The Role of Extracellular Amino Acids in the Regulation of Macropinocytosis

by

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Dedication

To my wife, Laina

For always being there for me

MTA

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Abstract

Macropinocytosis is an endocytic pathway by which cells internalize extracellular solutes from the environment in vesicles known as macropinosomes. It is involved in a wide array of functions ranging from antigen presentation to cancer cell development. In bone marrow-derived macrophages, macropinocytosis can be induced by growth factors such as colony-stimulating factor-1 (CSF1). While much is known about the molecular mechanisms involved in the formation and internalization of macropinosomes, relatively little is known about its regulation. As macropinocytosis functions as a mechanism for internalizing nutrients from the environment, we tested whether nutrients modulate solute uptake by macropinocytosis. One category of nutrient that was relatively understudied is amino acids, and as such are the focus of this thesis. We show that nine amino acids, when present individually or together, can suppress macropinocytosis. Furthermore, we show that suppression only occurs when macrophages are stimulated with ligands of the CSF1 receptor (CSF1R), which include CSF1 and the cytokine IL-34. Suppressive amino acids had no effect when macropinocytosis was induced by lipopolysaccharide or phorbol myristate acetate. Mechanistically, suppressive amino acids activate the metalloproteinase ADAM17 which cleaves CSF1R, resulting in the release of CSF1R from the cell surface. This in turn leads to the formation of smaller macropinosomes and consequently less total accumulation by macropinocytosis. Our findings may have implications for macrophage polarization, especially in nutrient-poor environments such as the tumor microenvironment.

Chapter 1
Introduction

1.1 Statement of Research Problem

Macropinocytosis is a process by which cells internalize extracellular solutes in heterogenous vesicles known as macropinosomes. This process has been implicated in physiological processes such as cell growth and antigen presentation, in addition to disease processes such as tumor development and pathogen infection. While our lab and others have done extensive work elucidating the mechanism of macropinosome formation and internalization, the regulation of this process is relatively understudied. As macropinocytosis is a mechanism for internalizing nutrients into the cell, we hypothesized that nutrients themselves may play a regulatory role in this process. In this dissertation, I focus on the role of extracellular amino acids in the regulation of macropinocytosis in bone marrow-derived macrophages.

1.2 Introduction to Macrophage Biology

Macrophages were first identified by Ilya Metchnikoff in the early 1900's[1]. They function as critical immune sentinels, sensing and responding to tissue damage and infection. Macrophage development begins in the primary lymphoid organs, such as the bone marrow. There, hematopoietic stem cells give rise myeloid precursor cells, which in turn differentiate into

monocytes[2]. Monocytes circulate in the blood and eventually migrate into tissues where they differentiate into macrophages.

CSF1 is the growth factor responsible for the differentiation of monocytes into macrophages. It exists in three major isoforms; a secreted glycoprotein, a secreted proteoglycan (protein bound to glycosaminoglycan group), and a membrane-bound glycoprotein[3]. The secreted isoforms function at distant sites whereas the membrane-bound CSF-1 functions locally. The effects of CSF1 are mediated by signaling through CSF1 receptor (CSF1R). CSF1R was identified and later purified in the lab of Richard Stanley[4,5]. It is a transmembrane protein consisting of an intracellular domain, a transmembrane domain, and a heavily glycosylated extracellular region[6] . The known ligands of CSF1R are CSF1 and the cytokine IL-34, whose functional roles are not fully understood. Upon CSF1 binding to CSF1R, CSF1R dimerizes and forms a complex with signaling molecules such as Grb2, SFK, Cbl in addition to p85, the regulatory subunit of PI-3 Kinase (PI3K)[6].

CSF1 signaling can promote macrophage differentiation, survival, or proliferation. Which pathway is effectuated is based on the signal strength (CSF1 concentration) and/or duration, which can result in the phosphorylation of different residues on CSF1R[6]. Macrophage differentiation is predominately regulated by ERK and PLC-γ2 signaling[7,8]. Inhibiting PLC-γ2 binding to CSF1R by engineering a Y807F mutation in CSF1R, in addition to inhibiting PLC activity using the inhibitor U73122, prevents monocyte differentiation into macrophages[8]. CSF1 interaction with CSF1R promotes two waves of ERK activation, an early transient wave lasting the first 30 minutes, and a persistent later wave starting about 4 hours after stimulation. Inhibiting ERK activation during the early wave using the MEK inhibitor U0126 does not inhibit differentiation, whereas

ERK inhibition during the later wave inhibits differentiation. Thus, the later ERK signaling is responsible for promoting differentiation.

In addition to macrophage differentiation, CSF1/CSF1R signaling can promote macrophage survival or proliferation. Whereas high concentrations of CSF1 stimulate macrophage proliferation, low doses promote survival[9]. Survival is regulated by PI3K-Akt signaling. Akt signaling promotes survival by inhibiting the function pro-apoptotic proteins[10]. Pharmacological inhibition of PI3K using LY294002 prevents macrophage survival, which can be partially restored by inhibiting pro-apoptotic proteins such as caspase-9[11]. Macrophage proliferation also stems from PI3K-Akt signaling, but also involves signaling through RAS-ERK[12]. Two of the ERKs, ERK1/2 act as a sensor of CSF1 concentration. At low concentrations of CSF1, ERK activation is enhanced, while at high concentrations of CSF1, ERK activation is diminished, allowing the cell to promote survival rather than proliferation[13]. Lastly, the adaptor protein DAP12 has also been shown to be important for macrophage proliferation. Following CSF1R activation, DAP12 is phosphorylated and recruits the kinase Syk, which ultimately results in the nuclear translocation of the β-catenin, a protein that promotes the expression of cell cycle related genes[14].

Macrophages recognize pathogens through specialized receptors known as pattern recognition receptors (PRRs)[15]. PRRs recognize microbial components referred to as pathogen-associated molecular patterns (PAMPs), such as double-stranded RNA, lipopolysaccharide (LPS), or flagellin[15]. The best characterized PRRs are the toll like receptor (TLR) family, which are located both in endosomal compartments and on the cell surface[16]. One such TLR is TLR4, which responds to bacterial LPS to generate the production of type 1 interferons as well as inflammatory cytokines[16]. Depending on the stimuli macrophages encounter in their environment, they can differentiate into three main sub-populations: classically activated macrophages, wound-healing

macrophages, or regulatory macrophages[17]. Macrophage populations can also be defined as either M1 or M2, with M1 representing the classically activated macrophages and M2 representing alternatively activated macrophages such as the wound healing and regulatory macrophages.

Classically activated macrophages arise following exposure to the cytokines interferon-γ (IFN-γ), tumor-necrosis factor-α (TNFα), or microbial products such as LPS[17]. The microbicidal activities of classically activated macrophages include the generation of reactive oxygen species, and reactive nitrogen species[18]. M1 macrophages also secrete proinflammatory cytokines such as TNFα, IL-1β, IL-6, IL-12, and IL-23[19]. Wound healing macrophages arise in response to IL-4 and IL-13[17]. Wound healing macrophages secrete components of the extracellular matrix which are important for wound healing[18]. While these macrophages are typically less efficient at killing pathogens, studies have shown that they play an important role in the clearance of some pathogens such as helminths[20]. Lastly, regulatory macrophages arise in response to IL-10[17]. These macrophages secrete large amounts of IL-10 which polarize T cells to a T_H2 phenotype[17].

Macrophages play an important role in the tumor microenvironment[21]. Referred to as tumor-associated macrophages (TAMs), these macrophages are generally tumor promoting[21]. Cytokines such as IL-4, IL-10, IL-13, in addition to the growth factor CSF1, can polarize a macrophage towards an anti-inflammatory M2-like state[22]. Highlighting the role of CSF1/CSF1R signaling in the development of these tumor-promoting macrophages, overexpression of CSF1 and CSF1R correlates with poor prognosis in human breast cancers[23]. Genetic deletions of CSF1 in mice prevent the progression from benign to malignant tumor in a mouse breast cancer model[24,25]. Furthermore, pharmacologically inhibiting CSF1R using the selective inhibitor AZD7507 caused shrinkage of tumors and promoted mouse survival in a mouse model for pancreatic ductal adenocarcinoma (PDAC)[26]. M2-like TAMs release tumor promoting factors such as the growth

factor epidermal growth factor (EGF), and angiogenic factors such as vascular endothelial growth factor A (VEGFA). These macrophages also secrete prostaglandin E2 (PGE2), which can polarize nearby macrophages to this M2-like state, in addition to promoting cancer cell proliferation. M2-like TAMs will secrete the cytokines CCL5, CCL18, IL-6, and IL-10[22]. CCL5, CCL18 and IL-6 can enhance glycolysis in the tumor microenvironment, whereas IL-10 can further dampen the immune response[22,27]. Pro-tumor TAMs also upregulate programmed cell death protein 1 (PD-1), which decreases their phagocytic capacity. In vivo, this results in decreased phagocytosis of CT26 tumor cells[28]. TAMs also secrete MMP-9, a matrix metalloprotease that promotes the proliferation of tumors[29].

In our lab, we study bone marrow-derived macrophages (BMMs). These macrophages can be obtained by differentiating bone-marrow cell suspensions (obtained by flushing the bone marrow out of mouse tibias and fibulas) into macrophages using CSF1. After culturing the macrophages for 5-7 days in media containing CSF1, the macrophage populations can be identified using flow cytometry. BMMs can be identified as showing high expression of the monocyte/macrophage phagocytotic system (MPS) marker CD115 and low expression of the granulocyte marker GR-1[30]. BMMs can also be identified using the F4/F80 pan macrophage markers. Weakly positive staining for F4/80 indicates predominately monocytes, whereas strongly positive F4/80 cells are mature macrophages[30]. The F4/80 levels are highest at about 7 days of culture, with about 70% of cells exhibiting this mature macrophage phenotype[30].

1.3 Macropinocytosis

The process of macropinocytosis was first described in the 1930's by Warren Lewis, who used time-lapse microscopy to observe fluid engulfment in macrophages and sarcoma cells[31,32].

Lewis named this process pinocytosis, derived from the Greek *pino* (drink) and *cyto* (cell)[31]. This process was renamed macropinocytosis in the 1970s to distinguish it from ingestion mediated by smaller vesicles such as clathrin and caveolin-mediated endocytosis[33]. Whereas clathrin-coated vesicles are around 100 nm in diameter, macropinosomes are much larger, ranging between 0.2-10 μM in diameter[34].

1.3.1 Physiological Relevance of Macropinocytosis

Macropinocytosis has been implicated in a wide array of physiological functions. These include positive functions such as cell growth and antigen presentation, as well as detrimental functions such as pathogen infection and cancer cell development. With regards to cell growth, macropinocytosis has been shown to be required for T cell growth[35]. In macrophages, macropinocytosis has been shown to be a supply route for amino acids leading to the activation of mechanistic target of rapamycin complex-1 (mTORC1)[36]. Macropinocytosis is also a way for antigen presenting cells to internalize solutes from the environment. In dendritic cells, extracellular antigens are internalized via macropinocytosis where, after partial proteolysis, they can be loaded onto MHC Class II molecules for subsequent antigen presentation[37]. Highlighting the role of macropinocytosis in antigen presentation, stimulators of macropinocytosis such as phorbol myristate acetate (PMA) enhance antigen presentation in bone marrow-derived macrophages, whereas inhibitors macropinocytosis such as amiloride block presentation[38].

Many protozoan, bacterial, and viral pathogens exploit macropinocytosis as part of their life cycles. Viruses such as Vaccinia virus, adenovirus, and human immunodeficiency virus 1 (HIV-1) can be internalized via macropinocytosis[39]. Adenovirus Type 2 (Ad2) can stimulate macropinocytosis itself allowing the virus to enter cells and replicate[40]. While the canonical HIV-

1 lifecycle begins with fusion at the plasma membrane, studies have suggested that in primary macrophages entry may be dependent on macropinocytosis[41]. Maréchal et. al showed that uptake of HIV-1 virions was sensitive to dimethyl amiloride, a specific inhibitor of macropinocytosis[38,41].

Bacteria such as Salmonella, Legionella and Shigella use macropinocytosis to complete their life cycle[42]. *Legionella pneumophila*, the causative agent of Legionaire's disease, can replicate within alveolar macrophages following the inhalation of aerosolized bacteria from a contaminated water supply[43]. The products of the *L. pneumophila dot/icm* genes promote macropinocytic uptake of *L. pneumophila* into the macrophages[44]. Lastly, macropinocytosis is exploited by protozoans. The protozoan parasite Leishmania exists in two forms, a flagellar form (promastigote) that replicates in the gut of sandflies, and an aflagellar form (amastigote), that replicates in mammalian hosts[45]. Amastigotes of Leishmania induce macropinocytosis in macrophages by the presentation of phosphatidylserine (PS) on their surface, which can trigger macropinocytosis[46]. Wanderley et al. showed that masking of PS using Annexin V reduces macropinocytosis in macrophages[46]. Furthermore, the phagocytosis of amastigotes was significantly reduced by amiloride treatment[46].

A link between macropinocytosis and cancer was first demonstrated in studies which showed that oncogenic Ras and Src proteins stimulated macropinocytosis[47,48]. Recently, macropinocytosis has emerged as an important nutrient scavenging mechanism that supports cancer cell development. Macropinocytosis of protein and its subsequent degradation in lysosomes provides amino acids that support the growth of Ras-transformed cells[49]. Furthermore, pharmacological inhibition of macropinocytosis was shown to attenuate the growth of pancreatic tumor xenografts, and in some instances even caused tumor regression[49]. As tumor microenvironments are nutrient poor, the ability to scavenge nutrients may be essential for tumor

growth. Under conditions of glutamine deficiency, PDAC tumors upregulate macropinocytosis through an enhancement of EGFR-Pak signaling[50]. Highlighting the important role of macropinocytosis in PDAC, a genome-wide CRISPR screen performed in pancreatic cancer cells identified many key genes in macropinocytosis as essential for growth[51]. In addition to pancreatic tumors, macropinocytosis is important for the growth of bladder, colon, lung, and prostate cancers[52]. Regarding prostate cancers, many harbor deletions in PTEN, a negative regulator of PI3K signaling. Under nutrient limiting conditions, PTEN-deficient prostate cancer cells exhibited enhanced levels of macropinocytosis[53]. This enhanced macropinocytosis was only seen in conditions when the AMP-activated protein kinase (AMPK) was active[53]. As AMPK is a key energy sensor that is activated under glucose-starvation conditions, this represents a pathway that enables prostate cancer cells to survive nutrient poor environments by upregulating macropinocytosis[54].

1.3.2 Mechanism of Macropinocytosis

The process of macropinocytosis can occur in a signal-dependent manner, following stimulation by growth factors, cytokines, or bacterial products, or it can occur constitutively. Growth factors such as CSF1 or EGF, cytokines such as CXCL12, or bacterial products such as LPS, have been shown to induce macropinocytosis (**Figure 1.1**)[55–58].

In the context of CSF1, CSF1 interacts with CSF1R, which signals through phosphatidylinositol 3-kinase (PI3K) to generate a transient spike of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) at the base of the forming macropinosome[59]. This is followed by the generation of diacylglycerol (DAG), in a manner dependent on phospholipase C-γ (PLCγ)[60]. PMA works as a DAG mimetic, and thus can stimulate macropinocytosis independently of PI3K

signaling (**Figure 1.1**). Closure of the macropinosome is accomplished through the actions of protein kinase C (PKC) and the Ctb1/BARS complex[61,62]. Other molecules necessary for macropinocytosis include Rac1, the p21-activated-kinase-1 (Pak1), as well as Rabs 5, 10, and 35[63–65].

Macropinocytosis can also occur constitutively[66,67]. Compared to macropinosomes formed in response to stimuli, those formed through constitutive macropinocytosis are often much smaller[68]. The classic example of constitutive macropinocytosis can be observed in transformed cells. Cells expressing oncogenic Ras or Src proteins exhibit constitutive macropinocytosis[47,48]. While most non-transformed cells require induction of macropinocytosis, this is not the case with macrophages and dendritic cells[67]. Constitutive macropinocytosis equips these cells to sample their environment and to obtain antigens to present on MHC molecules[37,38,67]. Constitutive macropinocytosis in human monocyte-derived macrophages is calcium dependent and relies on signaling from the extracellular calcium receptor[68]. In mice, constitutive macropinocytosis in macrophages and dendritic cells is dependent on phosphatidic acid, which is involved in actin polymerization[69].

A common model organism for studying macropinocytosis is the amoeba *Dictyostelium discoideum*. Macropinocytosis was first described in amoebas around the same time that it was described in mammalian cells[70]. Wildtype cells of *Dictyostelium* are obligatory phagocytes; however, laboratory strains can survive axenically. Bloomfield et al. show that deletions in the RasGAP Neurofibromin-1 results in the formation of larger macropinosomes, allowing *Dictyostelium* to internalize sufficient nutrients for their survival[71]. One benefit to this model organism is the existence of both forward and reverse genetic systems[71,72]. Studies in *Dictyostelium* have gleaned much information about the regulation of macropinocytosis. For example, an Akt

(PkbA) and a SGK (PkbR1) kinase are essential for Dicytostelium growth axenically, and result in the formation of larger macropinosomes[73]. That study represents one of a few studies describing regulators of macropinosome size. Moreover, much of our knowledge about how nutrients regulate macropinocytosis come from studies in *Dictyostelium*. As macropinocytosis is an evolutionarily conserved process, there is overlap between macropinocytosis in *Dicytostelium* and in mammalian cells; thus it is plausible that findings in *Dictyostelium* may be applicable to mammalian cells and vice versa[74].

1.3.3 Nutrient Regulation of Macropinocytosis

Cells encounter a diverse array of material in the environment, including glucose, vitamins, proteins, as well as free amino acids, some of which regulate macropinocytosis. Vitamins do not affect macropinocytosis in either alveolar macrophages or in *Dictyostelium*[75,76]. While glucose depletion enhances macropinocytosis in certain cancer cell lines, it has not been shown to affect macropinocytosis in non-transformed cells[53]. Extracellular protein increases macropinocytosis in *Dictyostelium*[76]. With regards to amino acids, Besterman et al. showed that rates of pinocytosis in rabbit alveolar macrophages decreased in the presence of extracellular essential amino acids[75]. Single amino acids have also been shown to regulate macropinocytosis. In *Dictyostelium,* arginine, lysine, and glutamate have been shown to upregulate macropinocytosis[76].

1.4 CSF1R Dynamics

Numerous exogenous molecules modulate CSF1R. These include bacterial products such as LPS and bacterial DNA, cytokines such as IL-2 and IL-4, and growth factors such as CSF1[77–

[80]. This regulation can occur via distinct mechanisms. Whereas binding of CSF1 to CSF1R promotes the internalization and degradation of CSF1R, bacterial DNA promotes CSF1R shedding from the cell surface[78,81,82]. Endogenous molecules regulate CSF1R as well. One such protein is Eps15-homology domain containing protein 1 (EHD1). The EHD family of proteins are key regulators of endocytic transport[83]. Recent reports have shown that EHD1-knockout macrophages express reduced CSF1R levels on the cell surface, resulting in a decrease in macrophage proliferation[84]. Later studies have implicated EHD1 as a regulator of other growth factor receptors such as the EGF receptor (EGFR)[85].

1.4.1 The Release of CSF1R

The two major mechanisms of cell surface receptor release are secretion and shedding. Secretion of surface receptors can occur in extracellular vesicles (EV), which include exosomes and microvesicles[86]. Exosomes are derived from the endosomal system whereas microvesicles originate at the plasma membrane[86]. The sizes of both kinds of vesicle are similar, with exosome sizes ranging from 50 – 150 nm and microvesicles ranging from 50-500 nm in diameter. The secretion of surface receptors in microvesicles has been demonstrated for the chemokine receptor CCR5, in addition to an oncogenic form of EGFR, EGFRvIII[87,88]. With regards to EGFRvIII secretion, the secreted EGFRvIII-containing microvesicles can be internalized by cells lacking EGFRvIII, thereby transferring oncogenic activity to these naive cells[87]. The chemokine receptor, CCR5, is a central co-receptor involved in HIV-1 entry. Mack et al. showed that CCR5 could be secreted in microvesicles from peripheral blood mononuclear cells and Chinese hamster ovary cells[88]. CCR5-negative cells could internalize these microvesicles and become CCR5-positive. Physiologically, this could render CCR5-negative cells susceptible to infection by HIV-1[88].

To our knowledge, CSF1R has not yet been shown to be selectively secreted in EVs. Instead, CSF1R can be shed from the macrophage following a proteolytic cleavage reaction involving A Disintegrin and Metalloproteinase Domain 17 (ADAM17)[89,90]. ADAM17 was originally recognized as the enzyme responsible for cleaving TNF-α into its active form; hence, ADAM17 was first named TNF-α converting enzyme (TACE)[91,92]. There are over 80 substrates of ADAM17, including growth factors, cytokines, adhesion molecules, and their respective receptors[93]. ADAM17 is expressed in most tissues[94]. Functional ADAM17 activity is critical for health: people lacking ADAM17 can have been shown to have inflammatory skin and bowel disease[95].

ADAM17 is expressed in cells in an inactive form and an active form lacking the N terminal prodomain[96]. Most of the active form of ADAM17 localizes to membranous organelles in the perinuclear area, with only a small minority detectable at the plasma membrane[96,97]. ADAM17 is comprised of 5 major domains; a pro-domain, a metalloprotease-domain, a disintegrin domain, a membrane proximal domain, and a transmembrane cytoplasmic domain[93]. The N-terminal pro-domain inhibits the catalytic activity of ADAM17; it must be cleaved for ADAM17 to become active[98]. The metalloprotease domain is the catalytic site required for ectodomain shedding of target molecules. The disintegrin domain is thought to aid in adherence between cells[99]. The membrane proximal domain is involved in substrate recognition and is also a site of regulation[100]. When the membrane proximal domain switches from an open to a closed conformation, it prevents access to the transmembrane cytoplasmic domain, which is where ADAM17 is phosphorylated, leading to its activation[93]. ADAM17 can be activated by p38 MAP kinase and protein kinase C (PKC)[101,102]. IRhom2, a member of the Rhomboid family of proteases, is required for the activation of ADAM17 for some but not all substrates. While iRhom2 is required

for ADAM17-mediated shedding of CSF1R and TNFα, it is not required for ADAM17-mediated shedding of TGFα[103–105] .

Recently, links have been made between ADAM17 and SARS-CoV-2. Angiotensin-converting enzyme (ACE)-2 has been shown to both facilitate entry of SARS-CoV-2 into cells, and to mediate protection against severe disease by negatively regulating the renin-angiotensin system (RAS)[106]. RAS overactivation is a hallmark of cardiovascular disease[107]. ACE-2 exerts its effects by degrading angiotensin II, the main effector of RAS dysfunction, to angiotensin 1-7[108,109]. ADAM17 has been shown to cleave ACE-2 resulting in a loss of ACE-2-mediated protection against RAS dysfunction[110]. As patients with cardiovascular complications are at a significant risk for developing severe COVID-19, ADAM17 represents a potential therapeutic target[106,111].

