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Colony Stimulating Factor-1 Receptor (CSF-1R) Expression, Degradation, and Signaling in Lipid-Laden Macrophages

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COLONY STIMULATING FACTOR-1 RECEPTOR (CSF-1R) EXPRESSION, DEGRADATION, AND SIGNALING IN LIPID-LADEN MACROPHAGES

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

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This thesis is dedicated to my lab family here in Brookings for their guidance and friendship, my parents for their unwavering support, and my dear wife Kylie for everything in this journey.

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CONTENTS

ABBREVIATIONS

ABCA1	ATP-binding cassette transporter
ABCG1	ATP-binding cassette sub-family G
Akt	protein kinase B
Apo-E	apolipoprotein E
BMM	bone marrow media
BSA	bovine serum albumin
Cbl	an E3 ubiquitin ligase
CD115	cluster of differentiation 115, AKA CSF-1R and M-CSFR
CD11b	cluster of differentiation 11, Integrin alpha M
CD14	cluster of differentiation 14, Co-receptor for LPS
CD163	cluster of differentiation 163, Hemoglobin scavenger receptor
CD36	cluster of differentiation 36, Scavenger receptor
CD68	cluster of differentiation 68, Scavenger receptor that binds modified LDL
CD80	cluster of differentiation 80, Costimulatory molecule for T-cell activation
Cox-2	cyclooxygenase-2, enzyme that promotes formation of prostaglandins
CSF-1	colony stimulating factor one
CSF-1R	colony Stimulating Factor One Receptor
DAPI	4,6-Diamidino-2-phenylindole, dihydrochloride, Nuclear stain
DMEM	Dulbecco’s modification of Eagle’s medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco’s phosphate buffered saline
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
Fox	fork-head box transcription factor
GM-CSF	granulocyte macrophage colony stimulating factor
Grb2	growth factor receptor-bound protein 2

HA-mac	hemorrhage associated macrophage
HDL	high density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, buffer
Hmox1	heme oxygenase
IDL	intermediate density lipoprotein
IFN-γ	interferon gamma, cytokine
IL-10	interleukin 10, anti-inflammatory cytokine
IL-12	interleukin 12, cytokine produced in response to LPS
IL-1β	interleukin 1 beta, inflammatory cytokine
IL-4	interleukin 4, anti-inflammatory cytokine
IL-6	interleukin 6, Inflammatory cytokine
Ki67	protein expressed in proliferating cells
L-cell	murine fibroblast line used for CSF-1 production
LCIB	live cell imaging buffer
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPS	lipopolysaccharide
LXR	liver X receptor
M(Hb)	hemoglobin stimulated macrophage
M0	non-activated macrophage
M1	inflammatory macrophage
M2	anti-inflammatory macrophage
MDH	monodansylpentane, blue lipid stain
MDM2	mouse double minute 2 homolog, ubiquitin ligase
MHCII	major histocompatibility complex
MMP12	matrix metallopeptidase-12
MMP7	matrix metallopeptidase-7
NRF2	nuclear factor like 2, basic leucine zipper
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate

PKB	protein kinase B, Akt
Ras	small GTPase
TLR-2	toll-like receptor 2, recognizes bacterial lipoproteins
TLR-4	toll-like receptor 4, recognizes LPS
TNF	tumor necrosis factor, cytokines that cause apoptosis
Ub	ubiquitin, used to flag molecules for degradation
VLDL	very low density lipoprotein

