The distribution of the inositol phosphatase SHIP1 in a murine model of Amyotrophic Lateral Sclerosis (ALS)

by

Sapana M. Thakore

B.Sc. (Biomedical Physiology), Simon Fraser University, 2010

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the
Department of Biomedical Physiology and Kinesiology
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2012

Approval

Name: **Sapana M. Thakore**

Degree: **Master of Science**

Title of Thesis: The distribution of the inositol phosphatase SHIP1 in a murine model of Amyotrophic Lateral Sclerosis (ALS)

Examining Committee:

Chair: **Dr. Parveen Bawa**
Professor Emerita- Department of Biomedical Physiology & Kinesiology

---

Dr. Charles Krieger
Senior Supervisor
Professor- Department of Biomedical Physiology & Kinesiology

Dr. Miriam Rosin
Supervisor
Professor- Department of Biomedical Physiology & Kinesiology

Dr. Jonathan Choy
External Examiner
Assistant Professor- Department of Molecular Biology and Biochemistry

Date Defended/Approved: July 17, 2012

Partial Copyright Licence

Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics,

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University;

or has conducted the research

c. as a co-investigator, collaborator or research assistant in a research project approved in advance,

or

d. as a member of a course approved in advance for minimal risk human research, by the Office of Research Ethics.

A copy of the approval letter has been filed at the Theses Office of the University Library at the time of submission of this thesis or project.

The original application for approval and letter of approval are filed with the relevant offices. Inquiries may be directed to those authorities.

Simon Fraser University Library
Burnaby, British Columbia, Canada

update Spring 2010

Abstract

Src homology 2 domain-containing inositol-5' phosphatase (SHIP1) is a protein which suppresses the activation, proliferation, and survival of hematopoietic cells. Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the degeneration of specific neuron populations which leads to atrophy of skeletal muscles, paralysis, and death. Microgliosis is a common hallmark in ALS patients and in mouse models having over-expression of SOD1 (mSOD1); however, the association between microgliosis and disease progression has not yet been determined.

Using immunohistochemistry (IHC), SHIP1 expression in macrophages and microglia was investigated in the lumbar spinal cord of control and mSOD1 mice at 3 time points: 11 weeks (asymptomatic), 15 weeks (symptomatic), and 18 weeks (advanced stage). A significant increase in SHIP1 immunoreactivity was found between control mice and diseased mice at symptomatic and advanced stages. SHIP1 immunoreactivity also significantly increased throughout disease progression suggesting that SHIP1 is involved in regulating microgliosis in ALS.

Keywords: ALS; SHIP1; macrophages; microglia

To my family and friends for their infinite love and support in all of my endeavours.

Acknowledgements

I would like to first and foremost thank my senior supervisor and mentor, Dr. Charles Krieger. The completion of my thesis would not have been possible without your insight, wisdom, and encouragement. I would also like to thank my supervisory committee and examiners: Dr. Miriam Rosin, Dr. Jonathan Choy, and Dr. Parveen Bawa. I am extremely grateful to Dr. Coral-Ann Lewis who took the time to teach me numerous laboratory procedures. Your patience and guidance made my research experience unforgettable. I would also like to thank my fellow colleagues, John Manning, Christine Barr, and Dr. Kyle Peake for their encouragement and company throughout these past couple of years. Last but not least, I would like to thank my parents and my sister Suhani for their continuous encouragement and support throughout this process. Thank you all for making my experience truly memorable.

Table of Contents

List of Tables

List of Figures

List of Acronyms

AD	Alzheimer's disease
ArgI	Arginase I
ALS	Amyotrophic Lateral Sclerosis
BMDM	Bone-marrow derived macrophages
BSA	Bovine serum albumin
CNS	Central nervous system
DAMP	Danger-associated molecular patterns
DAP12	DNAX-activating protein of 12 kD
E.	Embryonic day
FcγR	Fcγ receptor
Gab	Grb2-associated binding protein
Grb2	Growth factor receptor-bound protein 2
HSC	Hematopoietic stem cells
Iba1	Ionized Ca^{2+}-binding adapter molecule
IFN-γ	Interferon-γ
IGF1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITAM/ITIM	Immunoreceptor tyrosine based activation/inhibition motifs
LBP	LPS binding protein
LPS	Lipopolysaccharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MR	Mannose receptor
MS	Multiple sclerosis
mSOD1	Mutant Cu^{2+}/Zn^{2+} superoxide dismutase
mTOR	Mammalian target of rapamycin
MYD88	Myeloid differentiation primary response protein 88
NDS	Normal donkey serum

NF-κB	Nuclear factor--κB
NK	Natural killer cells
NO	Nitric oxide
NOX2	NADPH oxidase complex
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PH	Pleckstrin homology domain
PKB	Protein kinase B
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PFA	Paraformaldehyde
PRR	Pattern recognition receptors
PTB	Phosphotyrosine binding domain
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RAGE	Receptor for advanced glycation end-products
ROS	Reactive oxygen species
RT	Room temperature
SH2	Src homology 2 domain
SHIP	Src homology 2 domain-containing inositol-5' phosphatase
sSHIP	stem-cell restricted SHIP
SLAM	signal lymphocyte activation molecule
TDP43	TAR DNA-binding protein
TGF-β	Transforming growth factor
T_h1	T helper cell Type 1
T_h2	T helper cell Type 2
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor
TREM-2	Triggering receptor expressed on myeloid cells 2
TRIF	TIR domain-containing adaptor protein inducing IFNβ
WT	Wild type

1. Introduction

1.1. Macrophages and Microglia

1.1.1. Role in the Central Nervous System (CNS)

Macrophages play a role in both the innate, and to a lesser extent, the adaptive immune systems (Galli et al., 2011). They are involved in the innate response by detecting, attacking or removing foreign particles through phagocytosis and the release of inflammatory cytokines (Lee et al., 2011). Macrophages also play a role in the adaptive immune response through their function as antigen-presenting cells (Galli et al., 2011). Macrophages are derived from hematopoietic stem cells (HSC) in the bone marrow where they mature into monocytes and are released into the circulation. Monocytes can then migrate into various tissues during steady state conditions or in response to inflammation where they differentiate into macrophages (Mosser & Edwards, 2008).

Microglia are the resident macrophages of the CNS that comprise approximately 5-12% of the total glial population (Ling et al., 1973). Microglia are of mesenchymal origin and have the capacity for local self-renewal within the CNS (Ajami et al., 2007). It was formerly believed that microglia invade the CNS at two different time points: during fetal development and during postnatal life (Hanisch & Kettenmann, 2007); however recent studies in mice suggest that

adult microglia derive almost exclusively from primitive myeloid progenitors from the embryonic yolk sac that appear before embryonic day 8 (E.8.0) (Ginhoux et al., 2010). These cells, which are genetically distinct from definitive HSCs, migrate from the yolk sac into the brain through blood vessels between E.8.5 and E.9.0 and become microglia (Ginhoux et al., 2010; Schulz et al., 2012). Although microglia have similar functions and express similar markers to macrophages, a recent study has found that macrophages require specific transcription factors, such as Myb, which are not required for microglial development (Schulz et al., 2012).

During the 'resting' or 'surveying' state, microglia regularly screen for any disturbance or change within the microenvironment (Hanisch & Kettenmann, 2007). Their long, thin processes are in constant motion allowing microglia to scan the environment without disturbing adjacent neurons (Kettenmann et al., 2011). Pathological changes or potential threats to the structural and/or functional integrity of the CNS, such as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), are recognized through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and non-TLR receptors, which activate microglia (Nakamura, 2002; Block et al., 2007; Colton, 2009). This recognition initiates downstream signalling cascades involving molecules such as myeloid differentiation primary response protein 88 (MYD88) and TIR domain-containing adaptor protein inducing IFNβ (TRIF). Non-

PRR signalling through purinergic receptors and receptors for advanced glycation end-products (RAGE) can also activate microglia (Saijo & Glass, 2011).

The activated state of microglia is characterized by a morphological change from ramified CD11b expressing cells with small cell bodies and long, thin processes to an amoeboid (rounded cells with sparse processes) morphology (Ransohoff & Cardona, 2010) (See Figure 1). Once activated, microglia undergo functional changes including phagocytosis, antigen presentation, and the production of cytokines and growth factors (Henkel et al., 2009). Activated microglia have been shown to have both cytotoxic and neuroprotective effects depending on the presence of pathogenic factors and interactions with the cellular microenvironment (Philips & Robberecht, 2011).

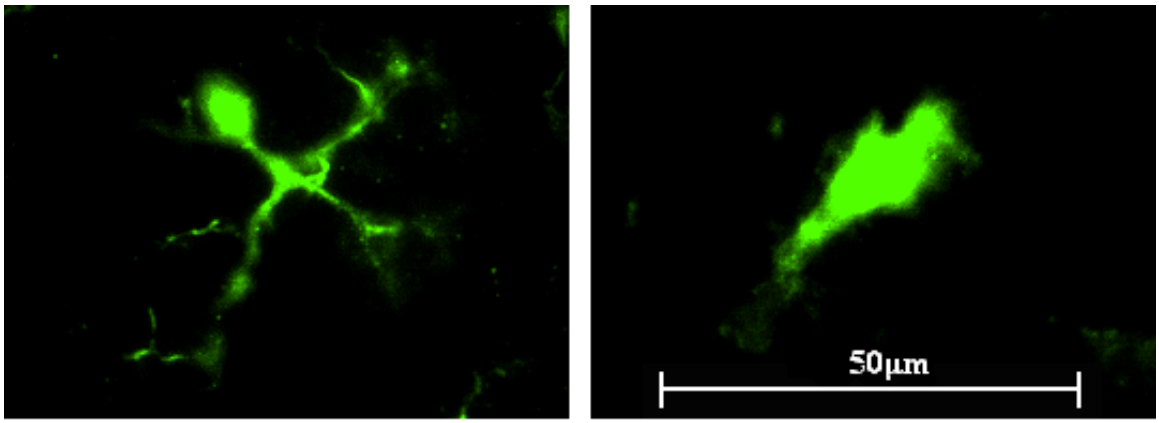

Figure 1. Morphological Changes in Macrophages

Microglia change shape in response to various stimuli. 'Ramified' microglia (left) have small cell bodies and long, thin processes; whereas, so called activated microglia can adopt an 'amoeboid' morphology (right) with rounded cell bodies and sparse processes. Magnification at 40x.

1.1.2. Distinct Macrophage Subsets within the CNS

Macrophages have significant plasticity and are able to respond to a variety of environmental cues by altering their phenotype (Mosser & Edwards, 2008). There are two main types of macrophages present in the CNS: the classically activated (M1) phenotype and alternatively activated (M2) phenotype. M1 macrophages secrete pro-inflammatory factors such as tumour necrosis factor (TNF-α), interleukin 1β (IL-1β), and reactive oxygen species (ROS) (Philips & Robberecht, 2011). The release of pro-inflammatory factors is important for the phagocytic function of macrophages and its antimicrobial activity (Colton, 2009; Henkel et al., 2009). The M1 phenotype is also characterized by high levels of inducible nitric oxide synthase (iNOS) which converts L-arginine to nitric oxide (NO) and citrulline (Varin & Gordon, 2009). Although NO is important for protection from invading organisms, high levels of NO can result in mitochondrial damage, and oxidative and nitrosative damage to cell proteins (Colton, 2009).

M2 macrophages are associated with tissue repair and inhibition of inflammation (Varin & Gordon, 2009). In order to restore normal tissue homeostasis, anti-inflammatory cytokines and neurotrophic factors such as insulin-like growth factor 1 (IGF1) and interleukins 4,10, and 13 (IL-4, IL-10, IL-13) are secreted by M2 macrophages (Henkel et al., 2009; Philips & Robberecht, 2011). The M2 phenotype is characterized by high Arginase I (ArgI) levels which convert L-arginine to ornithine (Rauh et al., 2005). Ornithine is metabolized and produces polyamines and proline, which promote tissue repair, collagen

production, cell proliferation and differentiation (Colton, 2009; Varin & Gordon, 2009). Within the M2 phenotype, three subtypes have been identified based on their gene expression profile and range of functional states (Benoit et al., 2008) (See Figure 2). The M2a phenotype, induced by IL-4 and IL-13, is involved with anti-inflammatory cytokine production and tissue repair (Appel et al., 2009; Colton, 2009). The M2b phenotype is induced by immune complexes and TLRs, which are important for the detection of bacterial, fungal, and viral structures (Colton, 2009). The M2b macrophages have an anti-inflammatory effect through the production of IL-10; however, they also produce modest amounts of pro-inflammatory cytokines (Appel et al., 2009; Rees, 2010). The M2c phenotype, induced by IL-10 or transforming growth factor (TGF-β), promotes immunosuppression and apoptotic cell uptake (Appel et al., 2009; Colton, 2009). The M2c macrophages also have a reduction in the major histocompatibility complex (MHC) class II antigens making them less effective in presenting antigen to T-cells (Colton, 2009). It is important to note that in an *in vivo* setting, macrophages have thousands of different surface receptors and are exposed to various competing stimuli. Therefore, it is likely that macrophages can display a range of overlapping phenotypes depending on the concentration and timing of various signals (Rees, 2010).

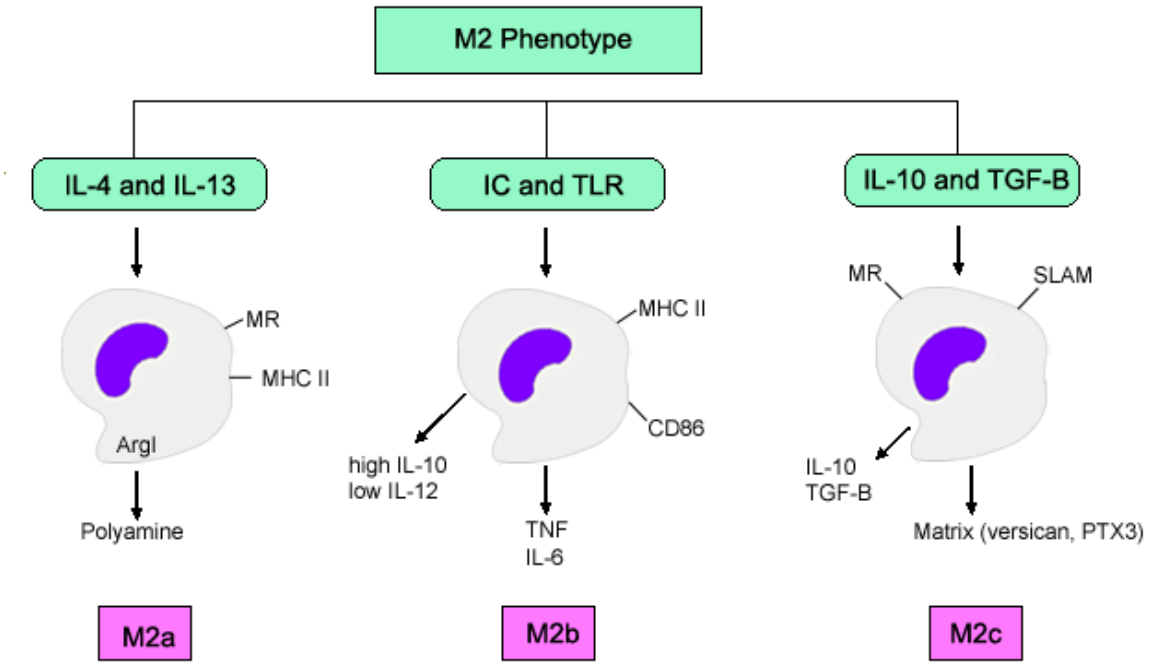

Figure 2. Factors Influencing M2 Macrophages

M2 macrophages can display a wide range of overlapping phenotypes depending on the timing and concentration of various signals. Three distinct subtypes have been identified in vitro based on exposure to specific factors. M2a macrophages (left), induced by IL-4 and IL-13, are involved in the production of anti-inflammatory cytokines. M2b macrophages (centre) are induced by TLRs and immune complexes. They secrete high levels of IL-10 and have both inflammatory and anti-inflammatory effects. M2c macrophages (right), induced by IL-10 and TGF-β, are involved in the down-regulation of immune responses. See text for details. Adapted from Mantovani et al. (2004).

1.1.3. Factors Influencing Macrophage Phenotypes

A number of factors are involved in inducing different macrophage phenotypes. IFN-γ is a cytokine that induces an M1 phenotype characterized by the release of pro-inflammatory factors (Dalton et al., 1993). IFN-γ is secreted by natural killer (NK) cells and T helper cells Type 1 (T_h1) and is involved in the initiation of the “killing” phase of macrophage function (Adams & Hamilton, 1987; Mosser & Edwards, 2008). LPS, a glycolipid that comprises the outer membrane of Gram-negative bacteria, also activates M1 macrophages inducing the release

of pro-inflammatory cytokines and chemokines (Colton, 2009; Bode et al., 2012). The LPS binding protein (LBP) and LPS form a high-affinity complex, which can bind to the TLR4/MD2/CD14 receptor complex on macrophages. This triggers the oligomerization of TLR4, which results in the activation of downstream signals and the release of pro-inflammatory factors (Bode et al., 2012).

