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IDENTIFICATION OF MACROPINOCYTOSIS REGULATING PROTEINS AND SIGNALING FROM MACROPINOSOMES

BY

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IDENTIFICATION OF MACROPINOCYTOSIS REGULATING PROTEINS AND SIGNALING FROM MACROPINOSOMES

This dissertation is approved as a creditable and independent investigation by a candidate for the Master in Biological Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Natalie Thiex, Ph.D. Date
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Dean, Graduate School Date
This work is dedicated to my lab family, Thiex lab, where I learned to appreciate science. To my parents, for their unconditional love and support throughout this process. To my fiancé for the encouragement and for believing in me even I when I didn’t. To my Brookings family, that have been there to make me feel home away from home and make this process more enjoyable, thank you!
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Understanding macrophage cell biology is important due to macrophages key roles in human health and diseases including proper immune function, wound healing, atherosclerosis, and cancer. Despite their importance, relatively little is understood about macrophage activation, growth factor signaling, and cytoskeletal regulation. This thesis presents data from investigations into mechanisms of macrophage growth factor signaling and actin polymerization for ruffling and macropinocytosis. Alt-R CRISPR-Cas9 method and dextran uptake assay were used to knock out individual genes (SHP-1, Lyn, Syk, BTK, Vav1) and determine their role in macropinocytosis and CSF-1R signaling. Dextran uptake was disrupted in SHP-1 and Lyn targeted knockout cells, while it was increased in Syk, BTK, and Vav1 targeted knockout. We showed that SHP-1 and Lyn knockout express more p-Y53 actin than wildtype. We propose that SHP-1 regulates macropinocytosis through dephosphorylation of actin at tyrosine 53. Lyn colocalized on macropinosomes with CSF-1R and Lyn knockout cells faster than wildtype, suggesting their negative role in regulating growth factor signaling. However, the absence of Lyn downregulated ERK phosphorylation, suggesting that Lyn might play a role of both positive and negative regulator of signaling.
Chapter I

LITERATURE REVIEW

I. Introduction

Macropinocytosis is an endocytic process used by different cell types to internalize extracellular fluid (Swanson and Watts 1995). The purpose of macropinocytosis can differ depending on the cell type (Swanson and Watts 1995). Cancer cells use macropinocytosis to take up nutrients in a nutrient-deprived environment such as the tumor microenvironment where they have to compete with other cells to acquire nutrients (Recouvreux and Commissio 2017). Macrophages and dendritic cells also use macropinocytosis for nutrient uptake and or to sample their environment for pathogens (Buckley and King 2017).

Emerging research suggests that endocytosis and macropinocytosis might be more than just an internalization process but can also serve as a signaling platform to control growth factor signaling (Sorkin and von Zastrow 2002, Huynh, Kwa et al. 2012). Different signaling proteins such as growth factor and their effector proteins are seen on macropinosomes (Bryant, Kerr et al. 2007, Huynh, Kwa et al. 2012).

Macropinocytosis is involved in pathogenesis such as cancer (Ha, Bidlingmaier et al. 2016, Recouvreux and Commissio 2017). Certain pathogens can trigger macropinocytosis to enter the cell (Pernet, Pohl et al. 2009). Despite the importance of macropinocytosis, little is known about how macropinocytosis is regulated in the cell. Macropinocytosis can be exploited to develop better therapeutics for diseases affected by macropinocytosis and can also be used as a drug delivery route (Kou, Sun et al. 2013). To be able to use macropinocytosis as a potential therapeutic, it is important to understand the mechanism that regulates macropinocytosis and also understand how macropinocytosis can control growth factor signaling.
Whole-genome screening of genes regulating macropinocytosis revealed the involvement of Src homology region 2 domain-containing phosphatase-1 (SHP-1), Lyn, Spleen Tyrosine kinase (Syk), Burton tyrosine kinase (BTK), and Vav1 in macropinocytosis. This is a novel function for these proteins that no other studies have shown. Lyn tyrosine kinase has also been found on macropinocytosis, but little is known about how Lyn regulates growth signaling or actin regulation in macrophages and macropinosomes. In this review, I will provide information necessary to understand the process of macropinocytosis and growth factor signaling in macrophages.

II. Macropinocytosis and cellular signaling

Macropinocytosis is an actin-dependent endocytic process used by cells to internalize large volumes of extracellular fluid containing nutrients and solutes through the formation of macropinosomes (Swanson and Watts 1995). Macropinosomes are heterogeneous in size and can range from 0.2 to 5 µM (Swanson and Watts 1995). Macropinocytosis can be spontaneous or induced by growth factor signaling such as colony stimulating factor 1 (CSF-1) or epidermal growth factor (EGF) (Bryant, Kerr et al. 2007, Yoshida, Pacitto et al. 2018).

In macrophages, the addition of CSF-1 induces actin cytoskeleton rearrangement which leads to irregular ruffling of the membrane (Yoshida, Gaeta et al. 2015). The ruffles will then close into internal vesicles called macropinosomes (Yoshida, Gaeta et al. 2015). The ruffles are in C-shaped at first, then turn into O-shaped ruffles, which then close to form a macropinosome (Yoshida, Gaeta et al. 2015). The first step from a C-shaped to an O-shaped is termed ruffle closure; and the latter step is called cup closure (Yoshida, Gaeta et al. 2015). Following their formation, macropinosomes mature and during the early stages of maturation, take on the early endosomes marker EEA1 and later the late endosomes marker Rab7 before fusing with the lysosomes (1993).

Studies of signaling molecules involved in CSF-1 stimulated macropinocytosis showed the involvement of Phosphoinositide 3-kinase (PI3K) and Ras in the early stage of
macropinocytosis (Welliver and Swanson 2012, Yoshida, Gaeta et al. 2015). PI3K is an enzyme known to phosphorylate and generate different types of phosphatidylinositol (a lipid in the cell membrane) (Engelman, Luo et al. 2006). Observation of the early stages of macropinocytosis revealed the transition of different types of phosphatidylinositol and proteins (Welliver and Swanson 2012). (1) phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P2) appears directly after ruffle closure, (2) phosphatidylinositol(3,4,5)-triphosphate (PIP3), Rac1 activation, and diglycerol (DAG; another type of membrane lipid) occur shortly after PI(4,5)P2 (3) phosphatidylinositol(3,4)-bisphosphate(PI(3,4)P2) appears transiently during cup closure, and (4) phosphatidylinositol3-phosphate (PI3P) appearance, Ras activation, Rab5a and PKCα localization to macropinosomes formation sites happen during or after cup closure (Welliver and Swanson 2012).

Comparison of CSF-1 and PMA induced macropinocytosis showed a correlation between macropinosomes formation and mammalian target of rapamycin (mTOR) activation (Racoosin and Swanson 1989, Yoshida, Gaeta et al. 2015). mTOR is a kinase that regulates cellular growth and metabolism in response to growth factor and nutrient availability (Hall 2008). CSF-1 stimulation of BMM induced mTOR activation within 5 min of stimulation, which coincides with macropinosomes formation (Yoshida, Gaeta et al. 2015). On the other hand, PMA activation of mTOR was delayed, 30 min post stimulation, correlating with the delay in macropinosomes formation in PMA stimulated cells (Yoshida, Gaeta et al. 2015). A different experiment showed that macropinosomes induced mTOR activation by bringing amino acids into the cell (Pacitto, Gaeta et al. 2017). Macrophages were stimulated with CXCL12 in amino acid-rich or amino acid-free medium. Cells stimulated with both treatments were able to form macropinosomes, but only cells in amino acid-rich medium were able to activate mTOR (Pacitto, Gaeta et al. 2017). This suggested that macropinocytosis is one of the main source of amino acids which are important for cellular growth.

In addition to macropinocytosis, there are other endocytic processes used by the cells to internalize outside materials. There are two main types of endocytosis, clathrin-
mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (Naslavsky, Weigert et al. 2004).

Clathrin-mediated endocytosis (CME) is the major endocytic pathway in mammalian cells. It is used for internalization of transmembrane proteins, regulation of signaling and remodeling of the plasma membrane. In CME, clathrin-coated vesicles bud off the plasma membrane and are taken up into the cell, forming endosomes. CIE encompasses internalization mechanisms that do not require clathrin and can be characterized by large-scale processes such as macropinocytosis and phagocytosis and small-scale processes such as caveolin. The large-scale processes involved internalization of large particle and membrane volume. Though phagocytosis and macropinocytosis have some similarities, some differences are important to note. Unlike macropinocytosis, phagocytosis involves internalization of large solid particles and is induced by receptor activation. Membrane-bound receptors rearrange the plasma membrane and actin cytoskeleton around the particle to facilitate ingestion. Because phagocytosis internalizes solid particles, the particle provides a template that determines the size of the phagosome (Buckley and King 2017).

Actin polymerization is involved in macropinocytosis (Lee and Knecht David 2002). Visualization of actin dynamics during macropinocytosis in Dictyostelium revealed an increase in F-actin surrounding macropinocytotic cup (Lee and Knecht David 2002). Actin polymerization resulted in protrusion of the membrane followed by bifurcation of the protrusion to form an invagination that increased in size (Lee and Knecht David 2002). Analysis of this enlargement in invagination showed that it might be due not only to actin polymerization but also due to inward pulling of actin-rich regions (Lee and Knecht David 2002). Because macropinocytosis requires actin polymerization, genes controlling actin polymerization must be important for macropinocytosis. Proteins regulating actin polymerization include Bruton tyrosine kinase (BTK), Spleen tyrosine kinase (Syk) and VAV1 (Mohammad, Nore et al. 2013).
Vav1 is Guanine exchange factor (GEF) for GTPases that activate actin cytoskeleton rearrangement such as rac1 and Cdc42 (Oberley, Wang et al. 2012, Bustelo 2014). Nonphosphorylated Vav1 display a closed conformation that leads to an inactive state (Bustelo 2014). The structure of Vav1 is composed of a PH domain that allows it to translocate to the plasma membrane by binding to PIP3, an SH2 and two SH3 domain allowing protein-protein interaction and a CH domain that serves as a calcium mobilization and an actin-binding domain (Oberley, Wang et al. 2012). Vav1 is activated by protein tyrosine kinases such as SFK, TFK, and Syk. CSF-1 stimulation of macrophages induced Vav1 phosphorylation and Rac1 activation, leading to chemotaxis (Vedham, Phee et al. 2005). Knockout of SHIP1, a phosphatase of PIP3, led to a constitutively active Vav1 and Rac1, which led to an increase chemotaxis in macrophages (Vedham, Phee et al. 2005). Macrophages with highly active Vav1 also showed an elevated level of F-actin, suggesting a role of Vav1 in actin polymerization (Vedham, Phee et al. 2005).

