γ are the major isoforms involved in upregulating TNFα production in macrophages stimulated with LPS[60].

1.7 Inositol phosphatases

Dysregulation of PI3K is commonly found in a variety of diseases, such as cancer[175], inflammatory disease[176], and Alzheimer disease[177], therefore regulating the activity of PI3K is crucial for proper vertebrae survival. PI3K activity is antagonized by inositol phosphatases, enzymes that remove phosphatase from PIP molecules. The most well studied inositol phosphatase is Phosphatase and Tensin Homolog (PTEN), which converts Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) into Phosphatidylinositol (4,5)-bisphosphate (PIP_2)[178], and is an important tumor suppressor found mutated in many cancer types[179]. The SH2-domain containing inositol 5´phosphatase 1 and 2 (SHIP1 and SHIP2) converts PIP_3 into Phosphatidylinositol (3,4)-bisphosphate (PIP_2)[180,181]. PTEN and SHIP2 are expressed in a variety of tissues, whereas SHIP1 is expressed predominantly in hematopoietic cells. Because of SHIP1's specific expression in this cell type, compounds that alter SHIP1 activity have been shown to treat hematological disorders involving PIP metabolism[182,183].

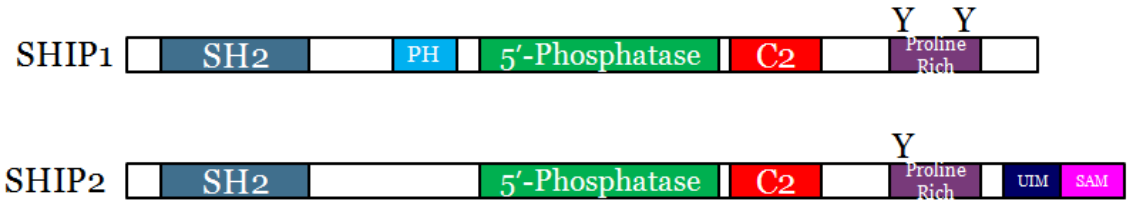

Illustration 3. Domains of SHIP1 and SHIP2

Unlike PTEN, the role of SHIP1 and SHIP2 in tumorigenesis has remained controversial. It was originally thought that they are tumor suppressors that inhibit the cell survival and proliferation pathway activated by PI3K/Akt signaling[182,184–186] However, growing body of evidence is showing that Phosphatidylinositol (3,4)-bisphosphate correlates with Akt activation[187] and a slew of studies have shown that SHIP2 play proto-oncogenic role in tumor initiation and progression[188–190]. Currently it is thought that a specific amount of PI(3, 4, 5)P_3 and PI(3, 4)P_2 is necessary to promote malignant state[191], and variation in their cellular levels between studies can potentially contribute to the variation of SHIP1 and 2's observed role in tumorigenesis.

1.7.1 SHIP1

SHIP1 was originally discovered as a 145 kDa protein that interacted with Shc and Grb upon activation of cytokine receptors[192]. SHIP1 contains six important features: SH2 domain, Pleckstrin Homology-Related (PH-R) domain, phosphatase domain, C2 domain, and proline-rich region (PRR) containing two NPXY motifs. The SH2 domain is involved in the recruitment of SHIP1 to FcγRII during B-cell cell fate determination[193,194]. PH-R domain is important for the recruitment of SHIP1 to the plasma membrane to mediate phagocytosis[195]. C2 domain is involved in binding of SHIP1's product, PIP_2, and it is also the allosteric binding site responsible for enhancing SHIP1's activity[182,183]. SHIP1 is capable of binding several SH3 domain-containing proteins such as Shc and Grb2 via its PRR[196,197]. When NPXY motifs are phosphorylated, SHIP1 can bind to proteins containing Phospho-tyrosine binding (PTB) domain, such as Shc, Dok1, and Dok2[198]. The phosphorylation of SHIP1 is triggered by the activation of various receptors such as B-cell receptor (BCR) and T-cell receptor (TCR)[199,200].

SHIP1 is a negative regulator of immune activity, parts of it due to SHIP1's ability to inhibit pathogen-stimulated PI3K signaling pathways. Consistent with this view, many studies have shown that knocking out SHIP1 results in increased phosphorylation of PI3K's downstream target such as Akt even in unstimulated conditions. Macrophages lacking SHIP1 also produce more pro-inflammatory cytokines than wild-type macrophages and lose endotoxin tolerance[201]. Furthermore, our laboratory showed that SHIP1 is necessary for IL10 inhibition of p38 MAPK substrate Mnk1 (MAPK-signal integrating kinase)[148] and miR-155 maturation[202], both of which upregulate production of TNFα.

1.7.2 SHIP2

SHIP2 was discovered after SHIP1, and although they have the same catalytic function, SHIP2 performs slightly different biological functions compared to SHIP1, owing to their difference in expression pattern and structure. SHIP1 and SHIP2 share 38% of the amino acid sequence (refer to Appendix A for sequence alignment of SHIP1 and SHIP2), and unlike SHIP1, SHIP2 lacks the PH-R domain, but it possesses at the C-terminus an ubiquitin interacting motif (UIM) and a sterile α motif (SAM). No notable functions have been attributed to SHIP2's UIM, but the SAM is known for interacting with SAM in Ephrin Type A Receptor 2 (EphA2)[193] and Arf-GAP With Rho-GAP Domain, Ankyrin Repeat and PH Domain 3 (Arap3)[203] (refer to Figure 4 for structures of SHIP1 and SHIP2) .

SHIP2 inhibits the insulin signaling pathway by competitively inhibiting the association of Shc to Grb2 which prevents the phosphorylation of MAPK p42/44 and Akt[204]. It is interesting to note that SHIP1 possesses these exact same functions in the context of

immune cells[198,201,205,206]. *In vivo* studies of SHIP2 knockout mice revealed two potential biological functions of SHIP2 in host metabolism. One study showed that SHIP2 necessary for retaining a certain level of glucose in the bloodstream, and mice lacking SHIP2 dies to hypoglycemia[207]. In another study, SHIP2 was shown to be necessary for maintaining a lean body in mice when fed with high-fat diet and that lack of SHIP2 do not affect blood glucose level as seen in the first study[208]. Despite the difference in physiological phenotype observed in the two studies, they both showed that SHIP2 is a negative regulator of insulin signaling.

Study of SHIP2 in the context of immune cells is limited to its function in FcγR-mediated signaling. SHIP2 is tyrosine phosphorylated upon FcγRIIa clustering and this leads to inhibition of NFκB activity[209]. SHIP2 also inhibits FcγR-mediated phagocytosis by disrupting the level of PIP_3 and inhibiting recruitment of Akt to the phagocytic cup[210].