IL-4 and IL-13 are produced in cellular and humoral responses to particular pathogens and induce an M2 phenotype (Varin & Gordon, 2009). IL-4 is a cytokine produced by T helper cells Type 2 (T_h2), a subpopulation of $CD4^+$ T cells (Varin & Gordon, 2009). The pathway, induced by IL-4, is dependent upon the expression of IL-4 receptors where IL-4 is believed to mediate the heterodimerisation of the IL-4α chain, which is also a component of the IL-13 receptor (Miloux et al., 1997; Murata et al., 1998). The stimulation of M2 macrophages by IL-4 results in the production of anti-inflammatory cytokines, inhibition of pro-inflammatory cytokine expression, and tissue repair (Varin & Gordon, 2009). Co-cultures treated with IL-4 were shown to promote an M2 phenotype by enhancing IGF-1 secretion and reducing the release of ROS (Zhao et al., 2006). IL-4 also controls L-arginine metabolism by increasing ArgI activity (Gratchev et al., 2001).

The mannose receptor (MR;CD206) is a member of the C-type lectin family which is not expressed on M1 macrophages (Colton, 2009). It is a phagocytic receptor that is important for the activation of the anti-inflammatory signalling pathway and the uptake of a range of mannosylated glycoproteins

(Colton, 2009; Varin & Gordon, 2009). IL-4 induces M2 macrophages to increase MR expression and activity on macrophages (Stein et al., 1992), as well as the expression of the triggering receptor expressed on myeloid cells 2 (TREM-2) (Turnbull et al., 2006). TREM-2 is primarily expressed in natural killer and myeloid cells and is a key regulator of macrophage inflammatory responses in the CNS (Whittaker et al., 2009; Saijo & Glass, 2011).

IL-10 also induces an M2 phenotype and is involved in tissue remodelling and the suppression of immune responses. It is important in the resolution phase of inflammation as it regulates and represses the expression of pro-inflammatory molecules (Ouyang et al., 2011). IL-10 binds to the IL-10 receptor complex consisting of IL-10R1 and IL-10R2 which activates STAT3, a key mediator in the anti-inflammatory response (Bode et al., 2012). IL-10 also increases the expression of MR in M2 macrophages (Mantovani et al., 2004).

1.2. Phosphoinositides

1.2.1. The Role of Phosphoinositides

Phosphoinositides are phospholipid components of cell membranes that consist of a glycerol backbone, two fatty acid tails, and a six-sided inositol ring which can be phosphorylated at the 3, 4, or 5 position (Ooms et al., 2009) (See Figure 3). These molecules are involved in signalling pathways which regulate various cellular processes including cell proliferation, differentiation, apoptosis, and migration (Krystal, 2000; Astle et al., 2007).

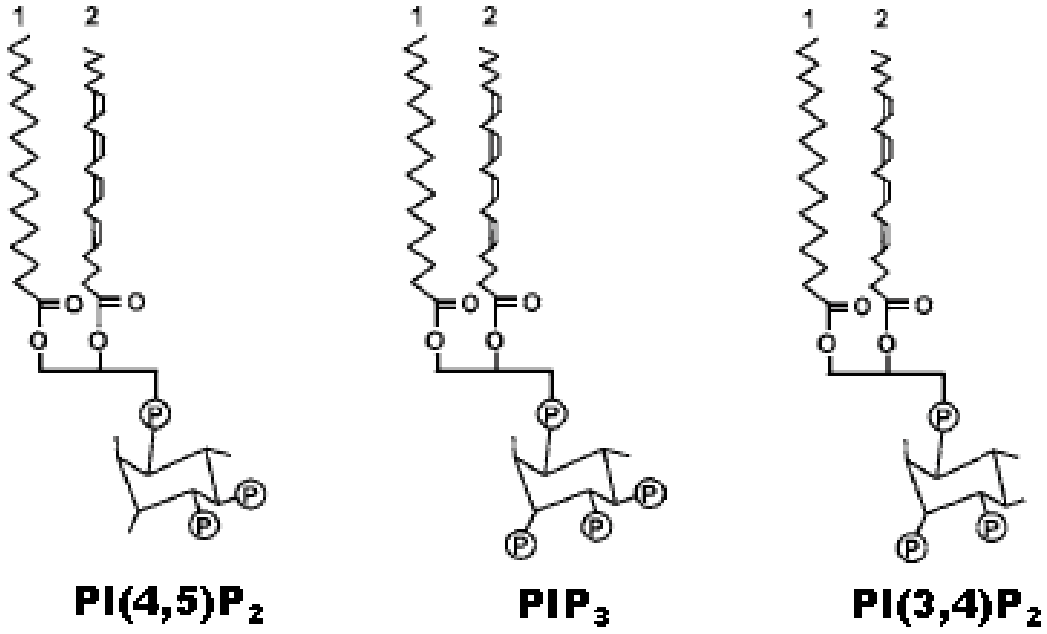

Figure 3. Structure of Phosphoinositides

Phosphoinositides have fatty acid tails allowing them to be anchored to the cell membrane. They also have an inositol ring which can be phosphorylated at the 3, 4, or 5 positions. When phosphorylated, they act as signaling molecules activating various downstream proteins and molecules. Adapted from Hawkins et al. (2006).

Phosphatidylinositol (3,4,5)-triphosphate (PIP_3) is a key second messenger which recruits and activates pleckstrin homology (PH) domain-containing proteins such as the serine/ threonine protein kinase Akt/PKB and the guanine nucleotide exchange factor, Vav (Rauh et al., 2003). Akt/PKB further activates downstream proteins such as mammalian target of rapamycin (mTOR) and plays a role in the activation of macrophages and is involved in promoting proliferation, cell metabolism, and survival (Lee et al., 2011).

1.2.2. The Regulation of PIP_3

PIP_3 is regulated by the addition or removal of phosphate groups in the inositol ring through various enzymes (Hawkins et al., 2006). Phosphoinositide 3-kinase (PI3K) is an enzyme that synthesizes PIP_3 by transferring a phosphate group from ATP onto the substrate $PI(4,5)P_2$. Three classes of PI3K have been identified according to their structure and function: class I, II, and III. Class I PI3Ks are divided into I_A and I_B and are believed to preferentially phosphorylate $PI(4,5)P_2$ to PIP_3. PI3Ks are heterodimers that consist of a catalytic subunit (p110α, p110β, p110δ or p110γ) and a regulatory subunit (p50-55, p85, p101 or p104) (Hawkins et al., 2006). Class I PI3Ks are stimulated through the activation of various cell-surface receptors including those of growth factors, hormones, inflammatory stimuli, and neurotransmitters (Hawkins et al., 2006). This interaction is regulated downstream by the activation of protein tyrosine kinases and heterotrimeric G-proteins (Hawkins et al., 2006).

There are two major enzymes involved in the de-phosphorylation, and thus the regulation, of PIP_3: phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Src homology 2 domain-containing inositol-5' phosphatase (SHIP). PTEN hydrolyzes the 3 position phosphate from PIP_3 to form $PI(4,5)P_2$ and SHIP hydrolyzes the 5-position phosphate from PIP_3 to form $PI(3,4)P_2$ (Ooms et al., 2009; See Figure 4). It is believed that both PIP_3 and $PI(3,4)P_2$ are involved in cellular signalling pathways and $PI(3,4)P_2$ may also be

involved in the activation of Akt/PKB and other effectors (Hawkins et al., 2006; Ooms et al., 2009).

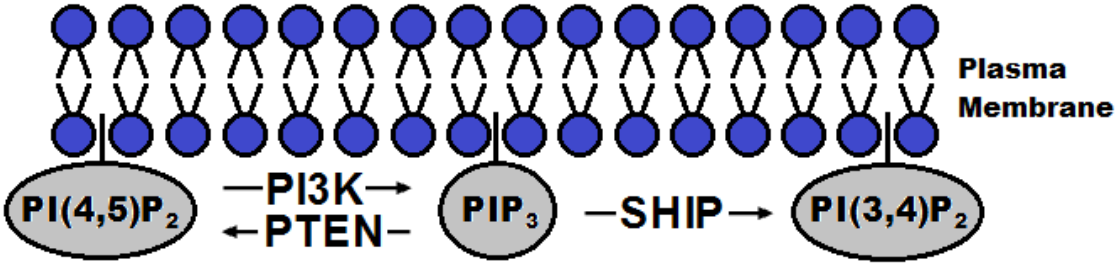

Figure 4. Regulation of PIP_3

The second messenger PIP_3 can be de-phosphorylated by PTEN to form $PI(4,5)P_2$ or by SHIP1 to form $PI(3,4)P_2$. Adapted from Rauh et al. (2003).

1.3. Src Homology 2 Domain-Containing Inositol-5' Phosphatase (SHIP1)

1.3.1. The Structure and Function of SHIP1

There are three types of SHIP proteins including SHIP1, SHIP2, and stem-cell restricted SHIP (sSHIP). SHIP1 is 145kDa protein which suppresses the activation, proliferation, and survival of hematopoietic cells (Sly et al., 2007). SHIP1 also has two isoforms with masses of 135kDa and 110kDa, which are produced through proteolytic degradation from the C-terminal end (Rohrschneider et al., 2000; March & Ravichandran, 2002). SHIP2 is a 142kDa homolog of SHIP1 and is expressed in the brain, skeletal muscle, heart, pancreas, placenta, and hematopoietic cells (Rohrschneider et al., 2000; Ooms et al., 2009). sSHIP is a 104kDa protein expressed in embryonic stem cells and

co-expressed with SHIP1 in hematopoietic stem cells which later disappears with differentiation (Sly et al., 2007).

SHIP1 has several functional domains which serve as binding sites for various signalling molecules and proteins (See Figure 5). The N-terminus contains a Src homology 2 (SH2) domain which preferentially binds to proteins with specific amino acid sequences which code for immunoreceptor tyrosine based inhibition motifs (ITIMs) or immunoreceptor tyrosine based activation motifs (ITAMs). The SH2 domain also binds to tyrosine phosphorylated forms of several proteins including Shc, SHP-2, and Doks (Damen et al., 1996; Liu et al., 1997; Sly et al., 2007; Leung et al., 2008). The central portion of SHIP1 contains an inositol 5'-phosphatase domain which de-phosphorylates the inositol ring of PIP_3 at the 5-position. The C-terminus contains two NPXY (asparagine, proline, any amino acid, tyrosine) motifs and proline-rich sequences. When tyrosine phosphorylated, the NPXY sequences provide binding sites for proteins with phosphotyrosine binding (PTB) and SH2 domains (Liu et al., 1997; Damen et al., 2001). The proline-rich sequences are docking sites for proteins containing an SH3 domain (March & Ravichandran, 2002). SHIP1 may also have a C2 domain adjacent to the phosphatase domain at the C-terminus which acts as an allosteric site to enhance phosphatase activity (Ong et al., 2007).

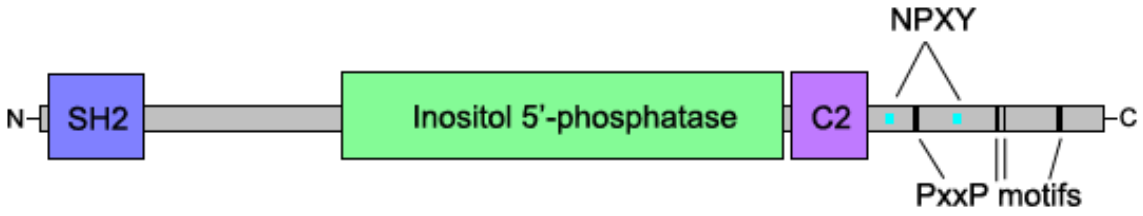

Figure 5. SHIP1 Structure

SHIP1 has several functional domains including the SH2 domain, inositol 5' phosphatase domain, NPXY sequences, and proline rich motifs. It has also been suggested that SHIP1 has a C2 domain which acts as an allosteric site. Various adaptor proteins and signaling molecules are able to bind to these sites. Adapted from Rohrschneider et al. (2000).

1.3.2. SHIP1 Experimental Models

The role of SHIP1 has been studied extensively in various immune cells including T-cells, B-cells, mast cells and macrophages (Leung et al., 2008). Various SHIP1-deficient cell lines have been created in order to understand the mechanism of SHIP1 action. In 1996, several independent groups created SHIP1 knockout ($SHIP^{-/-}$) mice (Krystal et al., 1999). Although viable and fertile, the $SHIP^{-/-}$ mice had increased numbers of granulocytes and macrophages, progressive splenomegaly, extramedullary haemopoiesis, osteoporosis, massive myeloid infiltration of the lungs, and a shortened lifespan (Rauh et al., 2004; Leung et al., 2008). Since SHIP1 is present in all hematopoietic cells, understanding the role of SHIP1 in the various immune cells was limited when SHIP1 was knocked out entirely in $SHIP^{-/-}$ mice. $SHIP^{-/-}$ mice were therefore generated by deleting SHIP1 in specific immune cell types using the Cre-Lox system (Leung et al., 2008). When the SHIP1 deletion was restricted to the macrophage-granulocyte lineage, the severity of the myeloid hyperproliferation

was reduced compared to that in the SHIP$^{-/-}$ mice suggesting that other cell types may contribute to the SHIP$^{-/-}$ phenotype. Also, since the SHIP1 deletion occurred only in mature macrophages, myeloid precursors expressing SHIP1 may have affected the response (Leung et al., 2008). The use of these various experimental models has expanded the knowledge about the role of SHIP1 in macrophages.

1.3.3. Mechanism of SHIP1 Activity

SHIP1 is activated by various stimuli including cytokines, chemokines, growth factors, and oxidative stress (Rauh et al., 2004). Studies have shown that the phosphatase activity of SHIP1 is not altered by extracellular stimulation by cytokines such as IL-3, tyrosine phosphorylation, or the binding of peptides to the SH2 domain (Damen et al., 1996; Phee et al., 2000). Upon stimulation, SHIP1 moves to the plasma membrane, decreasing PIP_3 levels suggesting that SHIP1 translocates to the plasma membrane where PIP_3 is synthesized and where SHIP1 mobility is restricted (Phee et al., 2000).

Although it is believed that the phosphatase activity of SHIP1 occurs at sites of PIP_3 synthesis, the exact mechanism of how SHIP1 translocates to the plasma membrane is not clearly understood. Studies have suggested that SHIP1 translocation to the plasma membrane involves direct association with receptor chains via its SH2 domain or indirect association with adapter proteins (e.g. Shc, Grb2) and scaffold proteins such as the Grb2-associated binding protein (Gab) (Kerr, 2011). Studies have shown that the binding of the adaptor protein, Shc, to

SHIP1 requires both the SH2 and the NPXpY domains of SHIP1 (Liu et al., 1997). Hematopoietic cell lines transfected with SHIP1 lacking a functional SH2 domain are unable to bind Shc or become tyrosine phosphorylated when stimulated by IL-3 (Liu et al., 1997). Damen et al. (2001) also found that $SHIP^{-/-}$ bone marrow derived mast cells infected with a C-terminally truncated SHIP1 mutant were unable to convert PIP_3 to $PI(3,4)P_2$ when stimulated, unlike WT-SHIP1. They also found that this mutant SHIP1 had significantly lower tyrosine phosphorylation levels compared to WT-SHIP1. This evidence suggests that the SH2 domain, NPXY domain, and the proline rich C-terminus are required for tyrosine phosphorylation and the translocation of SHIP1 to the plasma membrane.

Although SHIP1 has been primarily known for its enzymatic function, it is also believed to have a "masking function" where it can block recruitment of key signalling molecules (Kerr, 2011). TREM-2 associates with the adaptor protein DNAX-activating protein of 12 kD (DAP12) and is involved in the downregulation of TLR-induced production of inflammatory cytokines in vivo (Peng et al., 2010). The cross-linking of TREM-2 results in the binding of SHIP1 via its SH2 domain to DAP12, inhibiting the activation of TREM-2 and DAP12 signalling. When TREM-2 is cross-linked in $SHIP^{-/-}$ macrophages, there is an increase in the recruitment of PI3K to DAP12, suggesting that SHIP1 negatively regulates PI3K signalling by inhibiting the association between DAP12 and PI3K (Peng et al., 2010). SHIP1 has also been shown to negatively regulate the tyrosine

phosphatase SHP-1 recruitment to 2B4, a member of the signal lymphocyte activation molecule (SLAM) receptors, in natural killer cells (Wahle et al., 2007). This evidence suggests that SHIP1 can alter downstream signalling through its "masking function".

1.4. SHIP1 Involvement in Macrophages

1.4.1. Phagocytosis and Superoxide Production

SHIP1 plays a role in differentiation, phagocytosis, apoptosis, and production of superoxide and pro-inflammatory cytokines in macrophages. Phagocytosis and superoxide generation can occur through the PI3K pathway (Maresco et al., 1999). Fcγ receptor (FcγR)IIa and FcγRI are the major FcγRs found on monocytes which contain ITAMs that are involved in the promotion of phagocytosis (Maresco et al., 1999; Nakamura et al., 2002). Activation of FcγR results in the recruitment of Syc-family kinases which activate various downstream targets including PI3K (Okun et al., 2010). SHIP1$^{-/-}$ macrophages have been found to have an increase in the rate and extent of FcγR-mediated phagocytosis; whereas, over-expression of WT-SHIP1 resulted in the inhibition of phagocytosis (Cox et al., 2000). Phagocytosis is also significantly enhanced in monocytes only expressing FcγRIIa receptors with a dominant-negative form of SHIP1 (Nakamura et al., 2002).