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ABSTRACT

COLONY STIMULATING FACTOR-1 RECEPTOR (CSF-1R) EXPRESSION, DEGRADATION, AND SIGNALING IN LIPID-LADEN MACROPHAGES

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Atherosclerosis is a serious disease affecting a large portion of the world's population. In atherosclerosis, macrophages become filled with lipid droplets and form fatty plaques in artery walls. The formation of these atherosclerotic plaques is dependent on the macrophage growth factor colony stimulating factor-1 (CSF-1); however, understanding the changes in CSF-1 signaling and related pathways in foam cells remain poorly investigated. Lipid-laden macrophages were formed through exposure of murine bone marrow derived macrophages to 100 μg/ml acetylated LDL for 24 h. Non-LDL treated macrophages were grown in parallel for each experiment and used for comparison. Immunofluorescent staining was used to visualize and quantify CSF-1R, pAkt, pERK, pCSF-1R, and Ki67. To synchronize the signaling process, macrophages were deprived of CSF-1 supplemented media before experiments. The lipid-laden macrophages had a decreased rate of CSF-1R expression. The degradation of CSF-1R was unchanged after the initial CSF-1 stimulation, but the level of phosphorylated receptor was lower in lipid-laden macrophages. The phosphorylation of Akt was unchanged during the first hour but increased in non-LDL exposed cells after 10 h. The phosphorylation of ERK spiked in non-LDL exposed cells at 7 min indicating a possible

differential activation of pathways. Macrophage proliferation, as measured by Ki67 staining, increased while the cells had lipid droplets then leveled as lipid droplets cleared from the cells. Lipid-laden macrophages also did not display differences in CSF-1 stimulated macropinocytosis as observed with live-cell microscopy. Flow cytometry analysis revealed lipid laden macrophages had an increase in CD68, with decreased expression of CD11B and CD80. Differences in activation of signaling molecules highlight cellular functions affected by lipid droplets that can be targeted to prevent the proliferation of macrophages in plaques and promote plaque clearance.

Chapter 1

Literature Review

1. Introduction

The focus of this literature review is to present material pertinent to the understanding of atherosclerosis on a cellular level. I begin by presenting the major players involved in atherosclerosis; macrophages, serum lipoproteins, and the growth factor colony stimulating factor one (CSF-1). Understanding these different players provides insight into the widespread pathology of atherosclerosis and highlights potential directions for further research.

2. Macrophages

a. An introduction

Macrophages have an interesting history and are a critical part of the immune system. Macrophages were discovered by Ilya Mechnikov who observed them attacking the thorns of a tangerine tree that had been introduced into a starfish larva. For his observation, Mechnikov received the 1908 Nobel prize (Ehrlich, 1967). The function of macrophages is highlighted within in their name: the prefix “macro” meaning large and the root “phage” meaning to eat, make the literal name of these cells “large eaters.” Macrophages live up to their name as their classic function is to phagocytose pathogens and cellular debris (Alberts, 2008). As phagocytic cells, macrophages are not dependent on any prior exposure to pathogens and are part of the innate immune system (Alberts, 2008). From their initial discovery, macrophages have been found to have important and increasingly complex role in atherosclerosis, cancer, and infection.

One source of macrophages are hematopoietic stem cells in the bone marrow (Geissmann et al., 2010). These macrophages are generated through the process of hematopoiesis. For the generation of macrophages, hematopoietic stem cells differentiate into myeloid progenitor cells, which further differentiate into monocytes that enter into circulation (Alberts, 2008). Once in circulation, monocytes can be differentiated into macrophages by the growth factor CSF-1, which is critical to the proliferation and differentiation of macrophages (Geissmann et al., 2010). The ability of CSF-1 to differentiate hematopoietic stem cells into macrophages was demonstrated in vitro in the 1970's (Metcalf, 1970; Stanley et al., 1978). CSF-1 is constitutively expressed within the serum from fibroblasts, macrophages, smooth muscle cells, and osteoclasts (Hamilton, 2008) and is also upregulated along with other signaling molecules at sites of injury such as radiation (Xu et al., 2013). The generation of macrophages from hematopoietic stem cells provides a circulating pool of monocytes that can differentiate into macrophages.

Tissue specific macrophages provide another source of macrophages. These macrophages differentiate from the yolk sac during embryogenesis and proliferate within specific tissues. Yolk sac macrophages make up the specialized populations such as the Kupffer cells of the liver, microglia of the brain, and Langerhans cells of the epidermis (Perdiguero et al., 2015). The ability of Langerhans cells to proliferate is demonstrated in a case study of a surgical patient with a hand transplant. The Langerhans cells of the donor hand continued to populate the hand and were not replaced by the hematopoietic stem cell-derived macrophages of the recipient (Kanitakis et al., 2004).