1.7.3 Non-catalytic and PI3K-independent functions of SHIP1 and SHIP2

Numerous reports have shown that SHIP1 and SHIP2 do not function exclusively by antagonizing PI3K. SHIP1 and SHIP2 both contain SH2 domain, phosphorylatable tyrosines, and proline rich regions that can interact with other proteins. This property of SHIP comes into play when they become tyrosine phosphorylated by receptor tyrosine kinases and interact with Shc[204,206,211,212], leading to inhibition of Grb2-mediated Ras/Erk signaling pathway. Erk1 can prevent the translocation of TNFα mRNA to the cytoplasm[81] and also inhibit the production of IL-6 and IL-1β[205], and thus protein-protein interaction of SHIP proteins may inhibit production of pro-inflammatory cytokines. In support of this, studies that reconstituted cell lines with catalytic or non-catalytic mutants of SHIP proteins showed that the two regions outside of the phosphatase domain are necessary for

SHIP's function in FcγR[196], TLR4[63], IR[213], PDGFR[213], and HGFR[214] signaling in a manner that is independent of their catalytic activity. Furthermore, studies that identified interaction partners of SHIP1 and SHIP2 have shown that they frequently come into contact with proteins associated with cytoskeleton[211], which suggests that SHIP proteins do not function exclusively at the region of the cytoplasm rich in PIP.

1.7.4 Overlapping functions of SHIP1 and SHIP2

Despite the difference in their amino acid sequence, SHIP1 and SHIP2 possess similar domain structures, which allow them to play many overlapping functions in the immune system. The SH2 domain in each protein is necessary for its recruitment to FcγRIIB[212,215] and they have similar binding affinities for the ITIM motif on FcγRIIB[216]. SHIP1 and SHIP2's proline rich regions are capable of binding Grb2 in association with phosphorylated FcγRIIB[217] and they are both involved in FcγRIIB-mediated phagocytosis[210,218]. Because of their similarities, it is possible that SHIP2 may perform a similar function as SHIP1 in IL10 signaling.

1.8 CRISPR/Cas9 genome editing

Clustered, regularly interspaced, short palindromic repeat (CRISPR) is a segment of DNA found in prokaryotic genome whose function is to defend against invading virus and plasmid[219,220]. The sequence contains repeats of a short base sequence, where between each repeat is segment of DNA obtained from previous exposure to foreign genetic material. CRISPR is closely associated with Cas, nuclease that utilizes the sequences within CRISPR for targeted cleavage of previously exposed foreign DNA.

CRISPR/Cas9 system has been engineered to perform genome editing. The components in this system are the CRISPR-RNA (crRNA) that contains the complementary sequence of the DNA that is targeted, transactivating CRISPR-RNA (tracrRNA) that helps the maturation of crRNA and basepairs with crRNA, and the Cas9 endonuclease. The crRNA:tracrRNA duplex is incorporated into the nuclease to act as the guide sequence.

CRISPR/Cas9 system of genome editing has been used extensively to study the function of genes in cell lines[221] and many model systems, such as zebrafish[222], yeast[223], and mice[224]. This is accomplished by generating double stranded break (DSB) in a genomic sequence to induce gene deletion. DSB can be repaired by joining the two broken ends of the DNA by non-homologous end joining (NHEJ) or by copying existing homologous DNA by homology directed repair (HDR). During NHEJ, a segment of the 5′ DNA strand at each of the two broken ends of the DNA is excised, leaving a 3′ overhang. The overhangs seek for sequence complementarities between each other, and once they form basepairs, and gaps between 3′ and 5′ ends of each strand of DNA are filled in with DNA polymerase. This will result in insertion or deletion of DNA sequence at the site of DSB. This change can potentially introduce frameshift mutations that completely disrupt the amino acid sequence of the protein encoded by the targeted gene, effectively nullifying its function.

1.9 Hypothesis

The overall objective of this study is to study the role of SHIP2 in IL10 mediated anti-inflammation. This study will primarily be done in immortalized mouse bone-marrow derived macrophage cell line J2M. We will knockout the SHIP2 gene in J2M cells derived from wild-type and SHIP1-knockout mice using CRISPR/Cas9 technology. We

predict that SHIP2 plays a similar role as SHIP1 in mediating IL10 anti-inflammation by inhibiting the the production of pro-inflammatory cytokines. If SHIP2 promote anti-inflammation by IL10 signaling like SHIP1, then drug developers may consider creating compounds that activate SHIP2 as another alternative for treating inflammatory diseases, since this was already done for SHIP1[182,183,225].

2 Method

2.1 Cell culture

J2M cells and J2M cells knocked out in SHIP1 were kindly provided by Dr. Gerald Krystal (British Columbia Cancer Agency, Vancouver, British Columbia, Canada) and maintained in Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Nepean, ON), supplemented with 10% (v/v) Fetal Calf Serum (FCS, Fisher Scientific, Ottawa, ON), 10 μM β-mercaptoethanol (Sigma Aldrich, Oakville ON), 150 μM monothioglyerol, and 1 mM L-glutamine (BD Scientific, Mississauga, ON). HEK293T cells were maintained in High Glucose Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Nepean, ON) supplemented with 9% (v/v) Fetal Calf Serum. All cells were cultured at 37^{o}C, 5% CO2, 95% humidity.

2.2 Plasmids/primers

The pLentiCRISPR vector expressing Cas9 was kindly provided by Dr. Keith Humphries (British Columbia Cancer Agency, Vancouver, British Columbia, Canada), and it was digested with BsmBI and inserted with oligonucleotides (Life Technologies, Pleasanton, California, USA) containing coding sequence for sgRNA targeting SHIP2 exon 4, 9, 14, and 19. sgRNA oligonucleotides were designed using CRISPR Design Tool (Zhang laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). The following sgRNA primers were used. For sgRNA targeting exon 4, CACCGAGGGTCTGGAAGCGACGCAC and AAACGTGCGTCGCTTCCAGACCC TC. For sgRNA targeting exon 9, CACCGGGCCAAGACCATCCCCGTGC and AAACGCACGGGGATGGTCTTGGCCC. For sgRNA targeting exon 14, CACCG TCACACTGGACGTACTGACG and AAACCGTCAGTACGTCCAGTGTGAC. For

sgRNA targeting exon 19, CACCGAAAGATGAAGTCCTTGCGCT and AAACAGC GCAAGGACTTCATCTTTC.

2.3 Generation of cell lines

3.2×10^5 HEK293T cells were plated per well in a 6-well format, and allowed to adhere to the plate overnight. The following day, cells in each well were transfected with 62 µg of polyethylemine pre-incubated in 320 µL of Opti-MEM® I Reduced Serum Medium (Life Technologies, Pleasanton, California, USA) for 15 minutes, which was further pre-incubated with 0.32 µg of Vesicular Stomatitis Virus Envelope Protein expression vector, 0.96 µg of R8.9 plasmid, and 1.29 µg of pLentiCRISPR vector plasmid for 15 minutes. Cells were transfected for four hours in 37^{o}C, 5% CO2, and 95% humidity, and incubated for 48 hours. 0.5×10^6 parental J2M cells and 1×10^6 J2M ΔSHIP1 cells were plated per well in a 6-well format prior to transduction. Viral supernatant obtained from the transfected HEK293T cells was supplemented with 8 µg/mL of protamine sulfate (Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) and used to incubate J2M cells for 16 hours. 48 hours later, clones were selected by supplementing culture media with 6 µg/mL puromycin.