SHIP1 is activated by binding directly or indirectly to the ITAMs of FcγRs. Cross-linking of these receptors induces tyrosine phosphorylation of SHIP1 and

association with the adaptor protein, Shc (Maresco et al., 1999). Phosphorylated SHIP1 is able to associate with the phospho-ITAM of the FcγRIIa-associating γ-chain, or bind indirectly to FcγRIIa through Shc, which is involved in SHIP1 tyrosine phosphorylation (Maresco et al., 1999; Nakamura et al., 2002; Tridandapani et al., 2002). This suggests that SHIP1 is a negative regulator of phagocytosis through its association with ITAM-containing phagocytic receptors (Maresco et al., 1999; Nakamura et al., 2002). Although an increase in the FcγR has been observed in spinal cord tissue of mice that develop similar features to ALS patients, the association between FcYR expression and disease progression is unknown (Alexianu et al., 2001; See below for details).

Phagocytosis results in the production of superoxide and inflammatory cytokines (Ganesan et al., 2006). Superoxide production is catalyzed by NADPH oxidase and requires the GTPase Rac, which is essential for NADPH oxidase activation (Ganesan et al., 2006). When $SHIP^{+/+}$ and $SHIP^{-/-}$ bone-marrow derived macrophages (BMDM) are stimulated resulting in FcγR clustering, $SHIP^{-/-}$ BMDM produce a significantly higher amount of superoxide, IL-1β, and IL-6 compared to $SHIP^{+/+}$ BMDM at later time points of phagocytosis (Ganesan et al., 2006). Nuclear factor (NF)-κB is a transcription factor activated downstream of the PIP_3/PI3K pathway (Lee et al., 2011). NF-κB activation is required for the induction of various inflammatory cytokine genes during phagocytosis (Tridandapani et al., 2002). NF-κB-dependent gene transcription is present after FcγR clustering; however over-expression of WT-SHIP down-regulates gene

transcription suggesting that SHIP1 negatively regulates NF-κB-dependent gene transcription (Tridandapani, 2002). $SHIP^{-/-}$ BMDMs also have significantly higher Ras activity compared to their WT counterparts suggesting that SHIP1 negatively regulates Ras activity (Ganesan et al., 2006). During earlier stages of phagocytosis, however, $SHIP^{-/-}$ macrophages release lower amounts of reactive oxygen intermediates (Kamen et al., 2008). This suggests that SHIP1 is both a positive and negative regulator of superoxide and pro-inflammatory cytokine production depending on the stage of phagocytosis (Ganesan et al., 2006; Kamen et al., 2008).

1.4.2. Apoptosis

SHIP1 is also involved in the apoptosis of hematopoietic cells. Hematopoietic murine cell lines over-expressing WT-SHIP1 had a reduction in viability compared to their control counterparts (Liu et al., 1997). Also, SHIP1 expressing an inactive SH2 domain had reduced apoptosis and an increase in viability (Liu et al., 1997). Myeloid cells from $SHIP^{-/-}$ mice are resistant to apoptotic stimuli and exhibit enhanced resistance with the presence of growth factors (Liu et al., 1999). IL-3 stimulation resulted in enhanced and prolonged activation and phosphorylation in Akt/PKB and downstream targets in $SHIP^{-/-}$ myeloid cells, and increased PIP_3 levels compared to $SHIP^{+/+}$ cells (Liu et al., 1999). This suggests that SHIP1 is a negative regulator of Akt/PKB activation and myeloid cell survival.

1.4.3. M2 Macrophage Skewing

SHIP1 is also involved in repressing the activation of M2 macrophages (Rauh et al., 2005). LPS and IFN-γ stimulation of $SHIP^{-/-}$ peritoneal macrophages resulted in a decrease in NO production and a constitutive increase in ArgI activity compared to $SHIP^{+/+}$ macrophages which increased NO release in a dose dependent manner (Rauh et al., 2005). These observations demonstrate that SHIP1 is responsible for increasing NO production and decreasing ArgI activity indicating the involvement of SHIP1 in M1 macrophage skewing. The use of ArgI inhibitors in $SHIP^{-/-}$ macrophages also significantly increased LPS induced NO production, but not in $SHIP^{+/+}$ macrophages, suggesting that these constitutively high ArgI levels are responsible for the low levels of NO production. The molecular pathways involved in this effect are unknown.

It is important to note that in vitro macrophage colony-stimulating factor (M-CSF) derived macrophages from $SHIP^{-/-}$ mice do not express M2 markers or reduce NO levels (Rauh et al., 2005; Kuroda et al., 2009). This suggests that extracellular factors are also required for M2 skewing. Researchers have found that both IL-4, and to a lesser extent IL-13, induce ArgI activity in both $SHIP^{+/+}$ and $SHIP^{-/-}$ progenitors and mature macrophages suggesting IL-4 and IL-13 involvement in M2 skewing (Rauh et al., 2005; Weisser et al., 2011). Studies have shown that ArgI activity in $SHIP^{-/-}$ macrophages significantly increases, whereas SHIP1 activity in $SHIP^{+/+}$ macrophages decreases in response to IL-4 (Weisser et al., 2011). Modifications in SHIP1 expression also alter ArgI

expression and thus affect M2 skewing. An over-expression of SHIP1 leads to a significant decrease in IL-4 induced ArgI activity (Weisser et al., 2011). LPS induced macrophages had increased SHIP1 protein levels compared to macrophages treated with IL-4 (Weisser et al., 2011). A correlation between decreased SHIP1 protein levels and increased M2 markers in response to IL-4 treatment was also found in $SHIP^{+/+}$ macrophages suggesting that SHIP1 is a negative regulator of ArgI activity and IL-4 induced M2 skewing (Weisser et al., 2011).

The PI3K pathway is also believed to be involved in M2 skewing. In the presence of PI3K inhibitors, $SHIP^{+/+}$ peritoneal macrophages had reduced ArgI and SHIP1 levels in IL-4 induced macrophages (Weisser et al., 2011). $SHIP^{+/+}$ macrophages were also transfected with constitutively active PI3K which had a dose-dependent increase in ArgI levels and activity without LPS stimulation (Rauh et al., 2005). LPS-induced $SHIP^{-/-}$ macrophages also resulted in an enhanced phosphorylation of Akt (Fang et al., 2004; Rauh et al., 2005). These results suggest that PIP_3 is a positive regulator of ArgI levels and the M2 phenotype in $SHIP^{-/-}$ macrophages is associated with the activation of the PI3K/PIP_3/Akt pathway.

1.5. Amyotrophic Lateral Sclerosis (ALS)

1.5.1. Pathogenesis of ALS

ALS is a progressive neurodegenerative disease characterized by the degeneration of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord (Ferraiuolo et al., 2011). This leads to atrophy of skeletal muscles, paralysis, and ultimately death from respiratory failure (Gowing et al., 2008). The incidence of ALS is approximately 2 per 100,000 individuals and is relatively uniform worldwide (Ferraiuolo et al., 2011). The mean age of disease onset is between 55-60 years, and the average survival after symptom onset is between 3-5 years (Ferraiuolo et al., 2011; Wijesekera & Leigh, 2009). Familial ALS (fALS), refers to an inherited from of ALS, which is generally transmitted as an autosomal dominant disease and accounts for 5-10% of ALS patients. Sporadic ALS (sALS) has no clear familial transmission and accounts for 90-95% of ALS patients (Williams, 1991). Although there is currently no known causal factors associated with sALS, it is hypothesized that complex genetic-environmental factors are involved (Wijesekera & Leigh, 2009).

Although the exact molecular and cellular pathways involved in ALS remain unclear, there are multiple mechanisms that are believed to be involved. Oxidative stress, glutamate induced excitotoxity, mitochondrial dysfunction, impaired axonal transport, neuroinflammation, and protein aggregates are also believed to play a role in the pathogenesis of ALS (Ferraiuolo et al., 2011). Many

genetic subtypes have been discovered and have assisted in providing clues about the pathogenic mechanisms of ALS.

One mutation found in fALS patients is in the Cu^{2+}/Zn^{2+} superoxide dismutase (mSOD1) which occurs in 20% of fALS patients (Rosen et al., 1993). This ubiquitously expressed enzyme is involved in the conversion of superoxide radicals into hydrogen peroxide, which is then converted to H_2O by glutathione peroxidase in the CNS (Reaume et al., 1996; Dringen, 2000). Studies have shown that the absence of SOD1 does not produce neuronal loss or influence life span. Since no clinical or pathological features of ALS are observed in mice lacking SOD1, it is believed that the ALS pathology is due to a gain of function of mSOD1 which may be due to the misfolding of the protein (Reaume et al., 1996; Boillée et al., 2006).

The discovery of these SOD1 mutations has lead to the development of mSOD1 transgenic animal models which have similar clinical and pathological features to fALS patients (Wong et al., 1995). Although mSOD1 mutations are not found in sALS patients, or the majority of fALS patients, these forms of ALS are both clinically and pathologically similar (Wong et al., 1995). This has allowed researchers to gain a more thorough understanding of the pathogenesis of ALS (Rosen et al., 1993; Boillée et al., 2006).

1.5.2. The Role of Microglia in ALS

Microgliosis is a common hallmark in ALS patients and in mSOD1 mouse models (Ransohoff, 2010). Microgliosis occurs in regions of the brain, along the corticospinal tract, and in the ventral horn of the spinal cord (Phillips & Robberecht, 2011). Various studies have examined the role of microglia in ALS through cell-type specific expression of mSOD1 in microglia and neurons (Pramatarova et al., 2001; Clement et al., 2003; Beers et al., 2006). Researchers have shown that mSOD1 expressed in only motor neurons or microglia alters the onset and progression of neurodegeneration suggesting the involvement of microglia in ALS (Pramatarova et al., 2001; Beers et al., 2006). In transgenic mouse models, bone marrow transplants from mSOD1 mouse donors into PU.1$^{-/-}$ mice (which prevents mice from producing myeloid or lymphoid cells) do not induce an ALS-like disease (Beers et al., 2006). mSOD1 expression in neurons with a high proportion of WT neighbouring non-neuronal cells results in the reduction in neurodegeneration (Clement et al., 2003). This evidence suggests that ALS is a non-cell autonomous disease that requires the involvement of both motor neurons and non-neuronal cells (Henkel et al., 2009).

Studies examining the temporal changes in the CNS of mSOD1 transgenic mice have shown that the number and activation of microglia continue to increase until the end stage of the disease (Alexianu et al., 2001; Gowing et al., 2008; Yang et al., 2011). Microgliosis has also been shown to occur prior to motor neuron loss as well as before clinical disease onset (Hall et al., 1998;

Alexianu et al., 2001). In order to further understand the role of microglia in ALS, researchers reduced mSOD1 expression in microglia of mice by 25% (Boillée et al., 2006b). The Cre-Lox system was used to express Cre recombinase selectively in microglia using the promoter for CD11b in LoxSOD1^{G37R} mice to regulate the deletion of SOD1 in microglia. This resulted in a significant increase in survival mainly due to a slowing in disease progression (Boillée et al., 2006b). Also, bone marrow transplants in mSOD/PU.1$^{-/-}$ mice resulting in donor-derived microglia, also slowed down the progression of the disease suggesting that microgliosis is not involved in disease onset, but in the progression of the disease (Beers et al., 2006).

The role of microglia in ALS; however, is complex. A study conducted by Gowing et al. (2008) demonstrated that the ablation of 50% of microglia in an mSOD1 transgenic mouse model did not affect neuronal loss. Yang et al. (2011) also found that the number of activated microglia was only significant at the end stage of ALS in a transgenic mouse model. These discrepancies may be due to different experimental designs as well as differences in the roles and numbers of activated microglial subtypes within the CNS. Although ALS has been established as non-cell autonomous, the specific role of microglia and the association between microglial activation and the rate of disease progression in ALS has yet to be defined. In particular, the role of SHIP1 in microglial activation in ALS must be addressed in order to gain a more accurate and comprehensive understanding of how microgliosis influences the pathogenesis of ALS.

2. Objectives and Hypotheses

2.1 Justification of Research

SHIP1 is a negative regulator of the PI3K pathway and suppresses the activation, proliferation, and survival of hematopoietic cells (Sly et al., 2007). Microglia/ macrophages are of hematopoietic origin and microgliosis is a common hallmark in ALS patients and in mSOD1 mouse models (Ransohoff, 2010). Characterizing and quantifying SHIP1 in microglia/ macrophages in a murine mSOD1 model will give us a better understanding of the role of microglia/ macrophages and SHIP1 in the pathogenesis of ALS. By further understanding the role of SHIP1 in the progression of ALS, alterations in SHIP1 expression in ALS patients could potentially be used to control the number and phenotype of microglia/ macrophages as a future therapy for ALS patients.

2.2 Objectives and Hypotheses

1) Objective: To determine whether SHIP1 is present in microglia/ macrophages in the CNS.

Hypothesis: SHIP1 is present in hematopoietic cells. Since microglia/ macrophages are of hematopoietic origin, SHIP1 will be expressed in microglia/ macrophages in the spinal cord.

2) Objective: To determine whether there is a difference in SHIP1 immunoreactivity between mSOD1 mice and controls and whether SHIP1 positive (SHIP1+) staining in microglia/ macrophages is altered throughout the progression of the murine mSOD1 model.

Hypothesis: An increase in the number of microglia is a hallmark of ALS and therefore SHIP1+ microglia/ macrophages will be present in mSOD1 mice, and will increase throughout disease progression as a potential mechanism to control the growth and survival of microglia/ macrophages.

3. Methods

3.1. Mouse Model

The transgenic mouse line B6SJL-Tg(SOD1*G93A), and age- and gender-matched WT control mice were used for this study. The transgenic mouse line expresses endogenous WT mouse SOD1 as well as over-expresses a mutant form of human SOD1 (mSOD1) by approximately 25-fold which has a glycine to alanine substitution (Gurney et al., 1994). The mSOD1 mice develop motor neuron degeneration which results in muscle atrophy and eventually paralysis in one or more limbs. The mean survival of mSOD1 mice is 128.9±9.1 days (Wooley et al., 2005). Although there are differences between humans and mice, both ALS patients and mSOD1 mice display similar pathological features such as motor neuron loss and microgliosis (Wong et al., 1995). The animals received food and water *ad libitum* and all protocols were approved by the SFU review committees and in accordance with the guidelines of the SFU Animal Care Committee and the Canadian Council for Animal Care.

3.2. Genotyping

3.2.1. DNA Extraction

Ear tissue punches were taken from each mouse in order to determine whether a mouse had the mSOD1 mutation. The samples were placed in 1.5mL microcentrifuge tubes filled with 150µL of a Chelex solution consisting of 5% Chelex in Tris-EDTA buffer (5% w/v; 125µL), Ribonuclease (12.5µL), and Proteinase K (12.5µL). The tubes were then placed in a 55°C water bath for 15 minutes, vortexed, and then placed back into the bath for another 15 minutes. The tubes were then incubated in a 100°C water bath for 8 minutes and centrifuged for 5 minutes at 12,000 RPM to complete the DNA extraction.

3.2.2. Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA in order to differentiate between mice with and without the mSOD1 gene. 1µL of the extracted DNA from each mouse was added to a 24µL solution consisting of distilled H_2O (17.2µL), 2 primers (0.5µL of each), $MgCl_2$ (0.5µL), dNTPs (2.5 µL), 10x PCR buffer (2.5 µL), and Taq polymerase (0.3µL) in PCR tubes. The primers included mSOD1 (sense)- CAT CAG CCC TAA TCC ATC TGA and mSOD2 (antisense)- CGC GAC TAA CAA TCA AAG TGA. 1µL of DNA from a confirmed mSOD1 mouse was used as a positive control and 1µL of H_2O was added to the 24µL solution as a negative control. The tubes were then placed in the PCR machine (Techne TC-3000). The cycling protocol was as follows: a 5 minute cycle at 95°C; 30 cycles of 30

seconds each at 94℃, 56℃, and 72℃; a 10 minute cycle at 72℃. The samples then remained at 4℃.

3.2.3. Gel Electrophoresis

The presence or absence of the mSOD1 gene was determined by running a 1% agarose gel electrophoresis. The gel was created by heating a mixture of distilled H_2O (90mL), 10x Tris-Borate EDTA (10mL), and Agarose (1g) at 250℃ for approximately 8 minutes or until solution was clear. Ethidium Bromide (7.5μL) was then added to the mixture. The liquid mixture was poured into a gel plate and set for 30 minutes. The agarose gel was covered with a running buffer consisting of distilled H_2O (712.5mL) and Tris-Borate EDTA (37.5mL). A mixture of 10μL of the PCR product and approximately 3μL of blue dye were loaded into the gel lanes. The gel was then run at a constant voltage of 90V for 30 minutes. It was then placed under a UV light to determine the presence (fluorescent band) or absence (no band) of the gene coding for mSOD1.