BTK is a protein of the Tec family kinases (TFK) (Mohammad, Nore et al. 2013). It contains five distinct domains namely, a pleckstrin homology (PH) domain, that enables BTK to bind to phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (4,5)-bisphosphate; a Tec homology (TH) domain, a Src homology (SH) 2 and 3 domain and a C-terminal Kinase domain (Mohammad, Nore et al. 2013). Mutation in the PH domain, as well as inhibition of Pi3k, attenuates BTK activation, suggesting that translocation of BTK to plasma membrane domains is important for its activation (Takesono, Finkelstein et al. 2002). TFKs, including BTK, are known to be phosphorylated, thus activated by SFKs (Takesono, Finkelstein et al. 2002).

Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase expressed in all hematopoietic cells. It contains two SH2 domains and a kinase domain (Mócsai, Ruland et al. 2010). In macrophages, Syk is known to regulates inflammatory responses and is one of the upstream signaling proteins that phosphorylates several downstream effector proteins (Yi, Son et al. 2014). Syk is activated by autophosphorylation as well as
phosphorylation by other tyrosine kinases. Proteomic analysis showed several proteins that bind to Syk; among those are Vav1, BTK, PI3K, and ERK (Yi, Son et al. 2014).

Src homology domain-containing phosphatase-1 (SHP-1) has also been found to regulate actin polymerization by dephosphorylating actin at tyrosine residue 53. SHP-1 is a protein tyrosine phosphatase mainly expressed in hematopoietic cells (Lorenz 2009). It is encoded by the protein tyrosine phosphatase non-receptor type 6 (Ptpn6) gene. This phosphatase is mainly known as a negative regulator of signal transduction leading to cell growth and proliferation in hematopoietic cells (Yuan, Ma et al. 2017). Mice with loss of SHP-1 expression show hyperproliferation and activity of myeloid cells (Lorenz 2009). In addition to playing the role of a negative regulator of cell growth and function, SHP-1 has also been found to regulate actin reorganization (Baba, Fusaki et al. 2003). Immunoprecipitation analysis of SHP-1 and actin in B cells showed that SHP-1 directly binds to actin (Baba, Fusaki et al. 2003). In B cells with defective SHP-1, actin polymerization was normal while actin depolymerization was compromised (Baba, Fusaki et al. 2003). This experiment showed that SHP-1 was needed to dephosphorylate actin to enable proper actin depolymerization.

Lyn is a member of Src family kinase proteins (SFK) (Hibbs and Harder 2006). Most SFKs have been known to transduce signal for growth, proliferation, and survival in myeloid cells (Hibbs and Harder 2006). However, Lyn is different from most SFK in that it is known as both a positive and negative regulator of signals (Hibbs and Harder 2006). Mice deficient in Lyn showed an increasing number of myeloid cells, suggesting that Lyn plays the role of negative regulator of growth factor signaling (Harder, Parsons et al. 2001). In macrophages, Lyn negatively regulates CSF-1R induced AKT signaling by phosphorylating SHIP-1 (Baran, Tridandapani et al. 2003). In addition, immunoprecipitation analysis of Lyn showed that Lyn constitutively binds to CSF-1R before and after binding of CSF-1 ligand to CSF-1R (Dwyer, Mouchemore et al. 2016). However, Lyn phosphorylation did not change upon activation of CSF-1R (Dwyer, Mouchemore et al. 2016)
These proteins have been studied efficiently in B cells where they are known to positively or negatively regulate B cell signaling. SYk, BTK, and Vav1 were described as positive regulators of B cells (Ying, Li et al. 2011, Alsadeq, Hobeika et al. 2014). SHP-1 is known to negatively regulate B cell signaling (Alsadeq, Hobeika et al. 2014). Lyn-deficient B cells are hyperactive suggesting that Lyn is a negative regulator of B cell signaling (Lamagna, Hu et al. 2014).

III. Colony-stimulating factor receptor (CSF-1R)

Colony-stimulating factor-1 receptor (CSF-1R) is a macrophage growth factor receptor that mediates the growth, differentiation, and proliferation of macrophages by binding to the growth factor, colony stimulating factor-1 (CSF-1) (Huynh, Kwa et al. 2012). Binding of CSF-1 to the receptor causes CSF-1R to dimerize and trans-auto-phosphorylate, thus become activated (Huynh, Kwa et al. 2012)Figure 1.1). Upon its activation, CSF-1R is internalized into small endosomes and induces downstream signal transduction (Lou, Low-Nam et al. 2014) Figure 1.1). After CSF-1R internalization, the small endosomes containing CSF-1R mature into early endosomes, and later into late endosomes. These endosomes are characterized by rab5 and rab7 proteins respectively (Lou, Low-Nam et al. 2014). CSF-1R is trafficked to macropinosomes that will fuse with endolysosomes to facilitate degradation of the receptor (Lou, Low-Nam et al. 2014).
Phosphorylation of CSF-1R at different sites provides binding sites for many adaptor molecules such as grb2 and other signaling molecules such as Src family kinases (SFKs) (Stanley and Chitu 2014). These proteins contain a Src homology 2 (SH2) domain that allows them to bind to phospho-tyrosine residues of CSF-1R and other Tyrosines Kinases (Filippakopoulos, Müller et al. 2009). Binding of these signaling proteins to CSF-1R allows them to be activated and begin a downstream signaling cascade leading to cellular responses such as growth, proliferation, and survival. Some of the pathways include the Ras/MAPK pathway and the phosphatidylinositol 3 kinase(PI3K)/AKT pathway (Stanley and Chitu 2014). Binding of the adaptor molecule Grb2 that allows activation of son of sevenless (SOS). SOS is a guanine exchange factor (GEF) that activates Ras, a monomeric GTPase molecule, by promoting the exchange of GDP for GTP. Active Ras (Ras-GTP) induces a MAP kinase cascade which leads to phosphorylation of Erk1/2 (Li, Zhao et al. 2016). This pathway is important for cell proliferation (Stanley and Chitu 2014). The PI3K pathway can be activated either directly from active CSF-1R.
or through activation of Ras (Castellano and Downward 2011, Sampaio, Yu et al. 2011). Activation of PI3K leads to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-bisphosphate (PIP3). AKT has a pleckstrin homology (PH) domain that allows it to bind to PIP3 and translocate to the plasma membrane to be activated (Carnero and Paramio 2014). AKT activation, in turn, mediates activation of mammalian target of rapamycin (mTOR) (Gao, Flynn et al. 2004), which in turn regulates the metabolic processes of the cell (Yoshida, Pacitto et al. 2018). Many studies on RTK signaling have focused on signaling from the plasma membrane, implying that signaling occurs only at the plasma membrane and that internalization of these receptors was merely a mechanism for signal attenuation through degradation or recycling of the receptor (Pixley and Stanley 2004). However, recent studies suggest that internalization of CSF-1R is important for proper signaling (Huynh, Kwa et al. 2012). Huynh et al. demonstrated that impairing endocytosis of CSF-1R using a dynamin inhibitor led to improper signaling of downstream signaling proteins such as ERK 1/2 and AKT (Huynh, Kwa et al. 2012). They also showed that inactivating CSF-1R with CSF-1R inhibitor GW2580, following its internalization resulted in inactivation of ERK 1/2 as well. These results led to the conclusion that continuous CSF-1R signaling in endosomes was important for proper signaling.

Exposure of macrophages to CSF-1 upregulates the formation of macropinosomes within 2 to 5 minutes of stimulation (Lou, Low-Nam et al. 2014). In an experiment, Lou et al. showed that CSF-1R was first internalized through small endosomes before the endosomes containing CSF-1R were delivered to the macropinosomes. Moreover, other receptors such as CXCL12 induced macropinocytosis but these receptors were not trafficked to macropinosomes, showing a selectivity in recruiting only CSF-1R to the macropinosomes (Lou, Low-Nam et al. 2014). Inhibition of macropinocytosis delayed CSF-1R degradation (Lou, Low-Nam et al. 2014). These data raise the question of whether macropinosomes are a signaling platform of convergence for CSF-1R signaling amplification and attenuation.
In addition to signaling for growth, differentiation, and proliferation, CSF-1R also signals for cytoskeletal rearrangement through actin polymerization (Sampaio, Yu et al. 2011). Cells starved of CSF-1 showed a rounded morphology; when stimulated with CSF-1, actin reorganization was rapidly induced and cells formed membrane ruffles, lamellipodia, and filopodia (Allen, Jones et al. 1997). Analysis of Y721F CSF-1R mutant showed that cells with this mutation were unable to form ruffles (Sampaio, Yu et al. 2011). Immunoprecipitation was used to identify which effector protein bind to p-Y721, and it was found that PI3K was the major effector protein that binds to CSF-1R Y721 to signal for actin polymerization (Sampaio, Yu et al. 2011). To confirm that PI3K was indeed the effector protein through which CSF-1R induced actin polymerization, PI3K activity was inhibited with wortmannin; treatment of cells with wortmannin 30 min prior to CSF-1 stimulation prevented CSF-1 induced ruffling and actin polymerization (Sampaio, Yu et al. 2011).

Macropinocytosis is a cellular process that has not been studied thoroughly but has been identified in many disorders such as cancer, pathogenesis, neurodegenerative diseases and atherosclerosis (Aleksandrowicz, Marzi et al. 2011, Zeineddine and Yerbury 2015, Bloomfield and Kay 2016). Macropinosomes can be a potential signaling platform where signaling can either be amplified or downregulated, giving the cell the ability to control and organize different signaling pathways. Understanding the mechanism of macropinosomes formation, and how signaling is organized on macropinosomes is an important step to be able to exploit macropinosomes as a potential cure.
IV. References


14-3-3ζ, Regulates Shuttling, and Attenuates both Tonic and Induced Signaling in B Cells." Molecular and Cellular Biology 33(16): 3214-3226.


Chapter 2

IDENTIFICATION OF ACTIN-RELATED GENES THAT REGULATE MACROPINOCYTOSIS

I. Introduction

Macropinocytosis is an actin-dependent endocytic process used by cells to internalize large volumes of extracellular fluid through the formation of macropinosomes (Swanson and Watts 1995). Macropinocytosis is upregulated in macrophages where it can be used to capture nutrients and antigens (Lim and Gleeson 2011). Growth factor signaling is known to upregulate macropinocytosis, but macropinocytosis can also happen simultaneously (Yoshida, Gaeta et al. 2015). Despite the importance of macropinocytosis in both macrophages and other cell types, the genes and pathways that govern macropinocytosis are not known.