2.4 Immunoblotting

Cells lysates were obtained from lysing cells plated in 6-well dish format with plating density of 2×10^6 cells/well that were allowed to grow and adhere to plate overnight. Cells were lysed using 300 µL of 2×Laemmli's buffer, sonicated at 80% power output for 10 seconds using Microson Ultrasonic Cell Disruptor (Heat Systems Ultrasonics Inc., Farmingdale, New York, USA), and boiled for 5 minutes prior to loading onto 7.5% gel which was ran at constant 100V for 90 minutes. 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 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). Densitometry 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.

2.5 Antibodies

Primary antibodies used in the experiments include α-SHIP1 (P1C1) purchased from Santa Cruz (Dallas, Texas), α-SHIP2-2 kindly offered by Dr. Steven Pelech (Kinexus, Vancouver, British Columbia, Canada), and α-Actin purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The secondary antibodies, including Alexa Fluor®680 Goat Anti-Rabbit IgG, and Alexa Fluor®680 Goat Anti-Mouse IgG, were all purchased from Life Technology (Burlington, ON, Canada) unless otherwise stated.

2.6 TNFα ELISA

5×10^4 J2M cells were plated per well and allowed to the plate overnight. Cells were stimulated with 1 ng/mL LPS + IL-10 (ranged from 0 to 20 ng/mL IL-10) in a total volume of 150 μL. 50 μL of supernatant was removed from each well for TNFα

concentration analysis. TNFα protein concentration was assayed by enzyme-linked immunosorbant assay (ELISA) using BD OptEIA™ Mouse TNF ELISA Set II kit (BD Scientific, Mississauga, ON). Assay plate (Sigma Aldrich, Oakville ON) was precoated with 50 uL of capture antibody (α-TNFα 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 three times using the wash buffer (0.05% Tween in PBS). 50 µL of the supernatant was loaded on 96 well plate, and incubated at 23°C for two hours or overnight. Then the supernatant was removed and the plate was then incubated with the 50 µL of detection antibody (biotinylated α-TNFα antibody, diluted 1:250 in assay diluent) for 1 hour at 23°C. The detection antibody solution was removed by washing three times 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 removal of the solution by washing seven times 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 adding 50 µL of 2 N HCl. The plate was then read by the Epoch® Microplate Spectrophotometer at an absorbance of 450 nm.

3 Result

3.1 CRISPR/Cas9 constructs design and rationale

In order to investigate the function of SHIP2, we generated cell lines lacking SHIP2 using the CRISPR/Cas9 system of gene knockout[226]. Prior to this project, one study reported the knockout of SHIP2 gene expression by deleting the region of SHIP2 locus containing exon 19 – 29. In order to mimic this SHIP2-knockout effect in the J2M cell

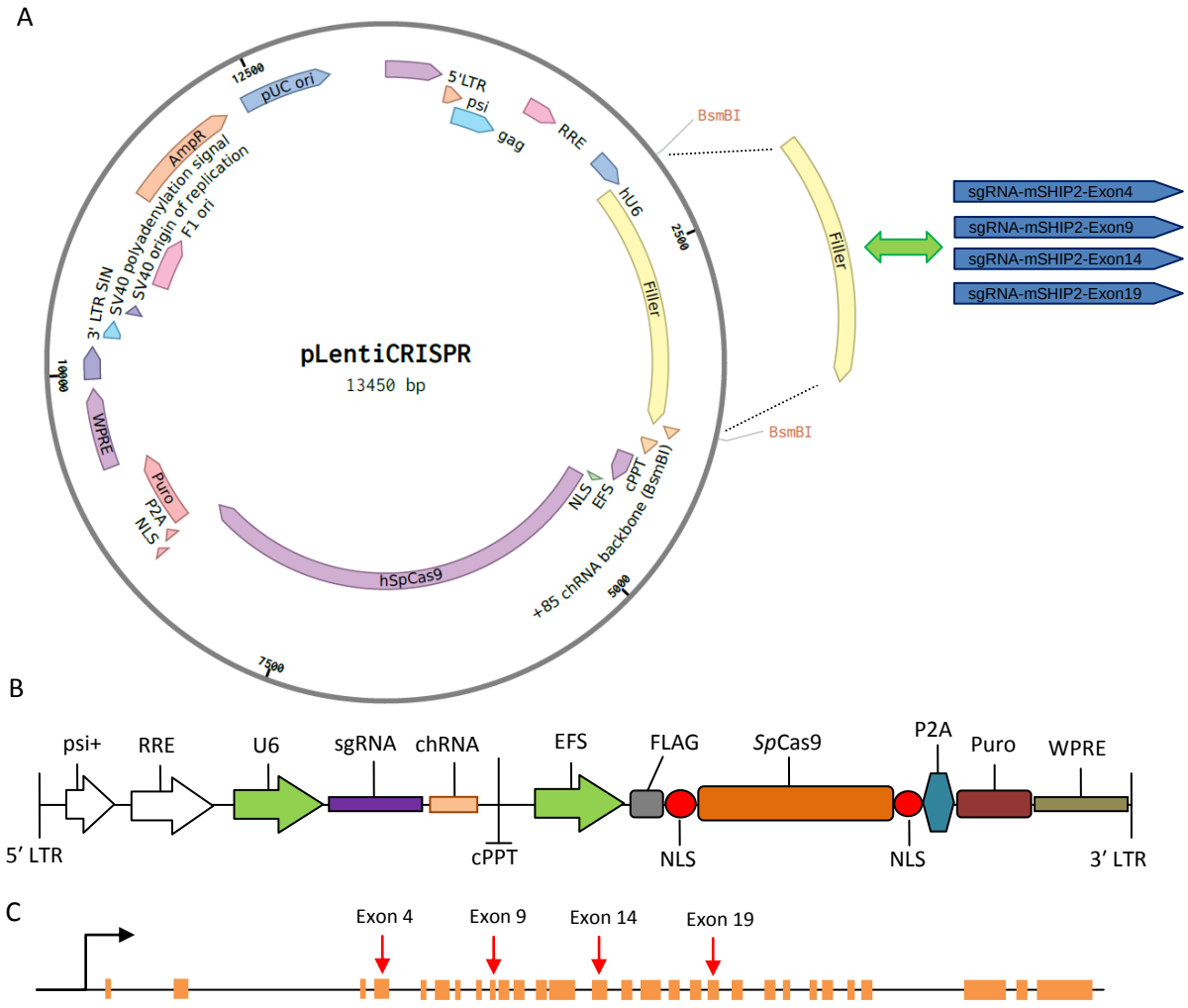

Illustration 4. SHIP2 knockout strategy using CRISPR/Cas9 genome editing

A: Plasmid map containing the lentiviral expression cassette of CRISPR/Cas9. **B**: Lentiviral expression cassette for *Streptococcus pyogenes* Cas9 and sgRNA in the lentiCRISPR v1. Psi packaging signal (psi+), rev response element (RRE), U6 promoter (U6), central polypurine tract (cPPT), FLAG octapeptide tag (FLAG), 2A self-cleaving peptide (P2A), puromycin selection marker (puro), posttranscriptional regulatory element (WPRE), and elongation factor-1α promoter (EFP). **C**: Diagram of SHIP2 gene, orange boxes represent exons, black line represent introns, and red arrows indicate the exons targeted by the various sgRNA.