1.5 Amino Acid Entry and Sensing

Amino acids are the building blocks of proteins. There are twenty amino acids that comprise proteins. Nine of these amino acids are termed essential amino acids, as mammalian cells do not possess the machinery to synthesize these amino acids *de novo*. As such, these amino acids must be obtained from the diet, and internalized by cells from the extracellular environment. These amino acids include leucine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The eleven remaining amino acids are termed non-essential amino acids, as the cell possesses the machinery to generate these amino acids from other metabolic precursors. These include asparagine, aspartate, glutamate, serine, alanine, arginine, cysteine, glutamine, glycine, proline, and tyrosine. Some non-essential amino acids are classified as “conditionally essential”, as there are physiological or pathological conditions where they must be obtained from

the diet. Tyrosine is a conditionally essential amino acid. As its synthesis requires phenylalanine, tyrosine can no longer be synthesized by the cell if stores of phenylalanine are depleted[112].

Amino acids can be internalized into cells either through transporters in the plasma membrane or they can be internalized via endocytosis[113]. Endocytic mechanisms of internalization include clathrin-mediated endocytosis and macropinocytosis[113]. Amino acids are either internalized as free amino acids or as proteins. However, as free amino acids represent a minor component of plasma, most amino acids obtained by endocytosis will be derived from internalized extracellular proteins such as albumin[114]. Amino acid transporters (AATs) are membrane-bound proteins that mediate movement of amino acids across membranes into the cytoplasm[115]. AATs are located on the plasma membrane in addition to organelles such as the lysosome. In macrophages, CD98 (LAT1), CAT2B, ASCT2, and SNAT are the major transporters[116]. LAT1 transports large neutral amino acids which include histidine, leucine, isoleucine, tryptophan, tyrosine, valine, phenylalanine, methionine, and threonine[117]. ASCT2 and SNAT transport serine and glutamine[118]. CAT2B is the primary transporter for arginine[116]. Once internalized, amino acids can be detected by dedicated amino acid sensing machinery.

The two major mammalian amino acid sensors are general control non-depressible-2 (GCN2), and mTORC1[119]. GCN2 is a protein kinase that senses uncharged (amino acid-free) tRNAs, which accumulate under amino acid starvation. GCN2 binds uncharged tRNA, resulting in a confirmational change that leads to GCN2 activation. Active GCN2 phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2), a key protein involved the initiation of translation, which ultimately leads to a global downregulation of protein synthesis[120]. GCN2 responds to decreasing levels of the twenty proteinogenic amino acids[121]. While active GCN2 generally inhibits protein synthesis, certain gene products are upregulated in response to GCN2. One such

product is the transcription factor ATF4[122]. ATF4 expresses genes involved in amino acid transport and metabolism such as AATs and amino acyl tRNA synthetases[123]. ATF4 can also lead to the expression of Sestrin2 which functions as a negative regulator of mTORC1[123]. Thus, GCN2 can function to inhibit mTORC1 indirectly.

MTORC1 is the master regulator of cell growth[124]. While GCN2 responds to the absence of amino acids, mTORC1 is activated by their presence. Unlike GCN2, which can respond to decreases in any of the twenty amino acids, mTORC1 responds to certain amino acids preferentially[125]. Leucine and arginine have important roles in the activation of mTORC1. Both Sestrin2 and CASTOR1 can function to inhibit GATOR2, a positive regulator of mTORC1[125]. The binding of leucine to Sestrin2, or the binding of arginine to CASTOR1, prevents these proteins from inhibiting GATOR2, thus activating mTORC1[126,127]. While arginine and leucine have profound effects on mTORC1 activation, the removal of most amino acids will also reduce mTORC1 activity[128]. While mTORC1 can sense and respond to amino acids, other inputs such as growth factors, energy levels, and cellular stress signals can activate mTORC1 as well.

MTORC1 is defined by its five components: mTOR, regulator protein associated with mTOR (RAPTOR), mammalian lethal with sec13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (DEPTOR), and 40 kDa Pro-rich Akt substrate (PRAS40)[129] . MTOR is a kinase that functions as the catalytic unit of mTORC1 and the related complex mTORC2. Whereas, mTORC1 contains five components, mTORC2 contains six. Like mTORC1, mTORC2 contains mTOR, DEPTOR, and mLST8[129]. Instead of RAPTOR and PRAS40, mTORC2 contains rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and protein observed with RICTOR (PROTOR)[129]. While the predominant function of mTORC1 is to regulate cell growth, mTORC2 regulates actin

cytoskeletal organization and cell survival[129–131]. The activity of mTORC1 is regulated by the Rheb and Rag GTPases in addition to Ragulator. In response to amino acids, the Rag GTPases associate with mTORC1 and promote its translocation to lysosomal compartments[132,133]. Once at lysosomal membranes mTORC1 can be activated by Rheb[134]. Lastly, the pentameric protein complex Ragulator is essential for mTORC1 activation, as it functions to tether the Rag-mTORC1 complex to the lysosomal membrane[133,135]. Crosstalk between mTORC1 and mTORC2 is thought to be mediated by Akt. Akt, which can be activated by mTORC2, inhibits the tuberous sclerosis complex 2 (TSC2), an inhibitor of mTORC1[136].

Interestingly, mTORC1 has also been shown to influence macropinocytosis. Shao et al. showed that mTORC1 inhibition in human trophoblasts increased macropinocytosis[137]. While GCN2 and mTORC1 are responsible for sensing intracellular amino acid levels, extracellular sensors have been reported as well. The calcium-sensing receptor (CaR) which canonically functions as a sensor of extracellular calcium can also sense extracellular amino acids under certain environmental contexts[138].

1.6 In this thesis

Macropinocytosis is an important physiological process with both beneficial and detrimental roles for the host. While much is known about the molecular mechanisms involved in the formation, internalization, and trafficking of macropinosomes, relatively little is known about its regulation, especially by nutrients. As macropinocytosis is a process by which cells can internalize nutrients from their environment, it is plausible that nutrients play an important regulatory role. One category of nutrient that is relatively understudied is amino acids; so we made them the focus of this investigation.

The following chapters investigate the role of amino acids in the regulation of macropinocytosis. In Chapter 2, we explore how amino acids, both individually and combined, affect macropinocytosis. We find that certain amino acids, termed suppressive amino acids or suppressors, can decrease total solute accumulation by macropinocytosis. Furthermore, we begin to uncover how this occurs mechanistically. We show that suppressors promote the release of CSF1R, which results in the formation of smaller macropinosomes. In Chapter 3, the mechanisms underlying this process are further explored. We investigate how suppressors enter the macrophage and are sensed, in addition to the mechanism underlying CSF1R release. We conclude this dissertation in Chapter 4 with a discussion of the data, major implications of the research, and plans for future experiments. This work advances our understanding of how nutrients regulate macropinocytosis, which has consequences on macrophage differentiation and polarization.

1.7 References

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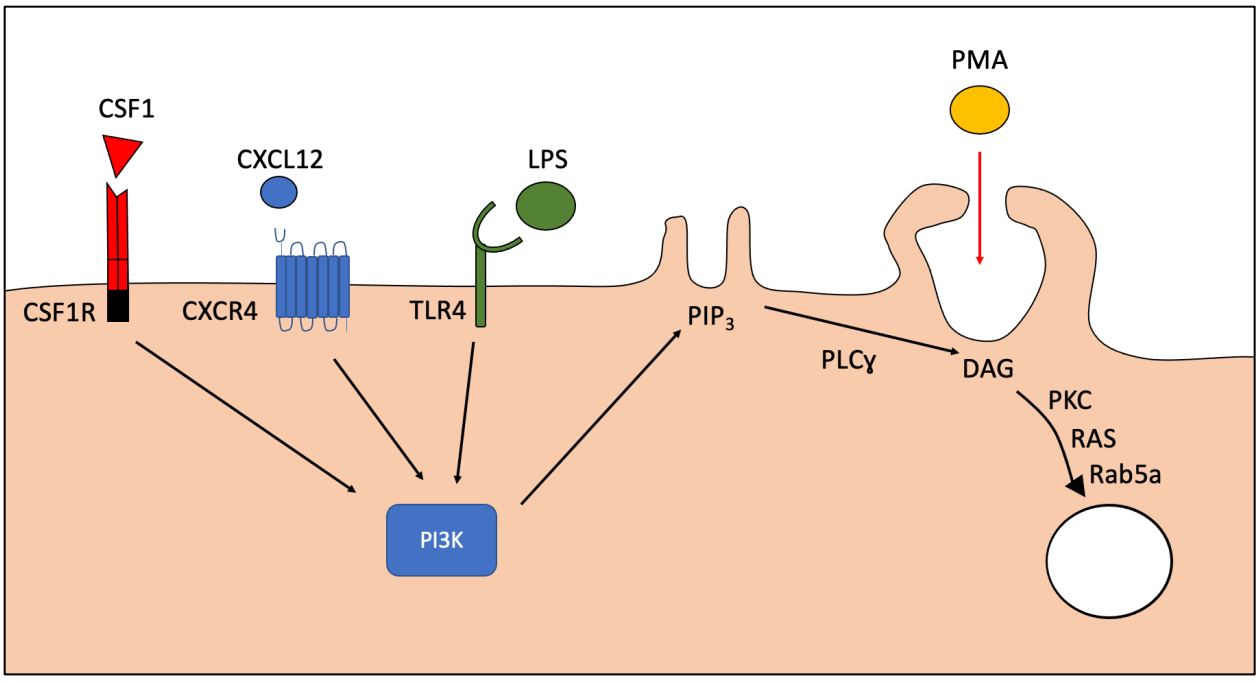

Figure 1.1: **Macropinocytosis can be stimulated by different molecules**: Macropinocytosis can be stimulated in a PI3K-dependent manner, such as with CSF1, LPS, or CXCL12, or in a PI3K-independent manner, such as with PMA. For the PI3K-dependent mechanisms, the respective receptors for the ligands are shown. PMA functions as a DAG mimetic and does not signal through a receptor.

Chapter 2
Amino Acids Suppress Macropinocytosis and Promote Loss of CSF1 Receptor in Macrophages

2.1 Abstract

The internalization of solutes by macropinocytosis provides an essential route for nutrient uptake in many cells. Macrophages increase macropinocytosis in response to growth factors and other stimuli. To test the hypothesis that nutrient environments modulate solute uptake by macropinocytosis, this study analyzed the effects of extracellular amino acids on the accumulation of fluorescent fluid-phase probes in murine macrophages. Nine amino acids, added individually or together, were capable of suppressing macropinocytosis in macrophages stimulated with the growth factors colony-stimulating factor-1 (CSF1) or interleukin 34, both ligands of the CSF1 receptor (CSF1R). The suppressive amino acids did not inhibit macropinocytosis in response to lipopolysaccharide, the chemokine CXCL12, or the tumor promoter phorbol myristate acetate. Suppressive amino acids promoted release of CSF1R from cells and resulted in the formation of smaller macropinosomes in response to CSF1. This suppression of growth factor-stimulated macropinocytosis indicates that different nutrient environments modulate CSF1R levels and bulk ingestion by macropinocytosis, with likely consequences for macrophage growth and function.

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2.2 Introduction

Macropinocytosis is an actin-driven cellular process in which cells internalize relatively large volumes of fluid into plasma membrane-derived vesicles known as macropinosomes. It has been implicated in antigen presentation, cell growth, and metabolic regulation[1–4]. Macropinocytosis may occur constitutively or following stimulation by growth factors, chemokines, or microbial products[5]. In macrophages, the growth factor colony-stimulating factor-1 (CSF1) stimulates macropinocytosis[6]. Although extensive work has defined the molecular mechanisms involved in the formation, internalization, and trafficking of macropinosomes[5], the regulation of macropinocytosis has been relatively understudied.

Macropinocytosis provides a mechanism for nutrient uptake that can support growth for tumor cells and lymphocytes[3,4,7]. Following stimulation of macrophages with CSF1, macropinocytosis delivers extracellular leucine into lysosomes to activate the mechanistic target of rapamycin complex-1 (mTORC1), a nutrient sensing complex that functions as a central regulator of cell growth[2,8]. As macropinocytosis provides nutrients to the cell, we hypothesized that nutrients themselves may regulate this process.

Several studies have indicated such regulation. Glucose depletion increases macropinocytosis in some cancer cell lines, but not in non-transformed cells[9]. Besterman et al.[10] showed that constitutive pinocytosis in rabbit alveolar macrophages decreased in the presence of essential amino acids; however, the mechanism of pinocytosis or its inhibition by amino acids was not determined. Specific single amino acids can alter a variety of cellular processes[11], including macropinocytosis[12]. In *Dictyostelium*, arginine, lysine, and glutamate can individually upregulate macropinocytosis[13]. In pancreatic ductal adenocarcinoma tumors, glutamine depletion enhances

macropinocytosis by potentiating epidermal growth factor receptor signaling[12]. Here we examined the effects of single amino acids on macropinocytosis in murine bone marrow-derived macrophages (BMM) and discovered that some amino acids suppress CSF1-stimulated macropinocytosis by promoting the loss of CSF1 receptor (CSF1R).

2.3 Results

2.3.1 Nine amino acids suppress macropinocytosis

We first examined the effect of leucine on macropinocytosis, as leucine has a well characterized role as an activator of mTORC1[14,15]. BMM were deprived of CSF1 overnight, then incubated 30 min in phosphate-buffered saline (PBS) with or without 0.25 mM leucine, a physiologically relevant concentration[16]. Cells were then incubated 60 min with 70 kDa fluorescein-isothiocyanate dextran (FDx), a specific marker for macropinocytosis[17,18], with or without CSF1 or leucine, before analysis by flow cytometry. Leucine inhibited CSF1-stimulated uptake of FDx by 40% (**Fig. 2.1A**). To further explore the physiological relevance of this finding, we performed these experiments in the presence of albumin, the major protein in circulation[19], as well as glucose. While both bovine serum albumin (BSA) and glucose slightly increased macropinocytosis, leucine still suppressed macropinocytosis in those conditions (**Fig. 2.2**).

In dendritic cells, FDx endocytosis is mediated in part by the mannose receptor[1]. In BMM, FDx is a valid probe for fluid-phase endocytosis[17]. However, as macrophages sometimes express high levels of the mannose receptor[20], we sought to confirm that leucine was specifically affecting fluid-phase macropinocytosis and not mannose receptor-mediated endocytosis. Lucifer yellow (LY) is a fluid-phase probe whose internalization is not mediated by the mannose receptor[1,17,21]. CSF1-stimulated uptake of LY was measured in the presence or absence of leucine. Similar to

FDx, CSF1-stimulated uptake of LY was reduced in the presence of leucine (**Fig. 2.1B**). This indicates that mannose-receptor-mediated endocytosis did not contribute to the accumulation of FDx by macrophages.

We next asked whether other amino acids could suppress macropinocytosis. We performed similar experiments as in Figure 2.1A, incubating BMM in PBS containing each of the other 19 amino acids that comprise proteins. To be consistent with the leucine experiments, the concentration for each amino acid was 0.25 mM. We defined any amino acid that reduced total macropinocytic uptake by at least 20% as a suppressor, and one that did not as a non-suppressor. Nine of the twenty amino acids were suppressors (**Fig. 2.1C**). Most of the essential amino acids were suppressors, with the exception of histidine and lysine. The non-essential amino acids were non-suppressors, with the exception of cysteine and tyrosine. We next sought to determine the minimal concentration by which leucine suppresses by macropinocytosis. To this end, cells were incubated in PBS with or without leucine at different concentrations and CSF1-stimulated uptake was quantified. Maximal suppression of macropinocytosis occurred at leucine concentrations greater than 125 μM, with intermediate suppression at concentrations around 25 μM. No suppression occurred at concentrations below 2.5 μM (**Fig. 2.1D**).

Combined suppressors and non-suppressors behaved like the individual amino acids: macropinocytosis was suppressed in cells incubated with the 9 suppressive amino acids but was not suppressed when incubated with the 11 non-suppressive amino acids (**Fig. 2.1E**). However, when macrophages were incubated in a mixture containing all twenty amino acids, no suppression was observed (**Fig. 2.1E**). From this we hypothesized that either the non-suppressive amino acids were dominant in the "all amino acid" mixture, or particular non-suppressive amino acids could inhibit the action of the suppressive amino acids. To address this, cells were incubated in a mixture

containing leucine plus the 11 non-suppressive amino acids. Leucine was sufficient to suppress macropinocytosis (**Fig. 2.1E**). This refutes the hypothesis that one or more non-suppressive amino acids can inhibit suppression. We cannot yet explain why leucine or the other suppressors were not dominant when all amino acids were present.

2.3.2 Amino acids selectively suppress CSF1R-dependent macropinocytosis.

To examine whether this amino acid-dependent suppression of uptake is present in other forms of endocytosis, we utilized leucine as a model suppressive amino acid and evaluated its effect on receptor-mediated endocytosis. We measured uptake of fluorescent acetylated low density lipoprotein (AcLDL), which binds to class A scavenger receptors and is internalized in clathrin-coated pits[22]. Cells were allowed to internalize either DiI-labeled AcLDL or FDx plus CSF1 in the presence or absence of leucine. Leucine did not inhibit uptake of DiI-AcLDL (**Fig. 2.3A**), suggesting that leucine specifically downregulates macropinocytosis. Macropinocytosis can be induced in macrophages by stimuli other than CSF1, including lipopolysaccharide (LPS)[23], the chemokine CXCL12[24], and the tumor promoter phorbol myristate acetate (PMA)[25]. To determine whether macropinocytosis is suppressed generally by amino acids, we compared the effects of leucine on macropinocytosis induced by CSF1, PMA, LPS or CXCL12. All increased macropinocytosis, with CXCL12 and LPS stimulating less than PMA and CSF1 (**Fig. 2.3B**). Leucine failed to suppress constitutive pinocytosis in unstimulated BMM, as well as macropinocytosis in response to PMA, CXCL12, or LPS (**Fig. 2.3C-F**). To test whether amino acid-dependent suppression was specific to CSF1R signaling, we utilized interleukin 34 (IL-34), which signals through CSF1R[26] to promote macrophage differentiation, growth and survival[27,28]. IL-34 stimulated macropinocytosis to the same extent as CSF1 (**Fig. 2.3B**). IL-34-stimulated

macropinocytosis was inhibited by leucine (**Fig. 2.3G**), suggesting that amino acid-dependent suppression of macropinocytosis is specific to CSF1R signaling.

2.3.3 Suppressive amino acids promote loss of CSF1R

To begin to define a mechanism by which leucine suppresses IL-34- and CSF1-stimulated macropinocytosis, we measured the effects of amino acids on cell surface expression of CSF1R. Compared to the PBS control, the suppressors leucine, phenylalanine and isoleucine significantly reduced the cell surface levels of CSF1R. The non-suppressors serine, asparagine and glutamate did not (**Fig. 2.4A**). As a control for specificity, we measured cell surface levels of CXCR4, the receptor for CXCL12[29]. As CXCL12-stimulated macropinocytosis was not sensitive to leucine, we expected that incubation with leucine would not reduce levels of CXCR4. Cell surface CXCR4 was unaffected by the presence of leucine (**Fig. 2.4B**), indicating that leucine and other suppressive amino acids specifically reduce surface levels of CSF1R.

To determine whether CSF1R was sequestered or degraded in response to suppressive amino acids, BMM were incubated in PBS with or without a suppressor (phenylalanine, leucine or isoleucine), or a non-suppressor (serine, glutamate, or asparagine). Cells were then fixed, permeabilized and stained to detect both intracellular and surface CSF1R. As a control for CSF1R degradation, cells were incubated in PBS + CSF1, as CSF1 promotes the internalization and degradation of CSF1R[30]. Cells were imaged by confocal microscopy and processed using a CellProfiler™ analytical pipeline, which allowed quantification of levels of CSF1R on a per cell basis for each of the conditions examined. Cells incubated with CSF1 exhibited about a 60% reduction in CSF1R levels (**Fig. 2.4C-D**). Cells incubated with a suppressor exhibited significant reductions in CSF1R levels, while those incubated with a non-suppressor did not (**Fig. 2.4C-D**).

The microscopy data were confirmed using western blotting. (**Fig. 2.4E**). Thus, our data suggest that CSF1R levels are reduced in response to suppressive amino acids.

To test the hypothesis that CSF1R was being degraded, we incubated cells with leucine in the presence of bafilomycin A1 (Baf). Baf is macrolide antibiotic which functions as potent inhibitor of the vacuolar ATPase, preventing lysosomal acidification and acid hydrolase-dependent protein degradation[31]. If leucine promotes lysosomal degradation of CSF1R, we would expect an increase in CSF1R levels in the Baf + leucine condition compared to the leucine alone condition. As a positive control for the effect of Baf on lysosomal degradation of CSF1R, cells were incubated with CSF1, which is known to promote lysosomal degradation of CSF1R, with or without Baf. We observed a significant increase in CSF1R levels, comparing the CSF1 + Baf-treated cells to the CSF1 alone cells (**Fig. 2.5A-B**). Contrary to our hypothesis, however, Baf did not increase CSF1R in the leucine-treated cells, which suggests that leucine does not promote lysosomal degradation of CSF1R (**Fig. 2.5A-B**).

As leucine appeared to promote neither sequestration nor degradation of CSF1R, we hypothesized that leucine was promoting the release of CSF1R from the macrophages. To test this, we assayed for the presence of CSF1R in the supernatants of cells incubated in either PBS alone, PBS with the suppressor leucine, or PBS with the non-suppressor serine. Significantly higher levels of CSF1R were detected in the supernatant of cells incubated in leucine compared to those incubated in serine or PBS alone (**Fig. 2.5C-E**). Thus, our data suggests that leucine promotes the release of CSF1R from macrophages.

To interrogate the dynamics of CSF1R release, we measured CSF1R levels of cells incubated in PBS with or without leucine at varying times from 1 min until 60 min. We also incubated cells in PBS with CSF1 to compare these dynamics to a degradative process. As

expected, cells incubated in PBS alone exhibited constant levels of CSF1R throughout the time course, whereas incubation with CSF1 caused rapid internalization and degradation of CSF1R. In contrast, cells incubated in leucine maintained steady levels of CSF1R for the first 15 min of incubation, followed by a slight decrease from 15 min to 30 min, and finally a large decrease from 30 min to 45 min (**Fig. 2.6**). CSF1R levels stayed constant from 45 min to 60 min (**Fig. 2.6)**. Moreover, as further evidence that leucine promotes release rather than internalization and degradation of CSF1R, we did not detect redistribution of CSF1R into punctate vesicles in the leucine condition, which was evident when CSF1R was downregulated by incubation in CSF1 (**Fig. 2.6).**

2.3.4 Leucine reduces the size of macropinosomes

Lastly, to identify the mechanism of reduced solute accumulation by suppressive amino acids, we examined cells microscopically in conditions with or without suppressive amino acids. In live cell imaging of cells incubated in CSF1 or CSF1 + leucine, we could not discern any obvious differences in ruffling or the process of macropinosome formation. This was likely due to the wide range of morphologies that characterize macropinocytosis[32]. Next, we quantified the numbers and sizes of macropinosomes formed in response to CSF1. Cells were incubated 30 min with or without leucine or serine, then were pulsed 5 min with CSF1 and FDx. Because macropinosomes shrink and fuse shortly after closing into the cell, it was necessary to image them for morphometry after only brief pulses with FDx to best approximate their initial sizes. Earlier work from this lab[33] and others[34] showed that 1-5-minute pulsed macropinosomes are enriched in markers of early endosomes, including Rab5. The cells were quickly washed and then imaged. An analytical pipeline was created using CellProfiler™ to quantify macropinosome numbers and sizes in the

micrographs (**Fig. 2.7A**). The number of macropinosomes generated did not change significantly in any of the conditions (**Fig. 2.7B**), but cells incubated with leucine made significantly smaller macropinosomes compared to cells incubated with serine or PBS alone (**Fig. 2.7C**). This indicated that the decreased FDx accumulation observed in the flow cytometry data resulted from the formation of smaller macropinosomes. From this we hypothesized that reducing CSF1R levels would result in smaller macropinosomes. To lower CSF1R levels another way, we incubated cells in CSF1 for 60 min, which lowers CSF1R levels in BMM significantly (**Fig. 2.4**). CSF1-treated cells and untreated control cells were pulsed for 5 min with CSF1 and FDx, then washed, fixed, imaged, and analyzed in the CellProfiler™ pipeline. Cells incubated in CSF1 exhibited fewer and smaller macropinosomes compared to those incubated in PBS alone. (**Fig. 2.8**). Thus, macrophages form smaller macropinosomes when CSF1R levels are decreased by exogenous molecules.