3.3. Tissue Processing

Mice were sacrificed at one of the three time points: 11 weeks (asymptomatic), 15 weeks (symptomatic) or advanced stage (18 weeks). Mice were defined as symptomatic when they were unable to splay their hind limbs outwards when lifted by their tails. Mice were defined as advanced stage when they exhibited dragging hind limbs or were unable to right themselves after being placed on their sides (Solomon et al., 2006). There were 3 mSOD1 mice in each

category and each mouse was paired with an age- and gender-matched control. Mice were sacrificed using a blend of CO_2 and O_2 and were immediately perfused transcardially with 30mL of 1% phosphate buffered saline (PBS; pH=7.4) and then 30mL of 4% paraformaldehyde (PFA; pH=7.0). The brain and spinal cord were carefully removed and fixed overnight in 4% PFA at 4℃. They were then dissected from the skull and vertebrae and placed in a sucrose solution (20% sucrose w/v in 1x PBS) overnight for cryoprotection.

3.4. Immunohistochemistry (IHC)

3.4.1. Cryosectioning

The spinal cord was transversely cut into three sections: cervical, thoracic, and lumbar, and encased in TissueTek O.C.T. medium in order to preserve it. The spinal cord and brain were stored at -80℃ until further analysis. The lumbar cord was then sliced transversely into 30µm sections using the Leica CM1900 UV Cryostat. The block temperature and chamber temperature were set between -11℃ to -13℃, and -14℃ to -16℃, respectively. Every 5^{th} lumbar section was collected, stored and suspended in 1.5mL microcentrifuge tubes of De Olmos at -20℃.

3.4.2. Immunofluorescense

The lumbar sections were suspended in a 0.3% Triton-X solution with PBS (PBST). They were then incubated in six-well cell culture plates for 10 minutes in an EDTA-Antigen retrieval buffer at 100℃, which uncovers any hidden antigenic

sites. The sections were then incubated in a blocking solution (25% NDS + 3% BSA in PBST) at room temperature (RT) for 1 hour to reduce background and unspecific staining. The sections were then incubated in primary antibody overnight at 4°C and then in secondary antibody for 2 hours at RT.

In order to detect SHIP1+ cells, antibody to the C-terminus of SHIP1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) was used and microglia/macrophages were detected using an antibody to the ionized Ca^{2+}-binding adapter molecule 1 (Iba1; 1:1000; Wako, Richmond, VA), which is specific to microglia and macrophages. The secondary antibodies used for SHIP1 and the microglia/ macrophages were either the Cy3 conjugate or Alexafluor568-conjugated donkey anti-goat IgG (1:1000; Molecular Probes, Eugene, OR) and Alexafluor488-conjugate goat anti-rabbit IgG (1:800; Molecular Probes), respectively. The stained sections were slide mounted and air dried. They were then covered with Vectashield mounting medium, which contained the nuclear dye, DAPI, and were then coverslipped. Negative controls were also created by only incubating sections in 2° antibodies to ensure that no unspecific labelling was present. Previous experiments conducted in our lab by Dr. Coral-Ann Lewis demonstrated that WT BMDMs stimulated with LPS expressed SHIP1 whereas $SHIP^{-/-}$ BMDMs did not have any SHIP1 labelling suggesting that the SHIP1 antibody is specific for SHIP1.

3.5. Image Analysis

The lumbar spinal cord sections were analyzed using a Leica DM4000B epifluorescence microscope. Images were captured with a Leica DFC 350FX camera and Leica Application Suite software. The ventral horn of five sections from each mSOD1 and control mouse were analyzed by counting the number of microglia/ macrophages and the number co-labelled with SHIP1 at a magnification of 10x. The ventral horn was defined by a 90° angle extending laterally from the central canal and vertically through the ventral median fissure to the outermost point of each section (See Figure 6). The cells were manually counted in both the grey and white matter. Microglia/ macrophages were identified through Iba1 labelling in the spinal cord. In order to ensure proper identification of cells, microglia/ macrophages were identified based on their morphological appearance as defined by Vallières and Sawchenko (2003). The nuclear dye, DAPI, was also used to ensure proper identification of microglia/ macrophages. Cells that were co-labelled with both Iba1 and SHIP1 antibodies were deemed as SHIP1+ cells.

3.6. Statistical Analysis

The average number of microglia/ macrophages and SHIP1+ cells were evaluated in the grey and white matter of the ventral horn. The average number of cells in each group was determined by counting 5 sections per mouse. Earlier work has shown that 5 sections that are separated by at least 150μm

demonstrate reliable cell counts (Solomon et al., 2006). Paired T-tests were used in order to determine whether there was a difference in SHIP1 and macrophage/ microglial expression between control and mSOD1 mice at the three time points. A one-way ANOVA test, followed by a Tukey Post Hoc Test was used to determine whether there was a significant difference in SHIP1 expression in mSOD1 mice at the 3 different time periods. Significance was defined as a P-value of less than 0.05. SPSS Statistics 19 Software was used for statistical analysis.

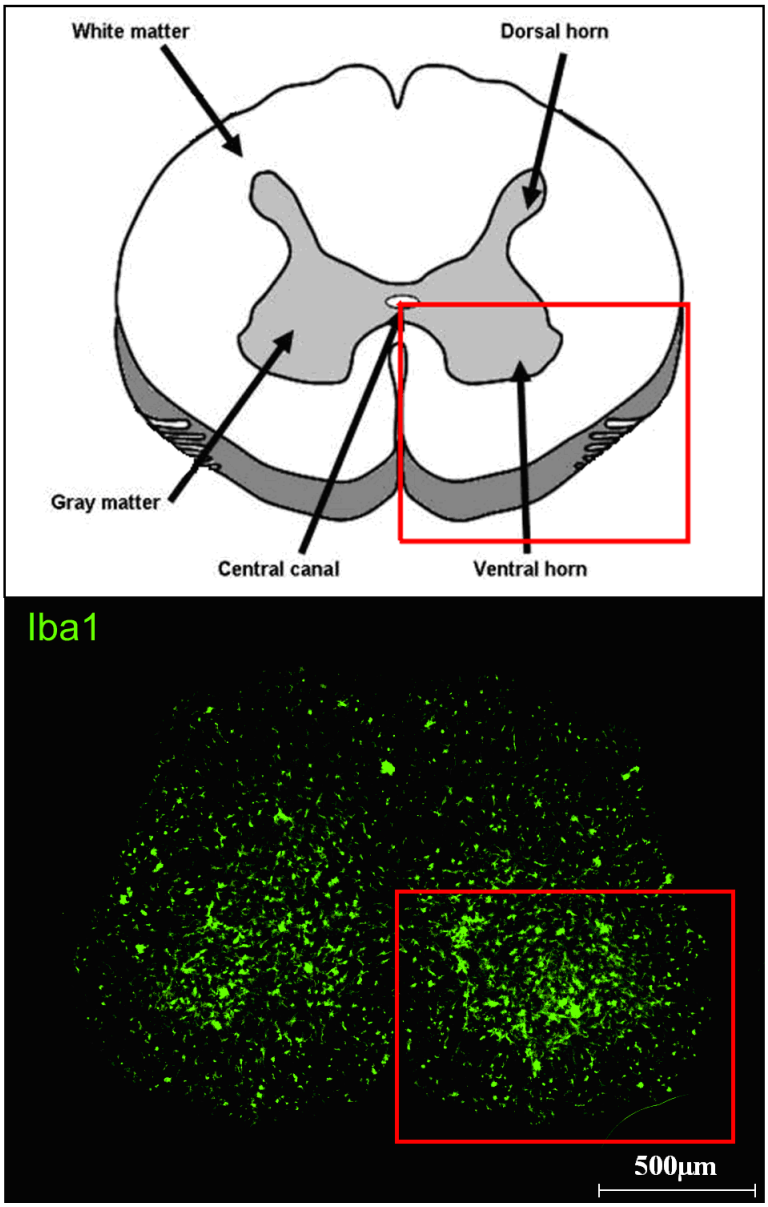

Figure 6. Defining the Ventral Quadrant

The ventral quadrant is defined as extending a line laterally through the central canal and vertically through the ventral median fissure extending to the outermost area of each section (red outline). The bottom image is labeled with the Iba-1 antibody which is specific to microglia and macrophages.

4. Results

4.1. mSOD1 Microglia/ Macrophage Immunoreactivity

In order to quantify microglia/ macrophage numbers during disease progression, the ventral horns of lumbar spinal cords were examined from mSOD1 mice at three different time points: 11 weeks (n=3), 15 weeks (n=3), and 18 weeks (n=3). The sections were labelled with anti-Iba1 antibody and stained with the nuclear dye DAPI to ensure proper microglial/ macrophage identification. The sections were analyzed under an epifluorescence microscope at a magnification of 10x. There was a significant increase in the number of cells in mSOD1 mice compared to the WT control mice at all three time points ($p<0.05$) (See Table 1). The number of microglia/ macrophages also significantly increased in mSOD1 mice throughout disease progression ($p<0.05$) (See Figure 7). There was no change in microglia/ macrophages numbers with age in control mice over this age range.

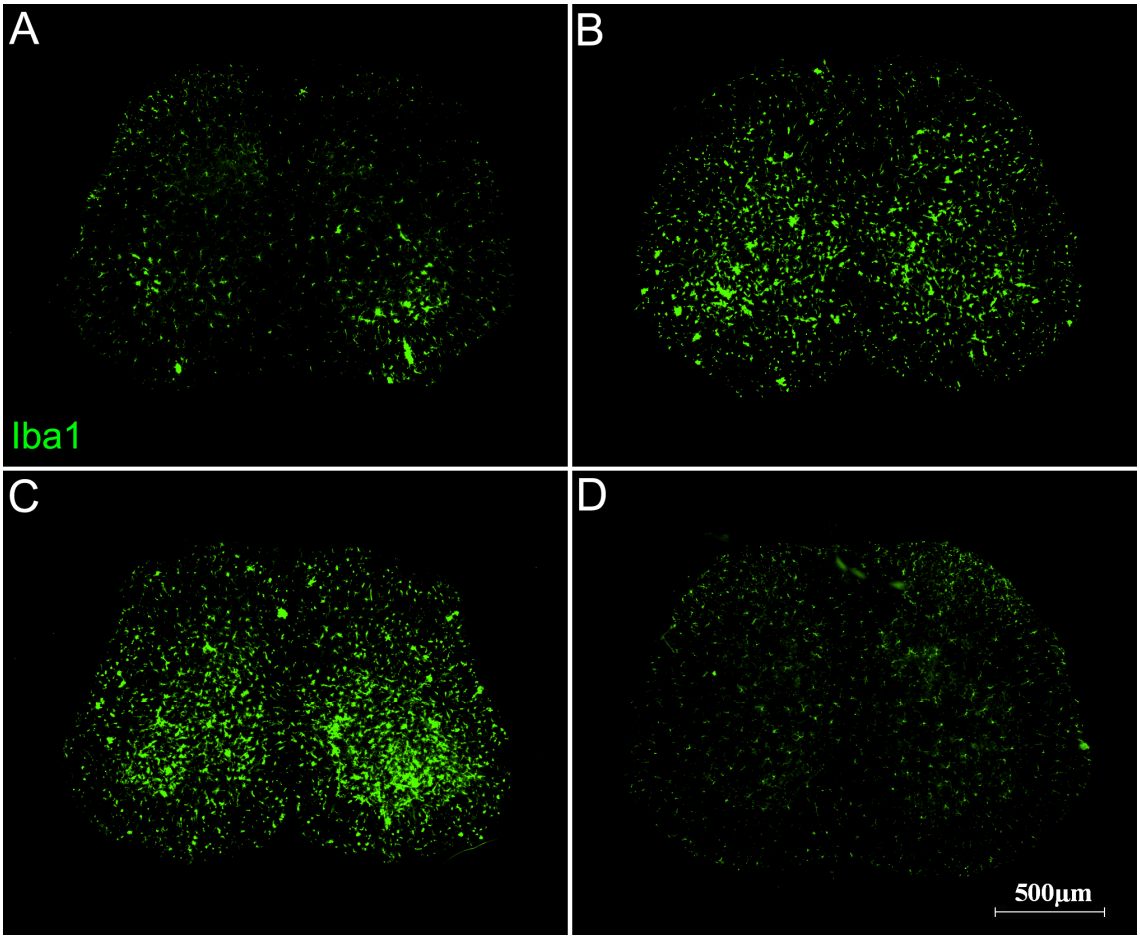

Figure 7. Characterization of Microglia and Macrophages in mSOD1 Mice

Macrophage and microglial immunoreactivity in an mSOD1 mouse at 11 weeks (A), 15 weeks (B), 18 weeks (C), and a control mouse at 18 weeks of age (D). Macrophage and microglial numbers significantly increase throughout disease progression in the ventral quadrant of lumbar spinal cord sections ($p<0.05$). Microglial activation is more prominent in the ventral horn where the motor neurons are located, compared to dorsal grey matter and white matter regions. Magnification at 4x.

Table 1. Mean Number of Microglia/ Macrophages and SHIP1+ Cells in the Ventral Quadrant

	Asymptomatic (*n*=6)		**Symptomatic (*n*=6)**		**Advanced (*n*=6)**	
	Control (*n*=3)	**mSOD1 (*n*=3)**	**Control (*n*=3)**	**mSOD1 (*n*=3)**	**Control (*n*=3)**	**mSOD1 (*n*=3)**
Iba1+ cells (mean±SEM)	144.9±3.3	226.5±0.8$^{*\#}$	157.3±2.3	341.7±23.4$^{*\#}$	142.7±3.5	426.5±5.2$^{*\#}$
SHIP1+ cells (mean±SEM)	0	5.3±1.9$^{\#\#}$	0.3±0.2	156.2±5.2$^{**\#\#}$	0.3±0.3	271.5±9.3$^{**\#\#}$

A significant increase in the number of microglia/ macrophages was observed between mSOD1 and control mice at all 3 time points (Paired T-test; $p<0.05^{*}$). A significant increase in the number of microglia/ macrophages was also observed at all 3 time points of disease progression (ANOVA; $p<0.05^{\#}$). There was a significant difference in SHIP1+ cells between controls and mSOD1 mice at symptomatic and advanced stages (Paired T-test; $p<0.01^{**}$).The number of SHIP1+ cells also continued to increase throughout disease progression (ANOVA; $p<0.001^{\#\#}$).

4.2. SHIP1 Immunoreactivity in Microglia and Macrophages

4.2.1. SHIP1 Immunoreactivity Is Present in the CNS

In order to determine whether SHIP1 is expressed in microglia/ macrophages in the CNS of mice, lumbar spinal cord sections of both WT and mSOD1 mice were labelled with anti-SHIP1 and anti-Iba1 antibodies. Co-labelling of anti-SHIP1 and anti-Iba1 antibodies was evident at all three stages of disease progression suggesting that SHIP1 is expressed in microglia/ macrophages in the CNS (See Figure 8). SHIP1 immunoreactivity was minimal in

WT mice (See Figure 9). Furthermore, essentially no cells were SHIP1+ that did not express Iba1.

4.2.2. SHIP1 Immunoreactivity in Controls and mSOD1 Mice

SHIP1 immunoreactivity varied throughout the different stages of disease progression (See Table 1). No specific SHIP1+ staining was seen in WT controls. At 11 weeks, when mice were asymptomatic, minimal SHIP1+ staining was observed in mSOD1 mice (n=3) where 5.3 ± 1.9 SHIP1+ cells (mean ± SEM) were seen. Although SHIP1 immunoreactivity was evident in the mSOD1 mice at 11 weeks, there was no significant difference between WT and mSOD1 mice ($p>0.05$), likely due to the relatively large SEM. At 15 weeks, when mSOD1 mice were symptomatic, there was a significant increase in SHIP1 immunoreactivity in microglia/ macrophages (156.2 ± 5.2, n=3) compared to WT mice ($p<0.01$). At 18 weeks, when mice reach advanced stages of disease, the number of SHIP1+ microglia/ macrophages continued to increase (271.5 ± 9.3, n=3), with a significant difference between WT and mSOD1 mice ($p<0.01$). There was no difference in SHIP1 immunoreactivity between WT mice at any time point.

4.2.3. SHIP1 Immunoreactivity throughout Disease Progression

SHIP1 immunoreactivity was evident at all three stages of disease progression in mSOD1 mice (See Figure 10) and significantly increased throughout disease progression at all 3 time points ($p<0.001$; See Figure 11). During the asymptomatic stage, the mean number of SHIP1+ cells was 5.3±1.9

(n=3). During the symptomatic stage, the number of SHIP1+ cells increased substantially by nearly 30-fold. By advanced stage, there was a 1.7-fold increase in SHIP1+ cells compared to symptomatic mice (15 weeks).

In order to further analyze SHIP1 expression, the percentage of microglia/ macrophages that were SHIP1+ was calculated. There was a significant increase in the percentage of SHIP1+ cells throughout disease progression ($p<0.01$; See Figure 12). At 11 weeks, 2.4±0.8% of microglia/ macrophages were labelled as SHIP1+. At both 15 weeks and advanced stage, the percentage of SHIP1+ cells continued to increase to 46.0±2.7% and 63.7±1.8%, respectively. A positive correlation between Iba1 and SHIP1 immunoreactivity in mSOD1 mice was also observed ($p<0.01$; $r=0.975$; See Figure 13). SHIP1 immunoreactivity was also analyzed in the grey and white matter (See Table 2). There was an increase in SHIP1 immunoreactivity in both the grey and white matter throughout disease progression (ANOVA; $p<0.05$).