The macrophage is the quintessential phagocytic cell of the body. These cells originally discovered in starfish, function in the innate immune system to phagocytose

debris and invading pathogens. Macrophages differentiate from bone marrow and multiply in tissues giving two sources of these critical cells. My focus will be on macrophages of hemopoietic origin and their role in supporting atherosclerosis.

b. Macrophage differentiation and activation

Just as circumstance can influence a person's actions, the external stimulation that a macrophage receives can influence the cell's functional role within the body. Macrophages are activated to particular transcription programs and phenotypes based on the conditions they encounter. Most frequently, macrophages are often characterized into M1 or M2 activation states based on the response to interferon gamma (IFN-γ) or lipopolysaccharide (LPS) (Mills et al., 000). In addition to M1 and M2 activation states, other categories for activation have been proposed and characterized (Gordon and Taylor, 2005). Cellular surface markers, secreted cytokines, and cellular phenotypes such as the ability to phagocytose opsonized red blood cells are indicators used to categorize these cells. Increasing evidence suggests that the spectrum of macrophage activation is a continuum rather than a dichotomy (Mosser and Edwards, 2008). Further, these phenotypes are very plastic, and Mosser and Edwards have likened the assignment of categories to macrophages to "assigning color to a chameleon" (Mosser and Edwards, 2008). Even with the growing theory of a continuum of differentiation, knowing the specific markers and secreted cytokines allows for predictions of cellular phenotype.

M0 macrophages are a group of macrophages that are yet to be activated. In vitro M0 macrophages are routinely cultured. Hemopoietic stem cells are isolated from the bone marrow and cultured in the presence of the growth factor colony stimulating factor-

1 (CSF-1). CSF-1 also stimulates the differentiation of monocytes into M0 macrophages (Chistiakov et al., 2015).

M1 macrophages are considered the classically activated macrophage. M1 stimulated macrophages promote inflammation and tissue damage along with inhibiting cell proliferation. The classic way to stimulate M1 polarization in macrophages is to expose the cells to endotoxin. Endotoxin, also known as lipopolysaccharide (LPS), is a component of gram-negative bacteria's outer membrane. Other major stimulators that generate M1 macrophages are interferon gamma (IFN-γ) and tumor necrosis factor (TNF) (Martinez and Gordon, 2014). Once the M1 phenotype has been stimulated, these macrophages can be identified by looking at the markers expressed on their surface along with the cytokines that are produced. Surface markers of M1 macrophages include cluster of differentiation 68 (CD68) and toll-like receptor 4 (TLR-4) (Chinetti-Gbaguidi et al., 2015). Cytokines expressed by these M1 macrophages include the pro-inflammatory cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-12 (IL-12), along with reactive oxygen and nitrogen species (Chinetti-Gbaguidi et al., 2015; Jones and Ricardo, 2013). M1 macrophages exhibit an inflammatory phenotype.

M2 macrophages are considered anti-inflammatory and are on the opposite end of the activation spectrum from M1. These macrophages are involved in tissue repair and cell proliferation. M2 macrophages, known as alternatively activated macrophages, are stimulated to form with exposure to interleukin-4 (IL-4), involved in T-cell differentiation and produce anti-inflammatory cytokine interleukin-10 (IL-10) (Chinetti-Gbaguidi et al., 2015). Further identification of M2 macrophages can be done with the surface markers CD163 and liver X receptor (LXR) (Chinetti-Gbaguidi et al., 2015). The

cytokine production profile of these cells varies but includes interleukin 10 (IL-10) and IL-4 (Chinetti-Gbaguidi et al., 2015). It is also possible to divide M2 macrophages into wound healing macrophages and regulatory macrophages based on expression level of IL-10 (Mosser and Edwards, 2008).