Actin polymerization is known to be involved in macropinosome formation (Lee and Knecht David 2002). In Dictyostelium, the presence of F-actin during the beginning stages of macropinosomes formation is transient, lasting only 30 to 50 s (Lee and Knecht David 2002). Macropinosome formation begins with the formation of a membrane ruffle that turns into a circular ruffle and closes upon itself to form the macropinosomes (Swanson 2008). This mechanism requires a tight regulation of actin polymerization. Actin polymerization must be restricted to the walls of the circular ruffle and inhibited at other sites (Bloomfield and Kay 2016). However, the way actin polymerization is regulated during macropinocytosis is not understood (Bloomfield and Kay 2016).

A CRISPR/Cas9 whole genome screening was used to determine genes and pathways necessary for macropinocytosis in macrophages. Ptpn6, which encodes the non-receptor tyrosine phosphatase SHP-1, was a major hit required for CSF-1 stimulated and unstimulated macropinocytosis (Fig 3.1). SHP-1 guide RNAs (Table 3.1) were enriched in cells (LFC= 3.3363 FDR= 0.000087) unable to properly internalize dextran (low drinkers) while the guide RNA inserts read count for SHP-1 was much lower in cells able to
internalize a higher concentration of dextran (high drinkers) (figure 3.1). SHP-1 is known to dephosphorylate actin at tyrosine 53 and therefore regulate actin polymerization (Baba, Fusaki et al. 2003). Although SHP-1 is known to be implicated in actin polymerization, no studies have shown the involvement of SHP-1 in macropinocytosis. In addition to SHP-1, other genes that were hit in the macropinosome screen included Lyn, Syk, BTK, and Vav1. The guide RNAs for Syk, BTK, and Vav1 were enriched in high drinkers, while the guide RNA for Lyn were enriched in low drinkers (figure 2.1 b-e; Table 3.1). These genes are known to be important for actin polymerization (Strijbis, Tafesse et al. 2013, Jaumouillé, Farkash et al. 2014), however, their specific roles in macropinocytosis are not known.

Macropinocytosis requires a tight regulation of actin polymerization (Lee and Knecht David 2002). Understanding how actin polymerization is regulated in macropinocytosis could give an additional insight into the mechanism of macropinocytosis...

II. Materials and methods

Reagents: Dulbecco's modified eagle medium (DMEM) (Corning, Manassas, VA), DPBS (2.67 mM potassium chloride, 1.47 mM Potassium Phosphate Monobasic, 136.9 mM NaCl, 8.1 mM Sodium phosphate dibasic; #SH30028.02, GE Healthcare Life Sciences, Pittsburgh, PA), HBSS (#BW10-543F, ThermoFisher Scientific, Waltham, MA), Fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), bovine serum albumin powder (ThermoFisher Scientific, Waltham, MA), normal goat serum (NGS; #5425S; Cell Signaling Technology), normal chicken serum (NCS; #16110082; ThermoFisher Scientific, Waltham, MA), NucBlue Fixed stain cell stain ReadyProbe (Invitrogen), NucBlue live cell Stain ReadyProbe (Invitrogen) (ThermoFisher Scientific, Waltham, MA), 12 mm glass coverslips (ThermoFisher Scientific, Waltham, MA), CSF-1R antibody (AFS98, eBioscience), p-ERK antibody (#9101, Cell Signaling Technology), pCSF-1R(Y721) antibody (#49C10, Cell Signaling Technology) SHP-1 antibody (#ab60268, abcam), pY536-SHP-1 antibody (#ab41437, abcam), pY53-actin antibody (#bs-12581R,
BiossAntibodies) were used for immunofluorescence, goat anti-Rat Dylight 594 and Goat anti-Rabbit Dylight 488 conjugated secondary antibodies (ThermoFisher Scientific) were used for detecting primary antibodies. Texas-Red Dextran (40 kD; #D1864, ThermoFisher Scientific) was used to label macropinosomes. Phalloidin (ThermoFisher Scientific, Waltham, MA), was used as a cell mask and to label filamentous actin, Fluoromount-G (Southern Biotech, Birmingham, AL) to mount coverslips.

**Bone marrow media preparation:** Bone marrow media (BMM) was prepared using 20% heat-inactivated fetal bovine serum (HI FBS), 30% L-cell conditioned media, 0.7% penicillin/streptomycin 100X (pen-strep) and 0.0004% β mercaptoethanol (ThermoFisher Scientific, Waltham, MA) in Dulbecco's modified eagle medium (DMEM). The mixture was filtered using a vacuum filter (EMD Millipore Darmstadt, Germany).

**Bone marrow macrophage isolation and culture:** Mice were euthanized with CO₂, and femurs were collected, being careful to maintain the integrity of the femur. Both ends of the femur were cut to collect the bone marrow by flushing with phosphate buffered saline containing mM (DPBS) in a 0.5 inch, 26-gauge needle and 5 ml sterile Luer Lock syringe. The collected bone marrow was centrifuged at 500 g for 5 minutes and the cell pellet resuspended in bone marrow medium (BMM). Cells were plated on a sterile 10 cm non-tissue culture dish at 10⁶ cells per dish, in 10 ml of BMM. Cells were placed in an incubator at 37°C and 5% CO₂ for two days. On day 2, an additional 10 ml of fresh pre-warmed BMM was added to the dish. After an additional 2 days, the media was removed and replaced with fresh pre-warmed BMM as the macrophages were adherent. Every 2 days, the media was replaced with fresh BMM to maintain an optimal level of nutrients. For experiments, cells were detached with ice-cold PBS lacking calcium and magnesium and re-plated to appropriate plates or coverslips (Weischenfeldt and Porse 2008).

**Plating cells:** Cells previously frozen in liquid nitrogen were thawed at 37°C and resuspended in 10 ml of BMM. After resuspension, cells were centrifuged at 300 g for 5
min and resuspended in BMM. Cells were counted using a hemocytometer and Trypan blue. Cells were then plated in a 96-well plate at a density of $3 \times 10^4$ to $3.5 \times 10^4$ cells per well in 100 μl of BMM; in a 24-well plate at a density of $7.5 \times 10^4$ cells per well in 1 ml of BMM; in a 6 cm dish at $3 \times 10^5$ cells per plate in 3 ml of BMM; in a 10 cm dish at $10^6$ cells in 10 ml of media.

For microscopy experiments, cells were plated on 12 mm glass coverslips in a 24-well plate. The coverslips were sterilized by flaming with 95% ethanol.

**Alt-R CRISPR-Cas9 gRNA transfection:** Guide RNA sequences targeting genes of interest were selected from the GeCKO or Brie libraries (Sanjana, Shalem et al. 2014, Doench, Fusi et al. 2016). SHP-1, Vav1, Lyn, Syk, and BTK Synthetic CrRNA oligos (Table 2.1; Integrated DNA Technologies) were complexed with Tracer RNA (enables association with Cas9) in a 1:10 molar ratio to make the guide RNA (ref). To transfect BMDM, the 10 picomoles guide RNA was complexed with INTERFERin siRNA transfection reagent (Polyplus) and added to Cas9-expressing BMDM. The RNA complex was incubated with cells for 6 to 18 hours before removing the transfection media. Cells were incubated for 7 to 10 days to allow for the majority of proteins to be degraded.

**Detaching cells from culture:** Cells were detached from the plate with cold modified DPBS -Ca-Mg. Bone marrow media was first removed from culture, cells were washed with 5 ml of cold DPBS to remove extra BMM. 10 ml of 4°C DPBS -Ca/-Mg was added to the dish and cells were then incubated at 4°C for 15 minutes. Macrophages were further detached by pipetting the media up and down. The solution was then placed in a 15 ml tube and centrifuged for 5 min at 300 g. the pellet was resuspended in BMM.

**CSF-1R trafficking:** To synchronize trafficking and signaling and to upregulate CSF-1R on the plasma membrane, cells were deprived of CSF-1. 24h prior to the experiment, BMM was removed and replaced with DMEM plus 10% FBS to upregulate CSF-1R expression. CSF-1 was added at a concentration of 0.2 μg/ml in DMEM 10% FBS or LCIB for different
time points and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature or 100% ice-cold methanol for 10 min at -20°C (Lou, Low-Nam et al. 2014).

Dextran uptake assay by flow cytometry: To measure macropinocytosis, dextran uptake was measured by flow cytometry. Transfected cells were stimulated with 40 kDa Texas-red dextran and 100 ng/ml CSF-1 for 15, 30 or 60 min. Negative control cells were exposed to dextran for the same times on ice (cold block). Cells were washed and detached with cold DPBS. Cells were analyzed by flow cytometry for the ability to internalize dextran. Cells unable to internalize dextran correctly and cells over-internalizing dextran compared to non-transfected cells were successfully knocked out.

Dextran uptake and macropinosomes phenotype by high content microscopy: Cells were plated on an optical-quality 96 well plate. Once cells adhered to the plate and recovered from plating, cells were exposed to 250 µg/ml 40 kDa Alexa 647 conjugated dextran and nuclear stain for 3 min and the dextran was washed off the cells 10 times with HBSS. Cells were imaged with high content microscopy.

Immunofluorescence and phalloidin staining: Immunofluorescence was done to visualize protein expression, location in the cell, and phosphorylation. Bone marrow macrophages plated on coverslips were stimulated with CSF-1 for different time points. Cells were fixed with 4% PFA for 15 minutes (100% methanol for 1 min). blocking for non-specific binding was done using 5% NGS plus 0.3% triton-X if fixed with PFA, or 5% NGS if fixed with methanol. Primary antibodies were added and incubated for 1h to 18h, and secondary antibodies were incubated for 1 to 2h. Primary and secondary antibodies were diluted in 1% BSA plus 0.3% triton-X (no triton-X if fixed with methanol). Phalloidin was added for 20 min to stain for filamentous actin.