2.4 Discussion

The modulation of CSF1R expression described here suggests roles for amino acids in the regulation of macrophage physiology. CSF1R signaling is critical for macrophage proliferation, survival and differentiation[35]. Many exogenous molecules have been shown to modulate CSF1R[36,37]. CSF1 promotes the internalization and degradation of CSF1R, mainly via clathrin-coated vesicles[24,30], while LPS induces secretion of factors that downregulate CSF1R[36]. The present study shows that suppressive amino acids promote release of CSF1R, resulting in the formation of smaller macropinosomes and consequently less ingestion of extracellular solutes and nutrients.

The secretion of surface receptors in microvesicles has been shown in the context of an oncogenic form of the epidermal growth factor receptor, EGFRvIII, and of CCR5, the co-receptor

for HIV-1[38,39]. With regards to EGFRvIII secretion, microvesicles containing EGFRvIII can be internalized by cells lacking EGFRvIII, which transfers oncogenic activity to the cells lacking the mutant receptor[38]. Future studies aim to elucidate whether CSF1R is secreted in microvesicles or shed from the cell surface in another form.

In vivo, an imbalance of suppressive and non-suppressive amino acids in different nutrient environments, such as nutrient replete healthy tissue or nutrient deficient tumor microenvironments, could regulate surface expression of CSF1R, with consequent effects on macrophage growth, differentiation, and function. Tumor-associated macrophages (TAMs) are anti-inflammatory and tumor promoting[40]. One of the molecules responsible for polarizing macrophages to this subset is CSF1[41]. As the presence of TAMs is associated with poor prognosis in many cancer types[42], therapies that modulate CSF1R signaling have garnered attention[43]. The regulation described here may be relevant to macrophage differentiation in tumors.

The nine suppressors of CSF1R macropinocytosis were predominately essential amino acids, thus the detection system may be directed toward these amino acids. However, two non-essential amino acids, cysteine and tyrosine, suppressed macropinocytosis and two essential amino acids, histidine and lysine, did not. Cysteine and tyrosine require or substantially rely on an essential amino acid for their synthesis. Tyrosine requires phenylalanine, while cysteine substantially relies on methionine for its synthesis through the transsulfuration pathway[44,45]. Thus, the requirement of essential amino acids for synthesis of cysteine and tyrosine may explain why they are included among the suppressors.

How suppressive amino acids promote release of CSF1R remains unknown, but the process may involve known metabolic sensors of amino acids. General control non-depressible-2 (GCN2) kinase senses uncharged tRNA molecules, which are abundant under amino acid starvation, and

in turn phosphorylates eIF2α resulting in a global downregulation of protein synthesis[46]. Similarly, in amino acid-deficient environments, mTORC1 is dissociated from lysosomes, preventing its function as a metabolic hub to promote various anabolic processes[47]. Inhibition of mTORC1 in human trophoblasts was recently shown to enhance macropinocytosis[48]. GCN2 or mTORC1 may regulate CSF1R expression at the cell surface. Either the detection system or the mechanism of CSF1R release must be responsive to the composition of amino acids in the environment, as suppression is lost when all 20 amino acids are present.

The mechanisms regulating macropinosome size in metazoan cells are largely unknown. Unlike phagocytosis, in which the dimensions of a particle determine the size of the phagosome, there is no structure to guide macropinosome formation. Rather, macropinosomes are self-organized structures that assemble from cell surface ruffles that close into cups, which then close into macropinosomes[5,49]. What is known about the regulation of macropinosome size largely comes from genetic studies. In *Dictyostelium*, an Akt (PkbA), and an SGK (PkbR1), were shown to regulate macropinosome size, with PkbA-/PkbR1- double knockouts forming smaller macropinosomes[50]. Also in Dictyostelium, the RasGAP Neurofibromin-1 was shown to regulate macropinosome size with knockout mutants forming larger macropinosomes[51]. This study reveals exogenous regulation of macropinosome size by amino acids, resulting from a decrease in cell surface CSF1R levels.

If amino acids in the environment modulate growth factor receptor levels in other cell types, then this could have implications for the regulation of cell growth in tissues and the upregulation of macropinocytosis in some kinds of cancer cells. Accordingly, constitutive macropinocytosis in tumor cells may be due to loss of feedback inhibition by amino acids of

growth factor-related signals. A more thorough understanding of the effects of nutrient environments on growth factor function could guide the design of therapies.

2.5 Materials and Methods

2.5.1 Materials

RPMI-1640, fetal bovine serum (certified; FBS), GlutaMAX, penicillin-streptomycin (P/S), 70 kDa Fluorescein-isothiocyanate dextran (Fdx), Lucifer yellow CH, DiI-AcLDL, unlabeled AcLDL, bovine serum albumin, goat serum, DAPI solution, paraformaldehyde, 0.25% Trypsin-EDTA with phenol red, Prolong™ Diamond Antifade Mountant, DPBS, Hoechst 33342, CellTracker™ Red CMTPX Dye, Halt™ Protease Inhibitor Cocktail, and phycoerythrin CXCR4 antibody (2B11) were purchased from ThermoFisher. IL-34 and recombinant mouse CSF1 were purchased from R&D Systems. HEPES, 2-Mercaptoethanol, sucrose, Amicon Ultra-2 mL centrifugal filters, and all amino acids were purchased from Sigma. Bafilomycin A1 from *Streptomyces griseus* was purchased from Cell Signaling Technology. Phorbol 12-myristate 13-acetate (PMA), allophycocyanin anti-CSF1 receptor antibody (ab210247) for immunofluorescence and flow cytometry, Phalloidin-iFluor 488, and recombinant anti-CSF1 receptor antibody (ab221684) for western blotting were purchased from Abcam. CXCL12 was purchased from Peprotech. LPS from *Salmonella Typhimurium* was purchased from List Biological Laboratories. 35mm dishes with 14mm coverglass were purchased from MatTek Corporation. IRDye 680RD Goat anti-Rabbit IgG and IRDye 800CW Goat anti-Mouse IgG secondary antibodies for western blotting were purchased from LI-CORE Biosciences.

2.5.2 Bone Marrow Macrophage Isolation and Culture

Macrophages were generated from C57BL6/J mice (Jackson Laboratory). Both male and female mice between the ages of 3 – 12 months were used. Bone marrow flushed from mouse femurs were differentiated into macrophages by culture for 5 days in RPMI supplemented with 20% FBS, 50 ng/mL recombinant CSF1, 1% glutamax, 0.1% penicillin-streptomycin, 37 μM 2-mercaptoethanol. Macrophages were detached using cold PBS lacking calcium and magnesium. 3-4 x 10^6 cells/ml were frozen in the culture media described above with 10% DMSO and stored in liquid nitrogen. All animal-related procedures were approved by the University of Michigan Committee on Use and Care of Animals.

2.5.3 Cell culture and stimulation

PBS used for all cell incubations contained the following ingredients: 0.90 mM calcium chloride, 0.49 mM magnesium chloride, 2.67 mM potassium chloride, 1.47mM potassium phosphate monobasic, 137.93 mM sodium chloride, 8.06 mM sodium phosphate dibasic, containing 15 mM HEPES buffer, pH 7.2 **For macropinocytosis assays**, 1E6 BMM were plated on 60 mm treated dishes in RPMI containing 10% FBS, 1% glutamax, 0.1% P/S, 50 ng/mL CSF1, and 37 μM 2-Mercaptoethanol. The medium was replaced with fresh medium 4 hours after seeding. At the end of the following day, media was aspirated and replaced with RPMI containing 10% FBS, 1% glutamax, 0.1% P/S. The following day, cells were incubated with 300 ng/mL CSF1 and 0.5 mg/mL FDx or 0.5 mg/mL LY. For LPS treatments, cells were pretreated with 100 ng/mL LPS for 30 min, followed by addition of FDx. For PMA treatments, cells were pretreated 15 min with 100 nM PMA, followed by addition of FDx. For IL-34 and CXCL12 treatments, cells were treated with FDx and 100 ng/mL IL-34 or 50 nM CXCL-12. **For flow cytometry-based measurements**

of cell surface receptor levels, cells were plated on 60 mm untreated dishes in RPMI containing 10% FBS, 1% glutamax, 0.1% P/S, 37 μM 2-Mercaptoethanol, and 300 ng/mL CSF1. The medium was replaced with fresh medium four hours after seeding. The experiments were performed the following day. Cells were stained with either 10 ng/μl anti-CSF1 receptor antibody or anti-CXCR4 antibody. **For assays examining receptor-mediated endocytosis**, cells were incubated 15 min with 5 μg/mL DiI-AcLDL with or without 50 μg/mL unlabeled Ac-LDL, followed by rinsing and analysis by flow cytometry. **For western blot experiments**, 2E6 BMM were seeded in 60mm tissue culture treated dishes in RPMI containing 10% FBS, 1% glutamax, 0.1% P/S, 37 μM 2-Mercaptoethanol, and 50 ng/mL CSF1. Four hours later, media was aspirated and replaced with fresh media. At the end of the following day the media was aspirated and replaced with RPMI containing 10% FBS, 1% glutamax, and 0.1% P/S overnight. For experiments involving Bafilomycin A1 (Baf), the overnight media was aspirated and replaced with fresh media containing 500 nM Baf. For conditions not receiving Baf, the media was replaced with fresh media. Cells were pre-treated in Baf for 60 min followed by another 60 min in the assay conditions.

2.5.4 Flow cytometry-based assays

For measuring macropinocytosis, BMM were seeded in 60 mm tissue culture dishes at 1E6/dish. On the day of the experiment, cells were washed 4x with PBS, then incubated in PBS containing 0.25 mM of the specified amino acid(s) or in buffer alone for 30 min at 37°C. 70 kDa FDx and either CSF1, LPS, CXCL12, IL-34, or PMA were added for 60 min at 37°C. To remove cells from the dish, 0.25% trypsin-EDTA was added to the cells for 3 min at 37°C, at which point RPMI containing 10% serum was added to the cells. Cells were removed from the dish by gentle scraping. **For measuring cell surface receptor levels**, BMM were seeded in untreated 60 mm culture dishes

at 2E6/dish. To begin the experiment, cells were washed 4x with PBS, and then incubated for 60 min in PBS containing 0.25 mM of the specified amino acid, or in PBS alone at 37°C. Cells were removed from the dishes by gentle scraping. Cells were pelleted by centrifugation and then resuspended in 100 µl of PBS. 1 µg of anti-CSF1 receptor antibody or anti-CXCR4 antibody was added to the cells. **For measuring receptor-mediated endocytosis**, BMM were seeded in 60 mm tissue culture dishes at 1E6/dish. On the day of the experiment, cells were washed 4x with PBS, and then incubated 30 min at 37°C in PBS only, or PBS containing 0.25mM of the specified amino acid. Following this, cells were incubated with 5 µg/mL DiI-AcLDL, with or without 50 µg/mL unlabeled Ac-LDL, for 15 min at 37°C. Cells were washed with PBS. To remove cells from the dish, 0.25% trypsin-EDTA was added to the cells for 3 min, followed by the addition of RPMI containing FBS. Cells were removed from the dish by gentle scraping. All flow cytometric analysis was done using either a BD LTRFortessa or BD Canto (Becton-Dickenson)

2.5.5 Microscopy

For quantifying macropinosome area and number in live cells, 6E4 BMM were seeded in a MatTek dish and cultured as detailed above. Cells were washed 4x with PBS and incubated in PBS containing 0.25 mM of the specified amino acid, or PBS alone for 30 min at 37°C. CSF1 and FDx were added to the cells for 5 min, after which the cells were washed with PBS, placed in PBS containing Hoechst 33342 (1000x), and imaged for 4 min. Images were acquired on a Nikon TE300 inverted microscope equipped with a mercury arc lamp, Plan-Apochromat 60×, 1.4 NA objective, cooled digital CCD camera (Photometrics Coolsnap HQ^2), temperature-controlled stage set at 37°C, and a DAPI-FITC-Texas Red dichroic mirror (Chroma Technology). For each field of view, phase-contrast, exc.400-em.455nm, and exc.490-em.535nm images were taken using

Metamorph Image Analysis Software (Molecular Devices). **For quantifying macropinosome area and number in fixed cells,** 1E6 BMM were seeded on 18 x 18 mm glass coverslips placed in a 35mm dish and cultured as detailed in the text. Following the 30 min incubations, the media was aspirated and replaced with PBS containing FDx, Hoechst 33342, and CSF1. Following a 5 min pulse, cells were fixed using fixation buffer 1 (20 mM HEPES pH 7.4, 2% paraformaldehyde, 4.5% sucrose, 70 mM NaCl, 10 mM KCl, 10 mM MgCl2, 2 mM EGTA, 70 mM lysine-HCl) at RT for 15 min. Cells were washed over 15 minutes then incubated for 30 min at RT with CellTracker™ Red CMTPX Dye (1000x). Cells were then washed, mounted, and imaged 48hrs later using a Nikon X1 Yokogawa Spinning Disk Confocal microscope equipped with an iXon Ultra 888 camera, with Plan Apo 100x/1.45 oil objective. **For quantifying CSF1 receptor levels**, 1E6 BMM were seeded on 18 x 18 mm glass coverslips placed in a 6-well plate. The cells were cultured in the same manner as for standard macropinocytosis assays. Cells were incubated 60 min in their respective conditions, and then fixed for 15 min using 4% PFA in PBS. The cells were then washed for 15 min using PBS (containing 0.1% Triton X), then incubated 45 min in blocking buffer (PBS containing 0.1% Triton X, 5% BSA w/v, 10% goat serum v/v). CSF1R antibody, DAPI, and Fluorescein-Phalloidin dyes were then added for 30 min at RT. Cells were washed and mounted in Prolong Diamond, then imaged at least 48 hours later using a Nikon X1 Yokogawa Spinning Disk Confocal microscope equipped with an iXon Ultra 888 camera, with Plan Apo 100x/1.45 oil objective.

2.5.6 Western blotting:

For performing western blots on cell lysates, media was aspirated and 100 µl of lysis buffer (1% NP-40 lysis buffer with 1x complete mini protease inhibitor) was added to the cells. Cells were

scraped, collected, and incubated on ice for 15 min. After centrifugation, 4x Laemmli buffer with 2-Mercaptoethanol was added to the supernatant. Cell lysates were separated by SDS-PAGE, and protein was transferred to a nitrocellulose membrane by a semidry transfer method. The membrane was blocked with blocking buffer (5% BSA w/v and 0.1% Tween-20 v/v in PBS) for 30 min at RT. Primary antibodies diluted in blocking buffer were added at 4°C overnight. Membranes were washed with PBS and then incubated with secondary antibodies (Licor #926-68071, and #926-32210) in blocking buffer for 30 min, followed by a wash in PBS. Western blots were visualized using the LI-COR Odyssey infrared imaging system. Gels were quantified according to the ImageJ densitometric gel analysis protocol for 1D gels (https://imagej.nih.gov/ij/docs/menus/analyze.html#gels). **For performing western blots on cell supernatants**, protease inhibitor (100x) was first added to the collected supernatants, which were then spun at 1000xg for 10 min at 4°C. Supernatants were concentrated using Amicon Ultra 30K centrifugal filter devices. In brief, supernatants were spun at 4000xg for 15 min using a Sorvall ST 16R centrifuge with a swinging bucket rotor. The flow-through was discarded and the eluate was obtained by centrifugation at 1000xg for 2 minutes. 4x Laemmli buffer with 2-Mercaptoethanol was added to the concentrated supernatants and the western blotting was performed as described above.

2.5.7 Quantifying macropinocytosis and CSF1R using ilastik and CellProfiler

To measure the frequency and size of macropinosomes on a single-cell basis using microscopy, we developed an automated image analysis pipeline which utilizes the open-source software ilastik™ and CellProfiler™. Phase contrast images were processed to generate cell masks using the ilastik Pixel Classification workflow. This assigns the probability that pixels in an image fit

user-defined criteria in a Random Forest machine learning model. In these experiments, 10% of images were used for training, sampling 3-4 cells and 3-4 background regions in each image, and the remaining 90% of images were automatically analyzed. ilastik probability maps were exported as cell masks. Finally, a CellProfiler™ pipeline was developed to quantify the number and size of macropinosomes per cell. The CellProfiler™ pipelines used for this study are available upon request. In brief, single cells were defined by propagation of nuclear objects based on Hoechst 33342 staining to the cell periphery as defined by ilastik cell masks. Macropinosomes were defined based on object segmentation of intracellular FDx signal. Macropinosome number and area were measured and related to individual cells. The pipeline was validated using the control conditions (+/- CSF1) before being blindly applied to the remainder of experimental conditions. The average frequency and size of macropinosomes per cell from at least 4 experiments are reported for each condition. **To quantify the levels of CSF1 receptor** on single-cell basis using microscopy we developed an automated image analysis pipeline utilizing CellProfiler™. In brief, single cells were defined by propagation of nuclear objects using DAPI, to the cell periphery using Phalloidin-FITC. CSF1R was visualized using allophycocyanin anti-CSF1 receptor antibody. The average intensity of CSF1R on a per cell basis was quantified. The pipeline was validated using the control condition (+CSF1).

2.5.8 Statistical methods

Statistical analysis for all experiments was performed using GraphPad Prism software. At least 3 independent experiments were performed in all cases using cells from at least 2 different mice. In each graph, bars indicate mean ± SEM. Analysis was done using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control. For experiments

showing data relative to the PBS condition, statistics were applied using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*p < 0.05, **p<0.01, ***p <0.001, ****p<0.0001)

2.6 Acknowledgments

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The authors declare no competing financial interests.

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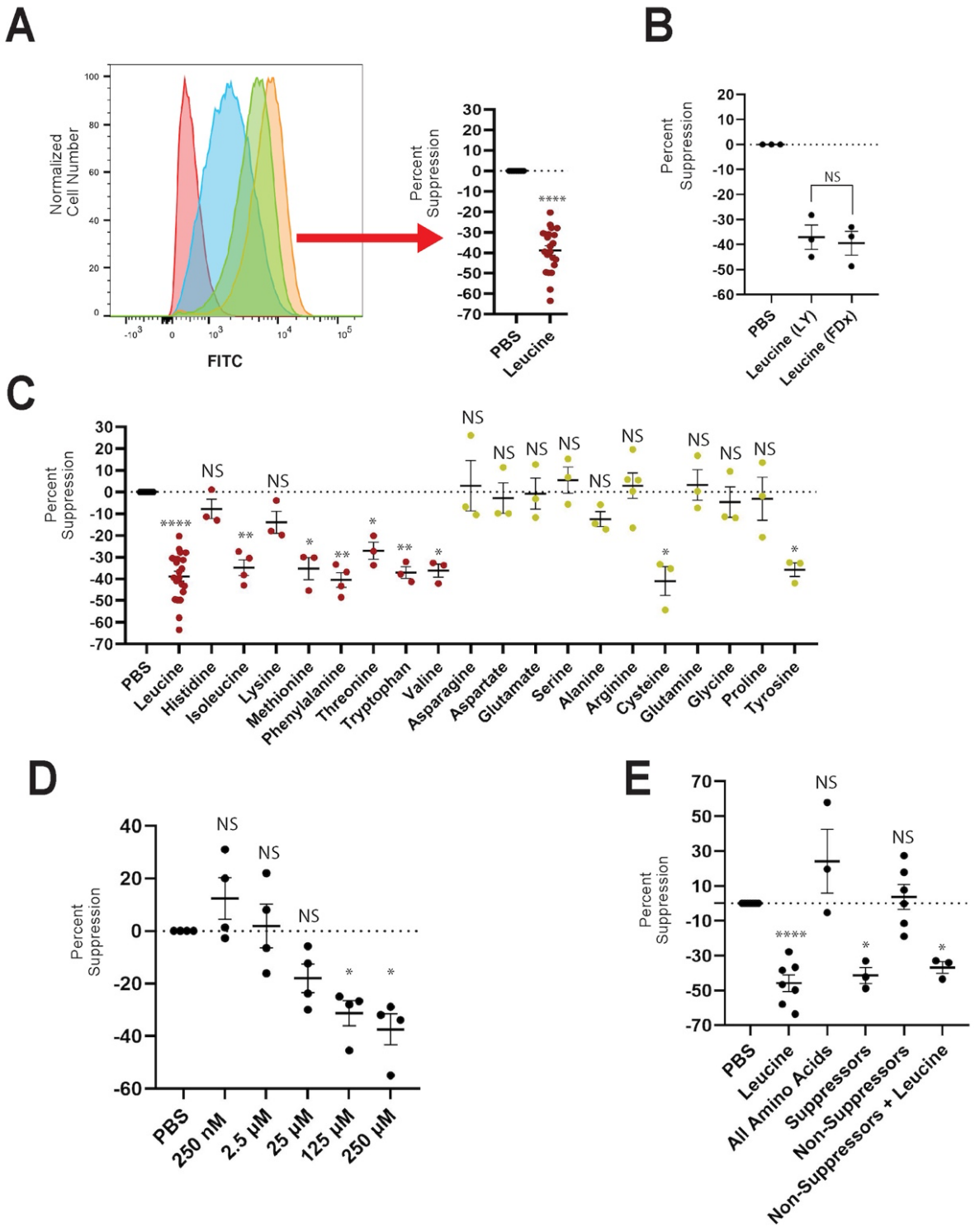
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Normalized Cell Number
FITC
Percent Suppression
PBS
Leucine
****
B
Percent Suppression
NS
PBS
Leucine (LY)
Leucine (FDx)
C
Percent Suppression
PBS
Leucine
Histidine
Isoleucine
Lysine
Methionine
Phenylalanine
Threonine
Tryptophan
Valine
Asparagine
Aspartate
Glutamate
Serine
Alanine
Arginine
Cysteine
Glutamine
Glycine
Proline
Tyrosine
D
Percent Suppression
PBS
250 nM
2.5 µM
25 µM
125 µM
250 µM
E
Percent Suppression
PBS
Leucine
All Amino Acids
Suppressors
Non-Suppressors
Non-Suppressors + Leucine

Figure 2.1: **Amino acids suppress macropinocytosis.** (**A**) BMM were incubated 30 min in PBS with or without leucine, then 60 min with FDx and CSF1. Solute accumulation was analyzed by flow cytometry. (*left*) Representative results, showing cells incubated with FDx at 4ºC (red), FDx at 37 ºC (blue), FDx + CSF1 + leucine at 37 ºC (green), and FDx + CSF1 at 37 ºC (orange). (*right*) The flow data is displayed as "percent suppression", in which FDx accumulation by cells incubated with CSF1 and leucine is compared to that in CSF1 alone (reference value). Each data point is calculated using the median of the fluorescence distributions for a single experiment. (**B**) BMM were incubated 30 min in either PBS containing 0.25 mM leucine or PBS alone. Cells were then incubated 30 min with CSF1 plus either FDx or LY. Percent suppression comparing cells incubated in leucine to those in PBS alone. (**C**) Cells were incubated for 30 min in PBS with or without the indicated amino acid, then for 60 min with FDx, CSF1 and the indicated amino acid. Percent suppression comparing macropinocytosis in CSF1 in PBS alone with that in CSF1 plus the indicated amino acid. Red = essential amino acids. Yellow = non-essential amino acids. (**D**) Percent suppression of macropinocytosis of cells incubated in PBS with or without leucine ranging in concentration from 250 nM to 250 μM. Cells were incubated for 30 min in PBS with or without the indicated concentration of leucine, then for 60 min with FDx, CSF1 and leucine. (**E**) Cells were incubated for 30 min in the indicated mixture of amino acids, then for 60 min with FDx and CSF1. Percent suppression by mixtures of amino acids compared to PBS alone. The non-suppressor group included histidine, lysine, asparagine, aspartate, glutamate, serine, alanine, arginine, glutamine, glycine and proline. The suppressor group included leucine, isoleucine, methionine, phenylalanine, threonine, tryptophan, valine, cysteine, and tyrosine. The "all amino acid" group included all twenty amino acids. pH was adjusted to 7.2-7.4 for all conditions. $N \geq 3$ independent experiments. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control, using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*$p < 0.05$, **$p<0.01$, ****$p<0.0001$).