Mox macrophages are stimulated by exposure to oxidized phospholipids (Chinetti-Gbaguidi et al., 2015), and express oxidant genes hem oxygenase 1 (Hmox1), sulfiredoxin 1 (Srxn1), Thioredoxin reductase (TXNRD), and glutathione reductase (GSR) (Kadl et al., 2010). The expression of these genes is not seen in the traditional M1 or M2 macrophage and is dependent on Nuclear factor-like 2 (Nrf2) (Chistiakov et al., 2015; Kadl et al., 2010). Mox macrophages express TLR-2 and Nrf2 and produce IL-1β and cyclooxygenase-2 (COX-2) (Chinetti-Gbaguidi et al., 2015). These macrophages are pro-atherogenic and make up 30% of macrophages in atherosclerotic lesions, in LDL receptor knockout mouse model (Chistiakov et al., 2015; Kadl et al., 2010).

M4 macrophage are another subset of macrophages found in atherosclerotic plaques of humans (Chistiakov et al., 2015). M4 macrophages are induced by ligand 4 (CXCL4), express CD206, matrix metalloproteinase-7 (MMP7) and calcium binding protein A8 (S100A8) while producing the cytokines IL-6, TNF-α, and MMP12 (Chistiakov et al., 2015).

Hemoglobin stimulated macrophages (M(hb)), hemorrhage associate macrophages (HA-mac) and a similar group of macrophages that respond to heme and hemoglobin (Mhem) macrophages are found in atherosclerotic plaques. M(hb), HA-mac, and Mhem macrophages are atheroprotective (Chistiakov et al., 2015). These macrophages express ATP-binding cassette transporter (ABCA1) and ATP-binding

cassette transporter sub-family G (ABCG1) transporters that allow them to efflux cholesterol (Chistiakov et al., 2015).

3. Plasma lipoproteins

Lipoproteins function to move hydrophobic cholesterol through the hydrophilic environment of the body. These molecules are made up of a monolayer of phospholipids with a hydrophobic core (Cox, 1990). Different types of apolipoproteins move cholesterol to and from different cells in the body. For example, low density lipoprotein (LDL) is responsible for bringing cholesterol to cells in the body from the liver. Because high serum concentrations of LDL have been associated with poor health outcomes, LDL has been coined "bad cholesterol". Alternatively, high density lipoprotein (HDL) is commonly referred to as "good cholesterol" as it carries cholesterol away from the cells of the body and high serum levels have been associated with good health outcomes (American Heart Association, 2016).

a. Structure

Lipoproteins are defined in part by their density and can be isolated from serum by ultracentrifugation. LDL is isolated in the density range of 1.019-1.063g/ml. The LDL particle is made up of a phospholipid monolayer with embedded proteins surrounding a core of cholesterol and triglycerides. The LDL particle is 50% cholesterol, 25% protein, 20% phospholipid, and 5% triglyceride. The mass of LDL varies from 2.4-3.9 MDa. Interestingly, the one molecule of apo-B100 makes up over 95% of the protein mass and is the signature protein of LDL (Rajmanet al., 1999). Lipoproteins also fall into other categories such as HDL, very low density lipoprotein (VLDL), and intermediate density lipoprotein (IDL). Besides having different densities, these lipoproteins have different

protein profiles. The embedded proteins allow the different lipoproteins to deliver their cargo to its target cell type.

b. Modifications

Chemical modifications to LDL occur and are relevant to plaque formation and pathogenesis. Oxidized LDL is the most important modification for the formation of atherosclerotic plaques, as oxidized LDL is readily taken up by macrophages (Steinberg and Witztum, 2010). Oxidation of LDL occurs naturally in the body via reactive oxygen species present due to metabolic process (Arai, 2014). Inflammation and infection also induce the formation of oxidized LDL in vivo (Memon et al., 2000). LDL can be acetylated through the exposure of LDL to saturated sodium acetate and acetate anhydride in vitro (Reza, 2010). Acetylated LDL is taken up by macrophage scavenger receptors; however, it has not been proven to exist in vivo (Steinberg and Witztum, 2010). Despite having different modifications, acetylated LDL and oxidized LDL are both taken up by macrophage scavenger receptors and delivered to the same lysosomes (Yancey et al.,2002). There are differences in the transport of cholesterol out of the cell and the oxidized components of oxidized LDL can impair degradation and stimulate inflammatory phenotype (Wang et al., 2007).