Image analysis: Immunofluorescence images were acquired using either a high content microscope or small inverted Leica CTR4000 microscope using a 60X oil lens EVOS air lens. image analysis and quantification were done using ImageJ (Schneider, Rasband et al. 2012) and CellProfiler (Carpenter, Jones et al. 2006) open-access software.
III. Results

*CRISPR/Cas9 deletion of Ptn6/SHP-1 in BMDM inhibits macropinocytosis*

To test whether SHP-1 was a promotor of macropinocytosis, dextran uptake by SHP-1 KO cells was compared to wildtype (WT) cells using flow cytometry and high content microscopy (HCM). SHP-1 KO cells were unable to internalize dextran in both assays (figure 2.2). In the flow assay, the mean fluorescence intensity in SHP-1 KO was 2-fold lower than the WT cells at 5, 15 and 60 minutes post dextran exposure to BMDM (p-value <10^{-4}, figure 2.2A and table 2.1). In the HCM assay, the phenotype of SHP-1 KO looked different than WT, with less dextran uptake than WT (figure 2.2.B)

Percent of cells inside of left-shifted low dextran uptake that recapitulate screen phenotype and may be due to SHP-1 deletion. Percentage of cells with low dextran-uptake correlates with SHP-1 immunostaining showing cells with less SHP-1 expression than WT (figure 2.2. C and D).

*CRISPR/Cas9 deletion of Lyn in BMDM disrupt macropinocytosis*

To determine the role of Lyn in macropinocytosis, dextran uptake by Lyn KO cells was compared to WT. the results were analyzed by flow cytometry and high content microscopy. The mean fluorescence intensity of Lyn KO cells was 0.14-fold lower than WT at 15 and 60 min post dextran exposure (p-value < 10^{-4}; figure 2.3). At 5 min post dextran exposure, the mean fluorescence of Lyn was higher than WT (p-value < 10^{-4}). The phenotype of Lyn KO cells looked similar to WT in HCM assay (figure 2.3.B).

*SHP-1 KO cells are unable to close their circular ruffles*

Observation of SHP-1 KO cells with a brightfield microscope revealed a distinct phenotype of SHP-1 KO cells (figure 2.3.A). These cells appeared bigger with wide lamellipodia compared to the WT BMDM (figure 2.3.A). In addition, no macropinosomes were present in those cells, while macropinosomes could clearly be seen in the WT cells
To determine what stage of macropinosome formation was defective in those cells, live cell imaging movie was taken during macropinosomes formation. Cells were starved of CSF-1 to upregulate the expression of CSF-1R on the surface of the plasma membrane and synchronize the biology of the cells. Addition of CSF-1 to WT and SHP-1 KO cells caused the cells to ruffle (figure 2.3.B). WT formed linear ruffles at 1 min followed by circular ruffles (ruffle closure) at 1:45 min. The circular ruffles closed (cup closure) after 3 min (figure 2.3.B, top panel). On the other hand, SHP-1 began to ruffle and almost turned their linear ruffles into circular ruffles at 1 min (figure 2.3.B, bottom panel). In contrast to WT that mainly forms ruffles at polar ends of the cell, SHP-1 KO cells formed ruffles all around the cell (figure 2.3.B). At 1:45 min, the ruffle in SHP-1 KO flattened and did not turn into a circular ruffle in the subsequent min (figure 2.3.B, bottom panel). WT cells had many macropinosomes at 7 min while SHP-1 KO had no macropinosomes (figure 2.3.B).

Excessive filamentous actin (F-actin) gives SHP-1 KO cells a distinct phenotype

We suspected that excessive or spatially non-restricted actin polymerization was responsible for the large lamellipodia phenotype in SHP-1 KO BMDM. To test our hypothesis, we compared the amount, distribution and location of F-actin in SHP-1 KO and WT BMDM by staining with phalloidin following the addition of CSF-1 to starved cells for different time points. In WT, the amount of F-actin was higher before CSF-1 stimulation and decreased after CSF-1 stimulation (p-value < 0.0001; figure 3.5 top panel). In SHP-1 KO cells, the amount of F-actin was lower before CSF-1 stimulation and increased at 5 min post CSF-1 addition (P-value < 0.10^-4; figure 2.5 middle panel). The distribution of F-actin was also different for SHP-1 KO cells compared to WT (figure 2.5). Before the addition of CSF-1, the distribution of F-actin was polarized in WT; in SHP-1 KO cells, F-actin was distributed throughout the cell (figure 2.5). At 2.5 min post-CSF-1 stimulation, both WT and SHP-1 KO have F-actin concentrated on membrane ruffles. However, the SHP-1 KO cells had mainly bigger ruffles that looked almost as if the whole cell was ruffling (figure 2.5). The WT cells had smaller ruffles and the ruffles were
polarized to either side of the cell (figure 2.5). consistent with the brightfield images (figure 2.4.B).

We also observed the distribution of F-actin in Lyn KO cells (figure 2.5, lower panel). Before CSF-1 stimulation, Lyn KO cells had less F-actin than WT (p-value < 0.001; figure 2.5). At 2.5 min and 5 min post-CSF-1 addition, the amount of F-actin in Lyn KO cells increased and was higher than WT (p-value < 0.0001; figure 2.5). at 7.5 min, the amount of F-actin was similar to WT (figure 2.5). the distribution of F-actin in Lyn KO cells similar to WT with polarized ruffles (figure 2.5).

*SHP-1 KO cells contain more pY53 actin than wildtype*

SHP-1 is phosphatase that has been described to dephosphorylate actin at tyrosine 53, leading to more F-actin concentration (Baba, Fusaki et al. 2003, Bertling, Englund et al. 2016). To test whether SHP-1 KO cells had a higher concentration of F-actin due to excessive actin phosphorylation at tyrosine 53 (Y53), we compared the concentration of pY53 in SHP-1 KO and WT. Overall, SHP-1 KO had more pY53 actin compared to WT cells (P value < 10^-4; figure 2.4). The concentration of pY53 actin in WT was constant through the CSF-1 time course, while the concentration of pY53 actin in SHP-1 KO increased at 2.5 min post-CSF-1 stimulation (P-value < 0.0001) and stayed constant up to 7.5 min post stimulation (figure 2.4.B). pY53 localized more in the perinuclear region on both WT and SHP-1 KO, with some localization on membrane ruffles (figure 2.4.A and C).

Lyn is a kinase that has been described to phosphorylate SHP-1 at tyrosine 536 and activate it phosphatase activity (Xiao, Ando et al. 2010). We hypothesized that Lyn activates SHP-1 and that in the absence of Lyn, SHP-1 would be inactive and thus unable to dephosphorylate actin. To determine if Lyn was involved in the actin regulation through SHP-1, an immunostaining of pY53 actin was done in cells constitutively exposed to CSF-1. Lyn KO cells had more pY53 actin than WT (p-value < 0.001; figure 2.7).
**SHP-1 localizes on membrane ruffles and macropinosomes in BMDM**

To better understand the action of SHP-1 in macropinocytosis, we did an immunofluorescence assay, staining SHP-1 in BMDM. SHP-1 was located on macropinosomes, membrane ruffles and on perinuclear compartment (figure 2.8). Unstimulated cells showed the presence of SHP-1 on the cell surface (figure 2.8). from 2.5 to 7.5 min, SHP-1 localized on membrane ruffles and macropinosomes (figure 2.8).

**Other actin-related proteins are negative regulators of macropinocytosis**

The MP screen revealed the involvement of Vav1, Syk, and BTK in macropinocytosis regulation. These proteins were categorized as high drinkers, suggesting that they are negative regulators of CSF-1 stimulated macropinocytosis. To confirm that these genes were indeed involved in the negative regulation of macropinocytosis, each gene was individually knocked out in macrophages. To confirm that these genes were knockout and were high drinkers we performed a dextran uptake assay and analyzed by flow cytometry and high content microscopy. Flow cytometry analysis of dextran uptake assay showed that all four target knockouts internalized more dextran than WT at 5, 15, and 60 min dextran exposure (p-values < 10^{-4}; figure 2.9). For dextran uptake assay analysis by high content, Syk and Vav1 show similar results as the flow, while BTK internalized less dextran than the WT (figure 2.10). To determine the distribution and the amount of F-actin in these Alt-R CRISPR Cas 9 targeted knockouts, a phalloidin staining was done. Each of the targeted knockouts had a difference F-actin structure (figure 2.11)

**IV. Discussion**

Alt-r Crispr-cas 9 genome editing method was used to knockout SHP-1 in BMDM and determine its role in macropinocytosis. We have shown that SHP-1 is a critical regulator of macropinocytosis. In absence of SHP-1, macrophages were unable to take up dextran, suggesting that SHP-1 is a positive regulator of macropinocytosis. These knockout cells
also exhibited a phenotype with large lamellipodia compared to the WT. Phalloidin staining of these cells revealed that SHP-1 KO cells had more F-actin than the WT and more pY53 actin than WT. Studies on actin regulation showed that SHP-1 regulates actin dynamics by dephosphorylating actin (Baba, Fusaki et al. 2003). This study suggested that dephosphorylation of actin at tyrosine 53 was important for actin depolymerization because cells with defective SHP-1 contained more F-actin (Baba, Fusaki et al. 2003). In neurons, p-Y53 actin was shown to enhance the development of lamellipodia (Bertling, Englund et al. 2016), coinciding with SHP-1 KO phenotype with large lamellipodia.

Tyrosine 53 phosphorylation of actin might either serve as a binding site for actin remodeling protein to bind or prevent actin remodeling proteins to bind to actin. Studies on the role of Y53 phosphorylation of actin have shown that phosphorylation at Y53, promotes hydrogen bond between Y53 of actin with the DNAsa binding loop (D-loop) (Baek, Liu et al. 2008). This conformation of actin might be responsible for pY53 actin properties such as higher critical concentration for polymerization and slower rate of nucleation (Liu, Shu et al. 2006). Studies in neurons and Dictyostelium suggested that pY53 actin causes actin filament to be less stable and break to form short strand of F-actin than normal (Liu, Shu et al. 2006, Bertling, Englund et al. 2016). These studies also suggested that phosphorylation of actin contributes to the dynamics of actin (Bertling, Englund et al. 2016).

Growth factors binding to their receptors initiate actin polymerization (Bi and Zigmond 1999). Likewise, CSF-1 stimulation of BMDM stimulate actin polymerization in macrophages (Pixley and Stanley 2004). When cells were starved, WT cells had more F-actin than SHP-1 KO. Once exposed to CSF-1, SHP-1 KO showed a higher amount of F-actin than WT at 5 min post-CSF-1 stimulation. Overall, SHP-1 KO had more pY53 actin than WT cells. This suggested that actin phosphorylation is triggered by CSF-1. These results suggest that SHP-1 cells polymerize actin slower than their control, probably due the higher concentration of pY53 actin. As noted above, a higher pY53 concentration leads to a slower nucleation rate and high critical concentration for actin
polymerization. All of these factors might lead to a delay in actin polymerization as shown in figure 3.5.