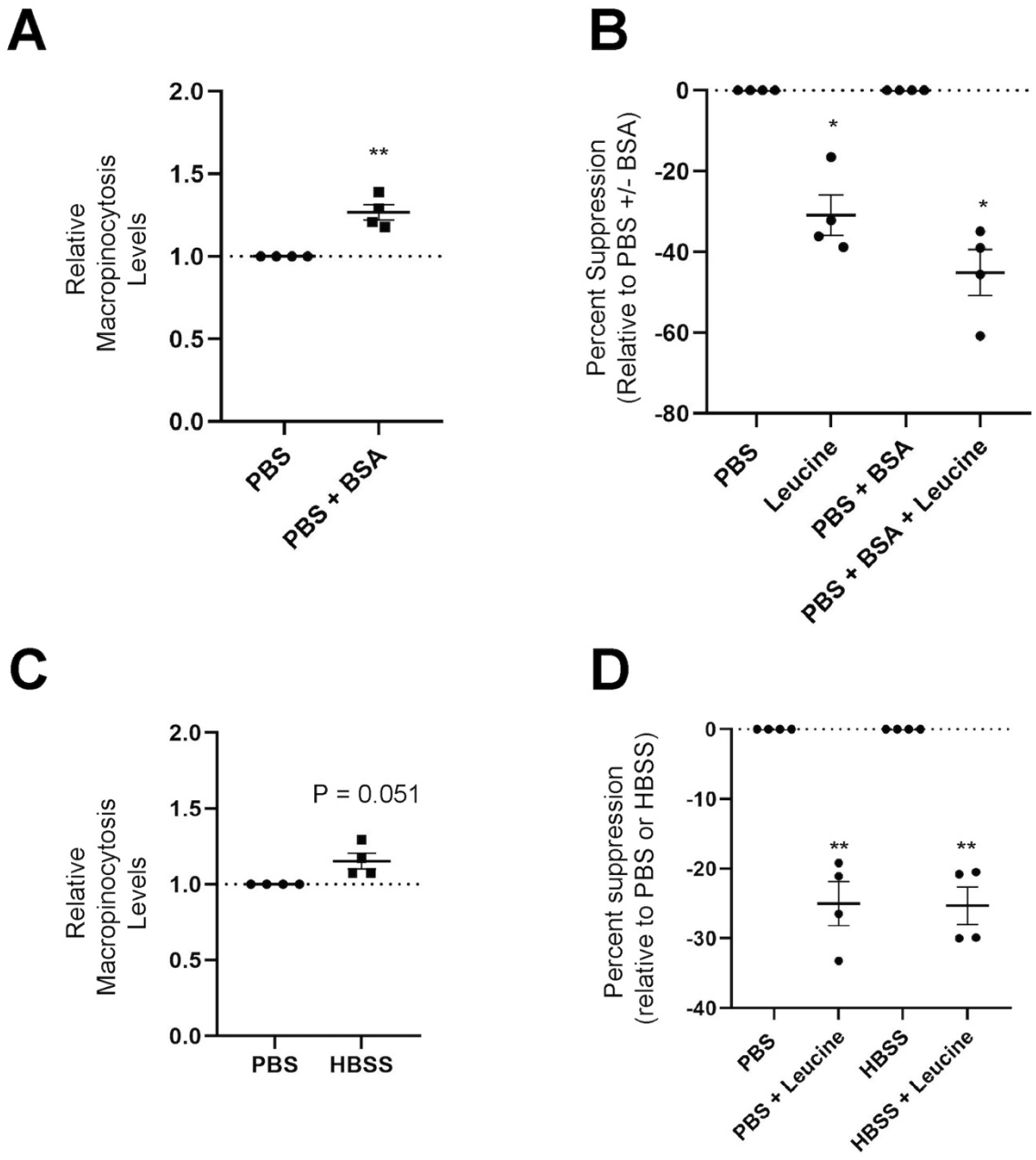

A
Relative Macropinocytosis Levels
2.0
1.5
1.0
0.5
0.0
**
PBS
PBS + BSA
B
Percent Suppression (Relative to PBS +/- BSA)
0
-20
-40
-60
-80
*
*
PBS
Leucine
PBS + BSA
PBS + BSA + Leucine
C
Relative Macropinocytosis Levels
2.0
1.5
1.0
0.5
0.0
P = 0.051
PBS
HBSS
D
Percent suppression (relative to PBS or HBSS)
0
-10
-20
-30
-40
**
**
PBS
PBS + Leucine
HBSS
HBSS + Leucine

Figure 2.2: **The effect of glucose and protein on leucine-induced suppression of macropinocytosis**. (**A-B**) BMM were incubated for 30 min in PBS +/- 3% BSA in the presence or absence of leucine, then for 60 min with FDx and CSF1. Solute accumulation was analyzed by flow cytometry. (**A**) Relative macropinocytosis are determined by comparing the median fluorescence in the PBS + BSA condition to that in the PBS alone condition. (**B**) Percent suppression in the leucine conditions relative to those without leucine. Statistics are performed comparing conditions with leucine to their respective conditions without leucine (**C-D**) Cells were incubated for 30 min in either PBS or HBSS, with or without leucine, then 60 min with FDx and CSF1. Solute accumulation was analyzed by flow cytometry. (**C**) Relative levels of macropinocytosis in HBSS as compared to PBS. (**D**) Percent suppression in the leucine conditions relative to those without leucine. Statistics are performed comparing conditions with leucine to their respective conditions without leucine. $N \geq 3$ independent experiments. Each data point represents a single experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control, using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*$p < 0.05$,**$p<0.01$)

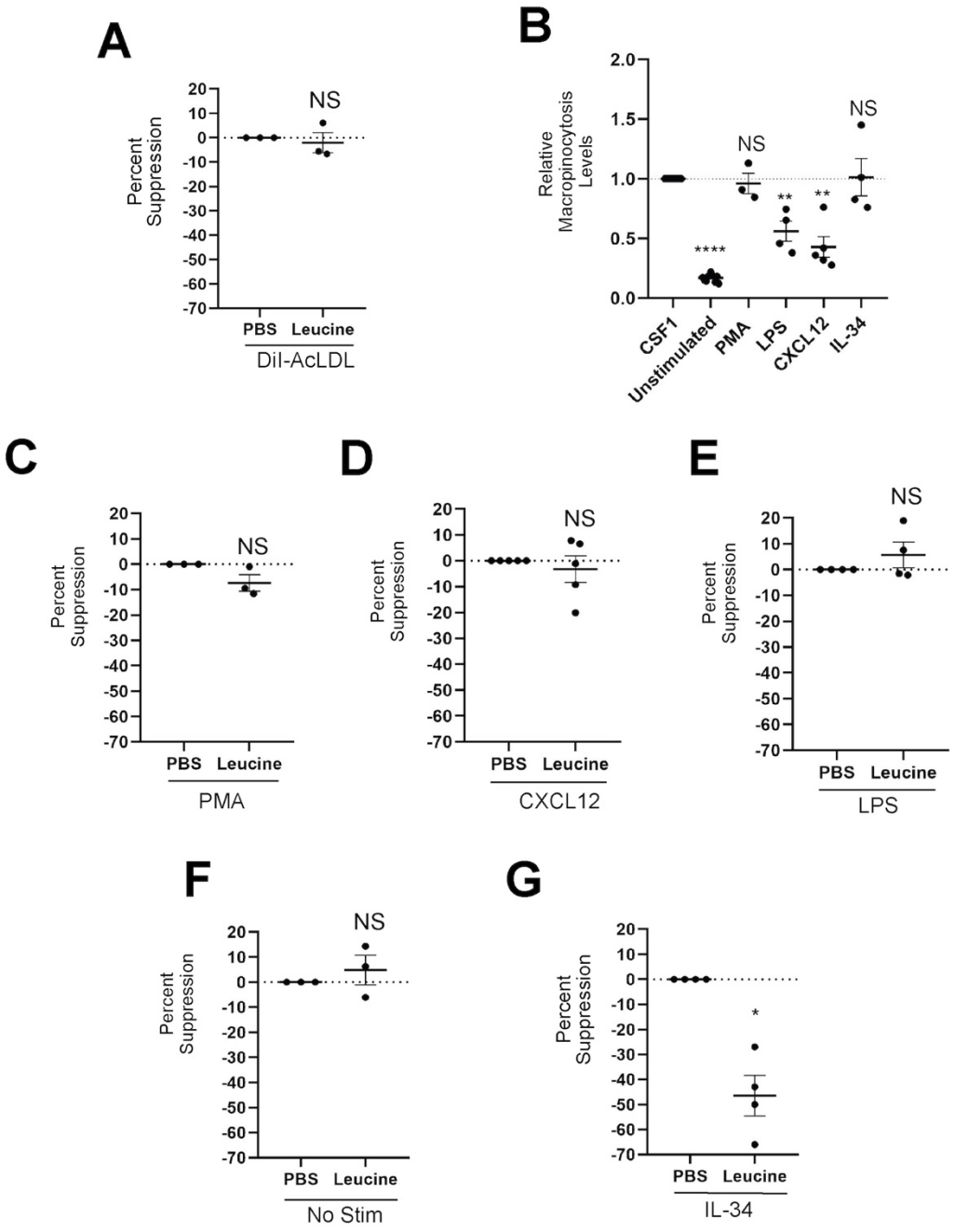
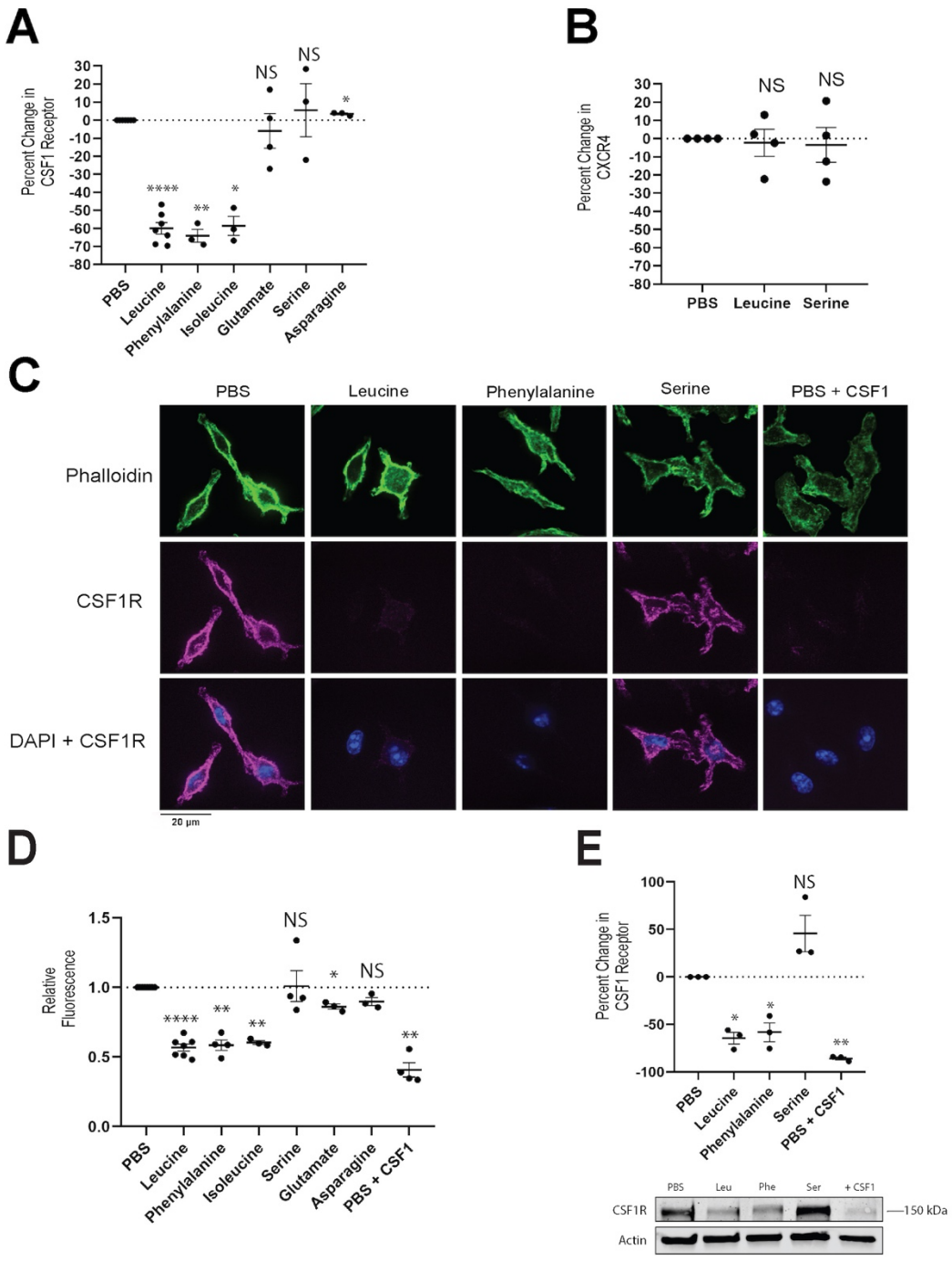

A
Percent Suppression
NS
PBS
Leucine
DiI-AcLDL
B
Relative Macropinocytosis Levels
NS
****
**
**
NS
CSF1
Unstimulated
PMA
LPS
CXCL12
IL-34
C
Percent Suppression
NS
PBS
Leucine
PMA
D
Percent Suppression
NS
PBS
Leucine
CXCL12
E
Percent Suppression
NS
PBS
Leucine
LPS
F
Percent Suppression
NS
PBS
Leucine
No Stim
G
Percent Suppression
*
PBS
Leucine
IL-34

Figure 2.3: **CSF1R is necessary for suppression of macropinocytosis by leucine.** (**A**) BMM were incubated 30 min in PBS with or without leucine, then 15 min with DiI-AcLDL or CSF1 plus FDx before analysis by flow cytometry. Shown is the percent suppression comparing cells incubated in leucine to those in PBS alone. (**B-G**) BMM were incubated 30 min in PBS with or without leucine, then 60 min with FDx and CSF1, CXCL12, PMA, LPS, IL-34, or no stimulation. Solute accumulation was analyzed by flow cytometry. (B) Relative levels of macropinocytosis are determined by normalizing the median fluorescence values in each condition to that in response to CSF1. (**C-G**) Shown is the percent suppression by leucine, comparing levels of macropinocytosis in cells incubated with PMA (**C**), CXCL12 (**D**), LPS (**E**), without stimulant (**F**), or with IL-34 (**G**), to cells incubated in the indicated stimulant without leucine. $N \geq 3$ independent experiments. Each data point represents an independent experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control, using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*$p < 0.05$, **$p<0.01$, ****$p<0.0001$).


A
Percent Change in CSF1 Receptor
NS
NS
*
****
**
*
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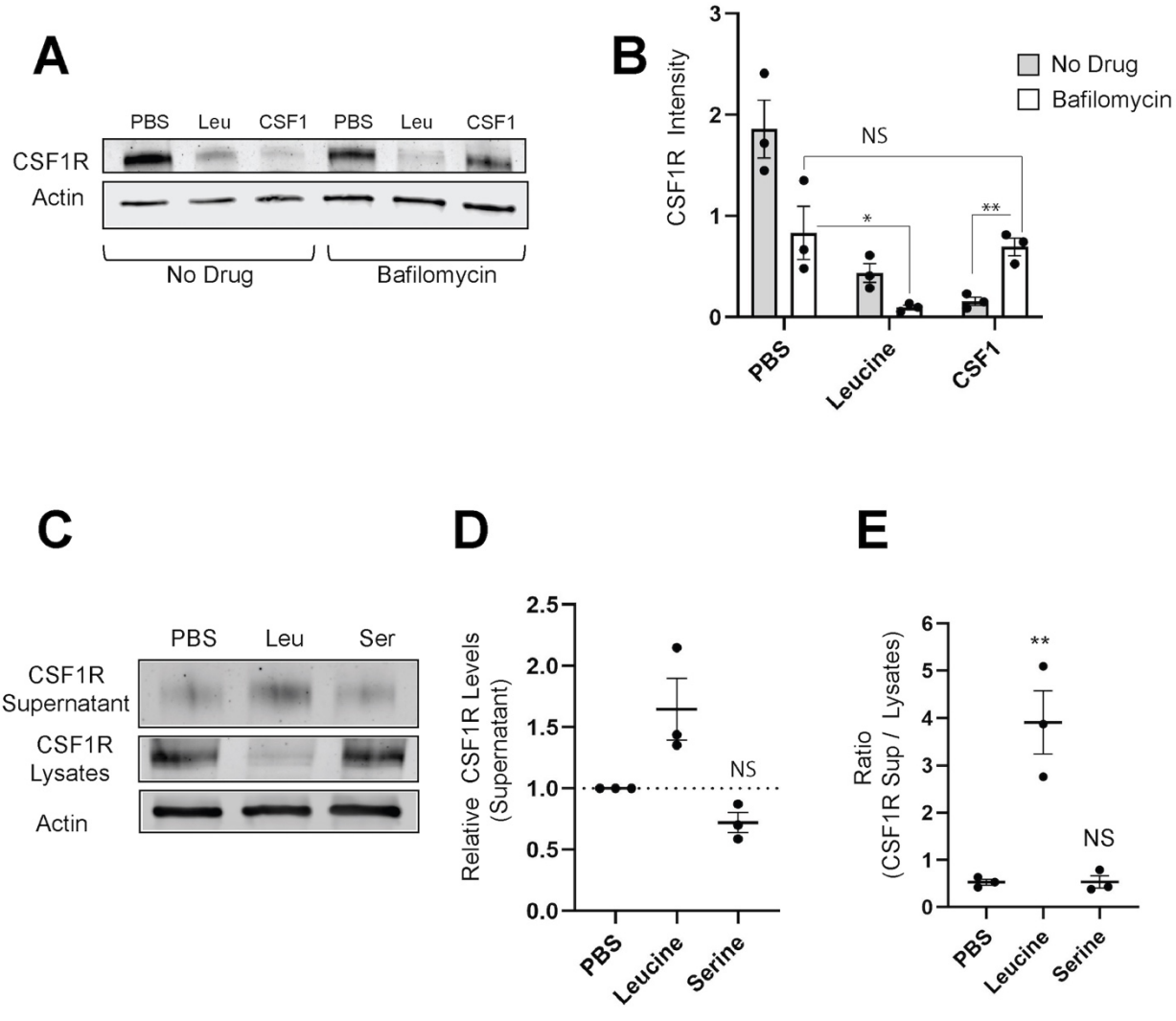
Leucine
Phenylalanine
Isoleucine
Glutamate
Serine
Asparagine
B
Percent Change in CXCR4
NS
NS
PBS
Leucine
Serine
C
PBS
Leucine
Phenylalanine
Serine
PBS + CSF1
Phalloidin
CSF1R
DAPI + CSF1R
20 µm
D
Relative Fluorescence
NS
*
NS
****
**
**
**
PBS
Leucine
Phenylalanine
Isoleucine
Serine
Glutamate
Asparagine
PBS + CSF1
E
Percent Change in CSF1 Receptor
NS
*
*
**
PBS
Leucine
Phenylalanine
Serine
PBS + CSF1
PBS
Leu
Phe
Ser
+ CSF1
CSF1R
Actin
150 kDa

Figure 2.4: **Suppressive amino acids reduce CSF1R levels.** (**A-B**) BMM were incubated 60 min in either PBS containing the indicated amino acid or in PBS alone. Cells were collected, stained using anti-CSF1R antibody or anti-CXCR4 antibody, and then analyzed by flow cytometry to measure cell surface receptor levels. Percent reduction in either CSF1R or CXCR4 was calculated by comparing the mean fluorescence of the population incubated with leucine to that incubated in PBS alone. (**A**) The percent change in CSF1R levels compared to PBS for three suppressive amino acids (leucine, phenylalanine, and isoleucine) and three non-suppressive amino acids (glutamate, serine, and asparagine). (**B**) The percent change in CXCR4 compared to PBS for cells incubated in either leucine or serine. (**C-D**) BMM were incubated 60 min in either PBS alone or PBS containing the indicated amino acid. Cells were permeabilized and stained using anti-CSF1R antibody to visualize total CSF1R. Actin was labeled using Phalloidin-iFlour 488, and nuclei were labeled using DAPI. Cells were imaged by confocal microscopy. (**C**) Representative images. (**D**) Quantification of the microscopy data showing the average CSF1R fluorescence of cells incubated in the various amino acid conditions. Data is normalized to the PBS condition. (**E**) BMM were incubated in either PBS containing the indicated amino acid or PBS alone for 60 min. Cells were lysed and blotted for CSF1R. (*Top*) quantification of the blots from 3 independent experiments. CSF1R levels are normalized to the actin loading control. (*Bottom*) representative western blot gel. $N \geq 3$ independent experiments. Each data point represents an independent experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control, using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*$p < 0.05$, **$p<0.01$, ****$p<0.0001$).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PBS
Leu
CSF1
PBS
Leu
CSF1
CSF1R
Actin
No Drug
Bafilomycin
B
CSF1R Intensity
No Drug
Bafilomycin
NS
*
**
PBS
Leucine
CSF1
C
PBS
Leu
Ser
CSF1R Supernatant
CSF1R Lysates
Actin
D
Relative CSF1R Levels (Supernatant)
NS
PBS
Leucine
Serine
E
Ratio (CSF1R Sup / Lysates)
**
NS
PBS
Leucine
Serine

Figure 2.5: **Leucine promotes secretion of CSF1R**. (**A-B**) Cells were either pre-treated for 60 min with 500 nM Baf or left untreated, then incubated 60 min in either PBS containing the indicated amino acid or in PBS alone +/- Baf. Cells were lysed and probed for CSF1R. (**A**) Representative western blot. (**B**) Quantification of western blots from 3 independent experiments. CSF1R levels are normalized to actin loading controls. (**C-E**) BMM were incubated 60 min in either PBS containing the indicated amino acid or PBS alone. Supernatants were collected and concentrated using Amicon Ultra-2 30K filters. Cells were lysed. Both concentrated supernatants and cell lysates were probed for CSF1R. (C) Representative western blot. (D) Quantification of the supernatant data from 3 independent experiments showing the relative amount of CSF1R in the supernatants of cells normalized to the PBS condition. For leucine P= 0.08. (E) Ratio of CSF1R levels in the supernatant compared to the levels in the cell lysate. CSF1R levels in each condition are normalized for amount loaded. Each data point represents an independent experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments. On figures showing relative amounts, statistics are performed using the raw values as opposed to the relative values. P values less than 0.05 were considered significant (*$p < 0.05$, **$p<0.01$).