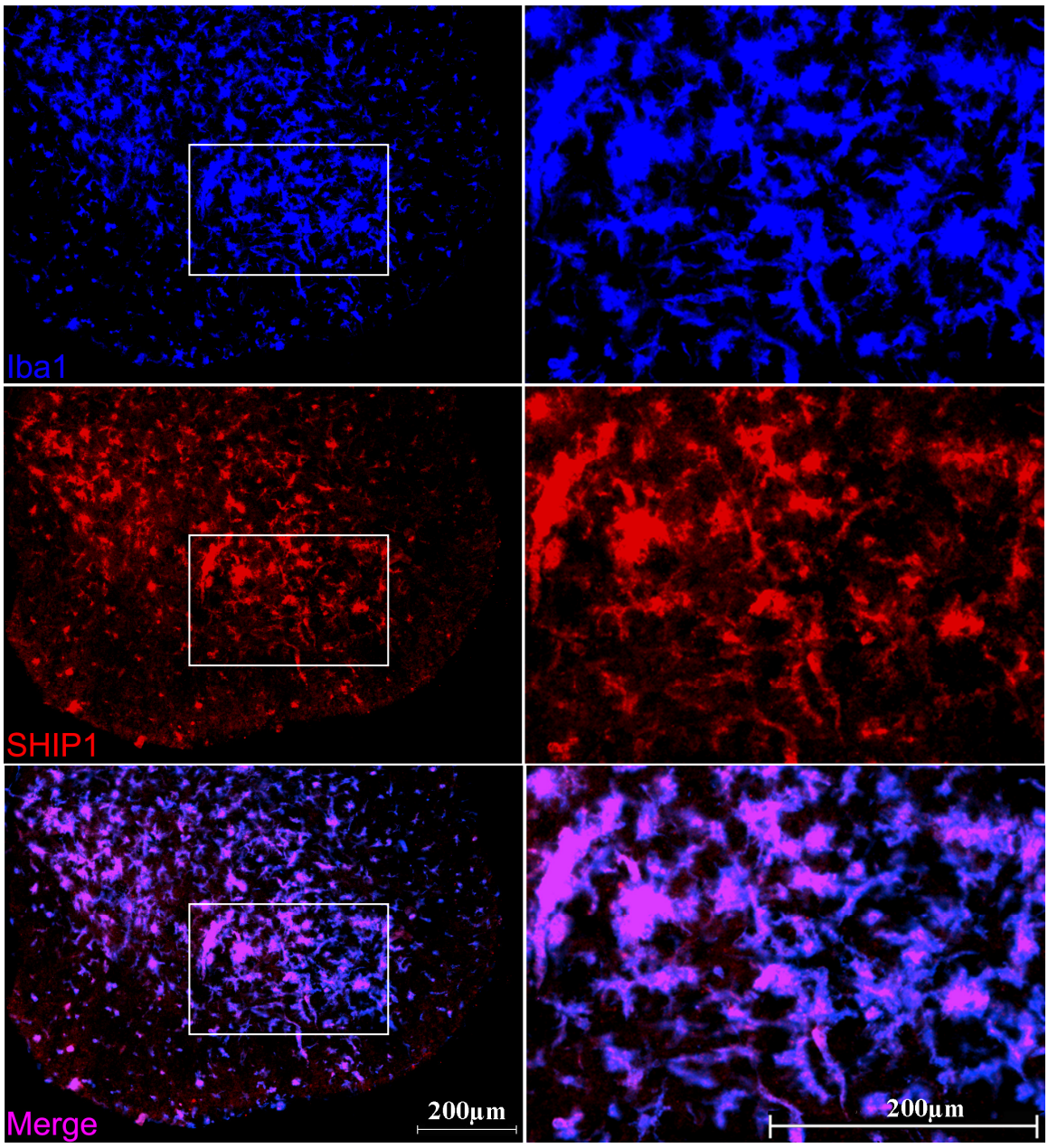

Figure 8. SHIP1 and Iba1 Co-labelling

Co-labelling of anti-Iba1 (top) and anti-SHIP1 (centre) antibodies in the ventral quadrant of a lumbar spinal cord section at 15 weeks. The nuclear dye, DAPI, (not shown above for clarity) was also used to identify individual cells. Magnification at 10x.

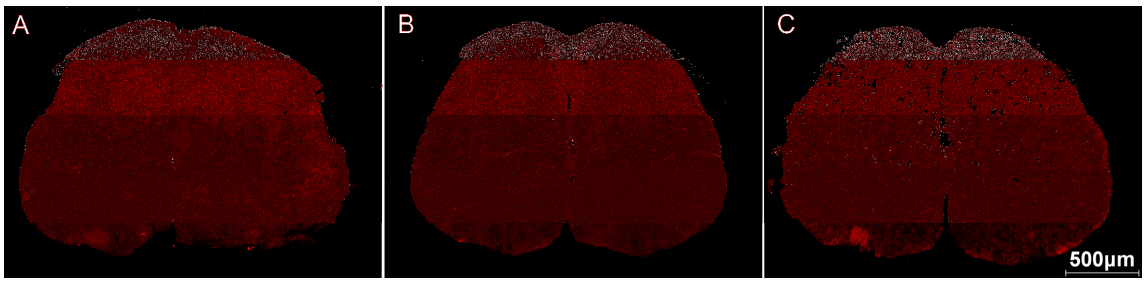

Figure 9. SHIP1 Immunoreactivity in Control Mice

There was no specific SHIP1 labelling in control mice at 11 weeks (A), 15 weeks (B) or advanced stage (C). Magnification at 4x.

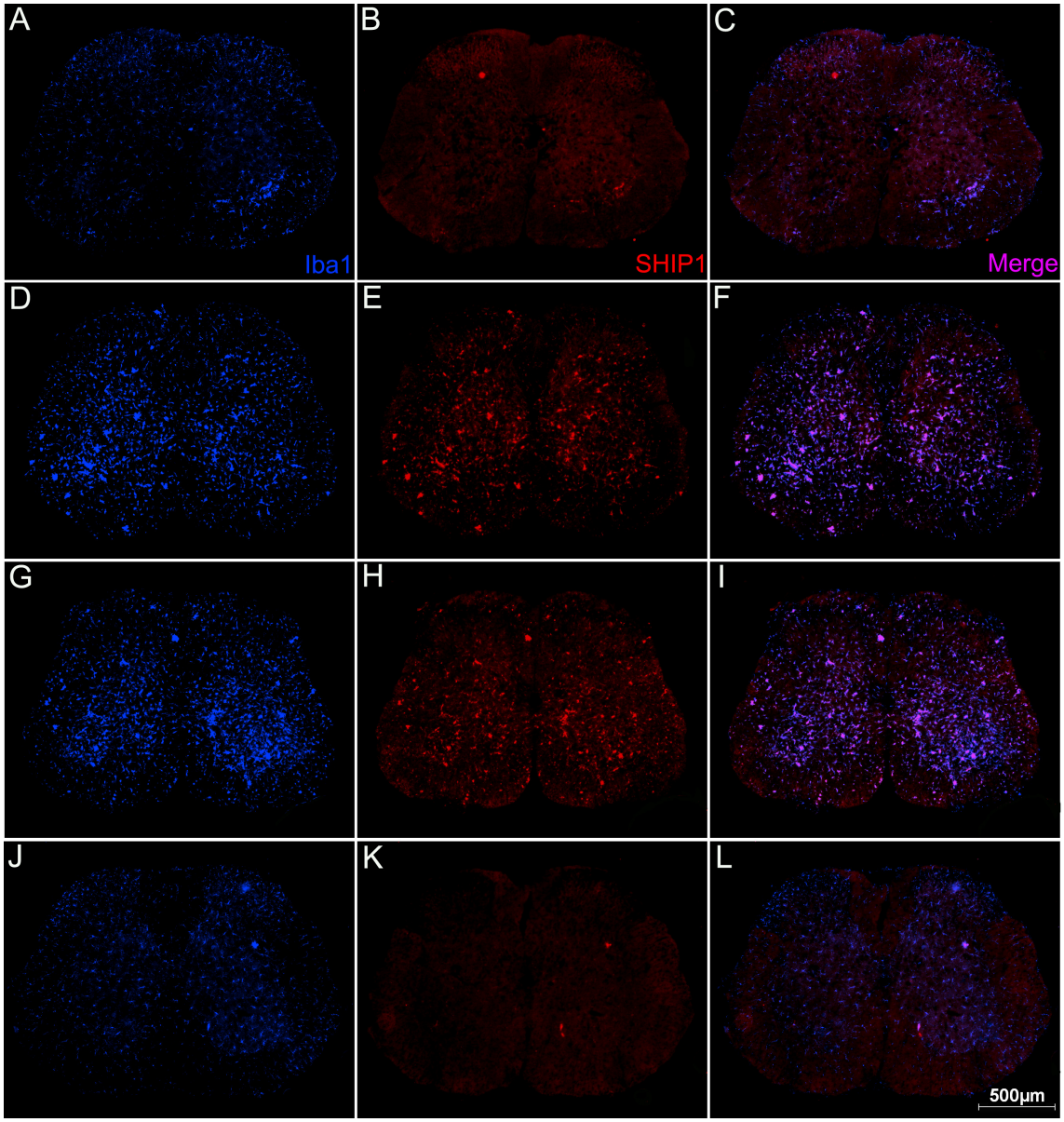

Figure 10. Co-labelling of SHIP1 and Iba1 in Mice

SHIP1 ($p<0.001$) and Iba1 immunoreactivity ($p<0.05$) significantly increase in the ventral quadrant of lumbar spinal cord sections of mSOD1 mice at 11 weeks (A-C), 15 weeks (D-F), and advanced stage (G-I) of disease progression. Age-matched control at advanced stage (J-L). Magnification at 4x.

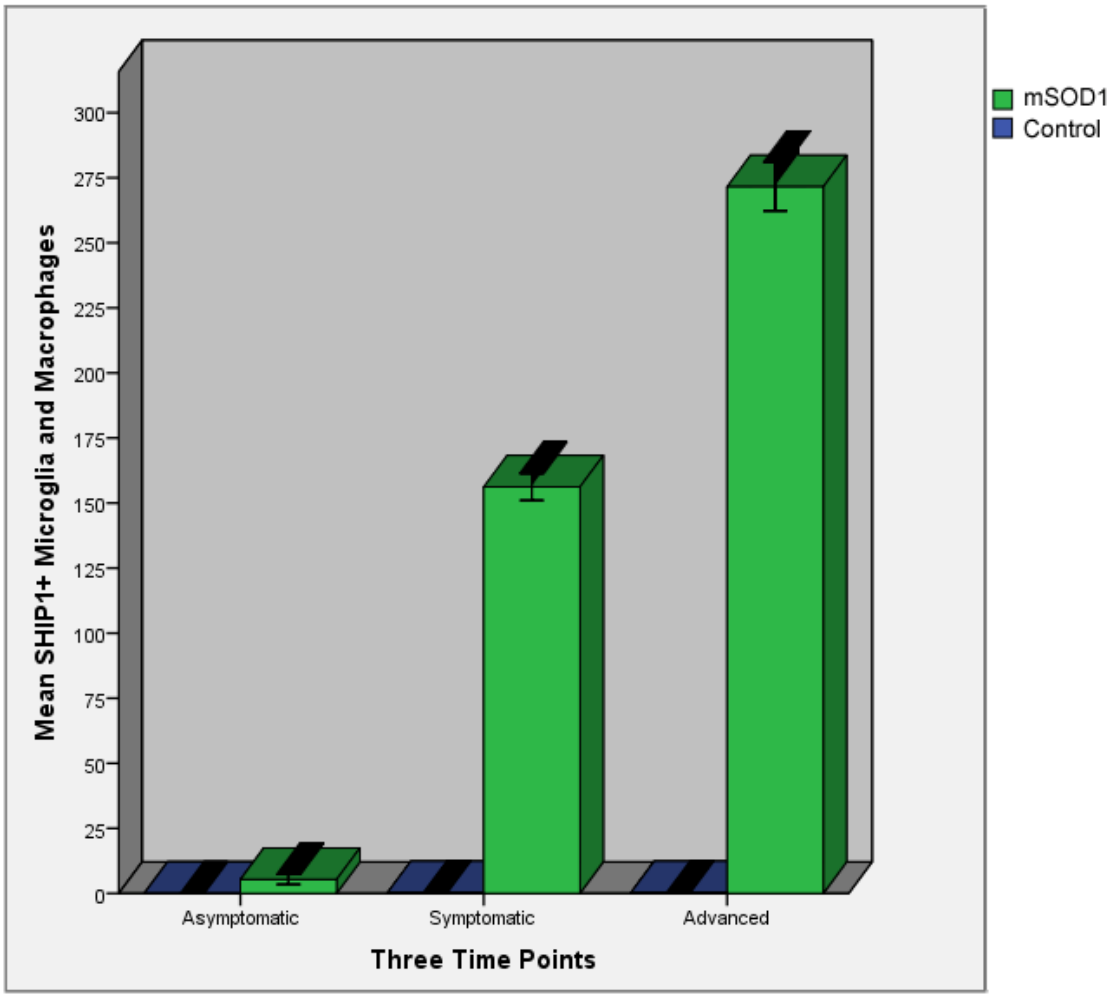

Figure 11. SHIP1 Expression at Three Time Points

The number of SHIP1+ cells was consistent across the control mice at all three time points. In the mSOD1 mice, the number of SHIP1+ cells increased throughout disease progression ($p < 0.001$). Error bars display SEM.

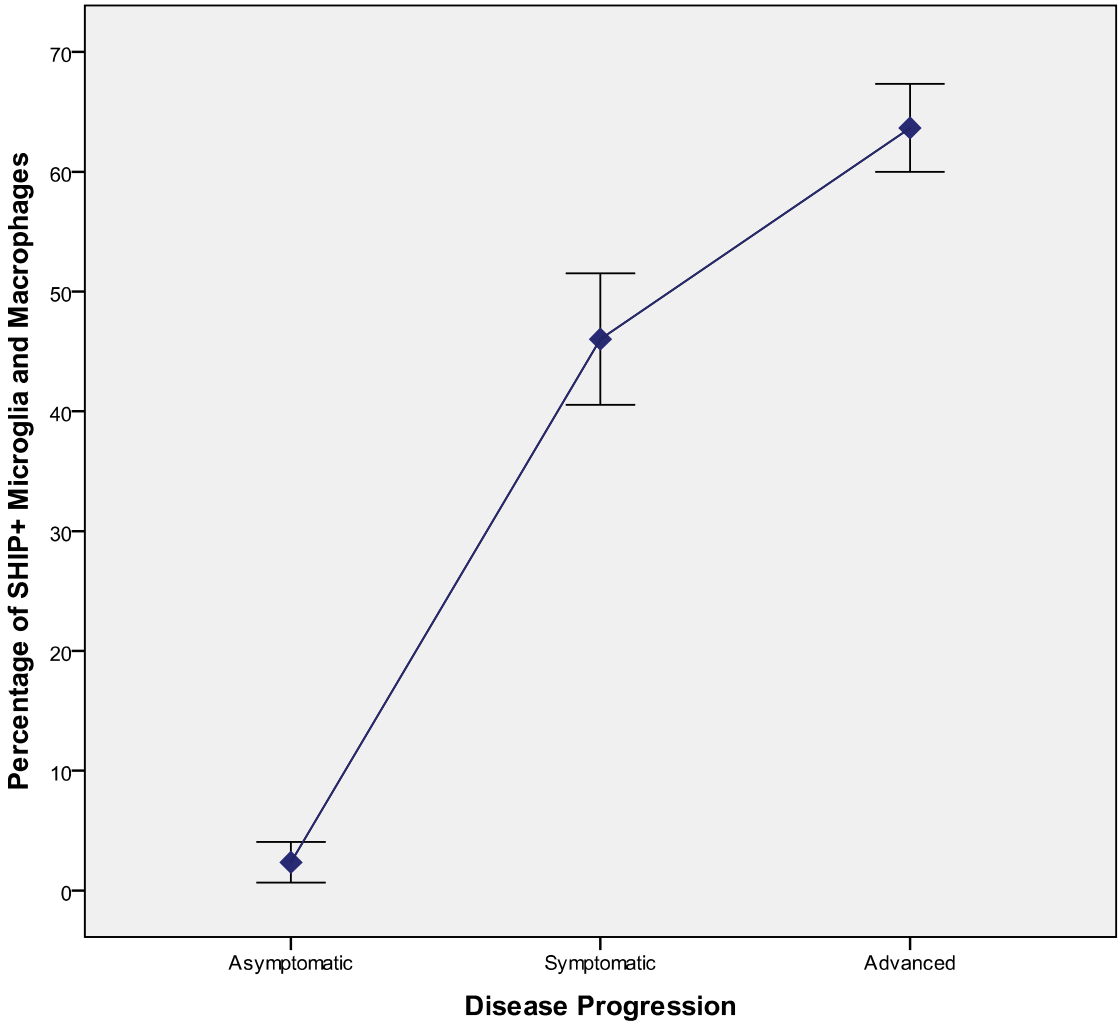

Figure 12. Percentage of Microglia and Macrophages that are SHIP1+

There was a significant increase in the percentage of SHIP1+ microglia/ macrophages throughout the progression of the disease ($p<0.01$). Error bars display SEM.