line, CRISPR/Cas9 constructs were designed to induce DSB in mouse SHIP2 exon 4, exon 9, exon 14, or exon 19 (Figure 5). Mechanistically, inducing DSB in these exons is more likely to disrupt expression of SHIP2 gene than any other combinations of exons. Targeting exons 19 – 29 for cleavage has the possibility of retaining an intact exon 19. Spreading the targeted exons across exons 1 – 19 rather than making multiple constructs targeting the same exon avoids the scenario of the chosen exon being resistant to nuclease cleavage.

After we constructed the plasmid that will be used to generate the lentiviral vectors, the presence of the inserted sgRNA sequence in the plasmids was verified using polymerase chain reaction (PCR). We chose to perform four different reactions, each reaction

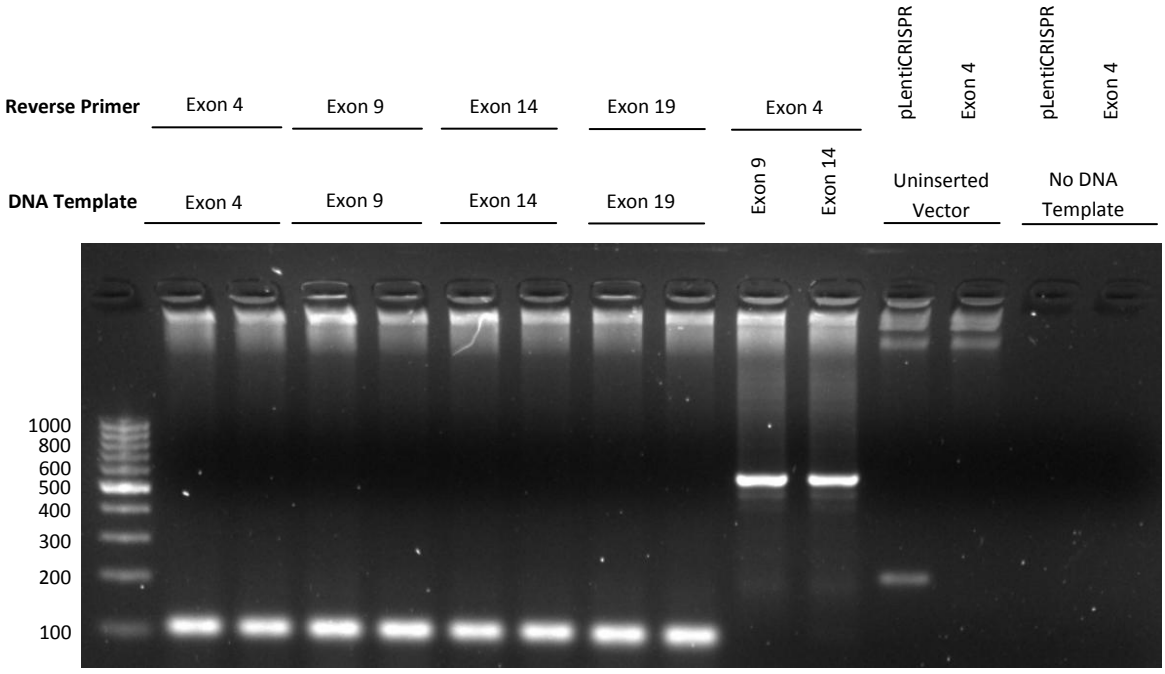

Figure 1. Validation of CRISPR/Cas9 plasmid constructs by PCR

All reactions used the same forward primer that binds to a region of the plasmid 130 bp upstream of the sgRNA insert sequence. Exon 4, 9, 14, and 19 reverse primers have the same sequence as the sgRNA that corresponds to each CRISPR/Cas9 construct. pLentiCRISPR reverse primer binds to a region 100 bp downstream of the sgRNA insert sequence. Exon 4, 9, 14 and 19 DNA templates are the CRISPR/Cas9 plasmids that contain sgRNA sequence complementary to regions within these exons. Uninserted vector is the parental pLentiCRISPR plasmid that was digested with BsmBI and re-ligated without any inserts.

duplicated. Each pair of duplicate reactions used plasmid DNA that were purified from two different bacterial colonies transformed with the same plasmid. Each plasmid DNA was amplified using a forward primer that bound to a constant region of the plasmid DNA that was 130 bp away from the sgRNA sequence and a reverse primer with the sequence of sgRNA or the sequence of a constant region 100 bp upstream of the sgRNA sequence. As shown in figure 6, all reactions that utilized the correct pairing of DNA template and reverse primer generated bands that migrated slightly higher than 100 bp, which corresponds to the expected size of the product. We believe that the unexpected bands that appeared in lanes 9 and 10 are caused by unintended interaction between the reverse primer and the DNA templates.

3.2 CRISPR/Cas9 reduces SHIP2 level in J2M cells

After generation of four CRISPR/Cas9 constructs targeting different exons of SHIP2, we packaged the CRISPR/Cas9 expression cassette into a lentiviral vector and transduced a retrovirally immortalized cell line generated from mouse bone marrow derived macrophage called J2M that either expresses or do not express SHIP1. After selection with puromycin the expression of SHIP2 protein in these cells were determined by analyzing cell lysates by Western Blot (Figure 7). In wild-type J2M cells, the level of SHIP2 band was knocked down when transduced with CRISPR/Cas9 constructs targeting exon 14 of SHIP2. In J2M ΔSHIP1 cells, the level of SHIP2 band was knocked down when transduced with CRISPR/Cas9 constructs targeting exon 19 of SHIP2. For the purpose of convenience, we will refer to parental J2M cells transduced with the pLentiCRISPR virus as J2M WW (no targeting virus), J2M WK_1 (exon 4), WK_2 (exon 9), WK_3 (exon 14), and WK_4 (exon 19). Simiarly, ΔSHIP1 J2M cells transduced with the