c. Epidemiology

High levels of cholesterol in blood are associated with higher risk for atherosclerosis. The National Cholesterol Education Program (NCEP) and the National Lipid Association have set the desirable total cholesterol level to be <200mg/dl (2,000 µg/ml). Approximately 15% of US adults over 20 have serum cholesterol levels over 240mg/dl (2,400 µg/ml) or greater and 69% of US adults have low density lipoprotein cholesterol (LDL-C) above 100mg/dl (1,000 µg/ml) (Jellinger et al., 2012). The high

levels of hypercholesterolemia and its significance in cardiovascular disease point to a need to understand disruptions in macrophages cell biology due to LDL uptake and lipid droplet formation.

4. Lipid droplets

Lipid droplets are organelles found within nearly all cells (Walther et al., 2012). The basic structure of lipid droplets is similar to that of lipoproteins in circulation, with a phospholipid monolayer surrounding a hydrophobic core. However, lipid droplets can be much larger - ranging in size from a few nanometers to 100 µm in white adipocytes (Guo et al., 2009). The formation of lipid droplets occurs at the endoplasmic reticulum (ER). Lipids are trafficked from the lysosome to the ER and build up between leaflets of the phospholipid bilayer until the monolayered droplets buds off. The exact process of lipid droplet formation from the ER is still being studied with the ER domain and budding, the bicelle, and vesicular budding models proposed (Guo et al., 2009). The structure and formation of lipid droplets in cells of the immune system still presents many exciting opportunities for discovery.

Lipid droplets are a dynamic organelle with changing morphology and internal membranes (Walther and Farese, 2009). Changes in morphology takes place in situations like infection and phagocytosis. The electron density of lipid droplets in activated macrophages changes due to the synthesis of eicosanoids (prostaglandins, leukotrienes, and lipoxins) within the lipid droplets (Bozza et al., 2011). Lipid droplets contain internal membranes that form a network of tubules resembling the ER (Melo et al., 2013). In macrophages lipid droplets are considered structural markers of activation (Bozza et al.,

2007). Research into lipid droplets has opened many more questions than have been answered unveiling an exciting and previously overlook organelle.

5. Colony stimulating factor one (CSF-1) and signaling

a. Structure and signaling

CSF-1R is a tyrosine protein kinase that promotes the growth and proliferation of macrophages. The receptor is activated when a homodimer is formed following binding of dimerized ligand and the receptors transphosphorylate each other. After phosphorylation, CSF-1R binds Grb2 and activates PI3K that in turn propagate and amplify the signal within the cell (Husson et al., 1997; Sampaio et al., 2011). This signaling supports cell growth, differentiation, survival, and stimulates macropinocytosis or chemotaxis.