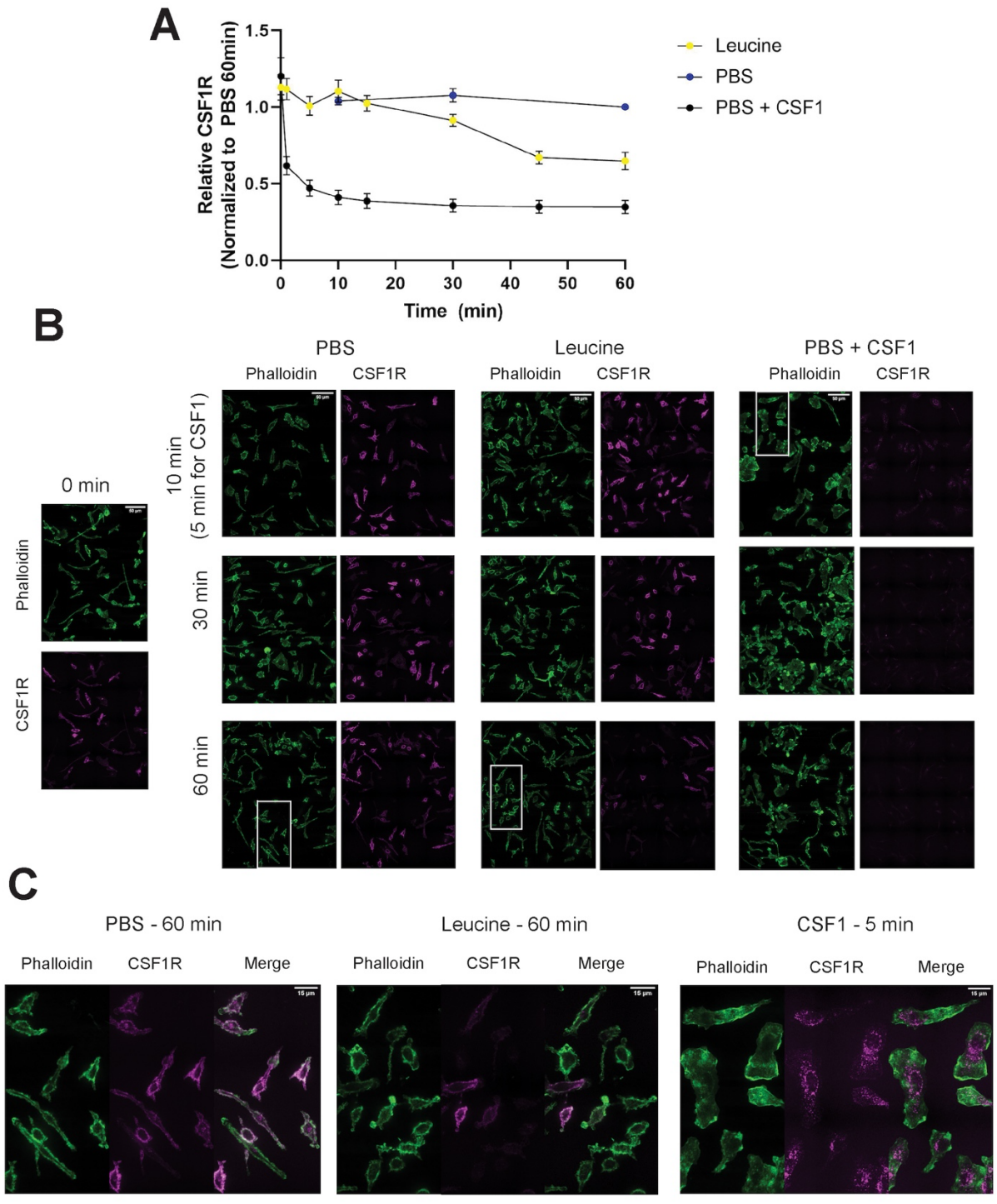
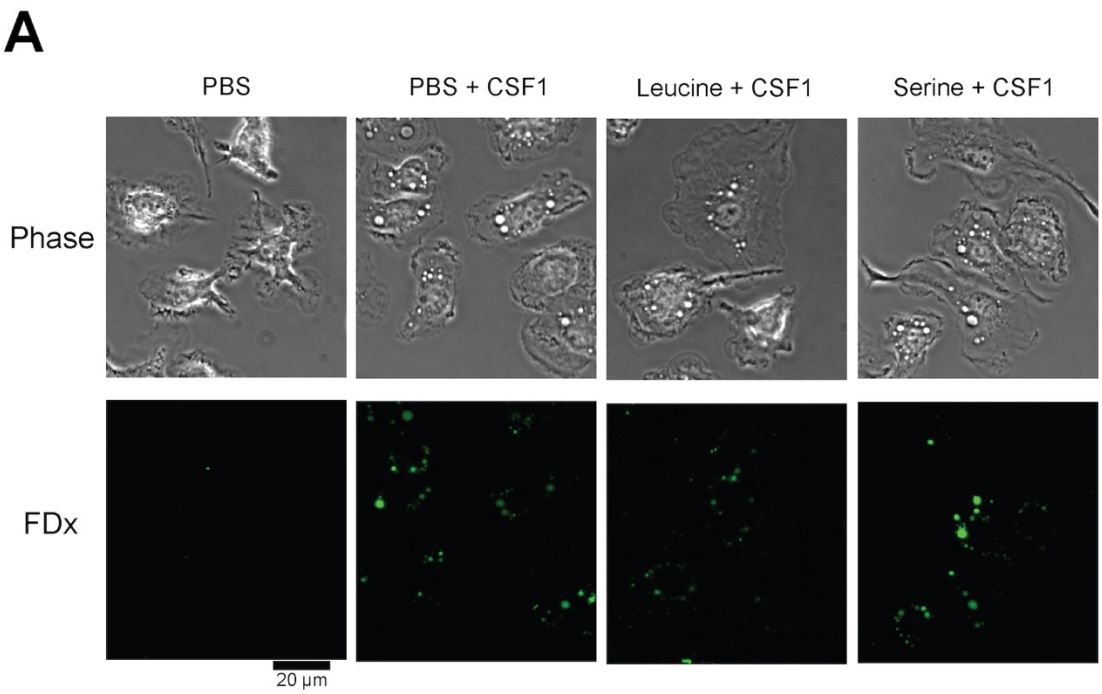

A
Relative CSF1R (Normalized to PBS 60min)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (min)
Leucine
PBS
PBS + CSF1
B
PBS
Leucine
PBS + CSF1
Phalloidin
CSF1R
0 min
10 min (5 min for CSF1)
30 min
60 min
C
PBS - 60 min
Leucine - 60 min
CSF1 - 5 min
Phalloidin
CSF1R
Merge

Figure 2.6: **Leucine-induced loss of CSF1R is a slow process.** A-C) Time course was performed in which BMM were incubated for 1 min to 60 min in either PBS alone, PBS containing leucine, or PBS + CSF1. Cells were permeabilized and stained using anti-CSF1R receptor antibody. Actin was labeled using Phalloidin-iFlour 488, and nuclei were labeled using DAPI. Cells were imaged by confocal microscopy. A) Quantification of the time course. All data were normalized to the 60-min/PBS condition. B) Representative images showing population level changes of cells incubated in the different conditions. White insets represent the area shown in greater detail in C. The actin and CSF1R signals were set to the same intensities in the different conditions. C) Enlarged micrographs of BMM in the different conditions. The CSF1R signals were set to the same intensities between the PBS +/- leucine conditions, but the CSF1R signal was greatly enhanced in the CSF1 condition to highlight the punctate intracellular localization of CSF1R. Actin signals were set to the same intensity between conditions. N = 3 independent experiments.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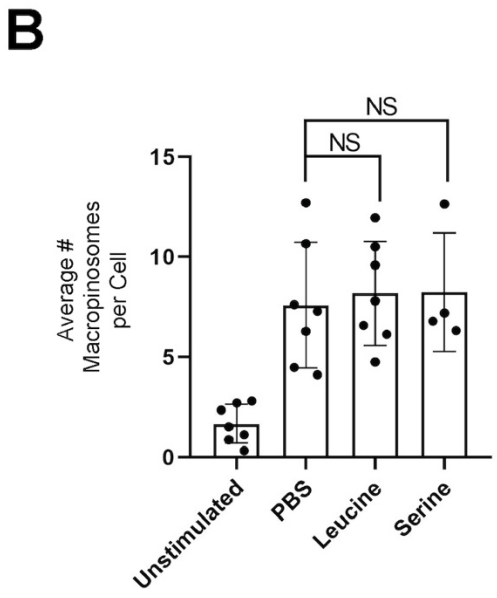
Leucine + CSF1
Serine + CSF1
Phase
FDx
20 µm


B
NS
NS
Average # Macropinosomes per Cell
15
10
5
0
Unstimulated
PBS
Leucine
Serine

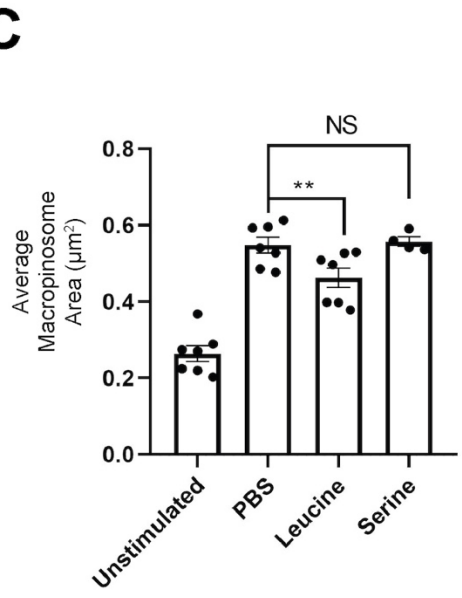
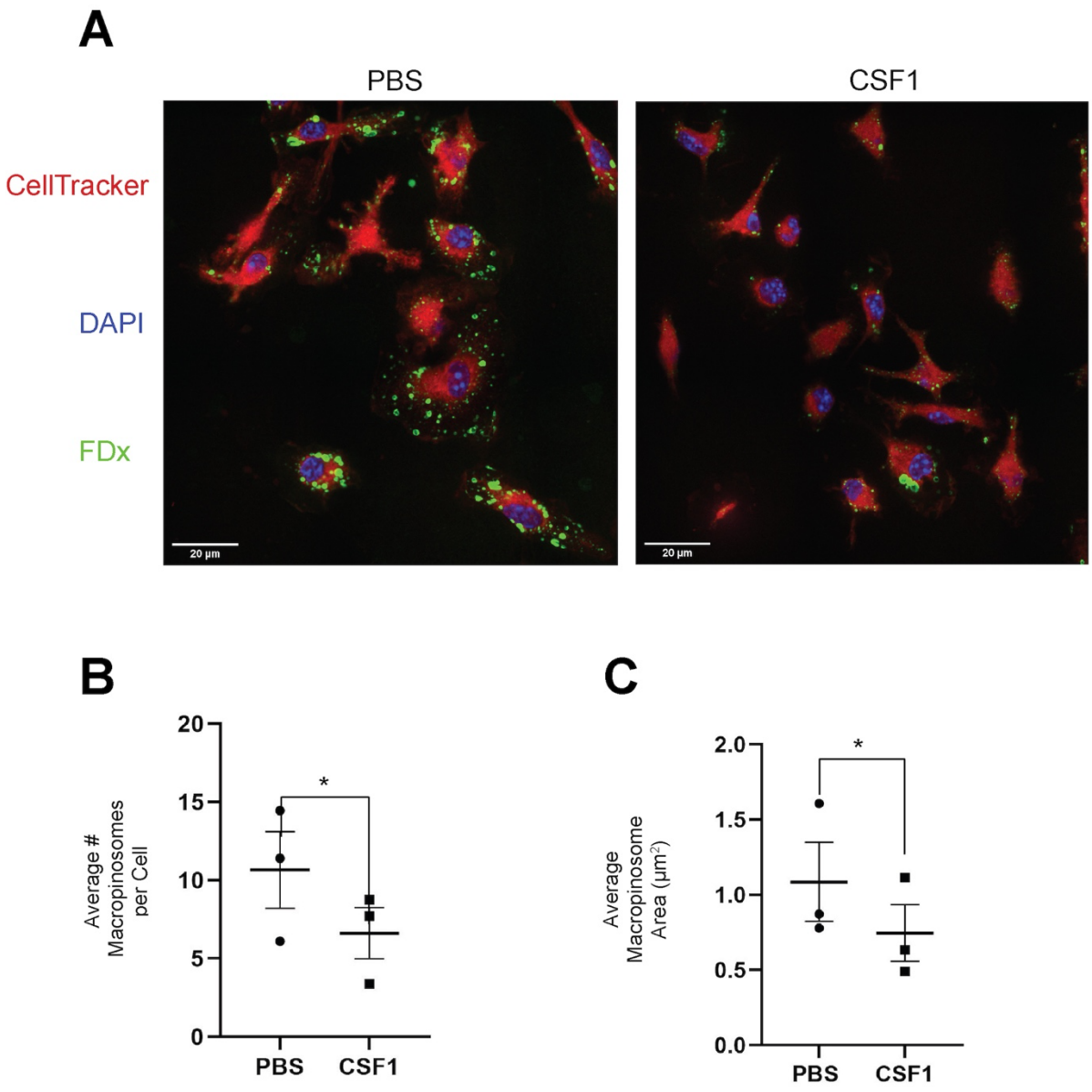

C
NS
**
Average Macropinosome Area (µm²)
0.8
0.6
0.4
0.2
0.0
Unstimulated
PBS
Leucine
Serine

Figure 2.7: **BMM incubated with leucine generate smaller macropinosomes**. (**A-C**) BMM were incubated in PBS with or without the indicated amino acid for 30 min. FDx and CSF1 were added to the cells for 5 min. After washing the cells and incubating them in PBS containing Hoechst dye to label nuclei, cells were imaged for 4 min. (**A**) Representative images of macrophages either left unstimulated, with CSF1, with CSF1 and leucine, or with CSF1 and serine. (**B**) The average number of macropinosomes per cell is shown for the indicated conditions. (**C**) The average area of the macropinosomes in the indicated conditions. N ≥ 4 independent experiments. Each data point represents a single experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control. P values less than 0.05 were considered significant (**p<0.01).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PBS
CSF1
CellTracker
DAPI
FDx
20 µm
20 µm
B
Average # Macropinosomes per Cell
20
15
10
5
0
*
PBS
CSF1
C
Average Macropinosome Area (µm²)
2.0
1.5
1.0
0.5
0.0
*
PBS
CSF1

Figure 2.8: **Downregulation of CSF1R by incubation in CSF1 results in fewer and smaller macropinosomes**. (**A-C**) For the CSF1 condition, BMM were incubated in CSF1 in cell culture media lacking serum for 30 minutes, at which point both the PBS control cells and CSF1 cells were washed and incubated either in PBS alone for the control cells or PBS + CSF1 for the CSF1 condition. Following another 30-minute incubation, all cells were pulsed with FDx, CSF1, and Hoescht dye for 5 min. Cells were then fixed, stained with CellTracker™, and then mounted on coverslips. Cells were later imaged and analyzed using the CellProfiler™ pipeline. (**A**) Representative images showing CSF1R levels in macrophages incubated for 60 min in CSF1 or 30 min in buffer. (**B**) The average number of macropinosomes per cell is shown for the indicated conditions. (**C**) The average area of the macropinosomes in the indicated conditions. N = 3 independent experiments. Each data point represents a single experiment. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control. P values less than 0.05 were considered significant (*$p < 0.05$).

Chapter 3

Mechanistic Studies of Amino Acid-Dependent Suppression of Macropinocytosis

3.1 Abstract

Cells such as macrophages can sense nutrients leading to a diverse range of physiological responses. We showed previously that certain extracellular amino acids, termed suppressive amino acids, can promote the release of colony-stimulating factor-1 receptor (CSF1R) from murine bone marrow-derived macrophages. Physiologically, the release of CSF1R results in a suppression of macropinocytosis, a form of bulk endocytosis. The mechanism underlying this regulation is poorly understood. Here, we address three major questions related to amino acid-dependent release of CSF1R. First, how do suppressive amino acids enter the macrophage to drive this phenotype? Second, how are these amino acids sensed by the macrophage? Lastly, what is the mechanism underlying CSF1R release? Using leucine as a model suppressive amino acid, preliminary studies suggest that macropinocytosis is not required for CSF1R release. Furthermore, the two major amino acid sensors, mTORC1 and GCN2, do not contribute to this effect. Lastly, we show that the metalloproteinase ADAM17 contributes to CSF1R release.

This chapter represents data that is a work in progress. As such, there are still more experiments that are needed to complete this story.

3.2 Introduction

Macropinocytosis is an endocytic mechanism by which extracellular nutrients are internalized into the cell in relatively large, heterogenous vesicles known as macropinosomes[1]. Nutrients obtained through macropinocytosis can support the growth of tumor cells and immune cells[2,3]. Moreover, this process has been implicated in a wide array of functions ranging from antigen presentation to cancer cell development[2,4]. Macropinocytosis can occur constitutively or following stimulation with growth factors such colony-stimulating factor-1 (CSF1). Following CSF1 binding to its cognate receptor, CSF1 receptor (CSF1R), macrophages rapidly undergo macropinocytosis[5].

In a previous study of bone marrow-derived macrophages (BMMs), we showed that nine amino acids, added individually or together, can suppress macropinocytosis and promote the release of CSF1R[6]. We termed these amino acids suppressors, or suppressive amino acids. The mechanisms underlying this process have yet to be elucidated. Here, we address the mechanism by which suppressive amino acids are internalized and sensed by the macrophage to drive this phenotype, and the mechanism of CSF1R release.

Cells can acquire exogenous amino acids through transport proteins located at the plasma membrane, or through internalization by endocytic mechanisms such as macropinocytosis or clathrin-mediated endocytosis[7]. With endocytosis, amino acids can be internalized either as free amino acids or as proteins. However, as free amino acids are a minor part of the biomass of plasma, most endocytic uptake *in vivo* will be of circulating proteins such as albumin[8]. Amino acid transporters (AATs) are membrane-bound proteins that mediate movement of amino acids within cells[9]. They reside on the plasma membrane in addition to the membranes of organelles such as the lysosome. AATs are members of the solute carrier (SLC) superfamily, of which there are at

least 66 transporters[9]. In macrophages, CD98 (LAT1), ASCT2, CAT2B, and SNAT are the major transporters of amino acids[10]. LAT1 transports large neutral amino acids which include histidine, isoleucine, leucine, tryptophan, tyrosine, phenylalanine, valine, methionine, and threonine[11]. CAT2B is the primary transporter for arginine[10], whereas ASCT2 and SNAT transport serine and glutamine[12].

In mammalian cells, the two major amino acid sensors are general control non-depressible-2 (GCN2) and mechanistic target of rapamycin complex-1 (mTORC1)[13]. GCN2, which is a member of the family of eIF2 kinases, senses uncharged tRNA molecules that accumulate in the absence of amino acids[14,15]. Activated GCN2 phosphorylates eukaryotic initiation factor 2α (eIF2α) resulting in a global downregulation of protein synthesis[16]. GCN2 can detect a decrease in any of the 20 amino acids that comprise proteins, as it has a high affinity for all uncharged tRNA molecules[17].

mTORC1 is a central regulator of cell growth, activating substrates that promote anabolic processes and inhibiting those that promote catabolic ones. The major component of mTORC1 is mTOR; a serine/threonine kinase that forms the major catalytic unit of mTORC1 complex in addition to the related complex mTORC2[18]. Whereas, mTORC1 regulates cell growth, mTORC2 has roles in cell proliferation and survival[18]. In addition to mTOR, mTORC1 is composed of Raptor, and mLST8[18]. mTORC1 can be activated by various growth factors, cellular stressors, and select amino acids[19]. In contrast to GCN2, mTORC1 does not sense all amino acids equally. While the removal of most single amino acids can reduce mTORC1 activity, the removal of either arginine or leucine has profound effects[20]. Later studies have shown that the mTORC1 apparatus contains distinct sensors for these amino acids: CASTORs, which detect arginine, and the Sestrins, which detect leucine[19].

The two major mechanisms of cell surface receptor release are secretion of extracellular vesicles (EV), through either microvesicles or exosomes, or direct shedding following proteolysis. The secretion of surface receptors in microvesicles was shown in the context of an oncogenic form of the epidermal growth factor receptor, EGFRvIII, and of CCR5, the co-receptor for HIV-1[21,22]. With regard to EGFRvIII secretion, microvesicles containing EGFRvIII are released from tumor cells and internalized by cells lacking EGFRvIII, thereby transferring oncogenic activity to the cells lacking the mutant receptor[21]. To our knowledge, CSF1R has not been shown to be released through vesicle-mediated secretion.

CSF1R, and a cell surface form of CSF1, have been shown to be cleaved by A Disintegrin and Metalloproteinase Domain 17 (ADAM17)[23–25]. ADAM17 was first identified as the enzyme responsible for proteolytically cleaving TNF-α to its active form[26]. The substrates of ADAM17 are numerous and include growth factors, cytokines, adhesion molecules, and their respective receptors; presently over 80 substrates have been shown to be cleaved by ADAM17[27]. ADAM17 occurs in two forms, a full-length inactive form, and an active form lacking the N-terminal pro-domain[28]. Most of the active form of ADAM17 is found in perinuclear region, with only a small amount present on the plasma membrane[28,29]. In this preliminary work, we investigate the mechanism amino acid entry and sensing leading to release of CSF1R, in addition to the mechanism of CSF1R release.