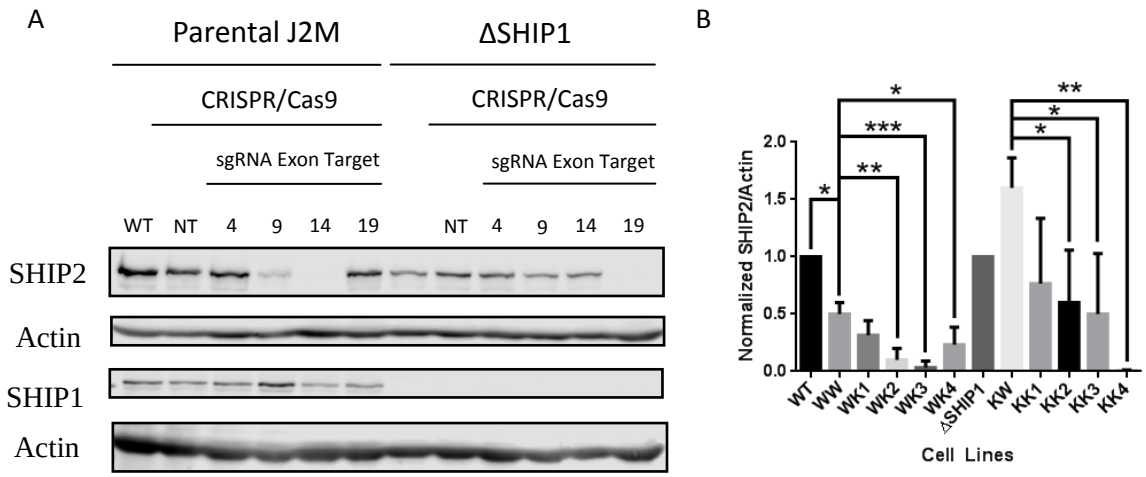

Figure 2. Western Blot analysis of SHIP2 expression in cells transduced with CRISPR/Cas9 virus

A. J2M cells and J2M cells lacking SHIP1 were transduced with CRISPR/Cas9 vector containing either no sgRNA (NT) or sgRNA complementary to part of SHIP2's exon 4, 9, 14, and 19. Membranes were probed with α-SHIP1, α-SHIP2-2, and α-Actin antibodies. **B**. The average SHIP2 band signal from three replicates of the experiment (N = 3). Dunette's multiple comparison test was used on the sets of Parental J2M cells and ΔSHIP1 J2M cells, with SHIP2 signal generated by J2M WW and KW acting as the controls for each set, respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

pLentiCRISPR virus will be referred as J2M KW (none targeting virus), J2M KK_1 (exon 4), KK_2 (exon 9), KK_3 (exon 14), and KK_4 (exon 19) (Refer to Appendix B for summary of nomenclature).

3.3 IL10 sensitivity of J2M cells lacking SHIP2

In response to LPS, macrophage activates an intracellular signaling cascade that leads to the production of pro-inflammatory cytokines such as TNFα and Interleukin-6 (IL6). IL10 is a well studied anti-inflammatory cytokine that is known to inhibit the production of TNFα at many regulatory steps[83,85,88,145–148,227]. SHIP1 is also important for inhibiting the pro-inflammatory response of macrophages, and it does this partly by inhibiting the production of TNFα[60,63,201,205,228,229]. Our laboratory has found that in macrophages, IL10 utilizes SHIP1 to inhibit p38[148] and miR-155-mediated expression of TNFα[202]. Furthermore, we have found through knocking out SHIP1 in mouse primary macrophages that IL10/SHIP1 specifically inhibits the burst of TNFα production at one hour after stimulation with LPS (Cheung ST et al, unpublished). Because evidence in the literature

suggests that SHIP2 may possess overlapping functions as SHIP1 and our laboratory often observe that removing SHIP1 expression from macrophages does not completely abrogate IL10's ability to inhibit TNFα production at one hour, we hypothesized that SHIP2 may play a dual role with SHIP1 in inhibiting TNFα production.

In order to test this hypothesis, we tested how well the J2M cells lacking SHIP2 were able to respond to IL10. The production of TNFα in all J2M cells expressing SHIP1 was inhibited by IL10 by 50% or more (Figure 8A, B and C). The cells transduced with the CRISPR/Cas9 virus produced more TNFα compared to the parental J2M cells, and this difference was not due to enhanced basal activation as all of the cells assayed produced less than 100 pg/mL of TNFα if not stimulated. The maximum inhibition of TNFα

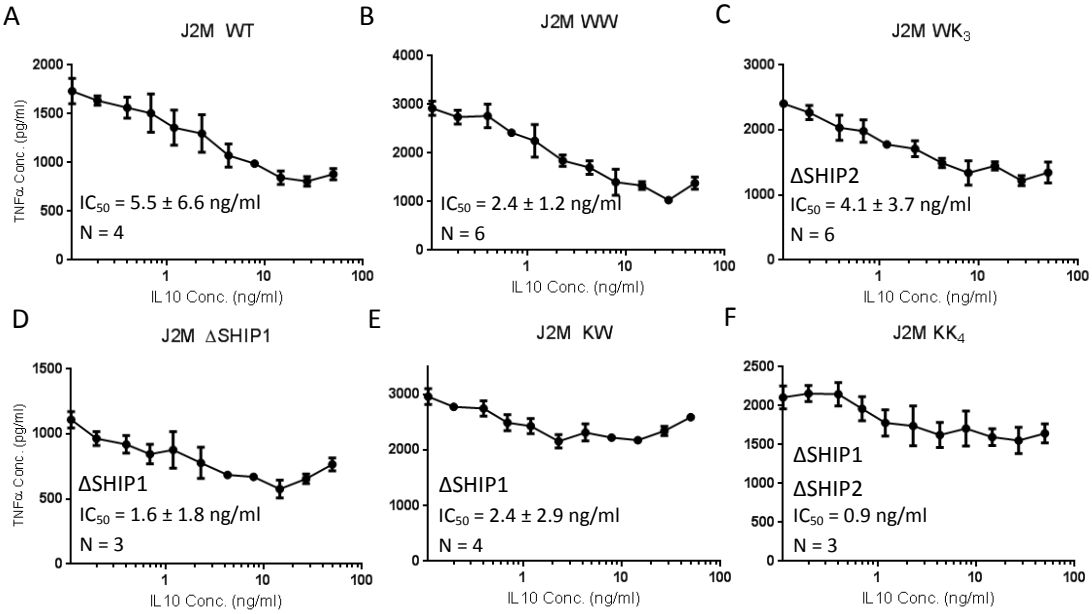

Figure 3. IL10 sensitivity of ΔSHIP2 and ΔSHIP1/ΔSHIP2 J2M cells

TNFα production assay was performed on each cell line at least three times. J2M WT transduced with control CRISPR/Cas9 virus (J2M WW), J2M WT transduced with CRISPR/Cas9 virus expressing SHIP2 exon 14 sgRNA and knocked out in SHIP2 (J2M WK_3), J2M ΔSHIP1 transduced with control CRISPR/Cas9 virus (J2M KW), and J2M ΔSHIP1 transduced with CRISPR/Cas9 virus expressing SHIP2 exon 19 sgRNA and knocked out in SHIP2 (J2M KK_4). Each graph is the representative IL10 inhibition profiles for its corresponding cell line. Cells were plated at a density of 30000/well in 96-well format and incubated in 37°C at 5% CO_2 for 20 hours. Cells were stimulated with media containing LPS and purified mouse IL10. LPS concentration was 10 ng/mL. TNFα level was analyzed using TNFα ELISA kit after 1 hour of stimulation. Cell line genotype, average IL10 IC_{50} and N indicated on each graph.

production by IL10 in the parental J2M ΔSHIP1 was about 50%, but it was less than 30% in cells transduced with the CRISPR/Cas9 virus that lack SHIP1 (Figure 8D, E and F).