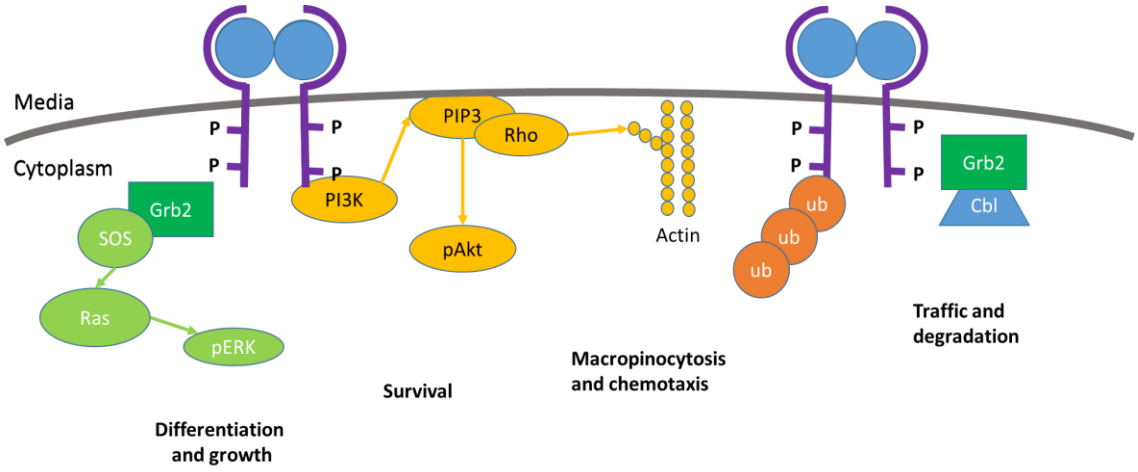

Figure 2. CSF-1R activated pathways within the cell, once the dimerized CSF-1 ligand binds to the receptor, the CSF-1R receptor also dimerizes. The receptors transphosphorylate each other binding Grb2 and activating PI3K. This in turn leads to differentiation and growth, survival, and macropinocytosis or chemotaxis. The receptor dimer is then ubiquitinated and degraded.

b. CSF1R Internalization and degradation

After the CSF-1 ligand binds to CSF-1R, the activated dimer is internalized by small vesicle endocytosis (Lou et al., 2014). Based on experimental addition of inhibitors

at time points after CSF-1 addition, CSF-1R continues signaling in early endosomes after being trafficked from the cell membrane (Huynh et al., 2012). These early endosomes are trafficked to macropinosomes (Lou et al., 2014). The timeline for this process is short with CSF-1R going from the cell surface to degradation in minutes. A disruption in the rate of degradation may lead to altered biology of the cell because of prolonged CSF-1R signaling.

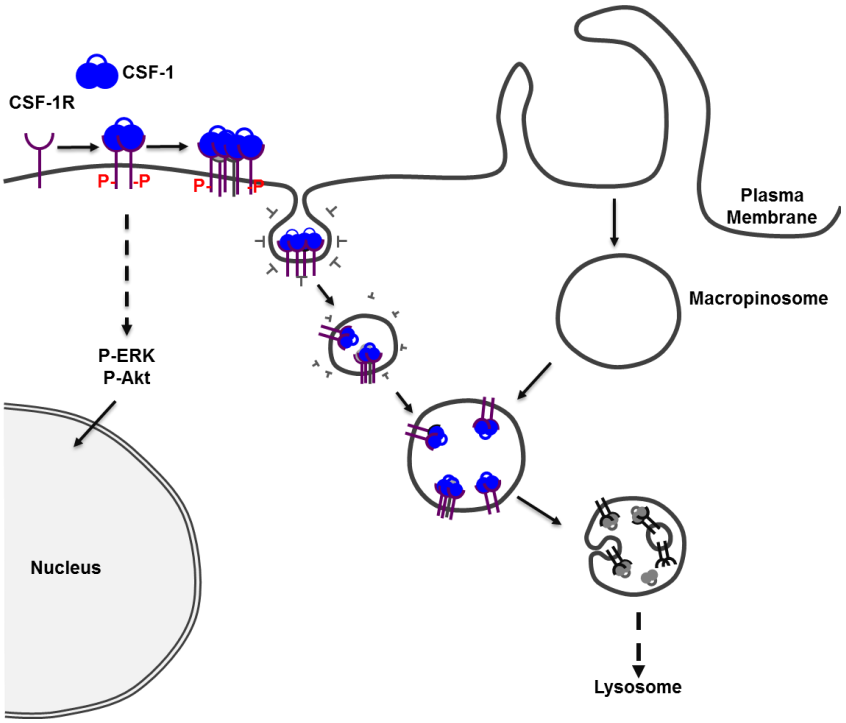

Figure 2. The degradation of the CSF-1R occurs quickly after ligand binding. The receptor is internalized into early endosomes that continue signaling. Early endosomes are trafficked to macropinosomes before degradation.