3.3 Results

Using leucine as a model suppressive amino acid, we investigated how it enters the macrophage to suppress macropinocytosis. However, instead of quantifying macropinocytosis as our readout, we quantified cell-associated CSF1R. As previously shown, following a 60-minute

incubation with 250 μM leucine, CSF1R levels were reduced about 50%[6]. We first examined whether leucine was being internalized through macropinocytosis before suppression. To inhibit macropinocytosis, we treated cells with either 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), or a combination treatment of jasplakinolide and blebbistatin (J/B). EIPA, which functions as an inhibitor of Na+/H+ exchange, inhibits macropinocytosis by increasing the submembranous pH[30]. Jasplakinolide inhibits actin depolymerization, whereas blebbistatin inhibits myosin II[31,32]. Used together, J/B treatment inhibits macropinocytosis effectively[3,33]. Cells were pretreated with EIPA or J/B for 30 min or left untreated, then incubated for 60 min in PBS, with or without leucine. Cells incubated in leucine plus an inhibitor of macropinocytosis still exhibited significant loss of CSF1R, suggesting that macropinocytosis of leucine was not a prerequisite to CSF1R loss (**Fig. 3.1A-C**). However, more experiments are needed to test this possibility. To start, these experiments were performed using 250 μM leucine, which we have previously shown is a saturating concentration[6]. As such, it is possible that incomplete inhibition of macropinocytosis might still allow enough leucine into the cell to signal CSF1R release. Maximum suppression of macropinocytosis can be observed using 125 μM leucine while an intermediate suppression can be observed at concentrations of 25 μM leucine[6]. These concentrations will need to be tested to ensure the validity of our findings. Moving forward we aim to validate our findings using another inhibitor of macropinocytosis IPA-3, which inhibits Pak-1[34,35]. We will also examine the role of clathrin-mediated endocytosis using the inhibitor Pitstop-2[36].

If endocytosis of leucine is not required to drive CSF1R release, this would suggest that amino acid transporters are required. To this end, future experiments will focus on inhibiting LAT1, the dominant transporter of leucine. We will use 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which is a selective inhibitor of system L transporters, which include

LAT1[37]. We will also utilize JPH203, which has been shown to inhibit LAT1 specifically, and works at concentrations much lower than that of BCH[38]. If neither transporters nor endocytosis appear to be required to drive CSF1R release, it is possible that the sensing might be extracellular. It has been reported that the extracellular calcium-sensing receptor can respond to amino acids in specific environmental contexts[39].

Next, we investigated how leucine was sensed by the macrophage. As a first step, we examined the roles of the major amino acid sensors in mammalian cells, mTORC1 and GCN2. To examine the role of mTORC1, we use the mTORC1 inhibitors Torin1 and rapamycin. Rapamycin selectively inhibits mTORC1, whereas Torin1 inhibits both mTORC1 and mTORC2[40]. Cells were pretreated with either Torin1 or rapamycin for 30 min, then washed and incubated in PBS with or without leucine, in addition to the inhibitors for another 30 min. This was followed by a 60-minute incubation in the presence of CSF1 and 70 kDa fluorescein-isothiocyanate dextran (FDx), a specific marker for macropinocytosis[41,42]. While Torin1 did not significantly reduce macropinocytosis, we observed a slight decrease in overall macropinocytosis in cells incubated with rapamycin (**Fig. 3.2A**). Cells incubated in leucine and either Torin1 or rapamycin exhibited reduced levels of macropinocytosis compared to those incubated in PBS, with or without inhibitors (**Fig. 3.2B**). This suggests that mTORC1 is not contributing to the sensing of leucine in our system. We still need to confirm our inhibitors are working. To this end, we will probe for phosphorylated S6K1. As S6K1 is phosphorylated when mTORC1 is active, inhibiting mTORC1 should prevent this phosphorylation, which can be detected via western blotting.

We next took a genetic approach to investigate the role of GCN2 in leucine-dependent suppression of macropinocytosis. We utilized a GCN2-KO mouse to obtain bone marrow-derived macrophages lacking GCN2[43]. The GCN2-KO mice have a deletion in the GCN2 locus, which

results in the deletion of exon 12 of the *EIF2AK4* gene, which encodes the GCN2 gene[43]. Confirmation of the GCN2-KO was confirmed by Dr. Rosario Labastida Conde in the lab of Dr. Katherine Spindler using PCR. Compared to WT-macrophages, GCN2-KO macrophages exhibited similar rates of macropinocytosis. (**Fig. 3.2C**). Similar to WT-macrophages, GCN2-KO macrophages incubated in leucine exhibited reduced levels of macropinocytosis compared to GCN2-KO macrophages incubated in PBS alone (**Fig. 3.2D**). Thus, GCN2 is likely not involved in the sensing of leucine in our system.

Next, we examined the mechanism of release of CSF1R from the surface of the macrophage. As CSF1R detected in the medium of macrophages incubated in leucine (supernatant) is a lower molecular weight than CSF1R detected in the cell lysates, we hypothesized that CSF1R was being shed from the cell surface by proteolytic cleavage of the extracellular domain. The metalloproteinase ADAM17 cleaves CSF1R in response to molecules such as phorbol myristate acetate (PMA) and bacterial DNA[25,44]. We therefore hypothesized that ADAM17 cleaves CSF1R in response to amino acids. To test this, we measured the effects of the ADAM10/17 inhibitor GW on leucine-stimulated loss of CSF1R. As a control for ADAM17 specificity, we measured the effects of the ADAM10 inhibitor GI. As positive controls for ADAM17 cleavage of CSF1R, we tested whether GW or GI could inhibit CSF1R release following stimulation with PMA. Cells were pretreated with GI or GW for 30 min, or left untreated, then incubated for 60 min in the presence or absence of PMA. As expected, cells incubated with PMA promoted the release of CSF1R and this process was inhibited by GW (**Fig. 3.3A**). GI had no effect, illustrating that this process is dependent on ADAM17 rather than ADAM10 (**Fig. 3.3A**). We then tested whether ADAM17 was responsible for cleavage of CSF1R following incubation with leucine. Cells were pretreated with GI or GW for 30 min, or left untreated, then washed and incubated in PBS for 60 min, with or

without leucine. Cells incubated with leucine + GW exhibited significantly higher levels of CSF1R in the cell lysates compared to cells incubated in leucine alone (**Fig. 3.3B**). GI did not inhibit CSF1R loss in response to leucine (**Fig. 3.3B**). Thus, ADAM17 is at least partially responsible for cleaving CSF1R, resulting in its release from the macrophage.

Lastly, we investigated the shedding of CSF1R in response to leucine on a per cell level. To do this we analyzed the time-course data presented in Chapter 2, Figure 5. The time course-summary of the microscopy data suggests that macrophages exhibit a significant drop in CSF1R levels over the 60 min incubation (**Fig. 2.5A**); however, examination of the microscopy data revealed that this drop was not uniform, with some macrophages expressing high levels of CSF1R and others expressing no CSF1R (**Fig. 2.5B**). This is in stark contrast to the CSF1-treated macrophages which show a uniform drop in CSF1R over all cells, with no CSF1R-positive cells at the end of the incubation. To better understand these dynamics, we analyzed the CSF1R levels on a per cell basis, categorizing cells as either CSF1R-positive or CSF1R-negative. The fluorescence threshold used to categorize a cell as either positive or negative was determined using the 60 min time point, as most cells in this condition were negative for CSF1R (**Fig. 3.4A**). The percent of cells that were CSF1R-positive at each time point was determined. Fewer than 20% of the cells were CSF1R-negative at early time points (1 to 15 min) (**Fig. 3.4B**). After 60 min, 60% of cells were CSF1R-negative (**Fig. 3.4B**). Thus, leucine promotes a heterogenous response in the macrophage population, with 40% of the macrophages still exhibiting CSF1R after a 60 min incubation. Further experiments are needed to determine if all the macrophages would eventually shed CSF1R or if some macrophages are insensitive to leucine.

3.4 Discussion

This chapter describes work in progress, and as such more experiments are needed to gain insight into the mechanism underlying amino acid-dependent loss of CSF1R. Our data suggest that macropinocytosis is not required for leucine suppression. However, as previously stated more experiments are needed to confidently make this claim. To determine if endocytosis of leucine is necessary to promote CSF1R release, future experiments will test effects of endocytosis inhibitors on CSF1R loss, using lower concentrations of leucine, as well as additional inhibitors of macropinocytosis and clathrin-mediated endocytosis.

Regarding the sensing of suppressive amino acids by macrophages, our data indicate the existence of an mTORC1 and GCN2-independent mechanism. A recent paper from the Overholtzer lab details a phenomenon by which starvation induces the storage of leucine within lysosomes[45]. Their data reveal that the mechanism underlying this is independent of mTORC1 but dependent on RAG-GTPases and the lysosomal protein complex Ragulator, which regulate mTORC1 activity on lysosomes[45]. Based on some of the similarities between our observations and theirs, the RAG-GTPases, in addition to Ragulator, are potential candidates for the sensing of leucine and other suppressive amino acids in our system.

Next, we uncover the mechanism of CSF1R release following incubation with suppressive amino acids. We show that the metalloprotease ADAM17 is partially responsible for the cleavage of CSF1R, resulting in its release from the cell. It is unclear if ADAM17 is the only protease acting on CSF1R in response to suppressive amino acids. The protease γ-secretase is a potential candidate as it has also been shown to cleave CSF1R[46]. Future studies are needed to better understand the mechanism underlying ADAM17-mediated cleavage of CSF1R. First, it is unclear why certain amino acids activate ADAM17, whereas presumably others do not. Moreover, the mechanism of

signaling from amino acids to ADAM17 has not been determined but plausibly involves p38 MAPK, as amino acids can activate p38 MAPK, and p38 MAPK can activate ADAM17[47,48].

Lastly, our analysis reveals that leucine promotes a heterogenous response in macrophages at the population level, with some cells CSF1R-negative and others CSF1R-positive after a 60 min incubation. Moreover, the switch from CSF1R-positive to CSF1R-negative is steady process starting around minute 15 and continuing until minute 60. We speculate that ADAM17 is activated once a threshold concentration of leucine is reached in the cell, at which point CSF1R is rapidly cleaved from the cells. This suggests that the threshold concentration varies within the population. To test this, we can perform time-course experiments at lower concentrations of leucine that still cause maximum suppression. If the threshold concentration for leucine varies, we might expect to see a less steep decline in percent of CSF1R-positive cells over time compared to those incubated in the higher concentration of leucine. Furthermore, it is unclear whether all macrophages respond to leucine, as roughly 40% of macrophages are CSF1R-positive after 60 min. We hypothesize that eventually all macrophages in the population would become CSF1R-negative, as this has been seen before in a similar context with regard to bacterial DNA. Bacterial DNA promotes the gradual release of CSF1R in BMMs in an ADAM17-dependent manner, which ultimately leads to loss of CSF1R in all cells[44,49]. Performing longer time-course experiments would allow us to test this hypothesis.

The physiological relevance of this regulation is unclear. We hypothesize that the downregulation of CSF1R might represent an adaptation to nutrient deficient tumor microenvironments. CSF1 is one molecule responsible for polarizing macrophages to the tumor-associated macrophage (TAM) phenotype[50]. As TAMs are tumor promoting, decreasing CSF1R signaling might represent a mechanism to prevent the macrophage from undergoing this

polarization[51]. In a mouse model of pancreatic ductal adenocarcima (PDAC), inhibition of CSF1R signaling using the selective inhibitor AZD7507, decreased tumor size, and increased mouse survival[52]. This type of regulation exists predominately with essential rather non-essential amino acids. As CSF1R signaling is critical for macropinocytosis, maintaining CSF1R signaling to obtain nutrients through macropinocytosis may be more favored than down-modulating signaling to prevent the polarization to the TAM phenotype. A more thorough understanding of the mechanism involved in amino acid-dependent release of CSF1R could guide future strategies for the development of cancer therapeutics.

3.5 Materials and Methods

3.5.1 Materials

RPMI-1640, fetal bovine serum (certified; FBS), GlutaMAX, penicillin-streptomycin (P/S), 70 kDa Fluorescein-isothiocyanate dextran (Fdx), bovine serum albumin, goat serum, Prolong™, DPBS, and Halt™ Protease Inhibitor Cocktail were purchased from ThermoFisher. Recombinant mouse CSF1 was purchased from R&D Systems. HEPES, 2-Mercaptoethanol, 60 mm treated dishes, and all amino acids were purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA), and recombinant anti-CSF1 receptor antibody (ab221684) for western blotting were purchased from Abcam. IRDye 680RD Goat anti-Rabbit IgG and IRDye 800CW Goat anti-Mouse IgG secondary antibodies for western blotting were purchased from LI-CORE Biosciences. GW 280264X (GW), GI 254023X (GI), and EIPA were purchased from Tocris.

3.5.2 Bone Marrow Macrophage Isolation and Culture

Macrophages were generated from male and female C57BL6/J mice (Jackson Laboratory) between the ages of 3 – 12 months. Bone marrow flushed from mouse femurs were differentiated into macrophages by culture for 5 days in RPMI supplemented with 20% FBS, 50 ng/mL recombinant CSF1, 1% glutamax, 0.1% penicillin-streptomycin, 37 μM 2-mercaptoethanol. Macrophages were detached using cold PBS lacking calcium and magnesium. 3-4 x 10^6 cells/ml were frozen in the culture media described above with 10% DMSO and stored in liquid nitrogen. All animal-related procedures were approved by the University of Michigan Committee on Use and Care of Animals.

3.5.3 Cell culture and stimulation

PBS used for all cell incubations contained the following ingredients: 0.90 mM calcium chloride, 0.49 mM magnesium chloride, 2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 138 mM sodium chloride, 8 mM sodium phosphate dibasic, containing 15 mM HEPES buffer, pH 7.2**. For western blot experiments**, 2E6 BMMs were seeded in 60mm tissue culture treated dishes in RPMI containing 10% FBS, 1% glutamax, 0.1% P/S, 37 μM 2-Mercaptoethanol, and 50 ng/mL CSF1. Four hours later, media was replaced with fresh media. At the end of the following day, the media was aspirated and replaced with RPMI containing 10% FBS, 1% glutamax, and 0.1% P/S for overnight incubation. **For macropinocytosis assays**, 1E6 BMMs were plated on 60 mm treated dishes in RPMI containing 10% FBS, 1% glutamax, 0.1% P/S, 50 ng/mL CSF1, and 37 μM 2-Mercaptoethanol. The medium was replaced with fresh medium 4 hours after seeding. At the end of the following day, media was replaced with RPMI containing 10% FBS, 1% glutamax, 0.1% P/S. The following day, cells were incubated with 300 ng/mL CSF1 and 0.5 mg/mL FDx. For Torin1 and rapamycin treatments, cells were pretreated with 250 nM Torin1 or

50 nM rapamycin for 30 min. For EIPA treatments cells were pretreated with 25 μM EIPA for 30 min. For J/B treatments, cells were pretreated with 75 μM jasplakinolide and 1 μM blebbistatin.

3.5.4 Flow cytometry-based assays

For measuring macropinocytosis, BMMs were seeded in 60 mm tissue culture dishes at 1E6/dish. On the day of the experiment, cells were washed 4x with PBS, then incubated in PBS containing 0.25 mM of the specified amino acid or in buffer alone for 30 min at 37ºC. 70 kDa FDx and CSF1 were added for 60 min at 37ºC. To remove cells from the dish, 0.25% trypsin-EDTA was added to the cells for 3 min at 37ºC, at which point RPMI containing 10% serum was added to the cells. Cells were removed from the dish by gentle scraping.

3.5.5 Western blotting:

For performing western blots on cell lysates, media was aspirated and 100 μl of lysis buffer (1% NP-40 lysis buffer with 100x Halt™ Protease Inhibitor Cocktail) was added to the cells. Cells were incubated on ice for 15 min, then scraped and collected. After centrifugation at 12,000xg for 10 min, 4x Laemmli buffer with 2-Mercaptoethanol was added to the supernatant. Cell lysates were separated by SDS-PAGE, and protein was transferred to a nitrocellulose membrane by a semidry transfer method. The membrane was blocked with blocking buffer (5% BSA w/v and 0.1% Tween-20 v/v in PBS) for 30 min at RT. Primary antibodies diluted in blocking buffer were added at 4ºC overnight. The following day membranes were washed with PBS and then incubated with secondary antibodies (Licor #926-68071, and #926-32210) in blocking buffer for 30 min, followed by a wash in PBS. Western blots were visualized using the LI-COR Odyssey infrared imaging

system. Gels were quantified according to the ImageJ densitometric gel analysis protocol for 1D gels (https://imagej.nih.gov/ij/docs/menus/analyze.html#gels).

3.5.6 Single-cell analysis of CSF1R levels over time

The data sets used to create the time-course figure (**Fig. 2.5**) was reanalyzed to generate data on a single-cell basis. In brief, the time-course was performed by incubating BMMs with either PBS +/- leucine for various time points ranging from 1 min to 60 min. Cells were fixed, permeabilized and stained using an anti-CSF1R antibody. A histogram was created for each condition in which CSF1R fluorescence for every cell was plotted. Using the 60 min time condition, a threshold was set to classify a cell as either CSF1R-positive or CSF1R-negative. The percent CSF1R-positive cells were calculated for each condition.

3.5.7 Statistical methods

Statistical analysis for all experiments was performed using GraphPad Prism software. In each graph, bars indicate mean ± SEM. Analysis was done using two-tailed ratio paired *t* tests for all experiments, comparing the experimental condition to the PBS control, unless stated otherwise. For experiments showing data relative to the PBS condition, statistics were applied using the raw values as opposed to the relative values, which are shown. P values less than 0.05 were considered significant (*p < 0.05, **p<0.01, ***p <0.001, ****p<0.0001)

3.6 Acknowledgments

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The authors declare no competing financial interests.

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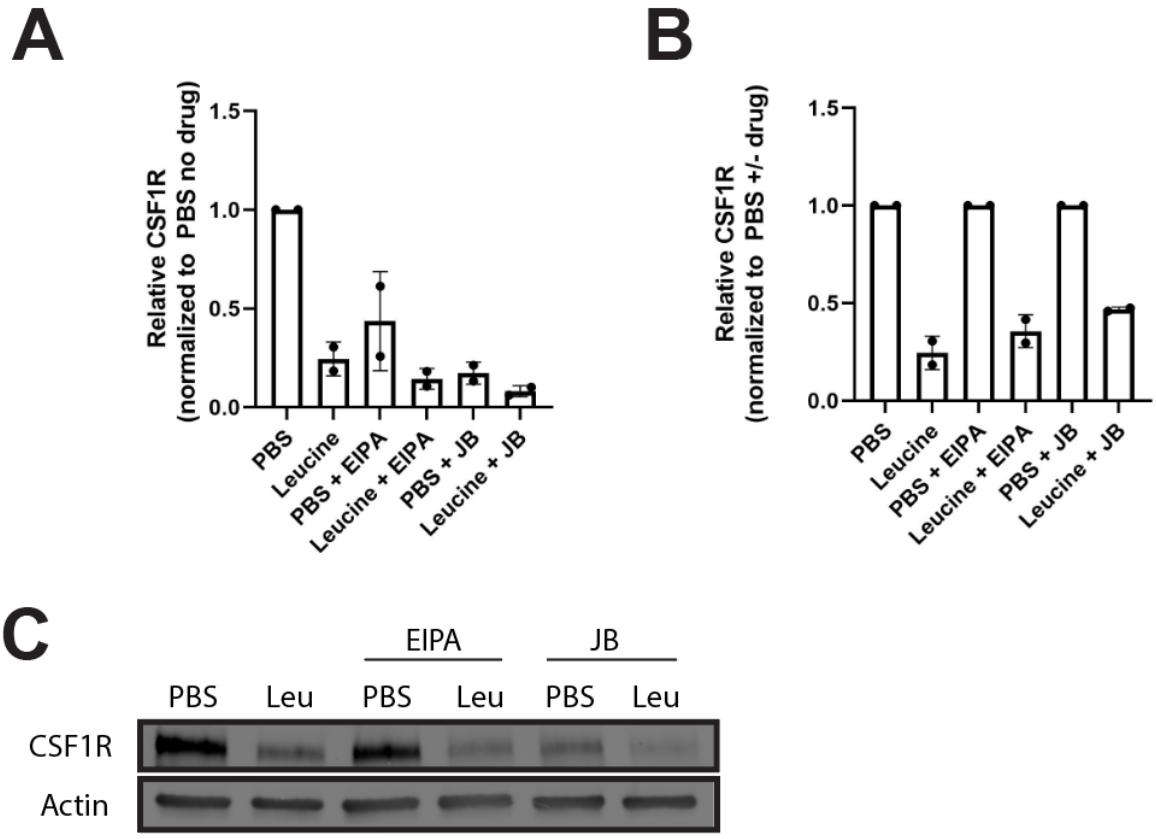

A
Relative CSF1R
(normalized to PBS no drug)
1.5
1.0
0.5
0.0
PBS
Leucine
PBS + EIPA
Leucine + EIPA
PBS + JB
Leucine + JB
B
Relative CSF1R
(normalized to PBS +/- drug)
1.5
1.0
0.5
0.0
PBS
Leucine
PBS + EIPA
Leucine + EIPA
PBS + JB
Leucine + JB
C
EIPA
JB
PBS
Leu
PBS
Leu
PBS
Leu
CSF1R
Actin

Figure 3.1: **Macropinocytosis of leucine is not required for leucine suppression**: (**A-C**) BMMs were either pretreated for 30 min with EIPA, J/B (jasplakinolide and blebbistatin), or left untreated. Cells were then incubated for 60 min in PBS with or without leucine. Cell lysates were collected and probed for CSF1R by western blotting. **A**) CSF1R levels in each condition relative to the untreated PBS condition. **B**) CSF1R levels in each condition, relative to the PBS condition with or without drug. **C**) Representative western blot. $N \geq 2$ independent experiments. Bars indicate mean ± SEM. Statistics were performed using two-tailed ratio paired *t* tests for all experiments comparing the experimental condition to the PBS control, using the raw values as opposed to the relative values, which are shown.