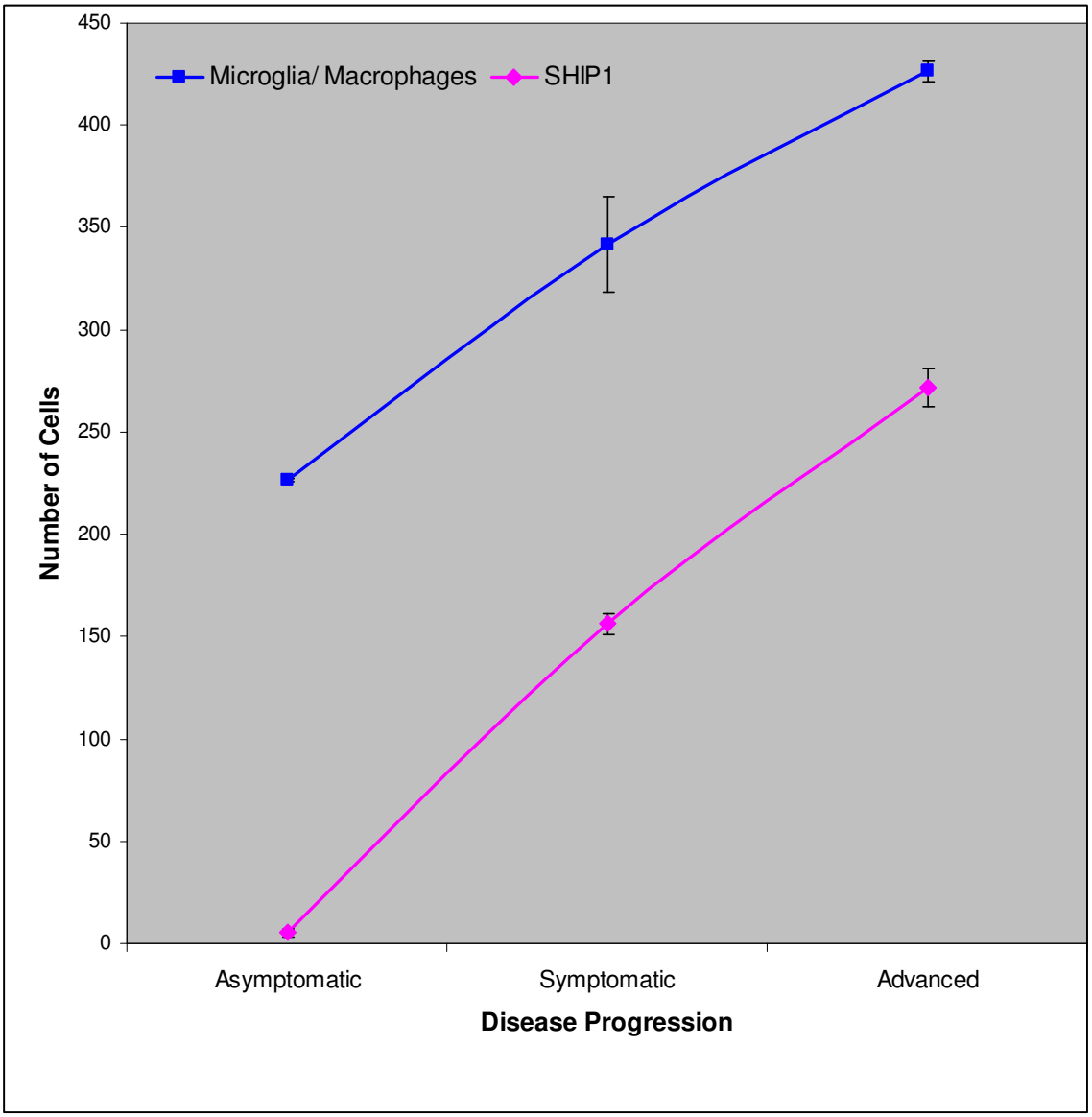

Figure 13. Number of Microglia/ Macrophages and SHIP1+ Cells in mSOD1 Mice

As the number of microglia/ macrophages increased, the number of SHIP1+ cells increased. There was a positive correlation between the number of microglia/ macrophages and SHIP1+ cells ($p<0.01$; $r=0.975$). Error bars display SEM; however due to the small SEM, some values are not distinguishable on the graph.

Table 2. Iba1 and SHIP1 Immunoreactivity in Grey and White Matter

		Asymptomatic (*n*=3)	Symptomatic (*n*=3)	Advanced stage (*n*=3)
Grey	Iba1+ Cells	128.6±2.8	225.6±13.9	279.0±1.8
	SHIP1+ Cells	4.7±1.6*	132.1±5.3*	186.5±3.6*
	% of SHIP1+ Cells	3.6±1.3%	58.9±3.7%	66.9±1.3%
White	Iba1+ Cells	97.9±2.1	116.1±10.5	147.5±7.0
	SHIP1+ Cells	0.7±0.3*	24.1±2.7*	85.0±6.9*
	% of SHIP1+ Cells	0.7±0.3%	20.7±0.4%	57.5±2.9%

The number of SHIP1+ cells in the grey and white matter of mSOD1 mice increased significantly throughout disease progression (ANOVA; $p<0.05$*).

5. Discussion and Conclusion

ALS is a neurodegenerative disease that results in motor neuron loss in the CNS. This debilitating disease leads to paresis and eventually paralysis and death within 3-5 years of symptom onset (Ferraiuolo et al., 2011). Microgliosis is a common hallmark observed in the CNS of both animal models and ALS patients (Alexianu et al., 2001; Henkel et al., 2004; Yang et al., 2011). Although neuroinflammation and proinflammatory factors have been detected in the CNS, little is known about how microglia/ macrophages are involved in neuronal loss in ALS or in murine models of ALS.

SHIP1 is an enzyme that negatively regulates the activation and survival of hematopoietic cells by hydrolyzing the 2nd messenger, PIP_3, to $PI(3,4)P_2$ (Sly et al., 2007; Ooms et al., 2009). This research was conducted in order to determine the presence of SHIP1 in microglia/ macrophages in the CNS and observe any changes in SHIP1 immunoreactivity throughout disease progression. This information is important to further understand the contribution of microglia/ macrophages to the pathogenesis of ALS.

5.1. Microglia and macrophage expression increases with disease progression

Changes in microglial/ macrophage number and activation throughout the progression of ALS have been examined in previous studies (Alexianu et al., 2001; Graber et al., 2010; Yang et al., 2011). In the current study, the number of microglia/ macrophages in the ventral quadrant of the spinal cord was significantly higher in mSOD1 mice compared to WT mice at all three time points. This observation is consistent with previous studies that found significant increases in microglial number and mRNA expression of cell surface markers for microglia/ macrophages in mSOD1 murine models and human ALS post-mortem spinal cord tissue (Yoshihara et al., 2002; Henkel et al., 2004; Beers et al., 2011).

In this study, Iba1 immunoreactivity in microglia/ macrophages in the ventral quadrant of mSOD1 mice also increased significantly throughout disease progression, which is consistent with previous research (Alexianu et al., 2001; Yang et al., 2011). The involvement of microglia in ALS is still largely unknown; however, various elegant studies have demonstrated that mSOD1 microglia/ macrophages are involved in neuronal loss, supporting the view that ALS is a non-cell autonomous disease. Chimeric mSOD1 mice expressing mSOD1 in neurons and having both WT SOD1 and mSOD1 in non-neuronal cells had reduced neurodegeneration compared to mice having both neuronal and non-neuronal cells expressing mSOD1 (Clement et al., 2003). The addition of WT microglia to mSOD1/PU.1$^{-/-}$ mice also reduced motor neuron loss and increased

survival compared to mSOD1/PU.1$^{-/-}$ with microglia expressing mSOD1 suggesting that microglia/ macrophages play a role in the pathogenesis of ALS (Beers et al., 2006). Furthermore, microglial aggregates were observed near neuronal cell bodies in the spinal cord of mouse models and post-mortem human ALS tissue suggesting microglial involvement in motor neuron loss. (Alexianu et al., 2001; Henkel et al., 2004; Sanagi et al., 2010).

5.2. SHIP1 immunoreactivity is present in microglia and macrophages in the CNS

In order to further understand the role of microgliosis in ALS, SHIP1 immunoreactivity was examined in spinal cord microglia/ macrophages. SHIP1 expression has been previously identified in hematopoietic cells including B-cells, T-cells, mast cells, and various tissue macrophages (Leung et al., 2008). Microglia, the resident macrophages of the CNS, are also of hematopoietic origin and share similar characteristics to various tissue macrophages in the body (Mosser & Edwards, 2008). It was therefore hypothesized that SHIP1 would be expressed in microglia/ macrophages in the CNS although there is no previous study that has yet demonstrated this finding. In this study, co-labelling of anti-SHIP1 and anti-Iba1 antibodies was achieved through IHC to analyze SHIP1 expression in the ventral quadrant of the lumbar spinal cord in mSOD1 and WT mice. My results show that SHIP1 immmunoreactivity is present in spinal cord tissue from mSOD1 mice, but there is limited labelling in WT spinal cord tissue. Furthermore, the number of SHIP1+ cells increases with disease progression. As

previous work has shown that SHIP1 immunoreactivity is restricted to hematopoetic cells, it was suspected that SHIP1 immunoreactivity would be restricted to microglia/ macrophages in the CNS (Sly et al., 2007; Leung et al., 2008). Consistent with this view, I found that all SHIP1+ cells also expressed Iba1, a microglia and macrophage-specific antigen (Imai et al., 1996). As previous work has clearly shown SHIP1 immunoreactivity in hematopoetic cells, the cell labelling I observed is specific for SHIP1. It is important to note that T-cells have also been observed in the spinal cord of mSOD1 mice and may also express SHIP1 (Alexianu et al., 2001). Although this was not examined in the current study, future studies should evaluate SHIP1 distribution and expression within T-cells.

5.3. SHIP1 immunoreactivity is greater in mSOD1 mice and increases throughout disease progression

My results showed a significant increase in SHIP1+ cells in mSOD1 mice at both 15 weeks and advanced stage compared to the WT controls. A significant increase in SHIP1+ cells was also found in mSOD1 mice throughout disease progression. The observation of an increase in SHIP1+ cells in mSOD1 mice raises the related questions of why SHIP1 expression should be increased and what the consequences of this increase will be in this murine model. SHIP1 is activated by various stimuli including cytokines, chemokines, growth factors, and oxidative stress (Rauh et al., 2004). Altered cytokine and chemokine expression has been observed in the spinal cord tissue of both ALS patients and mSOD1

animal models (Yoshihara et al., 2002; Henkel et al., 2004; Takeuchi et al., 2010; Beers et al., 2011) and I hypothesize that this elevation in cytokine and chemokine expression results in increased SHIP1 immunoreactivity. An increase in mRNA expression of pro-inflammatory cytokines and chemokines including TNF-α, IFN- γ, M-CSF, and monocyte chemotactic protein-1 (MCP-1) have also been observed in human ALS and mSOD1 mouse spinal cord tissue throughout disease progression (Henkel et al, 2004; Takeuchi et al., 2010; Beers et al., 2011). M-CSF is involved in the differentiation of most tissue macrophage populations (Ginhoux et al., 2010). MCP-1 is a chemoattractant that is involved in the recruitment of monocytes towards areas of injury and inflammation (Yoshihara et al., 2002; Henkel et al., 2004) and thus, if these cytokines are released in mSOD1 CNS tissue, it is possible that they could also act to attract monocyte entry into the CNS and microglial proliferation.

In the current study, a significant increase in microglia/ macrophage number was observed in mSOD1 spinal cord tissue beginning at the asymptomatic stage until advanced stages, which is consistent with previous studies (Hall et al., 1998; Alexianu et al., 2001). SHIP1 immunoreactivity also continued to increase throughout disease progression. Microglia/ macrophages are involved in the secretion of pro-inflammatory factors and have been shown to express significantly increased levels of pro-inflammatory cytokines including TNF-α and IFN- γ in mSOD1 murine models during the asymptomatic stage and throughout disease progression (Yoshihara et al., 2002; Henkel et al., 2009;

Takeuchi et al., 2010). This observation suggests that microglia/ macrophages are involved in the release of these pro-inflammatory factors throughout disease progression, and I hypothesize that the increased cytokine release from microglia/ macrophages assists in the activation of SHIP1. Future studies should examine cell-type specific release of pro-inflammatory cytokines and chemokines throughout disease progression.

In my study, although SHIP1 immunoreactivity was detected in asymptomatic mSOD1 mice, a significant increase was only observed in CNS tissue of symptomatic and advanced stage mice. This means that the significant increase in microglia/ macrophages precedes the increase in SHIP1 immunoreactivity. This increase in SHIP1 immunoreactivity at the symptomatic stage may, in part, be due to an increase in microglia/ macrophage number resulting in an increase in cytokine and chemokine production; however, it is likely that other factors, such as ROS, may play a role in the activation of SHIP1 in microglia/ macrophages. It is also possible that the expression of SHIP1 in individual cells is present at lower levels during the asymptomatic stage and increases with disease progression. This can be reflected by an increase in the immunofluorescence of SHIP1 throughout disease progression observed in the mSOD1 spinal cord sections.

The progressive increase in SHIP1 immunoreactivity in the microglia/ macrophages of mSOD1 mice may have several benefits or consequences in disease progression. An increase in SHIP1 expression in microglia/

macrophages suggests a decrease in PI3K mediated signalling pathways; however, the role of PI3K in disease pathology is unclear. Several studies have found an association between microglial activation and progression of the disease (Alexianu et al., 2001; Graber et al., 2010) and in the current study, a strong correlation between SHIP1 and microglia/ macrophage expression was observed in the mSOD1 mice. This suggests that SHIP1 may play a role in disease progression; however, it is unclear whether SHIP1 has a neuroprotective or neurodegenerative role in the pathogenesis of ALS.

Various studies have demonstrated SHIP1's involvement in hematopoietic cell apoptosis, phagocytosis, and macrophage phenotype skewing; therefore, SHIP1 may play a similar role in microglia/ macrophages in the CNS of mSOD1 mice (Liu et al., 1997; Cox et al., 2000; Nakamura et al., 2002; Rauh et al., 2005). SHIP1 is a negative regulator of FcγR phagocytosis. In my study, a significant increase in SHIP1 expression at 15 weeks and advanced stage was observed suggesting a decrease in FcγR-mediated phagocytosis. Various studies have demonstrated the involvement of FcγR expression in microglia/ macrophages in neurodegenerative diseases including Parkinson's Disease (PD), Alzheimer's Disease (AD), and Multiple Sclerosis (MS) (Okun et al., 2010). An increase in FcγR expression may have a neuroprotective role in AD through the clearance of Aβ deposits; whereas it may be involved in inducing lesions in the substantia nigra in PD (Bard et al., 2000; He et al., 2002). FcγR is up-regulated in mSOD1 mice, and increases throughout disease progression;

however, it is unclear whether an increase in phagocytic function would be beneficial or detrimental to ALS progression (Alexianu et al., 2001). Further research is required to assess the role of FcγRs in ALS.

SHIP1 is also involved in the negative regulation of cell survival. The over-expression of WT SHIP1 results in a reduction in cell viability (Liu et al., 1997). In the current study, a progressive increase in SHIP1 expression was observed; however, the number of microglia/ macrophages continued to increase throughout disease progression. This raises the question of whether mSOD1 microglia/ macrophages have an altered function compared to WT microglia/ macrophages. Studies have shown that the presence of WT microglia in mSOD1 mice slows down disease progression (Clement et al., 2003; Beers et al., 2006). In vitro, WT microglia release lower levels of neurotoxins and induce less neuronal death compared to mSOD1 microglia suggesting mSOD1 microglia are functionally abnormal (Wedyt et al., 2004; Beers et al., 2006). Due to such differences between WT and mSOD1 macrophages, I hypothesize that although SHIP1 is expressed in the microglia/ macrophages of mSOD1 mice, either other factors are involved in determining microglial/ macrophage survival or there is an impairment in signal transduction pathways in mSOD1 microglia/ macrophages. SHIP1 expression may also be elicited secondary to cytokines or other pro-inflammatory stimuli. Recent work has shown that the TAR DNA-binding protein 43 (TDP43), a protein found in inclusions of some ALS patients, leads to the overproduction of pro-inflammatory cytokines and neurotoxic mediators (Swarup

et al., 2011). Treatment with an inhibitor of NF-κB to mice over-expressing TDP43 lead to an improvement of neuromuscular function in these mice. Further research must be conducted in order to provide additional insight into the mechanisms of the involvement of SHIP1 in the apoptosis of microglia/ macrophages in the mSOD1 model or other models of ALS.

SHIP1 is also believed to be involved in repressing the activation of M2 macrophages (Rauh et al., 2005). LPS-induced $SHIP^{-/-}$ macrophages derived from mice have decreased levels of NO and an increase in ArgI activity compared to WT macrophages, which is typical of an M2 phenotype (Rauh et al., 2005). M2 skewing is also believed to be associated with the PI3K/PIP_3 pathway (Fang et al., 2004; Rauh et al., 2005). An increase in the activity of the downstream protein mTOR has been shown to bias macrophages to an M2 phenotype (Rees, 2010). The PI3K pathway enhances mTOR activity suggesting that SHIP1 is involved in negatively regulating M1 macrophage skewing (Rees, 2010).