SHP-1 KO cells grew faster than WT, suggesting that SHP-1 is a negative regulator of growth factor. Mice without SHP-1 displayed hyperproliferation of macrophages and granulocyte (Chen, Chang et al. 1996). In addition, studies in primary BMDM showed that macrophages lacking SHP-1 had a hyperphosphorylated CSF-1R, suggesting that SHP-1 directly dephosphorylate CSF-1R (Chen, Chang et al. 1996).

We've also shown that in the absence of Lyn, cells have more F-actin and more pY53 actin than control cells. This led to the hypothesis that Lyn is the kinase that activates SHP-1 phosphatase activity. Studies have shown that Lyn phosphorylates SHP-1 at tyrosine 536 and 546 to activate its phosphatase activity (Xiao, Ando et al. 2010). However, the morphological phenotype of Lyn KO is not similar to the phenotype of SHP-1 KO cells. Lyn KO cells formed macropinosomes, but less efficiently than WT cells. This may be explained by the fact that other kinases compensate for the loss of Lyn to activate SHP-1. Other kinases known to phosphorylate SHP-1 include Src (Frank, Burkhardt et al. 2004), Lck (Lorenz, Ravichandran et al. 1994), and insulin receptor (Uchida, Matozaki et al. 1994).

Syk, BTK, and Vav1 were shown to be high drinkers. The mean fluorescence of these targeted KOs was higher than the WT mean fluorescence. The results of repeated experiments were not consistent, and the experimental design needs to be optimized to obtain consistent results for each similar experiment. An explanation as to why these experiments were not consistent might be that the cells are susceptible to external factors such as oxidation and temperature (Lee and Repasky 2011). If the pathway in which these proteins are involved are susceptible to external conditions, this might affect the results each time the temperature or CO2 concentration is altered. Syk, BTK, and Vav1 have all been described as positive regulators of actin polymerization (Mohammad, Nore et al. 2013). Our initial hypothesis was that genes important for actin
polymerization would be positive regulators of macropinocytosis. Surprisingly, the screen suggests that these genes are negative regulators of macropinocytosis.

Many signaling pathways such as phagocytosis, inflammation, and growth factor signaling can trigger actin reorganization (Machesky and Insall 1999). These pathways require different organizations of actin to function properly and might compete for the machinery for actin polymerization. Studies have shown that cells that are able to move faster are slow at macropinocytosis (Veltman 2015). In another study, pro-inflammatory macrophages were slower at macropinocytosis while anti-inflammatory macrophages were more efficient at macropinocytosis (Redka, Gütschow et al. 2018). Syk and BTK are known to regulate the TLR4 pathway and are involved in the macrophage-mediated inflammatory response (Ní Gabhann, Hams et al. 2014, Yi, Son et al. 2014). Studies on SHP-1 deficient mice also showed that these mice have hyperactive immune cells that initiate a cascade of inflammatory responses, suggesting that SHP-1 is an inhibitor of inflammatory signaling (Sharma, Bashir et al. 2016). Our results suggest that SHP-1 is a positive regulator of macropinocytosis while Syk and BTK are negative regulators of macropinocytosis. These opposite functions of SHP-1, Syk, and BTK rise the question of whether genes regulating inflammatory responses are negative regulators of macropinocytosis.

In conclusion, our results suggest that the phosphatase activity of SHP-1 is a critical component in macropinocytosis and that Lyn activates SHP-1 to promote macropinocytosis. The other actin-regulating proteins, Syk, and Vav1 appear to be negative regulators of macropinocytosis, suggesting that actin polymerization needs to be tightly regulated to form macropinosomes.
V. References


VI. Figures

A  Stimulated Ptpn6 sgRNAs

![Graph showing stimulated Ptpn6 sgRNA expression levels with four guides, each represented by a different line color.](image)

B  Stimulated Lyn sgRNAs

![Graph showing stimulated Lyn sgRNA expression levels with four guides, each represented by a different line color.](image)

C  Stimulated Syk sgRNAs

![Graph showing stimulated Syk sgRNA expression levels with four guides, each represented by a different line color.](image)

D  Unstimulated Ptpn6 sgRNAs

![Graph showing unstimulated Ptpn6 sgRNA expression levels with four guides, each represented by a different line color.](image)

E  Unstimulated Lyn sgRNAs

![Graph showing unstimulated Lyn sgRNA expression levels with four guides, each represented by a different line color.](image)

F  Unstimulated Syk sgRNAs

![Graph showing unstimulated Syk sgRNA expression levels with four guides, each represented by a different line color.](image)
Figure 2: Guide RNA in low and high drinker pool (A-E)
Figure 2.2 Dextran uptake is disrupted in SHP-1 knockout cells. Cas9-BMDM were transfected with SHP-1 guide RNA for 18 hours and grown for 12 days post transfection. (A) Cells were stimulated with CSF-1 and 40 KDa Alexa 594 dextran for 5, 15 and 60 min and analyzed by flow cytometry (n= 5000). (B) BMM were stimulated with CSF-1 plus alexa 594 conjugated dextran for 5 min. A nuclear stain was added to facilitate cell identification. Cells were analyzed for internalization by high content microscopy (Blue: nucleus; red: dextran). Dextran uptake assay was done in WT
cells and SHP-1 KO cells, then cells were stained with SHP-1 antibodies (C) and (E). A correlation plot was done to compare SHP-1 fluorescence intensity to dextran fluorescence intensity (D) and (F).

**Figure 2.3.** Dextran uptake in Lyn KO cells. Cas9-BMDM were transfected with Lyn guide RNA for 18 hours and grown for 12 days post transfection. (A) Cells were stimulated with CSF-1 and 40 KDa Alexa 594 dextran for 5, 15 and 60 min and analyzed by flow cytometry (n=5000). (B) BMM were stimulated with CSF-1 plus alexa 594 conjugated dextran for
5 min. A nuclear stain was added to facilitate cell identification. Cells were analyzed for internalization by high content microscopy (Blue: nucleus; red: dextran)

A Wild type

SHP-1 knockout
Figure 2.4 formation of circular ruffle is disrupted in SHP-1 KO cells. Cells were grown in BMM and imaged under brightfield (A). Cells were starved overnight and stimulated with CSF-1R the next day WT cells and SHP-1 KO cells compared for macropinosomes formation and cell morphology. (arrow: macropinosomes; arrow head: lamellipodia) (B) A time lapse movie was taken under brightfield using a high content microscope (HCM) to observe ruffling and macropinocytosis (arrow: ruffle development).
Figure 2.5. SHP-1 and Lyn KO have more F-actin than control cells. Cells were starved overnight and stimulated the next day with CSF-1 for indicated time. Cells were stained with phalloidin to observe F-actin distribution and F-actin amount in time following CSF-1 addition (arrow: F-actin rich ruffles). (B) fluorescence intensity was quantified using...
CellProfiler; a two-way ANOVA was done to determine statistical significance (n=50; *** = p < 0.0001; + = different from zero min time point of the same cell type). Bars represent standard deviation.
Figure 2.6. SHP-1 KO have a higher concentration of pY53-actin compared to wildtype cells. (A) WT and SHP-1 KO BMDM were starved for 18 h and stimulated with CSF-1 for indicated time. Cells were fixed with 100% meOH and stained for pY53 actin. Images were taken with a HCM. (B) Fluorescent intensity was measured using cell profiler; a two-way ANOVA was done to determine statistical significance. (C) the 7.5 min timepoint in WT was analyzed by confocal microscopy to determine the location of pY53 actin in WT BMDM. (Arrow: p-actin on membrane ruffles; arrowhead: p-actin in nuclear/perinuclear region; n= 50; *** = p < 0.0001; ** = p < 0.001; * = p < 0.01; + = different from zero min time point of the same cell type)
Figure 2.7. Lyn KO cells have a higher pY53 expression than control cells. BMM were transfected with Lyn guide RNA for 18 hours and grown for 12 days post transfection. WT and Lyn KO BMDM were incubated in BMM and fix with 4% PFA. (A) cells were imaged with HCM. (B) fluorescence intensity was measured using CellProfiler software and statistical analysis by t-test (n=300; * = p< 0.05). Bars represent standard deviation.
Figure 2.8. SHP-1 localizes on membrane ruffles and on macropinosomes. (A) WT BMDM cells were starved for 18 h and stimulated with CSF-1 for indicated time. Cells were fixed with 100 % meOH and stained with SHP-1 antibody. Images were taken by HCM. (B) WT BMDM were constitutively exposed to CSF-1. Cells were fixed with 100% meOH and stained for SHP-1 ab. Images were taken with a confocal microscope (arrow: SHP-1 on macropinosomes).
A

Control
BTK
Negative control

5 min

15 min

60 min

FL4-H :: FL4-H

B

Control
Vav1
Negative control

5 min

15 min

60 min

FL4-H :: FL4-H
Figure 3.9 dextran uptake by flow cytometry in (A) BTK, (B) Vav1, and (C) Syk. Cells were exposed to fluorescent dextran plus CSF-1 for indicated time and analyzed by flow cytometry for dextran internalization (n= 5000).
Figure 2.10 Dextran uptake by HCM. Cells were exposed to fluorescent dextran for 5 minutes and imaged with HCM for dextran internalization (A) BTK, (B) Vav1, and (C) SYk. Blue, nucleus; red; alexa 594 conjugated dextran.
Chapter 3

CSF-1R SIGNALING FROM MACROPINOSOMES

I. Introduction

CSF-1R is an RTK that regulates the growth, proliferation, differentiation, and survival of macrophages (Caescu, Guo et al. 2015). Upon activation by its growth factor CSF-1, CSF-1R is trafficked to macropinosomes and is degraded in endolysosomes (Lou, Low-Nam et al. 2014). Phosphorylation of CSF-1R provides binding sites for different effector proteins. These effector proteins become activated upon binding to CSF-1R and continue a signaling cascade that leads to a range of cellular responses (Stanley and Chitu 2014).