c. Akt signaling

Akt, also known as protein kinase B (PKB), is a well-studied intermediate signaling molecule within cells critical for growth, survival, and proliferation (Hart and Vogt, 2011). Akt is activated through phosphorylation at different sights with S473 being important for a tenfold increase in the catalytic activity (Alessi et al., 1996). Once

phosphorylated, Akt passes the signal downstream to target of rapamycin complex 1 (TORC1), the forkhead box (FOX) transcription factors that are tumor suppressers, and murine double minute (MDM2) involved in metabolism (Hart and Vogt, 2011). The localization of Akt and level of phosphorylation observed in cells can be used to predict the cells' biology. Cells with nuclear localization of pAkt have extended lifespans (Martellia, 2012). Bone marrow derived macrophages exposed to oxidized LDL have decreased survival and proliferation that can be prevented by transfecting cells with constitutively active Akt (Chu et al., 2011).

d. ERK signaling

Extracellular-signal-regulated kinases (ERK) are classical MAP kinases that are phosphorylated when CSF-1R is activated and transmit the signal through the cytoplasm. The phosphorylation of ERK has been demonstrated to be important for the expression of the D2 cyclin genes (Dey et al., 2000). Cyclins are used by cells to regulate proliferation supporting the role of CSF-1R and pERK in cell proliferation.

6. Disease and lipid laden macrophages

Though macrophages are an important part of the immune system, they also can be involved in pathology. Lipid laden macrophages can be found with several diseases including infections and metabolic disorders. Infections agents that cause lipid droplet formation in macrophages include *Candida albicans*, *Chlamydia pneumonia*, *Leishmania amazonensis*, *Mycobacterium*, and *Trypanosoma cruzi* (Dias et al., 2014). Lipid droplet formation in macrophages is associated with diabetes and atherosclerosis, the hormones leptin and resistin, and inflammatory stimuli (Dias et al., 2014). Here, I will focus on lipid-laden macrophages and atherosclerosis.

a. LDL uptake by macrophages

LDL is taken up by macrophages through three mechanisms: LDLR mediated uptake, scavenger receptors, and macropinocytosis (Arai et al., 1989; Herijgers et al., 2000; Kruth et al., 2005). The LDLR mediated uptake can make a significant contribution to plaque formation in vivo (Herijgers et al., 2000). Macrophages express the LDL receptor that recognizes apoprotein B-100 found on LDL (Zaiou et al., 2000). Modified LDL such as oxidized and acetylated bind scavenger receptors CD36, scavenger receptor A, CD68, and LOX-1 (Boullier et al., 2001). Scavenger receptors are used by macrophages to remove foreign and damaged cellular material from the body (Peiser and Gordon, 2001). Besides receptor-mediated pathways of uptake, LDL can be taken up by macropinocytosis (Kruth, 2011; Kruth et al., 2005).

b. Epidemiology of atherosclerosis

Atherosclerosis is serious and widespread disease that affects a large percentage of people. Atherosclerosis is the underlying pathology for many serious diseases including coronary artery disease, carotid artery disease, peripheral artery disease, and chronic kidney disease (NIH, 2015). Coronary artery disease alone was responsible for 1 of every 6 deaths in 2007, in the United States (Jellinger et al., 2012). In 2012 the World Health Organization listed coronary artery disease as the top killer of people in the world with 7.4 million deaths (WHO, 2014). The grave outcome of this pathology along with its widespread nature presents great benefit to understanding the underlying causes and cell biology of pathogenesis.

The hallmark of atherosclerosis is the formation of plaques in the intima of the arteries. In coronary artery disease, the plaques form in the coronary arteries that supply oxygen to the heart. Carotid artery disease involves the buildup of plaques in the carotid

arteries in the neck, in peripheral artery disease the plaques occur in the arteries of arms and legs, and in chronic kidney disease, the plaques occur in the renal arteries (NIH, 2015). These plaques restrict blood flow and can destabilize and rupture causing blockage and clotting in the arteries. With atherosclerotic plaques being central to the pathology of atherosclerosis, understanding their formation is of utmost importance.