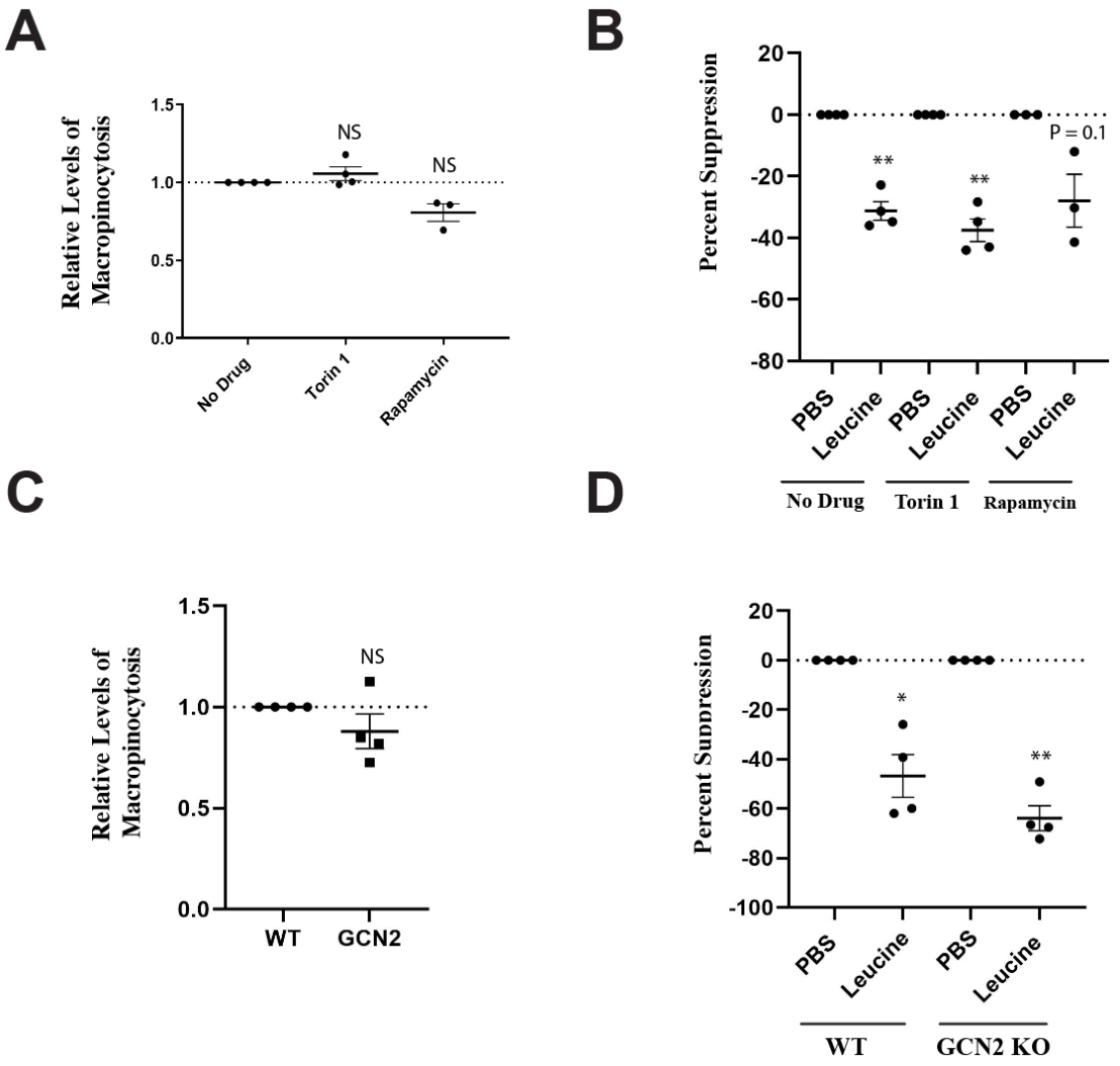

A
Relative Levels of Macropinocytosis
1.5
1.0
0.5
0.0
NS
NS
No Drug
Torin 1
Rapamycin
B
Percent Suppression
20
0
-20
-40
-60
-80
**
**
P = 0.1
PBS
Leucine
PBS
Leucine
PBS
Leucine
No Drug
Torin 1
Rapamycin
C
Relative Levels of Macropinocytosis
1.5
1.0
0.5
0.0
NS
WT
GCN2
D
Percent Suppression
20
0
-20
-40
-60
-80
-100
*
**
PBS
Leucine
PBS
Leucine
WT
GCN2 KO

Figure 3.2: **The role of mTORC1 and GCN2 in amino acid-dependent suppression of macropinocytosis.** (**A-B**) BMMs were pretreated with either 250 nM Torin1, 50 nM rapamycin, or left untreated in media for 30 min. Cells were washed and incubated in PBS +/- leucine for 30 min, then for 60 min with CSF1 plus FDx before analysis by flow cytometry. (**A**) Relative levels of macropinocytosis in the different PBS conditions. The data is normalized to the untreated condition. (**B**) The percent suppression by leucine, comparing levels of macropinocytosis in cells with leucine to those without. (**C-D**) WT or GCN2-KO macrophages were washed and then incubated in PBS +/- leucine for 30 min, then for 60 min with CSF1 plus FDx before analysis by flow cytometry. (**C**) Relative levels of macropinocytosis in the different PBS conditions. The data is normalized to the WT condition. (**D**) The percent suppression by leucine, comparing levels of macropinocytosis in cells with leucine to those without. P values less than 0.05 were considered significant (*$p < 0.05$, **$p<0.01$)

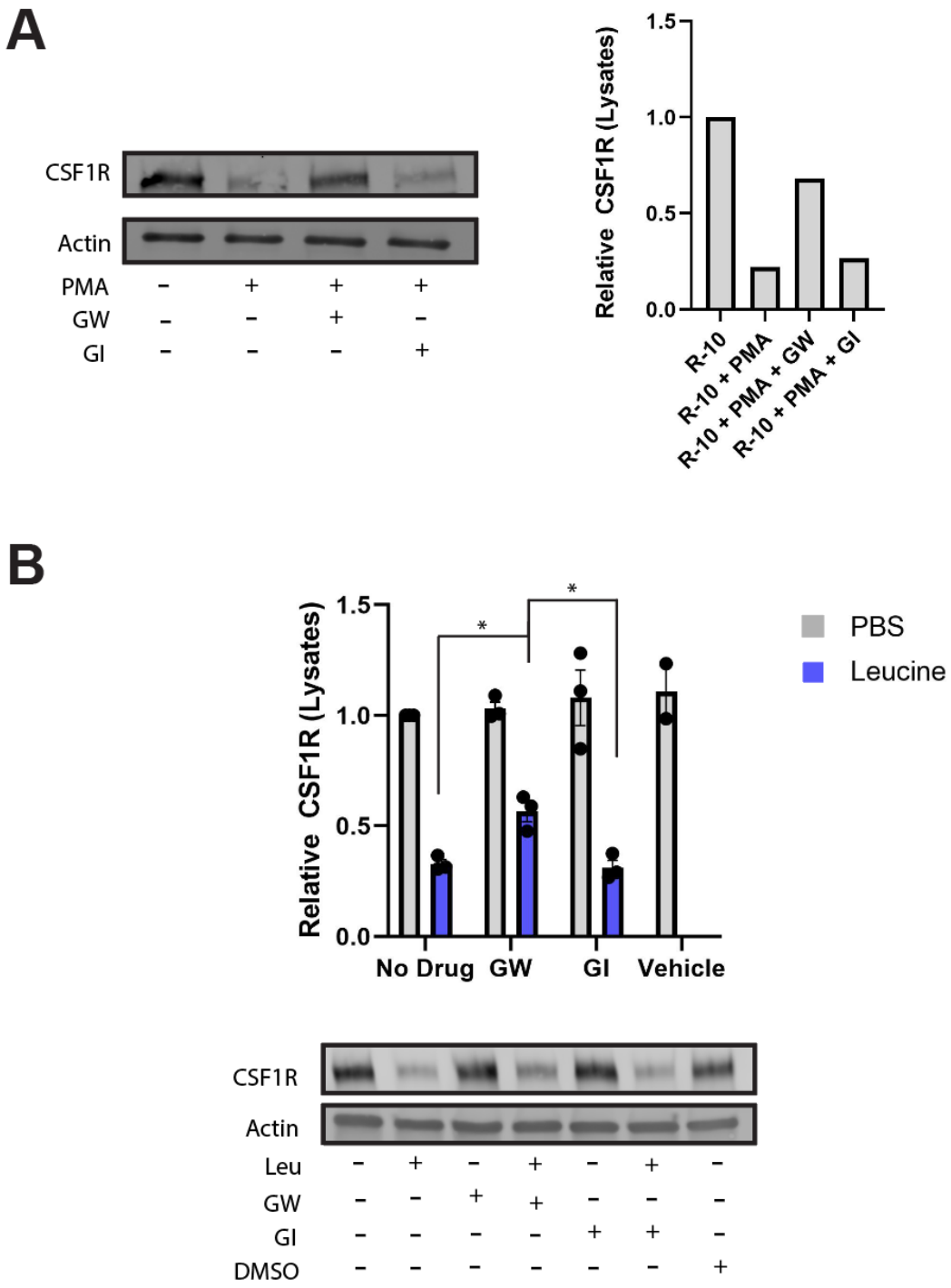
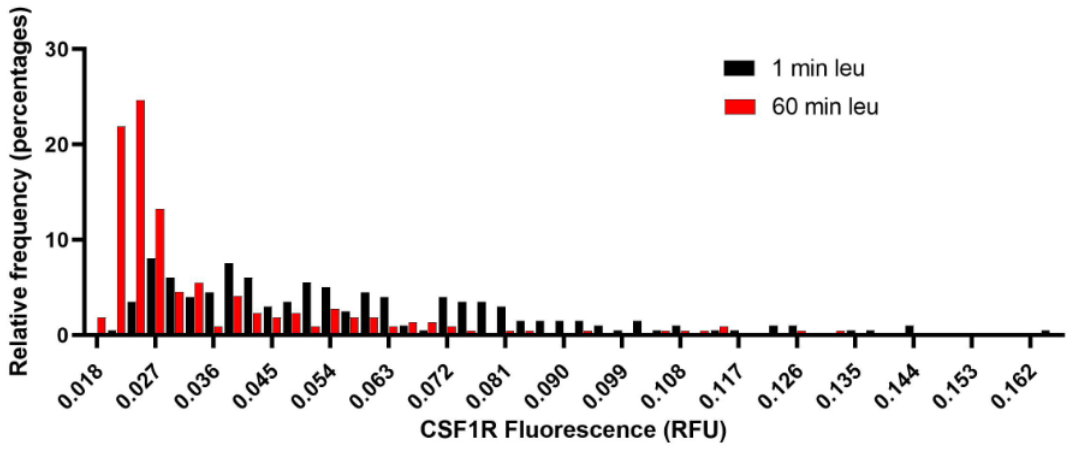

A
CSF1R
Actin
PMA - + + +
GW - - + -
GI - - - +
Relative CSF1R (Lysates)
1.5
1.0
0.5
0.0
R-10
R-10 + PMA
R-10 + PMA + GW
R-10 + PMA + GI
B
Relative CSF1R (Lysates)
1.5
1.0
0.5
0.0
*
*
No Drug
GW
GI
Vehicle
PBS
Leucine
CSF1R
Actin
Leu - + - + - + -
GW - - + + - - -
GI - - - - + + -
DMSO - - - - - - +

Figure 3.3: **ADAM17 cleaves CSF1R in response to leucine.** (**A**) BMMs were pretreated with either 2 μM ADAM10/17 inhibitor GW, 2 μM ADAM10 inhibitor GI, or left untreated. Cells were then either stimulated with PMA or left unstimulated for 60 min. Cell lysates were collected and probed for CSF1R by western blot. Shedding activity was normalized to the R-10 condition. Representative western blot (*left*). Quantification of the data showing relative CSF1R shedding in the different conditions (*right*). (**B**) BMMs were pretreated with either 2 μM ADAM10/17 inhibitor GW, 2 μM ADAM10 inhibitor GI, vehicle, or left untreated. Cells were washed and incubated with PBS +/- leucine in addition to the inhibitors or vehicle for 60 min. Cell lysates were collected and probed for CSF1R. Shedding activity was normalized to the untreated PBS condition. N = 1 for the control experiment performed in A, and N = 3 for the experiments performed in B. P values less than 0.05 were considered significant (*$p < 0.05$).

A


Relative frequency (percentages)
30
20
10
0
1 min leu
60 min leu
0.018
0.027
0.036
0.045
0.054
0.063
0.072
0.081
0.090
0.099
0.108
0.117
0.126
0.135
0.144
0.153
0.162
CSF1R Fluorescence (RFU)

B

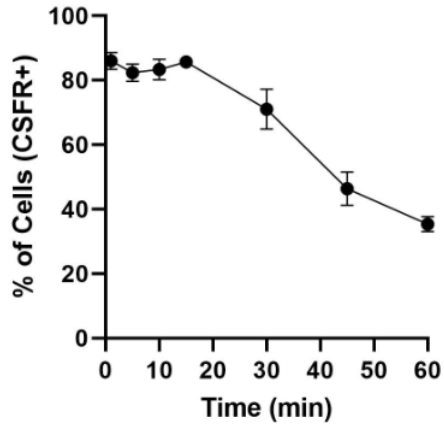

% of Cells (CSFR+)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)

Figure 3.4: **Leucine promotes release of CSF1R in an "all-or-none" manner**. (**A-B**) The data from Chapter 2, Figure 5, was reanalyzed to determine the percent of cells in each condition that was positive for CSF1R. In brief, a time course experiment was performed in which BMM were incubated for 1 min to 60 min in either PBS alone, PBS containing leucine, or PBS + CSF1. Cells were permeabilized and stained using anti-CSF1R receptor antibody. Cells were imaged by confocal microscopy. Raw fluorescence values for each cell in each condition was obtained. Using the 60 min leucine condition, the relative fluorescence value indicating a CSF1R-positive cell was determined. Using this value, the percent of cells in each condition that was positive for CSF1R was obtained. (**A**) Representative histogram for cells incubated for 1 min or 60 min in leucine. Line indicates the threshold fluorescence that defines a CSF1R-positive and CSF1R-negative cell. (**B**) Graph showing the percent CSF1R-positive cells in each condition over time.

Chapter 4
Discussion

4.1 Major Conclusions and Implications

The work presented here identifies a nutrient-based regulation of macropinocytosis based on modulation of CSF1R levels in macrophages (**Figure 4.1**). We show that suppressive amino acids inhibit macropinocytosis by stimulating the removal of CSF1 receptors from cells. The suppressive amino acids are predominately essential amino acids, whereas most non-suppressors are non-essential amino acids. There were two exceptions to these trends. The non-essential amino acids cysteine and tyrosine suppressed macropinocytosis, whereas the essential amino acids histidine and lysine did not. Although exogenous molecules such as bacterial DNA, LPS, and CSF1 have been shown to regulate CSF1R cell surface expression, the work presented here represents, to our knowledge, the first study showing that CSF1R expression can be regulated by nutrients[1,2]. CSF1R was released from the macrophage by the sheddase ADAM17. Loss of CSF1R resulted in the formation of smaller macropinosomes and consequently less solute accumulation by macropinocytosis. Compared to the rapid endocytosis and degradation of CSF1R following binding to CSF1, the release of CSF1R following incubation with suppressive amino acids was delayed. Interestingly, this regulation only occurred in nutrient-poor environments. Inhibition of macropinocytosis did not occur when all 20 proteinogenic amino acids were present. The regulatory pathway described here likely has consequences for macrophage differentiation and polarization and may represent a novel area for cancer therapeutics.

4.2 Major Questions and Future Directions

Why does this regulation exist in amino acid deplete but not replete environments?

The observation that suppressive amino acids do not suppress macropinocytosis when cells are incubated with all twenty proteinogenic amino acids (**Fig. 2.1E**), suggests that this regulation is active only under nutrient-limiting conditions. I speculate that this regulation is important in the tumor microenvironment (TME), which can be extremely nutrient deficient[3]. The tumor-associated macrophages (TAMs) that reside in these environments are often tumor promoting, as some of the molecules present in the TME can polarize macrophages towards an anti-inflammatory M2-like phenotype. M2-like TAMs secrete angiogenic factors such as vascular endothelial growth factor A (VEGFA) and immunosuppressive cytokines such as IL-10[3]. One of the molecules responsible for polarizing macrophages towards this phenotype is CSF1. Downregulating CSF1R in nutrient-poor tumor environments could prevent such polarization and would thus be beneficial. As evidence of this, drugs that target CSF1 and CSF1R are under investigation for their potential anti-cancer value[4].

A follow-up question might be why this regulation exists with most essential but not non-essential amino acids. I speculate that the macrophage must balance the requirement for CSF1R signaling for macropinocytosis with the need to downregulate CSF1R to prevent M2-like polarization. The macrophage accomplishes this by prioritizing essential amino acids, as it cannot synthesize these amino acids *de novo*. Once sufficient levels of essential amino acids are met, the cell prioritizes the prevention of polarization to an M2-like phenotype over obtaining nutrients via

macropinocytosis. Lastly, I speculate that this regulation is not active in nutrient replete environments, as there is no benefit to downmodulating CSF1R when amino acids are not limiting.

How CSF1R shedding in response to suppressive amino acids is turned off under nutrient replete conditions is unclear. Initially, we hypothesized either that non-suppressive amino acids were dominant over suppressive amino acids or that certain non-essential amino acids could function as anti-suppressors. However, when we examined macropinocytosis in a condition containing all 11 non-suppressors in addition to the suppressor leucine, we observed a suppressive phenotype (**Fig. 2.1E**); ie., leucine suppression was dominant. This excluded our initial hypothesis regarding anti-suppressors or the dominance of non-suppressors. We now hypothesize that some combination of amino acids is necessary to override the suppression of macropinocytosis. We speculated that perhaps the four amino acids that have been shown to regulate mTORC1 (glutamine, serine, arginine, and leucine) might represent this combination of amino acids[5]. However, macropinocytosis in these conditions was still suppressed (data not shown). Performing macropinocytosis experiments using various combinations of suppressors and non-suppressors will hopefully allow us to determine this combination. Such experiments could yield interesting results about how macrophages respond to nutrients in their environment.

Functionally, how are macrophages incubated with suppressors different from those incubated with non-suppressors?

Macrophages incubated with suppressors exhibit reduced CSF1R levels and decreased macropinocytosis in response to CSF1. We are interested in determining other phenotypic differences between suppressor and non-suppressors, which should eventually allow us to define the physiological relevance of this regulation. First, we would measure cytokines released from

macrophages exposed to suppressive amino acids, to determine if they are related to M1 or M2 phenotypes. M1 macrophages secrete IL-1, IL-12, and IL-23; M2 macrophages secrete IL-10 and TGF-β[6,7]. We can look for the presence of certain surface proteins such as PD-L1, in addition to the secretion of the metalloprotease MMP-9, both of which are features of M2-like TAMs[3,8]. Lastly, we can look for the expression of arginase 1, another marker of M2-like TAMs[9,10]. We speculate that macrophages incubated with suppressors would not exhibit an M2-like phenotype, whereas those incubated in PBS with or without a non-suppressor would.

Next, we can look for differences in motility and phagocytic capabilities. One approach would involve an Incucyte® imaging platform. Incucytes are automated live cell imaging and analysis platforms that allow for the quantification of specific cell behaviors over long periods of time. Incucytes have been used to quantify both phagocytosis and motility[11,12]. For comparing phagocytosis, we could incubate macrophages in either PBS +/- a suppressive amino acid for 60 min, at which point we would add pHrodo® bacterial bioparticles. Using the Incucyte analysis software, we could quantify the phagocytic events in macrophages incubated with or without suppressors. As M2-like TAMs have reduced phagocytic abilities, we speculate that macrophages incubated with suppressors would exhibit enhanced phagocytosis compared to those incubated in PBS or a non-suppressor[13]. A similar approach could be used to quantify cell motility or migration. Cells would be incubated in PBS +/- a suppressive amino acid for 60 min, at which point a scratch wound would be made. The rate of wound closure would be calculated using the Incucyte software. If we are unable to use an Incucyte, cell motility could be tracked using live cell microscopy coupled with tracking software such as CellTrack, an open-source software for tracking cell motility[14]. If a difference is observed regarding either motility or phagocytosis, we could perform experiments to confirm that the findings are dependent on CSF1R shedding. As ADAM17 is

required for CSF1R release, inhibiting ADAM17 using GW 280264X (as in Chapter 3 of this thesis) should abrogate any phenotype observed in the suppressor-treated cells if these phenotypes result from reduced CSF1R signaling.

It is also possible that macrophages incubated with suppressors or non-suppressors initially exhibit similar profiles, but then exhibit divergent profiles once in the TME. Boyer et al. demonstrate a method for testing this that involves incubating bone marrow-derived macrophages with conditioned medium from cancer cells generating "tumor-educated macrophages"[10]. We could use this approach to ask how the profiles of our two macrophage populations differ once they have been exposed to molecules in the TME. Overall, these experiments would allow us to start building a profile of both macrophage populations that would allow us to better hypothesize their role *in vivo*.

Is this regulation heterogenous within the macrophage population?

Suppressors promote the shedding of CSF1R in an all-or-none manner (**Figs. 2.5, 3.3**). As our time-course experiment, examining the percent of cells that are CSF1R-positive cells over time was only performed for 60 min, it is unclear whether some macrophages are resistant to this regulation, or if they would eventually shed CSF1R. Sester et al. described a process by which the unmethylated CpG motifs in bacterial DNA trigger the shedding of CSF1R in macrophages[2]. Their data showed a bimodal distribution of CSF1R on an individual cell basis, with cells expressing CSF1R in an all-or-none manner, similar to cells treated with suppressors in our system[2]. The cells shift from CSF1R-positive to entirely CSFR-negative over a 60 min incubation. Furthermore, later studies revealed that, as in our system, ADAM17 was responsible for the release of CSF1R[15]. Thus, based on the similarities between our findings with amino acids and those with bacterial

DNA, we speculate that eventually all macrophages in our system would shed CSF1R. Why the shedding of CSF1R in response to amino acids was slower compared to bacterial DNA is unclear and requires further investigation.

How are suppressive amino acids sensed by the macrophage leading to CSF1R secretion and the suppression of macropinocytosis? What does this signaling pathway look like?

Preliminary data reveal that neither GCN2 nor mTORC1 are involved in the sensing of suppressive amino acids (**Fig. 3.2**). As these are the two major known amino acid sensors within mammalian cells, our data suggest an alternative sensing mechanism. Bandyopadhyay et al. describe an mTORC1/GCN2-independent amino acid sensing pathway which involves the Rag GTPases and the Ragulator complex[16]. The Rag GTPases mediate the recruitment of mTORC1 from the cytoplasm to lysosomal membranes, where mTORC1 is activated, while the Ragulator tethers this Rag-mTORC1 complex to the lysosomal membrane[17]. We hypothesize that this sensing pathway might be involved in our system as well. This can be tested by generating RagA/B-KO macrophages in addition to knockouts in specific proteins of the Ragulator complex such as LAMTOR 1 and LAMTOR 2. If these proteins are required for sensing suppressors in our system, macrophages engineered with deletions of the genes encoding these proteins would not be responsive to suppressors. Furthermore, studies have shown that the expression of c17orf59, a Ragulator-interacting protein, disrupts the association between the Ragulator and Rag complexes[18]. By preventing Ragulator-Rag interactions, we could determine if this signaling is required for the sensing of suppressive amino acids in our system, which we hypothesize is required for this sensing. An interesting observation regarding the sensing of amino acids in our system is that we observe intermediate suppression at 25 μM. This concentration was determined

to be the K_d for the binding of leucine to Sestrin2[19]. However, as the only other amino acids reported to tightly bind Sestrin2 were methionine, isoleucine, and valine, I speculate that Sestrin2 is not responsible for the sensing of suppressive amino acids, despite this interesting observation[19].

If our sensing mechanism is similar to that described in the Bandyopadhyay et al. paper, this suggests that suppressive amino acids are sensed in the lysosome. Abu-Remaileh et al. show that under nutrient-poor conditions most essential amino acids are retained in lysosomes, whereas most non-essential amino acids are not[20]. Thus, perhaps most essential amino acids are suppressors, as they are present in sufficient concentrations in the lysosome to be sensed. Abu-Remaileh et al. show that non-essential amino acids can be retained in lysosomes by inhibiting the vacuolar ATPase (V-ATPase). Thus, perhaps we can turn non-suppressors into suppressors by increasing their concentration in lysosomes through the inhibition of the V-ATPase. To test this, we could incubate cells with a non-suppressor such as serine in the presence or absence of V-ATPase inhibitors bafilomycin A1 or concanamycin A, and then measure CSF1R levels. If retention in lysosomes is required to drive CSF1R release, then we would expect to observe CSF1R release in cells incubated with serine and V-ATPase inhibitors, but not in cells incubated in serine without the V-ATPase inhibitors.