In order to determine whether J2M WW and J2M WK_3 had differing sensitivity to IL10, we calculated the average IC_{50} of IL10 inhibition on these cell lines using GraphPad Prism 6 (refer to Appendix C for complete summary of IC_{50} values obtained from all repetitions of this experiment). The average IL10 IC_{50} of these cell lines ranged from 1.5 – 3 ng/ml and their standard deviations were widely dispersed. Therefore, the experimental technique and the variability of the cell line prevent us from distinguishing IL10 sensitivity between cells that express and lack SHIP2.

4 Discussion

IL10 is known for inhibiting the production of TNFα when macrophages are stimulated by Pathogen-associated molecular patterns such as LPS. Several groups have previously shown that SHIP1 is important for downregulating macrophage inflammatory response, and we were the first group to show that SHIP1 plays a significant role in the anti-inflammatory effect of IL10[148,202]. SHIP2 is the only known homologue of SHIP1, and in the immune system they are both recruited to FcγRIIB to inhibit B-cell proliferation[212,215–217] and phagocytosis[210,218]. Because of their similarities, we hypothesized that SHIP1 and SHIP2 have overlapping functions in IL10 signaling such that they compensate for each other. In order to test this hypothesis, we generated CRISPR/Cas9-mediated SHIP2 knockout macrophage cell lines in a wild-type and SHIP1 knockout background. By Western Blot, we verified that CRISPR/Cas9 construct that targeted exon 14 of SHIP2 was able to knockout its expression level in wild-type cells. In cells lacking SHIP1, CRISPR/Cas9 construct that targeted exon 19 was able to knockout SHIP2 expression.

Because it is well known that IL10 can inhibit the production of TNFα at many regulatory steps[83,85,88,145–148,227], a simple method for assaying IL10 responsiveness is to determine the IL10-inhibition sensitivity of TNFα production. To test whether SHIP2 plays a role in IL10 signaling, we stimulated cells lacking SHIP2 with LPS to induce the production of TNFα and simultaneously treated the cells with varying concentrations of IL10. We have observed the parental J2M cells that were transduced with CRISPR/Cas9 virus produced more TNFα than the parental J2M cells. We also observed the parental

ΔSHIP1 cells (J2M KW) that were transduced with CRISPR/Cas9 virus had weaker IL10 inhibition compared to the untransduced ΔSHIP1 cells (J2M ΔSHIP1).

This observation is likely due to two significant problems with the usage of CRISPR/Cas9 gene editing in macrophage cell lines. One is that macrophage expresses TLR3 that recognizes double-stranded RNA (dsRNA)[230]. Upon recognition of dsRNA, TLR3 activates adaptor protein TRIF which in turn activates TRAF6[67]. As mentioned in section 1.3, TRAF6 regulates the activity of several signaling proteins involved in the production of TNFα which are also utilized by the LPS/TLR4 signaling cascade[58,65]. The CRISPR/Cas9 expression cassette was packaged into a lentiviral vector in the form of dsRNA, so the vector may have triggered the signaling events downstream of TLR3 and altered the macrophage physiology. The second issue with CRISPR/Cas9 gene editing is Cas9 nuclease's off-target effect. After the cells were transduced with CRISPR/Cas9 virus, they were enriched by negative selection, a process that took approximately thirty days. By the end of the enrichment, Cas9 nuclease have likely caused significant amount of DNA damage to the genome, leading to the activation of DNA repair machinery that may have affected cellular physiology. However, this long-term mutagenesis may be avoided using more modern CRISPR/Cas9 products that significantly reduce the amount of time the Cas9 protein is active, such as transfecting cells with Cas9 proteins that could be degraded overtime rather than a CRISPR/Cas9 expression cassette that constantly expresses Cas9[231].

We observed in one experiment that IL10 IC_{50} of J2M WW (wild-type) was half of that of J2M WK_3 (ΔSHIP2), which suggested that wild-type J2M cells are slightly more sensitive to IL10 than compared to cells lacking SHIP2. We were unable to replicate this

finding, and in fact we were unable to determine whether SHIP2 plays a role in macrophage anti-inflammatory response due to the large variation in the IL10 IC_{50} between experiments. However, our laboratory was able to consistently observe that cells lacking SHIP1 had an IL10 IC_{50} four-fold higher than that of wild-type cells (unpublished data), therefore further work must be done to determine the origin of this variation.

Although in a static cell culture setting the difference in IL10 response between wild-type and ΔSHIP1 J2M cells may not be completely obvious, in an experimental system that more mimics the physiological condition of macrophages, the difference may be much clearer. Our laboratory have previously performed experiments using primary macrophage cells obtained directly from mouse and stimulated cells with LPS and IL10 in a continuous flow cell system, where the cells are placed in an environment analogous to that of the bloodstream by allowing the cell culture media to flow past the cell rather than keeping it in a static environment (unpublished data). In that experiment, the production of TNFα peaked at about 75 minutes after the first exposure of the cells to the stimulation media containing LPS. This peak is inhibited by approximately 90% if the stimulation media also contained IL10 in addition to LPS. The primary macrophage cells that lack SHIP1 responded to LPS in the same fashion as the wild-type cells, but IL10 no longer inhibited the production of TNFα at the 75-minute peak. Although this study was unable to detect a difference between wild-type and ΔSHIP2 J2M cells, future efforts should aim to perform the same experiments done in this study but with finer control of cell behavior and experimental technique. If knocking out SHIP2 does cause a shift in macrophage's IL10 IC_{50}, there is the possibility that primary macrophages that lack

SHIP2 will completely lose their sensitivity to IL10 when they are subjected to the flow cell environment.