Internalization of RTKs such as CSF-1R is a mechanism that the cells use to control their signaling (Sorkin and von Zastrow 2009, Miaczynska 2013). CSF-1 exerts a pleiotropic effect on macrophages through CSF-1R (Huynh, Kwa et al. 2012). However current understandings of CSF-1R cellular signaling does not explain how CSF-1 exerts a wide range of effects and induces a variety of biological responses in macrophages (Huynh, Kwa et al. 2012). Cellular responses due to growth stimulation of cells depend on a range of factors such as the spatiotemporal organization of signaling (Kholodenko 2006). More evidence now suggests the roles of endosomes and macropinosomes as signaling platforms (von Zastrow and Sorkin 2007). Huynh et al. demonstrated that CSF-1R might be signaling from endosomes. Preventing internalization of CSF-1R as well as inhibiting CSF-1R signaling post-CSF-1 stimulation resulted in disruption of ERK and AKT signaling in macrophages (Huynh, Kwa et al. 2012). Experiment attempting to demonstrate signaling from endosomes were mainly done by immunoblotting, leaving us blind to where the signaling complex is located (Huynh, Kwa et al. 2012). These experiments showed that CSF-1R might be signaling from macropinosomes, however, it failed to show how the macropinosomal or endosomal signaling complex is organized. It is not
clear whether the signaling complex at the plasma membrane is similar or different from the signaling from macropinosomes (von Zastrow and Sorkin 2007).

Organization of the signaling complex on macropinosomes could be a way in which macrophages control their cellular responses to CSF-1 (von Zastrow and Sorkin 2007). Understanding the spatiotemporal organization of endosomal signal could provide a way to understand how CSF-1 exert its pleiotropic effect and overlapping biological responses in macrophages. This knowledge could also be used to understand the involvement of macrophages in different disorders.

Src family kinases (SFKs) are activated by RTKs and also exert feedback on RTK signaling (Mezquita, Mezquita et al. 2014). Immunofluorescence analysis of macrophages showed that Lyn, an SFK was found on macropinosomes upon CSF-1 stimulation of macrophages (Dwyer, Mouchemore et al. 2016). Additionally, immunoprecipitation analysis of Lyn in CSF-1 starved and CSF-1 stimulated macrophages showed that Lyn binds to CSF-1 (Dwyer, Mouchemore et al. 2016). Lyn is known to exert both negative and positive regulation in myeloid cells (Scapini, Pereira et al. 2009). However, little is known about how Lyn might regulate CSF-1R signaling.

Here, we show that phosphorylated (active) CSF-1R (pY721) is present on macropinosomes suggesting active growth factor signaling from macropinosomes. Further, we show that Lyn colocalizes with CSF-1R on macropinosomes and may be involved in negative regulation of CSF-1R.

II. Materials and Methods

Reagents: Dulbecco's modified eagle medium (DMEM) (Corning, Manassas, VA), DPBS (#SH30028.02, GE Healthcare Life Sciences, Pittsburgh, PA), HBSS (#BW10-543F, ThermoFisher Scientific, Waltham, MA), Fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), bovine serum albumin powder (ThermoFisher Scientific, Waltham, MA), normal goat serum (NGS; #5425S; Cell Signaling Technology), normal chicken
serum (NCS; #16110082; ThermoFisher Scientific, Waltham, MA), NucBlue Fixed stain cell stain ReadyProbe (Invitrogen), NucBlue live cell Stain ReadyProbe (Invitrogen) (ThermoFisher Scientific, Waltham, MA), CSF-1R antibody(AFS98, eBioscience), p-ERK antibody (#9101, Cell Signaling Technology), pCSF-1R(Y721) antibody (#49C10, Cell Signaling Technology) SHP-1 antibody (#ab60268, abcam), pY536-SHP-1 antibody (#ab41437, abcam), pY53-actin antibody (#bs-12581R, BiossAntibodies) were used for immunofluorescence, goat anti-Rat Dylight 594 and Goat anti-Rabbit Dylight 488 conjugated secondary antibodies (ThermoFisher Scientific) were used for detecting primary antibodies. Texas-Red Dextran (40 kD; #D1864, ThermoFisher Scientific) was used to label macropinosomes. Phalloidin (ThermoFisher Scientific, Waltham, MA), was used as a cell mask and to label filamentous actin, Fluoromount-G (Southern Biotech, Birmingham, AL) to mount coverslips.

**Bone marrow media preparation:** Bone marrow media (BMM) was prepared using 20% heat-inactivated fetal bovine serum (HI FBS), 30% L-cell conditioned media, 0.7% penicillin/streptomycin 100X (pen-strep) and 0.0004% β mercaptoethanol (ThermoFisher Scientific, Waltham, MA) in Dulbecco’s modified eagle medium (DMEM). The mixture was filtered using a vacuum filter (EMD Millipore Darmstadt, Germany).

**Bone marrow macrophage isolation and culture:** Mice were euthanized with CO2, and femurs were collected, being careful to maintain the integrity of the femur. Both ends of the femur were cut to collect the bone marrow by flushing with phosphate buffered saline containing mM (DPBS) in a 0.5 inch, 26-gauge needle and 5 ml sterile Luer Lock syringe. The collected bone marrow was centrifuged at 500 g for 5 minutes and the cell pellet resuspended in bone marrow medium (BMM). Cells were plated on a sterile 10 cm non-tissue culture dish at $10^6$ cells per dish, in 10 ml of BMM. Cells were placed in an incubator at 37°C and 5% CO2 for two days. On day 2, an additional 10 ml of fresh pre-warmed BMM was added to the dish. After an additional 2 days, the media was removed and replaced with fresh pre-warmed BMM as the macrophages were
adherent. Every 2 days, the media was replaced with fresh BMM to maintain an optimal level of nutrients. For experiments, cells were detached with ice-cold PBS lacking calcium and magnesium and re-plated to appropriate plates or coverslips (Weischenfeldt and Porse 2008).

**Plating cells:** Cells previously frozen in liquid nitrogen were thawed at 37°C and resuspended in 10 ml of BMM. After resuspension, cells were centrifuged at 300 g for 5 min and resuspended in BMM. Cells were counted using a hemocytometer and Trypan blue. Cells were then plated in a 96-well plate at a density of 3 x 10^4 to 3.5 x 10^4 cells per well in 100 µl of BMM; in a 24-well plate at a density of 7.5 x 10^4 cells per well in 1 ml of BMM; in a 6 cm dish at 3 x 10^5 cells per plate in 3 ml of BMM; in a 10 cm dish at 10^6 cells in 10 ml of media.

For microscopy experiments, cells were plated on 12 mm glass coverslips in a 24-well plate. The coverslips were sterilized by flaming with 95% ethanol.

**Alt-R CRISPR-Cas9 gRNA transfection:** Guide RNA sequences targeting Lyn was designed using the Integrated DNA technology website. Synthetic CrRNA oligos (AUGCAGGGAAAAGUGAUGCG, Integrated DNA Technologies) were complexed with Tracer RNA (enables association with Cas9) in a 1:10 molar ratio to make the guide RNA (ref). To transfect BMDM, the 10 picomoles guide RNA was complexed with INTERFERin siRNA transfection reagent (Polyplus) and added to Cas9-expressing BMDM. The RNA complex was incubated with cells for 6 to 18 hours before removing the transfection media. Cells were incubated for 7 to 10 days to allow for the majority of proteins to be degraded.

**Detaching cells from culture:** Cells were detached from the plate with cold modified DPBS -Ca-Mg. Bone marrow media was first removed from culture, cells were washed with 5 ml of cold DPBS to remove extra BMM. 10 ml of 4°C DPBS -Ca/-Mg was added to the dish and cells were then incubated at 4°C for 15 minutes. Macrophages were further
detached by pipetting the media up and down. The solution was then placed in a 15 ml tube and centrifuged for 5 min at 300 g. the pellet was resuspended in BMM.

CSF-1 time course: To visualize CSF-1R trafficking and phosphorylation, cells were deprived of CSF-1. 24h prior to the experiment, BMM was removed and replaced with DMEM plus 10% FBS to upregulate CSF-1R expression. CSF-1 was added at a concentration of 0.2 μg/ml in DMEM 10% FBS or LCIB for different time points and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature or 100% ice-cold methanol for 10 min at -20°C (Lou, Low-Nam et al. 2014).

Dextran uptake and macropinosomes phenotype by high content microscopy: Cells were plated on an optical-quality 96 well plate. Once cells adhered to the plate and recovered from plating, cells were exposed to 250 μg/ml 40 kDa Alexa 647 conjugated dextran and nuclear stain for 3 min and the dextran was washed off the cells 10 times with HBSS. Cells were imaged with high content microscopy.

Immunofluorescence and phalloidin staining: immunofluorescence was done to visualize protein expression, location in the cell, and phosphorylation. Bone marrow macrophages plated on coverslips were stimulated with CSF-1 for different time points. Cells were fixed with 4% PFA for 15 minutes (100% methanol for 1 min). blocking for non-specific binding was done using 5% NGS plus 0.3% triton-X if fixed with PFA, or 5% NGS if fixed with methanol. Primary antibodies were added and incubated for 1h to 18h, and secondary antibodies were incubated for 1 to 2h. Primary and secondary antibodies were diluted in 1% BSA plus 0.3% triton-X (no triton-X if fixed with methanol). Phalloidin was added for 20 min to stain for filamentous actin.

Image analysis: immunofluorescence images were acquired using either a high content microscope or small inverted Leica CTR4000 microscope using a 60X oil lens EVOS air lens. image analysis and quantification were done using ImageJ (Schneider, Rasband et al. 2012) and CellProfiler (Carpenter, Jones et al. 2006) open-access software.
### III. Results

*CSF-1R is active on macropinosomes*

Emerging literature suggests signaling of RTK on endosomes, and that endosomal signaling might be important for proper RTK signaling (Villaseñor, Kalaidzidis et al. 2016). CSF-1R is one of the RTKs suggested to signal from endosomes and most specifically macropinosomes (Huynh, Kwa et al. 2012). Here, we took advantage of immunofluorescence technics to show the presence of total and phospho-CSF-1R on macropinosomes. To test whether CSF-1R was phosphorylated on macropinosomes, cells were starved of CSF-1 overnight and then restimulated with CSF-1 for 0-30 min. Within 5 min of CSF-1 stimulation, CSF-1R was internalized and localized 40 kDa dextran-labeled macropinosomes (Figure 3.1.A). Total receptor immunostaining declined by 30 min post stimulation (p<0.05, Figure 3.1.A). Phospho-pY721 CSF-1R was on macropinosomes at 5, 10 and 15 min post stimulation (Figure 3.1.B). The amount of pY721 CSF-1R decreases at 15 min and was mostly gone by 30 min post-stimulation. The total amount of CSF-1R lasted longer than the amount of pY721 CSF-1R (Figure 3.1.A).