While we know that ADAM17 is involved in the release of CSF1R, how ADAM17 is activated by amino acids is unclear. ADAM17 can be activated by p38 MAPK[21]. Whether p38 MAPK is activating ADAM17 in our system has yet to be determined. However, amino acids have been shown to activate p38 MAPK[22]. To test this plausible hypothesis, we can inhibit p38 MAPK using the specific inhibitor SB203580 or broad MEK inhibitors such as U0126 and see if CSF1R is still released in the presence of suppressive amino acids[21]. Furthermore, we can determine if p38 MAPK is activated by suppressive amino acids by measuring phosphorylated-p-38 MAPK using

antibodies which detect p38 MAPK when it is activated by phosphorylation at threonine 180 and tyrosine 182[21].

Lastly, we show that the loss of CSF1R by suppressive amino acids results in the formation of smaller macropinosomes. However, the mechanism underlying this is unclear. While the size of a phagosome is dictated by the size of the particle being ingested, the determinants of macropinosome size are not fully understood[23]. As stated in the introduction section of this dissertation, the RasGAP Neurofibromin-1 restricts the size of macropinosomes in Dictyostelium by modulating Ras activity at the base of the macropinocytic cup[24]. In macrophages macropinocytic cups form in areas with patches of PIP_3, Rac1 and Ras[25,26]. I speculate that decreasing CSF1R levels might reduce the surface area of these patches or perhaps interfere with the localization of PIP_3, Rac1 or Ras in the patches thereby limiting the size of the macropinosomes.

How do suppressive amino acids enter the macrophage?

Our preliminary data suggests that macropinocytosis of suppressors is not required for CSF1R release (**Fig. 3.1**). However, more experiments are needed to test this idea sufficiently. In the experiments thus far, we inhibited macropinocytosis and incubated cells with 250 μM leucine. As it is possible that incomplete inhibition of macropinocytosis could allow sufficient leucine to enter the cell to drive this phenotype, it would be prudent to perform these experiments at lower concentrations of leucine. We show that maximum suppression of macropinocytosis occurs at leucine concentrations of 125 μM and higher, and that intermediate levels of suppression occur around 25 μM leucine (**Fig. 2.1**). If macropinocytosis inhibitors do not affect CSF1R shedding at those lower concentrations of leucine, we can more confidently conclude that macropinocytosis of

leucine is not required to drive this phenotype. Moreover, as we have only examined the role of macropinocytosis, it is important to examine the role of other forms of endocytosis such as clathrin-mediated endocytosis. As stated in the previous section, we hypothesize that amino acids are sensed in the lysosome, leading to CSF1R release and subsequent suppression of macropinocytosis. As such, we hypothesize that endocytosis of amino acids is required.

If the inhibitor studies described above demonstrate that endocytosis is not required, we will probe the role of amino acid transporters such as LAT1, which transport large neutral amino acids such as leucine. As LAT1 can transport 8 out of the 9 suppressive amino acids it is plausible that is required in our system. LAT1 is present both on the plasma membrane and lysosomal membranes where it can facilitate movement from the extracellular environment into the cell and from the cytosol into the lysosome[27,28]. As I speculate that the sensing of suppressive amino acids occurs in the lysosome, perhaps LAT1 is required in our system to move suppressive amino acids into the lysosome.

Does this type of regulation exist in other cell types?

Currently we have shown that this type of regulation exists in macrophages. It would be interesting to determine if this type of regulation exists in other cell types as well. In addition to macrophages, CSF1R is expressed at high levels on monocytes, dendritic cells, osteoclasts, and Paneth cells[29]. Do suppressive amino acids also cause the release of CSF1R in those cell types? To test this, we can incubate other CSF1R-expressing cell types with suppressive amino acids and measure CSF1R levels in the lysate or supernatant. Moving forward, I would like to broaden these findings to other growth factor receptors such as epidermal growth factor receptor (EGFR), which is expressed in epithelial cells and is a key receptor involved in many cancers such as pancreatic

ductal adenocarcima and glioblastoma multiforme[30,31]. Exploring whether this regulation occurs with EGFR could yield insights for cancer biology.

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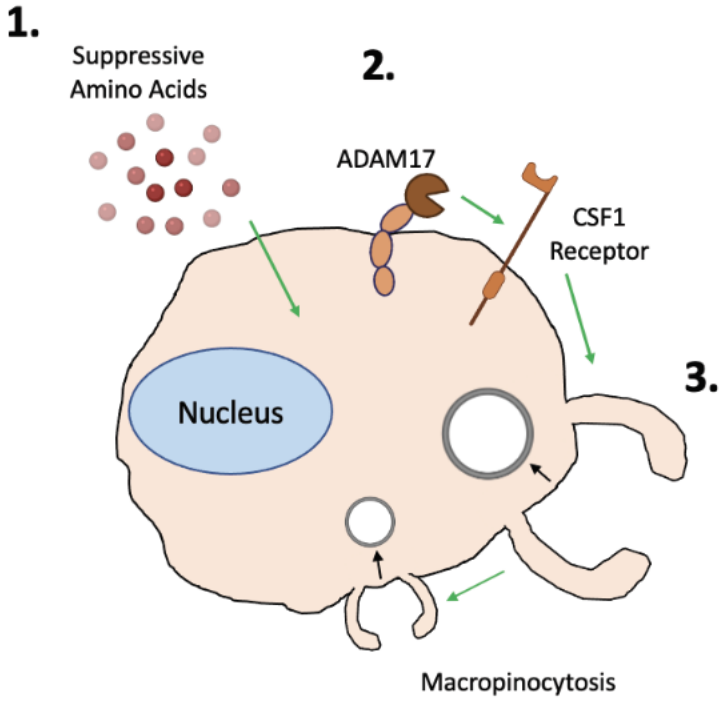
1.
Suppressive
Amino Acids
2.
ADAM17
CSF1
Receptor
3.
Nucleus
Macropinocytosis

Figure 4.1: **Model of amino acid-dependent suppression of macropinocytosis**. **1**.) Macrophages are incubated with suppressive amino acids, either individually or combined, which include leucine, isoleucine, methionine, phenylalanine, threonine, tryptophan, valine, cysteine and tyrosine. **2.**) Suppressive amino acids cause the activation of the metalloproteinase ADAM17, which cleaves the ectodomain of CSF1R, resulting in its release from the cell surface. **3.**) The loss of cell surface CSF1R results in the formation of smaller macropinosomes and thus less total accumulation by macropinocytosis.

Appendix

A.1 Introduction

I engaged in research that resulted in a published article titled "Renitence vacuoles facilitate protection against phagolysosomal damage in activated macrophages". This article was published in the journal *Molecular Biology of the Cell* in 2018 and explored the molecular mechanisms underlying a membrane damage response known as "inducible renitence"[1,2]. In brief, inducible renitence is a phenomenon by which lipopolysaccharide (LPS) protects the endolysosomal membranes within macrophages from damage following the ingestion of silica beads. In this study, Wong et al. describe vacuolar structures that are present in LPS-treated macrophages but absent in unstimulated macrophages. They termed these structures renitence vacuoles (RVs) because of their correlation with protection by inducible renitence. RVs formed coincident with silica bead uptake in a process associated with membrane ruffling and macropinocytosis. However, unlike normal macropinosomes, which shrink within 20 minutes of formation, RVs persisted around bead-containing phagosomes. RVs fused with lysosomes, whereas associated phagosomes typically did not. In this appendix, I describe the research I engaged in that became a part of this published article. Specifically, I generated Figure 7 of the manuscript (Figure A1.1 here), which examined

This appendix represents a modified version of the published article: Wong, AO. *et al.* Renitence vacuoles facilitate protection against phagolysosomal damage in activated macrophages. *Molecular Biology of the Cell* **29**, (2018).

whether RVs contained markers of early endosomes (Rab5) or lysosomes (LAMP1) at early and late time points following bead incubation. Data and discussion not related to the figure I generated are not included in this appendix.

A.2 Results

A.2.1 Renitence vacuoles transition from Rab5-positive to LAMP-1-positive structures within 30 minutes

To further characterize the identity of RVs and the intracellular organelles with which they interact, we assessed the timing of recruitment to RVs of molecular markers of major organelles of the endolysosomal pathway. As fixation and permeabilization methods for immunofluorescence staining failed to preserve RV structures in fixed cells, we performed these studies in live BMM transfected with fluorescent chimeras of Rab5a, a marker of early endosomes, and LAMP-1, a marker of late endosomes and lysosomes. LPS-treated BMM expressing YFP-Rab5a, CFP-LAMP-1, and mCherry were incubated with AW beads for 5 min or 20 min, and then mounted for imaging by phase contrast and ratiometric fluorescence microscopy. Still images of cells expressing all three probes were acquired over a 5-min imaging interval for coverslips exposed to beads for 5 min and over a 10-min imaging interval for coverslips exposed to beads for 20 min. Recruitment of Rab5a and/or LAMP-1 to RVs at either time point was determined using ratiometric images for each probe (i.e. YFP-Rab5a/mCherry and CFP-LAMP-1/mCherry).

In cells imaged between 5 and 10 min after AW bead incubation, a mixed population of RVs was observed, with a comparable proportion of RVs having acquired Rab5 only, LAMP-1 only, or both probes (**Figure A1.1**). After 20 to 30 min AW bead incubation, RVs were nearly uniformly LAMP-1-positive, Rab5-negative structures. These results suggest that RVs acquire Rab5 soon after their formation and transition into LAMP-1-positive, Rab5-negative

compartments within 30 min. The observation of LAMP-1-positive RVs within 5 to 10 min after AW bead incubation suggests rapid acquisition of LAMP-1 within a subset of RVs, consistent with the kinetics of macropinosome maturation and fusion with tubular lysosomes reported previously[3].

A.3 Discussion

This work reports the discovery of renitence vacuoles, damage-resistant structures formed in LPS-activated macrophages that protect against phagocytosis-mediated lysosomal injury. The rapid formation of RVs in the setting of membrane damage, their maintenance of low pH despite their proximity to a damaging particle, and their correlation with renitence provide evidence of a protective function. The dynamic interactions observed between RVs, macropinosomes, phagosomes, and lysosomes suggested a model for how RVs form and confer protection against lysosomal damage (**Figure A1.2**): In LPS-activated macrophages, macropinocytosis accompanies the phagocytosis of both damaging and non-damaging particles. Upon phagocytosis of a damaging particle, multiple MPs form around the phagosome, and in some cases enlarge and persist. These persistent, peri-phagosomal MPs (i.e. renitence vacuoles) fuse with lysosomes, whereas phagosomes associated with RVs typically do not. Thus, RVs prevent the fusion of their associated phagosomes with lysosomes. This activity, we propose, likely relates to the mechanism by which RVs protect against lysosomal damage. By preventing the fusion of damaged phagosomes with intact lysosomes, RVs would contain damage to early endocytic compartments and prevent the propagation of damage throughout the entire endolysosomal network. This strategy would represent an important protective mechanism not only for preserving the integrity of lysosomes, but also for restricting the release of lysosomal contents into the cytoplasm, a highly immunogenic event[4,5].

A notable feature of RVs is their persistence. The persistence of RVs likely relates to their functional role, as conventional MPs and vacuoles formed during uptake of non-damaging beads eventually shrink. Thus, understanding the factors that govern RV persistence may help to elucidate the mechanism of renitence. As MPs formed in macrophages stimulated with M-CSF shrink via fusion with lysosomes within 15 min after their formation, RV persistence may be caused by defects in their trafficking to lysosomes[3]. However, as RVs readily recruit LAMP-1 and acquire fluorescent probes pre-loaded into lysosomes, this possibility seems unlikely. Alternatively, RVs might persist due to an inhibition of MP shrinkage by other mechanisms or by an increase in fluid influx into the vacuole.

MPs themselves could be a source of extracellular fluid that maintains vacuole persistence. Additionally, as membrane-bound vesicles, MPs could contribute membrane necessary for the expansion of the compartment. This work introduces several concepts connecting LPS activation, macropinocytosis, and renitence that are consistent with such a model. Macropinocytosis is induced robustly in LPS-activated macrophages, and occurs both constitutively and following phagocytic challenge. In the case of damaging bead uptake, multiple MPs accumulate around the incoming phagosome. These MPs may function to supply a constant source of fluid to support the maintenance of a persistent RV. Consistent with this model, macropinocytosis is necessary for renitence. As analogous damage processes occur in the context of infection, we believe we have identified a general mechanism upregulated by macrophage activation or infection that preserves endolysosomal integrity following phagocytic encounter with membrane-damaging threats.

A.4 Methods

A.4.1 Plasmids and transfection

YFP-Rab5a and CFP-LAMP-1 plasmids were described previously[6]. The plasmid pmCherry-C1 (Takara Bio USA, Mountain View, CA) was used for expression of free mCherry. All plasmids were purified using an EndoFree Plasmid Purification Kit (Qiagen, Venlo, Netherlands). BMM were transfected with all three plasmids using a Nucleofector kit for Mouse Macrophages (Lonza, Basel, Switzerland) according to the manufacturer's protocol. Following transfection, macrophages were seeded onto glass-bottom microwell dishes in RPMI 1640 containing 10% FCS, 1% glutamax and 20 U/ml Penicillin-Streptomycin. Cells were incubated overnight in media containing 100 ng/mL LPS.

A.4.2 YFP-Rab5a and CFP-LAMP-1 imaging

Transfected LPS-treated BMM were incubated with AW beads for either 5 min or 20 min in RPMI 1640 media lacking phenol red. Cells were then washed to remove non-internalized beads and mounted for imaging. Still frames of phase contrast, mCherry, YFP, and CFP images were collected over a 5-min interval in coverslips incubated with AW beads for 5 min, and over a 10-min interval in coverslips incubated with AW beads for 20 min. Imaging was performed on a Nikon TE300 inverted microscope using an ECFP-EYFP-mCherry dichroic mirror and the following excitation and emission filter sets: mCherry (ex. 572 nm-em. 632 nm), YFP (ex. 500 nm-em. 535 nm), and CFP (ex. 440 nm- em. 470 nm).

A.4.3 Ratiometric imaging analysis

All fluorescence images used for ratiometric analysis (i.e. YFP-Rab5a, CFP-LAMP-1, and mCherry) were first corrected for camera bias and uneven illumination as previously described[7].To discard signal from regions outside of fluorescent cell areas, a binary threshold was applied over the cell using the mCherry component image.

The recruitment of YFP-Rab5a or CFP-LAMP-1 to RVs was assessed in ratiometric images generated by dividing the fluorescent signal in images of either fluorescent chimera by the fluorescent signal for mCherry (i.e. YFP-Rab5a/mCherry or CFP-LAMP-1/mCherry). This approach corrects for variations in optical path length owing to differences in cell thickness. The ratio images generated thus report the relative concentration of a specific fluorescent chimera in any given region of the cell normalized for cell thickness. Phase contrast images were used to identify the position of RVs. High intensity signal on the ratio image in regions corresponding to an RV was judged as positive recruitment of a given probe to the RV.

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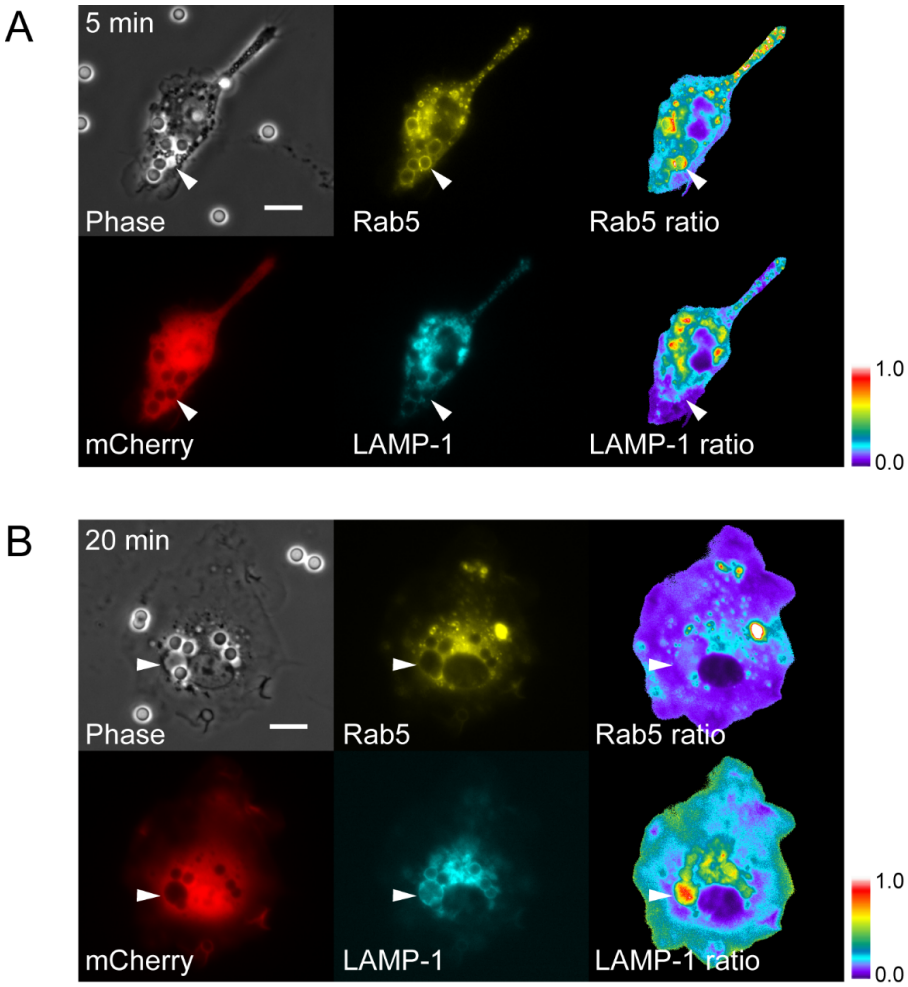
A
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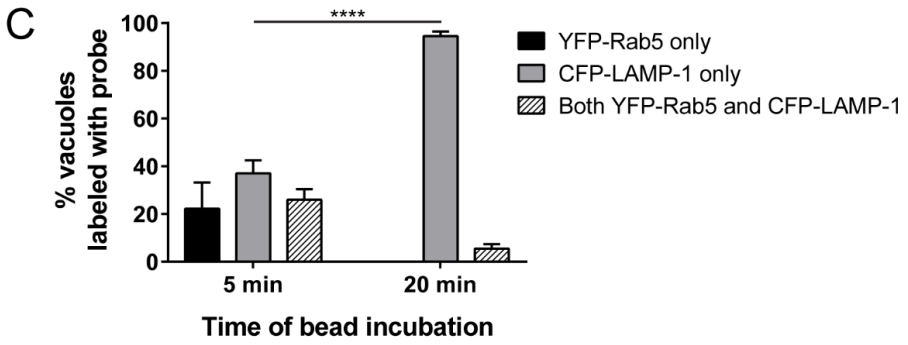
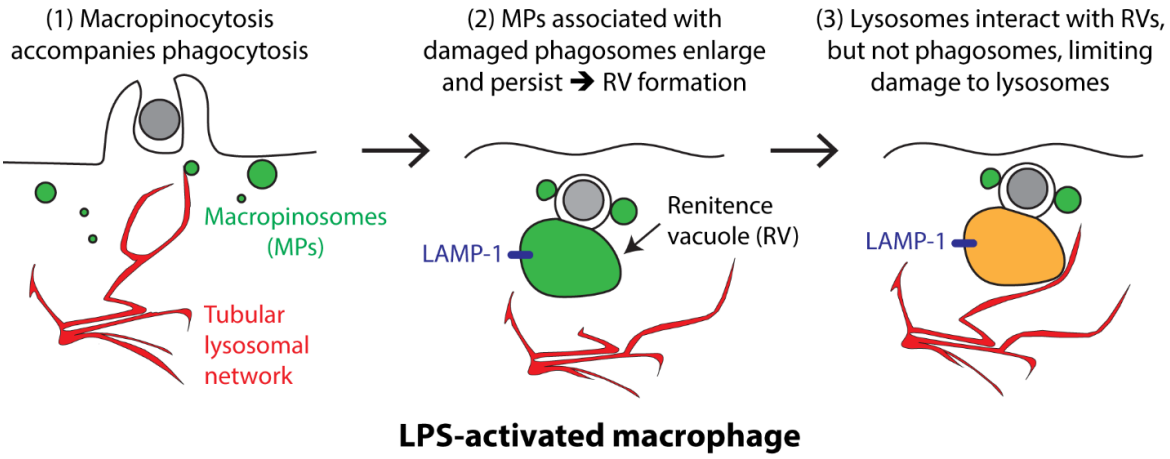

C
****
% vacuoles labeled with probe
100
80
60
40
20
0
5 min
20 min
Time of bead incubation
YFP-Rab5 only
CFP-LAMP-1 only
Both YFP-Rab5 and CFP-LAMP-1

Appendix Figure 1: **Renitence vacuoles transition from Rab5-positive to LAMP-1-positive structures within 30 minutes.** BMM transfected with YFP-Rab5a, CFP-LAMP-1, and mCherry were stimulated overnight with LPS. After 5 min (**A**) or 20 min (**B**) incubation with AW beads, cells were washed to remove non-internalized beads, then imaged by phase contrast and ratiometric fluorescence microscopy. Multiple still frames of cells expressing all three probes were imaged within a 5 or 10-minute time window following each period of bead incubation. Phase contrast images were used to identify renitence vacuoles. Ratiometric pseudocolor images (YFP-Rab5/mCherry and CFP-LAMP-1/mCherry) were used to assess probe recruitment to renitence vacuoles. (**A-B**) Representative phase contrast, fluorescence, and processed images of vacuole-containing LPS-treated BMM imaged between 5 to 10 min (**A**) or 20 to 30 min (**B**) after AW bead incubation. Pseudocolor images show recruitment of Rab5 but not LAMP-1 to the renitence vacuole (indicated with arrowhead) in (**A**) and, conversely, recruitment of LAMP-1 but not Rab5 to the renitence vacuole (indicated with arrowhead) in (**B**). Scale bar, 10 μm. (**C**) Proportion of renitence vacuoles that had acquired YFP-Rab5, CFP-LAMP-1, or both probes within LPS-treated BMM imaged after incubation with AW beads. Bars show the average percent ± SEM of vacuoles labeled with probes from 4 independent experiments. Weighted averages were calculated, where data from replicates in which more vacuoles were observed were proportionally given more weight. A total of 27 vacuoles were analyzed for cells imaged within the 5 to 10 min time interval, and a total of 55 vacuoles were analyzed for cells imaged within the 20 to 30 min time interval. ****$p \leq 0.0001$.



Appendix Figure 2: **Model of cellular events involved in renitence vacuole formation and protection against lysosomal injury.** Sequence of events leading to renitence vacuole formation and lysosomal damage protection in a representative LPS-activated macrophage. Lysosomes within LPS-activated macrophages assume a tubular lysosomal network (red). LPS-activated macrophages undergo macropinocytosis constitutively and upon particle phagocytosis. Upon internalization of a damaging particle, multiple MPs (green) accumulate around the incoming phagosome. In some cases, these MPs enlarge and persist. Persistent, peri-phagosomal MPs were identified as RVs. Whereas RVs fuse with lysosomes, as evidenced by their recruitment of LAMP-1 and acquisition of fluorescent probes pre-loaded into lysosomes, most phagosomes associated with RVs do not. Thus, RVs prevent the fusion of damaged phagosomes with intact lysosomes, and thereby preserve lysosomal integrity.