As of writing of this thesis, the complete mechanism of how SHIP1 is involved in IL10 signaling has remained elusive and no groups have published data suggesting a relationship between IL10 and SHIP2 signaling. One potential route of investigation that can shed light on whether SHIP2 is involved in macrophage inflammatory response is by determining the signaling proteins that are regulated by SHIP1 and SHIP2. There are multiple signaling targets downstream of TLR4 that become phosphorylated after induction by LPS, including p38, IκBα, Akt, and Erk1/2, and our laboratory have previously shown that at least the p38 branch of signaling is regulated by SHIP1[148]. To investigate the possibility of SHIP2 involvement in TLR4 signaling, we would stimulate wild-type, ΔSHIP1, and ΔSHIP2 J2M cells with LPS and test the effect of IL10 on the phosphorylation of TLR4 signaling proteins. In wild-type cells, we expect IL10 to inhibit the phoshorylation of all signaling proteins mentioned above, and IL10 will not be able to inhibit the phosphorylation of p38 in cells lacking SHIP1. The protein whose IL10 inhibition is abolished in the cells lacking SHIP2 is likely regulated by SHIP2.

5 Conclusion

SHIP2 is primarily known to be involved in insulin signaling, phagocytosis, and negative signaling under FcγRIIB. The study performed in this project was unable to determine whether SHIP2 is involved in IL10 signaling largely due to the variation in cell behavior between experiments. We also observed that macrophage cell lines transduced with virus containing the CRISPR/Cas9 expression cassette produced more TNFα than their parental counterpart, which may be the result of viral response activation in macrophages or long-term DNA damage stress induced by Cas9 protein. Future experiments should aim to interrogate the function of SHIP1 and SHIP2 in macrophage anti-inflammatory signaling by using more modern CRISPR/Cas9 technology that minimizes cell stress.

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Appendices

Appendix A - Amino acid sequence alignment of mouse SHIP1 and SHIP2 protein

```
SHIP1      -------------MPAMVPGWNHGNITRSKAEELLSRAGKDGSFLVRASESIPRAYALCV
SHIP2      MASVCGTPSPGGALGSPAPAWYHRDLSRAAAEELLARAGRDGSFLVRDSESVAGAFALCV
                        : : .*.* * :::*: *****:***:******* ***:  *:****

SHIP1      LFRNCVYTYRILPNEDDKFTVQASEGVPMRFFTKLDQLIDFYKKENMGLVTHLQYPVPLE
SHIP2      LYQKHVHTYRILPDGEDFLAVQTSQGVPVRRFQTLGELIGLYAQPNQGLVCALLLPVEGE
           *::: *:******: :* ::**:*:***:* * .* :** :* : * ***  *  **  *

SHIP1      EEDAIDEAEEDTESVMSPPELPPRNIP----MSAGPSEAKDLPLATENPRAPEVTRLSLS
SHIP2      REPDPPDDRDASDVEDEKPPLPPRSGSTSISAPVGPSSPLPTPE---TPTTPA-------
           .*    : .: ::   . * ****.        .***.    *    .* :*

SHIP1      ETLFQRLQSMDTSGLPEEHLKAIQDYLSTQLLLDSDFLKTGSSNLPHLKKLMSLLCKELH
SHIP2      -----------AESTPNGLSTVSHEYLKGSYGLDLEAVRGGASNLPHLTRTLVTSCRRLH
                      :.. *:   .. ::**. .  ** : :: *:******.: :   *:.**

SHIP1      GEVIRTLPSLESLQRLFDQQLSPGLRPRPQ-----VPGEASPITMVAKLSQLTSLLSSIE
SHIP2      SEVDKVLSGLEILSKVFDQQSSPMVTRLLQQQSLPQTGEQELESLVLKLSVLKDFLSGIQ
           .** :.* .** *.::**** ** :    *       ** .  ::* *** *..:**.*:

SHIP1      DKVKSLLHEGSEST---------NRRSLIPPVTFEVKS-------ESLGIPQKMHLKVDV
SHIP2      KKALKALQDMSSTAPPAPLQPSIRKAKTIPVQAFEVKLDVTLGDLTKIGKSQKFTLSVDV
           .*. . *:: *.::         .: . **  :****         .:*  **: *.***

SHIP1      ESGKLIVKKSKDGSED--KFYSHKKILQLIKSQKFLNKLVILVETEKEKILRKEYVFADS
SHIP2      EGGRLVLLRRQRDSQEDWTTFTHDRIRQLIKSQRVQNKLGVVFEKEKDRTQRKDFIFVSA
           *.*:*:: : :  *::  . ::*.:* ******:. *** ::.*.**::  **:::*..:

SHIP1      KKREGFCQLLQQMKNKHSEQPEPDMITIFIGTWNMGNAPPPKKITSWFLSKGQGKTRDDS
SHIP2      RKREAFCQLLQLMKNRHSKQDEPDMISVFIGTWNMGSVPPPKNVTSWFTSKGLGKALDEV
           :***.****** ***:**:* *****::********..****::**** *** **: *:

SHIP1      ADYIPHDIYVIGTQEDPLGEKEWLELLRHSLQEVTSMTFKTVAIHTLWNIRIVVLAKPEH
SHIP2      TVTIPHDIYVFGTQENSVGDREWLDLLRGGLKELTDLDYRPIAMQSLWNIKVAVLVKPEH
           :  *******:****: :*::***:*** .*:*:*.: :: :*:::****::.**.****

SHIP1      ENRISHICTDNVKTGIANTLGNKGAVGVSFMFNGTSLGFVNSHLTSGSEKKLRRNQNYMN
SHIP2      ENRISHVSTSSVKTGIANTLGNKGAVGVSFMFNGTSFGFVNCHLTSGNEKTTRRNQNYLD
           ******:.*..************************:****.*****.**. ******::

SHIP1      ILRFLALGDKKLSPFNITHRFTHLFWLGDLNYRVELPTWEAEAIIQKIKQQQYSDLLAHD
SHIP2      ILRLLSLGDRQLSAFDISLRFTHLFWFGDLNYRLDMDI---QEILNYISRREFEPLLRVD
           ***:*:***::** *:*: *******:******:::     : *:: *.::::. **  *

SHIP1      QLLLERKDQKVFLHFEEEEITFAPTYRFERLTRDKYAYTKQKATGMKYNLPSWCDRVLWK
SHIP2      QLNLEREKHKVFLRFSEEEISFPPTYRYERGSRDTYAWHKQKPTGVRTNVPSWCDRILWK
           ** ***:.:****:*.****:* ****:** :**.**: *** **:: *:******:***

SHIP1      SYPLVHVVCQSYGSTSDIMTSDHSPVFATFEAGVTSQFVSKNGPGTVDSQGQIEFLACYA
SHIP2      SYPETHIICNSYGCTDDIVTSDHSPVFGTFEVGVTSQFISKKGLSKTSDQAYIEFESIEA
           *** .*::*:***.*.**:********.***.******:**:* .....*. *** :  *

SHIP1      TLKTKSQTKFYLEFHSSCLESFVKSQEGENEEGSEGEL--VVRFGETLPKLKPIISDPEY
SHIP2      IVKTASRTKFFIEFYSTCLEEYKKSFENDAQSSDNINFLKVQWSSRQLPTLKPILADIEY
            :** *:***::**:*:***.: ** * : :...: ::  *   .. **.****::* **

SHIP1      LLDQHILISIKSSDSDESYGEGCIALRLETTEAQHPIYTPLTHHGEMTGHFRGEIKLQTS
SHIP2      LQDQHLLLTVKSMDGYESYGECVVALKSMIGSTAQQFLTFLSHRGEETGNIRGSMKVRVP
           * ***:*:::** *. *****  :**:    .: : : * *:*:** **.:**.:*::.

SHIP1      QG--KMREKLYDFVKTERDESSGMKCLKNLTSHD---PMRQWEPSGRVPACGVSSLNEMI
SHIP2      TERLGTRERLYEWISIDKDDTGAKSKVPSVSRGSQEHRSGSRKPASTETSCPLSKLFEEP
                 **:**:::. ::*::.. . : .::  .      . :*:.   :* :*.* *
```

```
SHIP1      NPNYIGMGPFGQPLHGKSTLSPDQQLTAWSYDQLPKDSSLGPGRGEGPPTPP----SQPP
SHIP2      ------------------------------------EKPPPTG-RPPAPPRAVPREEP
                                                .  *  *  **:**     : *

SHIP1      LSPKKFSSSTANRGPCPRVQEARPGDLGKVEALLQEDLLLTKPEMFENPLYGSVSSFPKL
SHIP2      LNPRLKSEGTSEQEG-----VAA----------------PPPKNSFNNPAYYVLEGVPHQ
           *.*:  *..*:::        *                    : *:** *  :...*:

SHIP1      VPRK-----------------------------------E------QESPK---MLRKEP
SHIP2      LLPLEPPSLARAPLPPATKNKVAITVPAPQLGRHRTPRVGEGSSSDEDSGGTLPPPDFPP
           :                                           ::*          *

SHIP1      PPCPDPGISSPSIVLPKAQEV-ES-VKGTSKQAPVPVLGPTPRIRSFT-CSSSAEGRMTS
SHIP2      PPLPDSAIFLPPNLDPLSMPVVRGRSGGEARGPPPPKAHPRPPLPPGTSPASTFLGEVAS
           ** ** .*  *  : * :  * ..   * ::  * *   * * :   *  :*:  *.::*

SHIP1      GDKSQGKPK-----ASASSQAPVPVKRPVKPSRSEMSQQTTPIPAPRPPLPVKSPAVL--
SHIP2      GDDRSCSVLQMAKTLSEVDYAPGPGRSALLPNPL-------ELQPPRGPSDYGRPLSFPP
           **. . .        *  . ** * :  : *.          :  ** *     *  :

SHIP1      -QLQHSKGRDYRDNTELPHHG------------------KHRQEEGLLGRTAMQ------
SHIP2      PRIRESIQEDLAEEAPCPQGGRASGLGEAGMGAWLRAIGLERYEEGLVHNGWDDLEFLSD
            :::.*  .*  :::  *: *                  .* ****: .   :

SHIP1      ----------------------------
SHIP2      ITEEDLEEAGVQDPAHKRLLLDTLQLSK
```