In the current study, there was a significant increase in SHIP1 immunoreactivity throughout disease progression suggesting that the microglia/ macrophages are skewed towards an M1 phenotype in the mSOD1 mouse model. Human ALS spinal cord tissue and mSOD1 animal models have an increase in the gene expression and levels of pro-inflammatory factors including TNF-α, IFN-γ, and NADPH oxidase complex (NOX2), which are characteristic of M1 macrophages (Hensley et al., 2003; Chen et al., 2004; Henkel et al., 2004;

Beers et al., 2011). LPS-induced stimulation of $SHIP^{-/-}$ cells also results in a decrease in TNF-α compared to WT cells (Fang et al., 2004). The ratio of IL-4 to TNF-α and IFN-γ is significantly lower in mSOD1 mice compared to WT mice suggesting a decrease in anti-inflammatory factors in the mSOD1 mouse model (Takeuchi et al., 2010). Based on the evidence above, I hypothesize that the increase in SHIP1 expression aids in maintaining an M1 phenotype in microglia/ macrophages resulting in the increase of pro-inflammatory factors in an mSOD1 mouse model of ALS.

Studies have also found that pro-inflammatory factors are involved in neuronal loss. LPS activated microglia reduce motor neuron survival; whereas, the anti-inflammatory cytokine IL-4 protects them (Zhao et al., 2006). In vitro derived mSOD1 microglia co-cultured with primary motor neurons also induce more neuronal death compared to WT microglia (Beers et al., 2006). I hypothesize that the combination of the cytotoxic potential of mSOD1 microglia/ macrophages and an increase in SHIP1 expression results in a shift towards an M1 phenotype resulting in an increase in pro-inflammatory factors and potentially neurodegeneration in the mSOD1 murine model. Reducing SHIP1 expression in the microglia/ macrophages of mSOD1 mice could potentially skew them towards an M2 phenotype which could reduce pro-inflammatory factors and increase neuroprotective effects in ALS patients.

In my study, an increase in the percentage of SHIP1+ microglia/ macrophages was observed in both the grey and white matter throughout

disease progression. This raises the question of why only certain microglia/ macrophages express SHIP1, and whether this difference in SHIP1 immunoreactivity may be due to phenotypic differences between microglia and macrophages. Recent studies have suggested that microglia and macrophages display different markers in their M1 and M2 phenotype (Durafourt et al., 2012). For instance, one in vitro study found that M2 treatment of microglia did not induce MR expression; whereas MR expression was observed in blood-derived macrophages (Durafourt et al., 2012). This is consistent with a study that found MR expression only within perivascular macrophages in the CNS of mSOD1 mice (Lewis et al., 2009). Further research should be conducted in order to determine whether SHIP1 is more likely to be expressed in either microglia or macrophages, and whether SHIP1+ cells are more likely to characterize an M1 or M2 phenotype.

It is important to note that the timing, concentration, and synergistic effects between extracellular factors play a role in determining the microglia/ macrophage phenotype. One study found that a combination of TNF-α, IL-6, and IFN-γ stimulation amplified nitrite release from macrophages compared to TNF-α stimulation alone (Hensley et al., 2003). Another study found that motor neuron survival was significantly higher in motor neuron and macrophage cell cultures treated with IL-4 first and then LPS, instead of vice-versa (Zhao et al., 2006). Also, $SHIP^{-/-}$ in vitro derived macrophages did not skew to an M2 phenotype unless mouse plasma was added (Rauh et al., 2005). Further research is

required to determine how different concentrations, the order in which factors stimulate macrophages, and extracellular interactions influence SHIP1 expression and the microglial/ macrophage phenotype in vivo.

5.4. Future Research

From the research that I have conducted, it is evident that there is an increase in SHIP1 expression throughout disease progression. In order to continue to understand the role of SHIP1 in ALS, it is important to further characterize SHIP1 expression by performing Western blot and RT-qPCR experiments to examine both the protein and gene expression of SHIP1. Murine models with altered phenotypes must also be examined. Although it has been proven to be somewhat challenging to breed mSOD1/SHIP$^{-/-}$ mice, these transgenic mice should be studied in order to determine how microglial/ macrophage expression is altered in the absence of SHIP1. I hypothesize that an increase in the number of microglia/ macrophages in mSOD1/SHIP$^{-/-}$ mice would suggest that SHIP1 is involved in suppressing microglial/ macrophage activation, proliferation, and survival in an mSOD1 mouse model of ALS. If this is the case, further research should be conducted to determine whether microglia/ macrophages in mSOD1/SHIP$^{-/-}$ mice express M2 markers and whether this reduces neuroinflammation and neuron loss. FcγR expression should also be examined to determine whether it has a neuroprotective or neurodegenerative role in ALS. If, however, the number of microglia/ macrophages in mSOD1/SHIP^{-}

$^{/-}$ is not altered, this suggests that SHIP1 may have a different function in mSOD1 mice. Levels of PI3K activity, PIP_3, and downstream molecules should be examined to determine whether SHIP1 expression in mSOD1 mice correlates to a decrease in PI3K activity and PIP_3 levels. This will also help to determine if there are impairments in the PI3K/PIP_3 signal transduction pathway in mSOD1 mice.

SHIP1 expression in chimeric mSOD1 mice with WT microglia/ macrophages should also be examined to determine whether SHIP1 in WT and mSOD1 microglia is expressed to the same extent. Several studies have shown that the presence of WT microglia in mSOD1 mouse models slows down disease progression; therefore, there is reason to believe that there may be differences in SHIP1 expression between WT and mSOD1 microglia/ macrophages (Clement et al., 2003; Beers et al., 2006). Also, SHIP1 immunoreactivity was only present in a certain percentage of microglia/ macrophages. Identifying markers to differentiate between macrophages and microglia, and an M1 and M2 phenotype would give further insight as to whether SHIP1+ cells have a unique phenotype compared to cells that do not express SHIP1.

Finally, in vitro experiments on the interactions between pro-inflammatory and anti-inflammatory stimuli, and their influence on microglial/ macrophage skewing and SHIP1 expression should also be examined to mimic in vivo settings. Further analysis will allow researchers to discover an optimal level of

SHIP1 expression to maximize its function as a potential therapeutic strategy in ALS.

5.5. Limitations

Due to differences between mice and humans, caution should be used when extrapolating data from mice to humans. mSOD1 murine models represent patients with fALS, whereas most ALS patients (90-95%) have sALS. It is therefore difficult to determine whether these results will translate to sALS patients (Philips & Robberecht, 2011). Mice also express different cell-surface markers compared to humans and sometimes have different responses to various signals. For example, mice only express one form of the FcγRII, whereas humans express two functionally different forms (Tridandapani et al., 2002). Also, in humans, M-CSF leads to the development of M2 macrophages; whereas GM-CSF leads to an M1 phenotype. In the murine model, GM-CSF leads to an increase in ArgI levels, which is characteristic of an M2 phenotype (Weisser et al., 2011). Finally, ALS patients usually have the disease long before treatment has started (Philips & Robberecht, 2011). When determining whether SHIP1 can be altered to slow down disease progression, it is important to alter and analyze protein levels at the symptomatic and advanced stages rather than the asymptomatic stage in order to more closely parallel disease progression in humans.

5.6. Conclusion

In conclusion, the present research examined the changes in SHIP1 immunoreactivity in microglia/ macrophages in the CNS of mSOD1 mice. This study demonstrated that co-labelling of SHIP1 and Iba1 was evident at all stages of disease progression and was not observed in the absence of the primary antibody or in WT mice. SHIP1 was highly expressed in mSOD1 mice at symptomatic and advanced stages and correlates with microgliosis in the CNS suggesting its involvement in the progression of the disease. SHIP1 immunoreactivity was present in only a percentage of microglia/ macrophages; therefore it is crucial to understand why only certain cells have the ability to express SHIP1, and whether this is due to microglia/ macrophages having different phenotypes. This will give researchers a further understanding of the different types of microglia and macrophages in the CNS and how they influence disease progression.

At this point, it is unclear whether SHIP1 expression in mSOD1 mice has a neuroprotective or neurodegenerative role in ALS; however, the increase in SHIP1 expression in the CNS suggests a role for SHIP1 in ALS. Further research in determining how SHIP1 affects phagocytosis, macrophage skewing, cell activation, and cell survival is required to determine how SHIP1 is involved in ALS. Future studies will give us further insight on how SHIP1 can be used as a potential target for therapeutic strategies in ALS.

References

Adams, D. O., & Hamilton, T. A. (1987). Molecular transductional mechanisms by which IFN gamma and other signals regulate macrophage development. *Immunological Reviews, 97*, 5-27. Retrieved from http://www.immunologicalreviews.dk/

Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., & Rossi, F. M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscience, 10*(12), 1538-1543. doi:10.1038/nn2014

Alexianu, M. E., Kozovska, M., & Appel, S. H. (2001). Immune reactivity in a mouse model of familial ALS correlates with disease progression. *Neurology, 57*(7), 1282-1289. Retrieved from http://www.neurology.org/

Appel, S. H., Beers, D. R., & Henkel, J. S. (2010). T cell-microglial dialogue in parkinson's disease and amyotrophic lateral sclerosis: Are we listening? *Trends in Immunology, 31*(1), 7-17. doi:10.1016/j.it.2009.09.003

Astle, M. V., Horan, K. A., Ooms, L. M., & Mitchell, C. A. (2007). The inositol polyphosphate 5-phosphatases: Traffic controllers, waistline watchers and tumour suppressors? *Biochemical Society Symposium, (74)*(74), 161-181. doi:10.1042/BSS0740161

Bard, F., Cannon, C., Barbour, R., Burke, R. L., Games, D., Grajeda, H., . . . Yednock, T. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of alzheimer disease. *Nature Medicine, 6*(8), 916-919. doi:10.1038/78682

Beers, D. R., Henkel, J. S., Xiao, Q., Zhao, W., Wang, J., Yen, A. A., . . . Appel, S. H. (2006). Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences of the United States of America, 103*(43), 16021-16026. doi:10.1073/pnas.0607423103

Beers, D. R., Zhao, W., Liao, B., Kano, O., Wang, J., Huang, A., . . . Henkel, J. S. (2011). Neuroinflammation modulates distinct regional and temporal

clinical responses in ALS mice. *Brain, Behavior, and Immunity, 25*(5), 1025-1035. doi:10.1016/j.bbi.2010.12.008

Benoit, M., Desnues, B., & Mege, J. L. (2008). Macrophage polarization in bacterial infections. *Journal of Immunology (Baltimore, Md.: 1950), 181*(6), 3733-3739. Retrieved from http://www.jimmunol.org/

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nature Reviews.Neuroscience, 8*(1), 57-69. doi:10.1038/nrn2038

Bode, J. G., Ehlting, C., & Haussinger, D. (2012). The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. *Cellular Signalling,* doi:10.1016/j.cellsig.2012.01.018

Boillee, S., Vande Velde, C., & Cleveland, D. W. (2006). ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron, 52*(1), 39-59. doi:10.1016/j.neuron.2006.09.018

Boillee, S., Yamanaka, K., Lobsiger, C. S., Copeland, N. G., Jenkins, N. A., Kassiotis, G., . . . Cleveland, D. W. (2006). Onset and progression in inherited ALS determined by motor neurons and microglia. *Science (New York, N.Y.), 312*(5778), 1389-1392. doi:10.1126/science.1123511

Chen, L. C., Smith, A., Ben, Y., Zukic, B., Ignacio, S., Moore, D., & Lee, N. (2004). Temporal gene expression patterns in G93A/SOD1 mouse. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders : Official Publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases, 5*(3), 164-171. doi:10.1080/14660820410017091

Clement, A. M., Nguyen, M. D., Roberts, E. A., Garcia, M. L., Boillee, S., Rule, M., . . . Cleveland, D. W. (2003). Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science (New York, N.Y.), 302*(5642), 113-117. doi:10.1126/science.1086071

Colton, C. A. (2009). Heterogeneity of microglial activation in the innate immune response in the brain. *Journal of Neuroimmune Pharmacology : The Official Journal of the Society on NeuroImmune Pharmacology, 4*(4), 399-418. doi:10.1007/s11481-009-9164-4

Cox, D., Dale, B. M., Kashiwada, M., Helgason, C. D., & Greenberg, S. (2001). A regulatory role for src homology 2 domain-containing inositol 5'-phosphatase (SHIP) in phagocytosis mediated by fc gamma receptors and complement receptor 3 (alpha(M)beta(2); CD11b/CD18). *The Journal of*

Experimental Medicine, 193(1), 61-71. Retrieved from http://jem.rupress.org/

Dalton, D. K., Pitts-Meek, S., Keshav, S., Figari, I. S., Bradley, A., & Stewart, T. A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science (New York, N.Y.), 259*(5102), 1739-1742. Retrieved from http://www.sciencemag.org/

Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., & Krystal, G. (1996). The 145-kDa protein induced to associate with shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proceedings of the National Academy of Sciences of the United States of America, 93*(4), 1689-1693. Retrieved from http://www.pnas.org/

Damen, J. E., Ware, M. D., Kalesnikoff, J., Hughes, M. R., & Krystal, G. (2001). SHIP's C-terminus is essential for its hydrolysis of PIP3 and inhibition of mast cell degranulation. *Blood, 97*(5), 1343-1351. Retrieved from http://bloodjournal.hematologylibrary.org/

Dringen, R. (2000). Metabolism and functions of glutathione in brain. *Progress in Neurobiology, 62*(6), 649-671. Retrieved from http://www.journals.elsevier.com/progress-in-neurobiology/

Durafourt, B. A., Moore, C. S., Zammit, D. A., Johnson, T. A., Zaguia, F., Guiot, M. C., . . . Antel, J. P. (2012). Comparison of polarization properties of human adult microglia and blood-derived macrophages. *Glia, 60*(5), 717-727. doi:10.1002/glia.22298

Fang, H., Pengal, R. A., Cao, X., Ganesan, L. P., Wewers, M. D., Marsh, C. B., & Tridandapani, S. (2004). Lipopolysaccharide-induced macrophage inflammatory response is regulated by SHIP. *Journal of Immunology (Baltimore, Md.: 1950), 173*(1), 360-366. Retrieved from http://www.jimmunol.org/

Ferraiuolo, L., Kirby, J., Grierson, A. J., Sendtner, M., & Shaw, P. J. (2011). Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. *Nature Reviews.Neurology, 7*(11), 616-630. doi:10.1038/nrneurol.2011.152

Galli, S. J., Borregaard, N., & Wynn, T. A. (2011). Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nature Immunology, 12*(11), 1035-1044. doi:10.1038/ni.2109

Ganesan, L. P., Joshi, T., Fang, H., Kutala, V. K., Roda, J., Trotta, R., . . . Tridandapani, S. (2006). FcgammaR-induced production of superoxide and

inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways. *Blood, 108*(2), 718-725. doi:10.1182/blood-2005-09-3889

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., . . . Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.), 330*(6005), 841-845. doi:10.1126/science.1194637

Gowing, G., Philips, T., Van Wijmeersch, B., Audet, J. N., Dewil, M., Van Den Bosch, L., . . . Julien, J. P. (2008). Ablation of proliferating microglia does not affect motor neuron degeneration in amyotrophic lateral sclerosis caused by mutant superoxide dismutase. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 28*(41), 10234-10244. doi:10.1523/JNEUROSCI.3494-08.2008

Gratchev, A., Guillot, P., Hakiy, N., Politz, O., Orfanos, C. E., Schledzewski, K., & Goerdt, S. (2001). Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. *Scandinavian Journal of Immunology, 53*(4), 386-392. Retrieved from http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-3083

Hall, E. D., Oostveen, J. A., & Gurney, M. E. (1998). Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. *Glia, 23*(3), 249-256. Retrieved from http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1098-1136

Hanisch, U. K., & Kettenmann, H. (2007). Microglia: Active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience, 10*(11), 1387-1394. doi:10.1038/nn1997

Hawkins, P. T., Anderson, K. E., Davidson, K., & Stephens, L. R. (2006). Signalling through class I PI3Ks in mammalian cells. *Biochemical Society Transactions, 34*(Pt 5), 647-662. doi:10.1042/BST0340647

He, Y., Le, W. D., & Appel, S. H. (2002). Role of fcgamma receptors in nigral cell injury induced by parkinson disease immunoglobulin injection into mouse substantia nigra. *Experimental Neurology, 176*(2), 322-327. Retrieved from http://www.journals.elsevier.com/experimental-neurology/

Henkel, J. S., Beers, D. R., Zhao, W., & Appel, S. H. (2009). Microglia in ALS: The good, the bad, and the resting. *Journal of Neuroimmune Pharmacology : The Official Journal of the Society on NeuroImmune Pharmacology, 4*(4), 389-398. doi:10.1007/s11481-009-9171-5

Henkel, J. S., Engelhardt, J. I., Siklos, L., Simpson, E. P., Kim, S. H., Pan, T., . . . Appel, S. H. (2004). Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Annals of Neurology, 55*(2), 221-235. doi:10.1002/ana.10805