*ERK and AKT activation correlates with CSF-1 activation and internalization*

Activation of CSF-1R leads to a downstream signaling cascade that leads to different cellular response (Stanley and Chitu 2014). Some downstream effector proteins of CSF-1R include ERK and AKT (Stanley and Chitu 2014). However, most experiments done to show the activation of these pathways have been done by immunoblot, not showing the exact location of these effector proteins in the cells. To confirm the correlation between CSF-1R activation and ERK and AKT activation, we exposed macrophages starved of CSF-1 to CSF-1. Using immunofluorescence assay, we attempted to determine the location and the duration of ERK and AKT signaling. pERK and pAKT activation were induced within a minute of CSF-1 stimulation (data not shown). Total ERK and AKT concentration did not change significantly, which is what was expected (Figure 3.2.B, 3.3.B). the phospho-AKT activity lasted at up to 30 minutes post CSF-1 stimulation, while ERK
activity decreased significantly at 30 minutes post stimulation (figure 3.2; figure 3.3). In addition, a confocal microscopy showed that phospho-ERK was localized around macropinosomes, some even around CSF-1 containing macropinosomes (figure 3.2.C). AKT was not localized to a specific area but propagated throughout the cytoplasm (figure 3.3).

**ERK and AKT phosphorylation is dependent on continuous CSF-1R signaling**

The involvement of CSF-1R and other RTKs signaling on macropinosomes has not been established yet. The presence of phosphorylated receptor on macropinosomes alone is not sufficient to establish signaling on macropinosomes. To determine if downstream signaling is dependent on continuous signaling from CSF-1R on macropinosomes, we used GW2580, an inhibitor of CSF-1R kinase activity. GW2580 inhibitor activity has been shown to be specific to CSF-1R (Huynh, Kwa et al. 2012). Before running a complete experiment, it was necessary to determine the concentration of GW2580 that would affect CSF-1R signaling without killing or making the cells sick. Cells were treated with 5, 10, 15, 20, 30 µM of GW2580 20 min prior CSF-1 stimulation and staining with CSF-1R and pERK. We found that 30 µM of GW2580 was the best concentration to see the desired results without killing the cells (figure 3.4.A). After determining that 30 µM was the optimal concentration, we exposed BMDM to 30µM of GW2580 5 min post-CSF-1 stimulation. The result showed a decreased in the concentration of pERK in GW2580 treated cells compared to non-treated cells (figure 3.4.B and C).

**Lyn is a negative regulator of CSF-1R**

Although CSF-1R is suggested to signal from macropinosomes, not much is known about the signaling complex on macropinosomes. Lyn has been found on macropinosomes following 5 min CSF-1 stimulation of macrophages (Dwyer, Mouchemore et al. 2016). However, it wasn’t determined whether CSF-1R and Lyn interacted on macropinosomes. To determine if Lyn colocalized with CSF-1R on macropinosomes, an immunofluorescence analysis staining CSF-1R and Lyn following CSF-1 stimulation of
BMDM was done. These data were analyzed on a confocal microscope to determine colocalization of both proteins on macropinosomes on an XYZ plane. At 5 minutes post-CSF-1 stimulation, we found that Lyn sparingly colocalized with CSF-1R on macropinosomes on the same XYZ plane (figure 3.5). However, Lyn localization is not restricted to macropinosomes, showing that Lyn might have different functions that might not be related to CSF-1 signaling in BMDM.

To determine the function of Lyn in CSF-1R signaling pathway, we knocked out Lyn expression by CRISPR Cas9 genome editing and subsequently determined the rate of proliferation of Lyn KO cells compared to wildtype BMDM. To determine the effect of Lyn deletion on BMDM, a proliferation assay was done using the hemocytometer. Lyn KO cells grew 2 times faster than wild-type, indicating that Lyn might be a negative regulator of growth factors such as CSF-1 (data not shown). In addition, CSF-1R expression and trafficking were observed in Lyn KO cells. Lyn KO cells expressed less surface CSF-1R compared to wildtype cells (figure 3.6). But the trafficking of CSF-1R did not change significantly in the Lyn knockout cells (figure 3.6).

In addition to growing faster than the wild-type, macropinocytosis formation (measured by dextran uptake) was also downregulated compared to wildtype BMDM (figure 3.7). This shows that in addition to being a CSF-1R negative regulator, Lyn is also a positive regulator of CSF-1R induced macropinocytosis.

IV. Discussion

CSF-1 induces pleiotropic effects in macrophages, but it is unclear how a single growth factor can induce different cellular responses (Huynh, Kwa et al. 2012). It has been suggested that CSF-1R and other RTK continue to signal following internalization (Wang, Nakayama et al. 2010, Huynh, Kwa et al. 2012, Miaczynska 2013). The location and the duration of signaling are factors that can influence the cell responses to growth factors (Traverse, Seedorf et al. 1994, Villaseñor, Kalaidzidis et al. 2016). Continuing signaling of CSF-1R could explain its various effects in macrophages. Here, using
immunofluorescence, we showed that CSF-1R is active on macropinosomes and we showed that pharmacological inhibition of CSF-1R signaling following its internalization affected the downstream signaling cascade which was measured by ERK phosphorylation. We also showed that CSF-1R colocalized with Lyn on macropinosomes and that BMDM with Lyn Knockout proliferated more rapidly than wildtype BMDM, suggesting that Lyn is a negative regulator of growth factor.

Inhibition of post internalization reduced the activation of ERK. GW2580 selectively inhibit CSF-1R activity while other tyrosine kinases are unaffected by GW2580 (Conway, McDonald et al. 2005). Treating BMDM with GW2580 5 min post-CSF-1 stimulation resulted in less phospho-ERK compared to non-treated cells. This result was similar to the previous study by immunoblot (Huynh, Kwa et al. 2012). This demonstrated that continuous CSF-1R signaling even after internalization may be necessary for proper signaling. However, Studies have shown that 90-95% of CSF-1R is internalized by 15 min (Huynh, Kwa et al. 2012). Because of that, it is not certain that at 5 min post-CSF-1 stimulation, all receptors at internalized, which leaves the possibility that ERK activity was impaired not due to inhibition of CSF-1R on macropinosomes, but due to inhibition of remaining CSF-1R at the plasma membrane. Treatment with GW2580 after CSF-1 stimulation did not affect CSF-1R trafficking and degradation. Cells treated with GW2580 trafficked CSF-1R similarly to non-treated cells. At 30 min post-CSF-1 stimulation, most CSF-1R were degraded in both GW2580 treated and non-treated cells.

We also see the presence of phospho-ERK around macropinosomes (Figure 3.2). As noted above, the location of signaling is an important factor determining cellular response (Kholodenko 2006). The presence of ERK in proximity to CSF-1R containing macropinosomes could be a way to amplify or modify ERK signaling, leading to different cellular response. In HeLa cells, ERK was found on tubular endosomes and was thought to regulate clathrin-independent endosomal trafficking (Robertson, Setty et al. 2006).
Many studies on RTKs signaling suggested continuous signaling on endosomes (Sorkin and von Zastrow 2009). Majority of the evidence suggesting signaling from endosomes include the presence of the component of the MAPK pathway on endosomes (Robertson, Setty et al. 2006, Nada, Hondo et al. 2009, Sorkin and von Zastrow 2009), and the presence of phosphorylated and active RTK such as EGFR on endosomes (Wang, Pennock et al. 2002, Sorkin and von Zastrow 2009). Studies demonstrated that EGFR can be activated in endosomes and endosomal activation of EGFR was sufficient to activate proliferation and survival signaling pathway (Wang, Pennock et al. 2002).

Although many evidence suggests growth factor signaling from endosomes, it is not clear whether the signaling complex on endosomes and at the plasma membrane is the same (Murphy, Padilla et al. 2009). We showed that Lyn translocated to macropinosomes and membrane ruffles following CSF-1 stimulation of BMDM. We also showed that Lyn and CSF-1R colocalized on macropinosomes. This suggests that Lyn might be involved in CSF-1R signaling in macropinosomes. Different studies have reported changes in cellular localization of SFKs following growth factor stimulation (Palacios-Moreno, Foltz et al. 2015). Lyn has been reposted to translocate to endosomes in neuroblastoma (Palacios-Moreno, Foltz et al. 2015) and to macropinosomes in macrophages (Dwyer, Mouchemore et al. 2016).

Lyn tyrosine kinase has been described as both a positive and negative regulator of growth factor signaling in myeloid cells (Hibbs and Harder 2006). Knocking out Lyn in BMDM resulted in more proliferative cells. Lyn KO cells grew two times faster than wildtype cells. In addition to growing faster, CSF-1R expression in Lyn KO cells was downregulated compared to wildtype cells. These two observations suggest that Lyn might be a negative regulator of CSF-1R and other growth factor receptors. If Lyn is a negative regulator of CSF-1R, the presence of Lyn on macropinosomes might be to attenuate CSF-1 signaling. Studies in B cells suggest that Lyn plays a role as a negative regulator of B cell receptor signaling and that Lyn-deficient mice suffered from autoimmunity (Lamagna, Hu et al. 2014).
The duration and location of signaling are important in dictating the cell fate. Signaling in cells must be just long enough to allow for the proper amount of cellular response (Sorkin and von Zastrow 2009). Binding of ligands to RTK lead to rapid phosphorylation and internalization into endosomes (Sorkin and von Zastrow 2002). For example activation ERK can contribute to different cellular responses such as differentiation, proliferation, migration, and survival (Ebisuya, Kondoh et al. 2005). The way that ERK is able to induce different cellular response is through signal duration, magnitude and spatiotemporal organization (Ebisuya, Kondoh et al. 2005). Thus, endosomes and macropinosomes might be a signaling platform where signaling might be either extended or attenuated and to organize different signaling complex to determine the cell fate.

In conclusion, we showed that CSF-1R is active on macropinosomes and that continuous signaling from CSF-1R is important for proper downstream signaling. We showed that Lyn is on macropinosomes and could interact with CSF-1R for it downregulation. Additional experiments could be to determine pERK and pAKT expression in Lyn knockout cells to confirm the involvement of Lyn in CSF-1R signaling.
V. References


Fig 3.1 CSF-1R is present on macropinosomes suggesting that signals emanate from internal vesicles. Bone marrow media were CSF-1 starved overnight. Next day, cells were stimulated with 70 KD Texas-red dextran and CSF-1 for 5 minutes and chased with DMEM for indicated time before fixing with 4% PFA. Cells were stained with CSF-1R and
imaged with high content microscope. CSF-1R is internalized and localizes to macropinosomes before being degraded.