Appendix B - Nomenclature of the cell lines used in this study

Cell Line Full Name	SHIP2 Targeting Site	Genotype		Cell Line Abbreviation
		SHIP1 locus	SHIP2 locus	
J2M WT	N/A	WT	WT	J2M WT
J2M WT CRISRP/Cas9 - control	None	WT	WT	J2M WW
J2M CRISRP/Cas9 - exon 4	Exon 4	WT	targeted	J2M WK_1
J2M CRISRP/Cas9 - exon 9	Exon 9	WT	targeted	J2M WK_2
J2M CRISRP/Cas9 -exon 14	Exon 14	WT	targeted	J2M WK_3
J2M CRISRP/Cas9 - exon 19	Exon 19	WT	targeted	J2M WK_4
J2M KO	N/A	KO	WT	J2M KO
J2M KO CRISRP/Cas9 - control	None	KO	WT	J2M KW
J2M KO CRISRP/Cas9 - exon 4	Exon 4	KO	targeted	J2M KK_1
J2M KO CRISRP/Cas9 - exon 9	Exon 9	KO	targeted	J2M KK_2
J2M KO CRISRP/Cas9 - exon 14	Exon 14	KO	targeted	J2M KK_3
J2M KO CRISRP/Cas9 - exon 19	Exon 19	KO	targeted	J2M KK_4

Appendix C - All IL10 IC_{50} values obtained for all cell lines

Note that the graphical data of figure 8 was obtained from FW124.

J2M WT			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW115	15	0.78	1.3 – 179
FW117	1.2	0.59	Very Wide
FW119	1.6	0.69	0.6 – 3.9
FW120	4.1	0.47	Very Wide
FW123	1.5	0.79	0.2 – 14.2
FW124	**2.1**	**0.89**	**1.3 – 3.4**
Average IC_{50} = 4.9 ± 5.8 ng/ml, N = 6			

J2M WW			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW115	3.6	0.95	2.6 – 5.1
FW117	4.4	0.93	Very Wide
FW118	1.2	0.93	0.7 – 2.1
FW119	4	0.85	2.5 – 6.4
FW120	1.5	0.94	0.9 – 2.5
FW122	2.2	0.85	0.9 – 5.7
FW123	1.8	0.95	1.2 – 2.6
FW124	**1.4**	**0.9**	**0.9 – 2.4**
Average IC_{50} = 2.5 ± 1.2 ng/ml, N = 8			

J2M WK_3			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW115	6.7	0.9	3.7 – 12
FW118	3.1	0.89	2.5 – 3.9
FW119	2.3	0.86	1 – 5.4
FW122	10.2	0.68	4.4 – 23.8
FW123	1.3	0.87	0.8 – 2.1
FW124	**0.8**	**0.79**	**0.3 – 2.6**
Average IC_{50} = 4.1 ± 3.7 ng/ml, N = 6			

J2M ΔSHIP1			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW115	3.7	0.9	2.3 – 5.9
FW119	0.1	0.56	(Very Wide)
FW124	**0.9**	**0.69**	**0.3 – 2.8**
Average IC_{50} = 1.6 ± 1.8 ng/ml, N = 3			

J2M KW			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW115	6.5	0.55	(Very Wide)
FW119	2.3	0.86	1 – 5.3
FW122	0.1	0.32	(Very Wide)
FW124	**0.5**	**0.71**	**0.3 – 0.9**
Average IC_{50} = 2.4 ± 2.9 ng/ml, N = 4			

J2M KK_4			
Exp. ID	IC_{50} (ng/ml)	R^2	95% C.I.
FW119	No Inhibition	N/A	N/A
FW122	No Inhibition	N/A	N/A
FW124	**0.9**	**0.70**	**0.5 – 1.7**
Average IC_{50} = 0.9 ng/ml, N = 3			