Hensley, K., Fedynyshyn, J., Ferrell, S., Floyd, R. A., Gordon, B., Grammas, P., . . . Williamson, K. S. (2003). Message and protein-level elevation of tumor necrosis factor alpha (TNF alpha) and TNF alpha-modulating cytokines in spinal cords of the G93A-SOD1 mouse model for amyotrophic lateral sclerosis. *Neurobiology of Disease, 14*(1), 74-80. Retrieved from http://www.journals.elsevier.com/neurobiology-of-disease/#

Imai, Y., Ibata, I., Ito, D., Ohsawa, K., & Kohsaka, S. (1996). A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochemical and Biophysical Research Communications, 224*(3), 855-862. doi:10.1006/bbrc.1996.1112

Kalesnikoff, J., Sly, L. M., Hughes, M. R., Buchse, T., Rauh, M. J., Cao, L. P., . . . Krystal, G. (2003). The role of SHIP in cytokine-induced signaling. *Reviews of Physiology, Biochemistry and Pharmacology, 149*, 87-103. doi:10.1007/s10254-003-0016-y

Kamen, L. A., Levinsohn, J., Cadwallader, A., Tridandapani, S., & Swanson, J. A. (2008). SHIP-1 increases early oxidative burst and regulates phagosome maturation in macrophages. *Journal of Immunology (Baltimore, Md.: 1950), 180*(11), 7497-7505. Retrieved from http://www.jimmunol.org/

Kettenmann, H., Hanisch, U. K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. *Physiological Reviews, 91*(2), 461-553. doi:10.1152/physrev.00011.2010

Krystal, G. (2000). Lipid phosphatases in the immune system. *Seminars in Immunology, 12*(4), 397-403. doi:10.1006/smim.2000.0222

Krystal, G., Damen, J. E., Helgason, C. D., Huber, M., Hughes, M. R., Kalesnikoff, J., . . . Humphries, R. K. (1999). SHIPs ahoy. *The International Journal of Biochemistry & Cell Biology, 31*(10), 1007-1010. Retrieved from http://www.journals.elsevier.com/the-international-journal-of-biochemistry-and-cell-biology/

Kuroda, E., Ho, V., Ruschmann, J., Antignano, F., Hamilton, M., Rauh, M. J., . . . Krystal, G. (2009). SHIP represses the generation of IL-3-induced M2 macrophages by inhibiting IL-4 production from basophils. *Journal of Immunology (Baltimore, Md.: 1950), 183*(6), 3652-3660. doi:10.4049/jimmunol.0900864

Lee, Y. G., Lee, J., Byeon, S. E., Yoo, D. S., Kim, M. H., Lee, S. Y., & Cho, J. Y. (2011). Functional role of akt in macrophage-mediated innate immunity. *Frontiers in Bioscience : A Journal and Virtual Library, 16*, 517-530. Retrieved from http://www.frontbiosci.org/

Leung, W. H., Tarasenko, T., & Bolland, S. (2009). Differential roles for the inositol phosphatase SHIP in the regulation of macrophages and lymphocytes. *Immunologic Research, 43*(1-3), 243-251. doi:10.1007/s12026-008-8078-1

Ling, E. A., Paterson, J. A., Privat, A., Mori, S., & Leblond, C. P. (1973). Investigation of glial cells in semithin sections. I. identification of glial cells in the brain of young rats. *The Journal of Comparative Neurology, 149*(1), 43-71. doi:10.1002/cne.901490104

Liu, L., Damen, J. E., Hughes, M. R., Babic, I., Jirik, F. R., & Krystal, G. (1997). The src homology 2 (SH2) domain of SH2-containing inositol phosphatase (SHIP) is essential for tyrosine phosphorylation of SHIP, its association with shc, and its induction of apoptosis. *The Journal of Biological Chemistry, 272*(14), 8983-8988. Retrieved from http://www.jbc.org/

Liu, Q., Sasaki, T., Kozieradzki, I., Wakeham, A., Itie, A., Dumont, D. J., & Penninger, J. M. (1999). SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes & Development, 13*(7), 786-791. Retrieved from http://genesdev.cshlp.org/

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology, 25*(12), 677-686. doi:10.1016/j.it.2004.09.015

March, M. E., & Ravichandran, K. (2002). Regulation of the immune response by SHIP. *Seminars in Immunology, 14*(1), 37-47. doi:10.1006/smim.2001.0340

Maresco, D. L., Osborne, J. M., Cooney, D., Coggeshall, K. M., & Anderson, C. L. (1999). The SH2-containing 5'-inositol phosphatase (SHIP) is tyrosine phosphorylated after fc gamma receptor clustering in monocytes. *Journal of Immunology (Baltimore, Md.: 1950), 162*(11), 6458-6465. http://www.jimmunol.org/

Miloux, B., Laurent, P., Bonnin, O., Lupker, J., Caput, D., Vita, N., & Ferrara, P. (1997). Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. *FEBS Letters, 401*(2-3), 163-166. Retrieved from http://www.febsletters.org/

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews.Immunology, 8*(12), 958-969. doi:10.1038/nri2448

Murata, T., Taguchi, J., & Puri, R. K. (1998). Interleukin-13 receptor alpha' but not alpha chain: A functional component of interleukin-4 receptors. *Blood, 91*(10), 3884-3891. Retrieved from http://bloodjournal.hematologylibrary.org/

Nakamura, K., Malykhin, A., & Coggeshall, K. M. (2002). The src homology 2 domain-containing inositol 5-phosphatase negatively regulates fcgamma receptor-mediated phagocytosis through immunoreceptor tyrosine-based activation motif-bearing phagocytic receptors. *Blood, 100*(9), 3374-3382. doi:10.1182/blood-2002-03-0787

Okun, E., Mattson, M. P., & Arumugam, T. V. (2010). Involvement of fc receptors in disorders of the central nervous system. *Neuromolecular Medicine, 12*(2), 164-178. doi:10.1007/s12017-009-8099-5

Ong, C. J., Ming-Lum, A., Nodwell, M., Ghanipour, A., Yang, L., Williams, D. E., . . . Mui, A. L. (2007). Small-molecule agonists of SHIP1 inhibit the phosphoinositide 3-kinase pathway in hematopoietic cells. *Blood, 110*(6), 1942-1949. doi:10.1182/blood-2007-03-079699

Ooms, L. M., Horan, K. A., Rahman, P., Seaton, G., Gurung, R., Kethesparan, D. S., & Mitchell, C. A. (2009). The role of the inositol polyphosphate 5-phosphatases in cellular function and human disease. *The Biochemical Journal, 419*(1), 29-49. doi:10.1042/BJ20081673

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A., & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology, 29*, 71-109. doi:10.1146/annurev-immunol-031210-101312

Peng, Q., Malhotra, S., Torchia, J. A., Kerr, W. G., Coggeshall, K. M., & Humphrey, M. B. (2010). TREM2- and DAP12-dependent activation of PI3K requires DAP10 and is inhibited by SHIP1. *Science Signaling, 3*(122), ra38. doi:10.1126/scisignal.2000500

Phee, H., Jacob, A., & Coggeshall, K. M. (2000). Enzymatic activity of the src homology 2 domain-containing inositol phosphatase is regulated by a plasma membrane location. *The Journal of Biological Chemistry, 275*(25), 19090-19097. doi:10.1074/jbc.M001093200

Philips, T., & Robberecht, W. (2011). Neuroinflammation in amyotrophic lateral sclerosis: Role of glial activation in motor neuron disease. *Lancet Neurology, 10*(3), 253-263. doi:10.1016/S1474-4422(11)70015-1

Pramatarova, A., Laganiere, J., Roussel, J., Brisebois, K., & Rouleau, G. A. (2001). Neuron-specific expression of mutant superoxide dismutase 1 in transgenic mice does not lead to motor impairment. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 21*(10), 3369-3374. Retrieved from http://www.jneurosci.org/

Ransohoff, R. M., & Cardona, A. E. (2010). The myeloid cells of the central nervous system parenchyma. *Nature, 468*(7321), 253-262. doi:10.1038/nature09615

Rauh, M. J., Ho, V., Pereira, C., Sham, A., Sly, L. M., Lam, V., . . . Krystal, G. (2005). SHIP represses the generation of alternatively activated macrophages. *Immunity, 23*(4), 361-374. doi:10.1016/j.immuni.2005.09.003

Rauh, M. J., Kalesnikoff, J., Hughes, M., Sly, L., Lam, V., & Krystal, G. (2003). Role of src homology 2-containing-inositol 5'-phosphatase (SHIP) in mast cells and macrophages. *Biochemical Society Transactions, 31*(Pt 1), 286-291. doi:10.1042/

Rauh, M. J., Sly, L. M., Kalesnikoff, J., Hughes, M. R., Cao, L. P., Lam, V., & Krystal, G. (2004). The role of SHIP1 in macrophage programming and activation. *Biochemical Society Transactions, 32*(Pt 5), 785-788. doi:10.1042/BST0320785

Reaume, A. G., Elliott, J. L., Hoffman, E. K., Kowall, N. W., Ferrante, R. J., Siwek, D. F., . . . Snider, W. D. (1996). Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nature Genetics, 13*(1), 43-47. doi:10.1038/ng0596-43

Rees, A. J. (2010). Monocyte and macrophage biology: An overview. *Seminars in Nephrology, 30*(3), 216-233. doi:10.1016/j.semnephrol.2010.03.002

Rohrschneider, L. R., Fuller, J. F., Wolf, I., Liu, Y., & Lucas, D. M. (2000). Structure, function, and biology of SHIP proteins. *Genes & Development, 14*(5), 505-520. Retrieved from http://genesdev.cshlp.org/

Rosen, D. R. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature, 364*(6435), 362. doi:10.1038/364362c0

Saijo, K., & Glass, C. K. (2011). Microglial cell origin and phenotypes in health and disease. *Nature Reviews.Immunology, 11*(11), 775-787. doi:10.1038/nri3086

Sanagi, T., Yuasa, S., Nakamura, Y., Suzuki, E., Aoki, M., Warita, H., . . . Ohsawa, K. (2010). Appearance of phagocytic microglia adjacent to motoneurons in spinal cord tissue from a presymptomatic transgenic rat model of amyotrophic lateral sclerosis. *Journal of Neuroscience Research, 88*(12), 2736-2746. doi:10.1002/jnr.22424

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., . . . Geissmann, F. (2012). A lineage of myeloid cells independent of myb and hematopoietic stem cells. *Science (New York, N.Y.), 336*(6077), 86-90. doi:10.1126/science.1219179

Sly, L. M., Ho, V., Antignano, F., Ruschmann, J., Hamilton, M., Lam, V., . . . Krystal, G. (2007). The role of SHIP in macrophages. *Frontiers in Bioscience: A Journal and Virtual Library, 12*, 2836-2848. Retrieved from http://www.frontbiosci.org/

Solomon, J. N., Lewis, C. A., Ajami, B., Corbel, S. Y., Rossi, F. M., & Krieger, C. (2006). Origin and distribution of bone marrow-derived cells in the central nervous system in a mouse model of amyotrophic lateral sclerosis. *Glia, 53*(7), 744-753. doi:10.1002/glia.20331

Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation. *The Journal of Experimental Medicine, 176*(1), 287-292. Retrieved from http://jem.rupress.org/

Swarup, V., Phaneuf, D., Dupre, N., Petri, S., Strong, M., Kriz, J., & Julien, J. P. (2011). Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor kappaB-mediated pathogenic pathways. *The Journal of Experimental Medicine, 208*(12), 2429-2447. doi:10.1084/jem.20111313

Takeuchi, S., Fujiwara, N., Ido, A., Oono, M., Takeuchi, Y., Tateno, M., . . . Urushitani, M. (2010). Induction of protective immunity by vaccination with wild-type apo superoxide dismutase 1 in mutant SOD1 transgenic mice. *Journal of Neuropathology and Experimental Neurology, 69*(10), 1044-1056. doi:10.1097/NEN.0b013e3181f4a90a

Tridandapani, S., Wang, Y., Marsh, C. B., & Anderson, C. L. (2002). Src homology 2 domain-containing inositol polyphosphate phosphatase regulates NF-kappa B-mediated gene transcription by phagocytic Fc

gamma Rs in human myeloid cells. *Journal of Immunology (Baltimore, Md.: 1950), 169*(8), 4370-4378. Retrieved from http://www.jimmunol.org/

Turnbull, I. R., Gilfillan, S., Cella, M., Aoshi, T., Miller, M., Piccio, L., . . . Colonna, M. (2006). Cutting edge: TREM-2 attenuates macrophage activation. *Journal of Immunology (Baltimore, Md.: 1950), 177*(6), 3520-3524. Retrieved from http://www.jimmunol.org/

Vallieres, L., & Sawchenko, P. E. (2003). Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 23*(12), 5197-5207. Retrieved from http://www.jneurosci.org/

Varin, A., & Gordon, S. (2009). Alternative activation of macrophages: Immune function and cellular biology. *Immunobiology, 214*(7), 630-641. doi:10.1016/j.imbio.2008.11.009

Wahle, J. A., Paraiso, K. H., Kendig, R. D., Lawrence, H. R., Chen, L., Wu, J., & Kerr, W. G. (2007). Inappropriate recruitment and activity by the src homology region 2 domain-containing phosphatase 1 (SHP1) is responsible for receptor dominance in the SHIP-deficient NK cell. *Journal of Immunology (Baltimore, Md.: 1950), 179*(12), 8009-8015. Retrieved from http://www.jimmunol.org/

Weisser, S. B., McLarren, K. W., Voglmaier, N., van Netten-Thomas, C. J., Antov, A., Flavell, R. A., & Sly, L. M. (2011). Alternative activation of macrophages by IL-4 requires SHIP degradation. *European Journal of Immunology, 41*(6), 1742-1753. doi:10.1002/eji.201041105

Weydt, P., Yuen, E. C., Ransom, B. R., & Moller, T. (2004). Increased cytotoxic potential of microglia from ALS-transgenic mice. *Glia, 48*(2), 179-182. doi:10.1002/glia.20062

Whittaker, G. C., Orr, S. J., Quigley, L., Hughes, L., Francischetti, I. M., Zhang, W., & McVicar, D. W. (2010). The linker for activation of B cells (LAB)/non-T cell activation linker (NTAL) regulates triggering receptor expressed on myeloid cells (TREM)-2 signaling and macrophage inflammatory responses independently of the linker for activation of T cells. *The Journal of Biological Chemistry, 285*(5), 2976-2985. doi:10.1074/jbc.M109.038398

Wijesekera, L. C., & Leigh, P. N. (2009). Amyotrophic lateral sclerosis. *Orphanet Journal of Rare Diseases, 4*, 3. doi:10.1186/1750-1172-4-3

Williams, D. B., & Windebank, A. J. (1991). Motor neuron disease (amyotrophic lateral sclerosis). *Mayo Clinic Proceedings.Mayo Clinic, 66*(1), 54-82. Retrieved from http://www.mayoclinicproceedings.org/

Wong, P. C., Pardo, C. A., Borchelt, D. R., Lee, M. K., Copeland, N. G., Jenkins, N. A., . . . Price, D. L. (1995). An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron, 14*(6), 1105-1116. Retrieved from http://www.cell.com/neuron/home

Wooley, C. M., Sher, R. B., Kale, A., Frankel, W. N., Cox, G. A., & Seburn, K. L. (2005). Gait analysis detects early changes in transgenic SOD1(G93A) mice. *Muscle & Nerve, 32*(1), 43-50. doi:10.1002/mus.20228

Yang, W. W., Sidman, R. L., Taksir, T. V., Treleaven, C. M., Fidler, J. A., Cheng, S. H., . . . Shihabuddin, L. S. (2011). Relationship between neuropathology and disease progression in the SOD1(G93A) ALS mouse. *Experimental Neurology, 227*(2), 287-295. doi:10.1016/j.expneurol.2010.11.019

Yoshihara, T., Ishigaki, S., Yamamoto, M., Liang, Y., Niwa, J., Takeuchi, H., . . . Sobue, G. (2002). Differential expression of inflammation- and apoptosis-related genes in spinal cords of a mutant SOD1 transgenic mouse model of familial amyotrophic lateral sclerosis. *Journal of Neurochemistry, 80*(1), 158-167. Retrieved from http://www.blackwellpublishing.com/jnc

Zhao, W., Xie, W., Xiao, Q., Beers, D. R., & Appel, S. H. (2006). Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia. *Journal of Neurochemistry, 99*(4), 1176-1187. doi:10.1111/j.1471-4159.2006.04172.x

Appendix A.

Solutions

PBST (500mL)

- 475mL distilled H2O
- 25mL PBS
- 1500µL Triton-X

EDTA-Antigen retrieval buffer

- 500mL ddH2O
- 0.185g EDTA

1) Adjust pH to 8.0 using NaOH
2) Add 0.25mL Tween20

1°and 2° Antibody Solution (4mL)

- 400µL NDS
- 0.12g BSA
- 3.48mL PBST

De Olmos

- 5g PVP40
- 150mL ethanediol
- 150g sucrose
- 25mL 20x PBS

4% PFA Solution

- 200mL 1x PBS
- 8g PFA

1) Stir @ 65℃
2) Add 12-14 NaOH pellets to dissolve

3) Wait ~15-20 minutes or until dissolved

4) Filter

5) Balance pH to 7.0