(Arrow: CSF-1R and pCSF-1R on macropinosomes; a-c: enlargement of CSF-1 and pCSF-1R colocalization on macropinosomes at 7.5 min; d-f enlargement of CSF-1 and pCSF-1R colocalization on macropinosomes at 10 min)
Figure 3.2: correlation of CSF-1R trafficking with ERK and AKT phosphorylation. (A) BMM were starved and stimulated with CSF-1 for indicated timepoints. Cells were fixed with 4% PFA. Cells were stained with CSF-1R and phospho-ERK and imaged under an inverted microscope (arrow: p-ERK rich macropinosomes). (B) Ratio of pERK/ERK. (C) Confocal analysis of ERK and CSF-1R colocalization on macropinosomes (lines: p-ERK and CSF-1R surrounded macropinosomes). (D) plot profile analysis of CSF-1R and pERK on macropinosomes.
Figure: correlation of CSF-1R trafficking with AKT phosphorylation. (A) BMM were starved and stimulated with CSF-1 for indicated timepoints. Cells were fixed with 4% PFA. Cells were stained with CSF-1R and phospho-AKT and imaged under an inverted microscope. (B) Ratio of pAKT/AKT.
Figure 3.4: Inhibition of CSF-1R tyrosine activity following its internalization disrupts phosphorylation of ERK. (A) Cells were treated with GW2580 for 15 min prior CSF-1 stimulation. Cells were treated or not treated 5 min post CSF-1 stimulation. Cells were fixed and stained for CSF-1R and pERK. (C) Quantification of ERK phosphorylation (n= 100; * = p < 0.05; ns = not significant). Bars represent standard deviation.
Figure 2.5. CSF-1R co-localize with Lyn at 2.5 and 5 min post stimulation with CSF-1. BMDM were starved of CSF-1 overnight. The following day cells were stimulated with CSF-1 and fixed with 4% PFA at indicated timepoints. Cells were immuno-stained with CSF-1R and Lyn. (B) colocalization on Z plane (C) Plot profile analysis.
Figure 3.6 Lyn KO cells have less pERK than wildtype: cells were starved of CSF-1 overnight and stimulated with CSF-1 the next day for 5 min. Cells were stained with pERK antibodies and analyzed with HCM (A). (B) statistical analysis (n=300; *** = p < 0.0001). Bars represent standard deviation.
Chapter 4

CONCLUSION

Understanding macrophage cell biology is important due to macrophages key roles in human health and diseases including proper immune function, wound healing, atherosclerosis, and cancer. Despite their importance, relatively little is understood about macrophage activation, growth factor signaling, and cytoskeletal regulation. This thesis presents data from investigations into mechanisms of macrophage growth factor signaling and actin polymerization for ruffling and macropinocytosis. Macrophage growth factor signaling is important because growth factor has various effects on cells including growth, proliferation, differentiation, and cell survival. Abnormal growth factor signaling in macrophages can lead to disorders like cancer, autoinflammation, and autoimmunity (Stanley and Chitu 2014, Cannarile, Weisser et al. 2017, Nissen, Thompson et al. 2018). Macrophage actin regulation is important in macropinocytosis, phagocytosis, and other endocytic and exocytosis processes as well as cellular motility. Here, we focused more on regulation of actin polymerization during macropinocytosis. Macropinocytosis is important to macrophage biology. Macrophages use macropinocytosis for nutrient uptake and to sample their environment. Growth factors upregulate the formation of macropinosomes, showing that growth factor signaling and macropinocytosis are related.

Signaling macropinosomes may coordinate macrophage function by integrating growth factor signaling, nutrient sensing, antigen recognition/presentation and inflammatory signaling (Marques, Grinstein et al. 2017). Spatio-temporal organization of signaling molecules is a mechanism that cells utilize to control the amplitude, duration, and location of signaling. Work on GPCR and other RTK signaling show the coordination of signaling and trafficking (Wang, Pennock et al. 2002, Pavlos and Friedman 2017). This coordination is important for signaling attenuation and/or amplification (Pavlos and Friedman 2017). For example, macropinocytosis is activated by growth factor to deliver
amino acid to the lysosomes for mTOR activation, suggesting that macropinosomes are a mechanism of nutrient sensing (Yoshida, Pacitto et al. 2015). We suggest that Lyn is a possible negative regulator of CSF-1R signaling, and colocalizes with CSF-1R on macropinosomes for CSF-1R signaling attenuation. Attenuation of CSF-1R signaling may promote different cellular responses. Data have shown that the amplitude of signaling is an important factor determining cellular response (Kholodenko 2006).

Mechanisms of macropinosomes formation in macrophages and other cells have historically not been studied or well understood. However, recent interest in this cellular process has increased due to better understanding of the importance of this process in cancer, pathogen uptake, and macrophage cell function. Data from Chapter 3 demonstrated that SHP-1 is an important regulator of macropinocytosis through actin regulation. Furthermore, Lyn might regulate actin phosphorylation by activating the phosphatase activity of SHP-1 (Xiao, Ando et al. 2010). Lyn might not be the only SHP-1 positive regulator as Lyn knockout does not affect macropinocytosis to the same extent as SHP-1 knockout.

Observation of actin polymerization following CSF-1 stimulation for various time showed that actin polymerization and depolymerization must be controlled during macropinocytosis. Slow or fast actin polymerization and depolymerization might be unfavorable for macropinocytosis. Upon CSF-1 stimulation, cells initiate ruffling to form macropinosomes. SHP-1 regulates actin reorganization by dephosphorylating actin (both G- and F-actin). In the absence of SHP-1, the concentration of p-actin increases. A high concentration of phospho-G-actin increases the critical concentration for actin polymerization, leading to a delay in actin polymerization (Liu, Shu et al. 2006). Once the critical concentration is reached, actin polymerization and depolymerization are in equilibrium, and when the concentration of G-actin is above the critical concentration, there is more polymerization than depolymerization. As seen in figure 3.5, absence of SHP-1 lead to an increase in p-actin concentration and to a delayed in actin polymerization. This suggests that a higher concentration of p-actin leads to a delayed in
polymerization due to an increase in the critical concentration. This delayed actin polymerization might be responsible for the inability of cells to form macropinosomes. Phosphorylation of actin also leads to unstable F-actin strands. These strands polymerize and break because of the unstable F-actin strand. This leads to a high concentration of G-actin that is always above the critical concentration, resulting in a very dynamic actin pool that is always polymerizing.

We propose a mechanism in which CSF-1 stimulation leads to activation of 1. a kinase that phosphorylates actin, 2. Kinases (Lyn and other kinases) that phosphorylate and activates SHP-1 which will, in turn, dephosphorylate actin (figure 4.1). Other signals might also be involved in the activation of SHP-1 and phosphorylation of actin (figure 4.1). A balance of actin kinase and SHP-1 is required to maintain a steady pool of p-actin for the cell to function normally. The absence of SHP-1 creates an unbalance in the concentration of p-actin which then disrupt actin polymerization in the cell.
**Figure 4.4:** Model illustrating the regulation of actin polymerization by SHP-1. Stimulation of cells by CSF-1 induces CSF-1R activation and phosphorylation. Phosphorylation of CSF-1R leads to activation of Lyn, which will in turn activate SHP-1. Activity of SHP-1 is important for actin depolymerization. An unknown kinase also activates SHP-1, and this activation might be independent of CSF-1. Accumulation of pY53 actin in the absence of SHP-1 leads to disruption in macropinocytosis and large lamellipodia phenotype.

The highly dynamic actin polymerization can also explain why SHP-1 KO had a phenotype with large lamellipodia. As noted above, an increase in p-actin leads to unstable F-actin and highly dynamic actin pool (Liu, Shu et al. 2006). Because actin constantly polymerizes when the concentration of p-actin is high, it leads to large lamellipodia as seen in SHP-1 KO cells (figure 3. 3. A). pY53 actin was found to increase the development of lamellipodia in neurons (Bertling, Englund et al. 2016), consistent with our results.

The opposite roles of positive regulators of macropinocytosis (SHP-1 and Lyn) and negative regulator of macropinocytosis (SYk, BTK, and Vav1) raise the question whether actin machinery required for other processes such as inflammation and motility compete with the actin machinery required for macropinocytosis. Syk, BTK, and Vav1 are part of actin regulator proteins but are also involved in other signaling pathways such as inflammatory response (Mohamed Abdalla, Yu et al. 2009, Mócsai, Ruland et al. 2010). Some studies have shown that Syk and BTK are part of the TLR4 pathway and are involved in the macrophage-mediated inflammatory response (Ni Gabhann, Hams et al. 2014, Yi, Son et al. 2014). The cell responses to inflammatory signaling involve actin reorganization. Our study shows that these proteins are negative regulators of macropinocytosis. Studies on macropinocytosis in proinflammatory and anti-inflammatory macrophages suggested that anti-inflammatory macrophages actively performed macropinocytosis while the proinflammatory macrophages lacked this ability (Redka, Gütschow et al. 2018). Studies on SHP-1 deficient mice also showed that these mice have hyperactive immune cells that initiate a cascade of inflammatory responses,
showing that SHP-1 is an inhibitor of inflammatory signaling (Sharma, Bashir et al. 2016). These studies show that SHP-1 is an antagonist to Syk that is known to promote inflammation (Deng, Kyttaris et al. 2016). Our data also show that SHP-1 is a positive regulator of macropinocytosis, which is the opposite of Syk, Btk, and Vav1 that are negative regulators of macropinocytosis. Other studies have also suggested that cells that do more macropinocytosis move much slower and that cells that move faster do less macropinocytosis (Veltman 2015).

Many studies on macropinocytosis have described macropinocytosis as a way of internalizing nutrients especially in cancer cells, *Dictyostelium*, and macrophages (Trajkovska 2013, Bloomfield and Kay 2016, Recouvreux and Commissio 2017, Yoshida, Pacitto et al. 2018). Our results, however, showed that macrophages can grow even faster without macropinocytosis, suggesting that macropinocytosis is not crucial for cellular growth and that macrophages possess other ways to uptake nutrients. Since macropinocytosis is not crucial for cellular growth or nutrient uptake, macrophages must use macropinocytosis for other purposes than nutrient uptake only. One other uses of macropinocytosis are the clearance of pathogens and cellular debris from an infected site (BoseDasgupta and Pieters 2014). Macropinosomes might also be a signaling platform for either signaling attenuation or amplification of growth factor signaling such as CSF-1R (Huynh, Kwa et al. 2012, Lou, Low-Nam et al. 2014).
References