c. Formation of atherosclerotic plaques

Plaque formation involves both cholesterol and the cells of the immune system. The plaque begins as a fatty streak that is the precursor to the atherosclerotic lesion. In some cases, and especially in younger people these streaks may simply disappear (Stary et al., 1994). The lesion itself is dominated by immune cells along with the buildup of lipids, calcium, and cellular materials (Stary et al., 1994). The buildup of these materials within the walls of the artery causes constriction of the vessels and limits blood flow (Stary et al., 1994). Macrophages and T-cells make up a majority of the immune cells in the fatty streak (Stary et al., 1994). LDL is a major component of the atherosclerotic plaque (Saxena and Goldberg, 1994). The center of the plaques become filled with both macrophages and extracellular lipid droplets and a cap of smooth muscle cells can form (Hansson, 2005). Under the cap, a necrotic core may develop leading to instability and the potential for destabilization and rupture. If the plaque does rupture, clotting in the artery leads to blockage of the vessels that can cause injury or death of the tissue (Van Der Wal and Becker, 1999).

d. CSF-1 in atherosclerosis

The importance of CSF-1R in atherosclerosis is highlighted in a knockout mouse experiment (Smith et al., 1995). Mice with an Apo-E and CSF-1 double knock out had significantly smaller plaques and with higher mean blood cholesterol levels than the just

Apo-E controls (Smith et al., 1995). Apo-E knockout mice cannot produce apolipoprotein E that is needed for all lipoproteins except LDL, it is synthesized by the liver, brain, monocytes, and macrophages (Meir and Leitersdorf, 2004). The deficiency of Apo-E causes high levels of blood cholesterol and atherosclerosis. The decrease in atherosclerosis in these double knockout mice could be due to decreased macrophage populations or changes in macrophage signaling in the absence of the CSF-1 ligand.

Oxidized LDL is able to promote the growth and proliferation of macrophages in the absence of growth factors CSF-1 and GM-CSF and increase the proliferative response to those growth factors (Hamilton et al., 1999). Exposure to low concentrations of oxidized LDL (50 µg/mL) led to growth and survival of macrophages in the absence of CSF-1 and GM-CSF while concentrations over 100 µg/ml lead to cell death (Hamilton et al., 1999).

e. Treatment and prevention

Treatments of atherosclerosis have primarily focused on lowering the amount of cholesterol in the blood. This can be done with changes in a person's diet or through the use of medications such as simvastatin that targets a reductase of LDL and pravastatin that also inhibits cholesterol synthesis (Pedersen et al., 1994; Shepherd et al., 1995). Vaccines for atherosclerosis are under development, and the first vaccines were against the ApoB-100 protein and heat shock proteins, as heat shock proteins induce atherosclerosis. These vaccines have shown to be effective in animals and are now approaching clinical testing (Nilsson et al., 2013). Though not currently in use, suppression of macrophage recruitment plaques in knock out mice has shown to b e a promising target for future therapies (Potteaux et al., 2011)

7. Conclusion

Atherosclerosis is a complex disorder that can benefit from a cellular level investigation of macrophage biology. Even though CSF-1 is significant to the formation of atherosclerosis and macrophages, the effect of lipid droplet load on receptor expression, signaling, and related pathways remains poorly characterized.

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Chapter 2

Lipid burden alters CSF-1 receptor signaling and traffic

Introduction

Atherosclerosis is a serious and widespread disease with a subset of atherosclerosis, coronary artery disease, being responsible for 1 in 6 deaths in the United States (Jellinger et al., 2012). Coronary artery disease alone was considered by the world health organization to be the top killer of people in the world (WHO, 2014). The hallmark of atherosclerosis is the formation of a fatty plaque in the arteries made up of macrophages filled with lipid droplets (Stary et al., 1994). The macrophages fill with lipid droplets and seemingly become trapped in the plaques, where they produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (Autieri, 2012). This inflammation can lead to plaque rupture that initiates clotting cascades within the arteries (Van Der Wal and Becker, 1999).

The growth factor, colony stimulating factor one (CSF-1), has both a positive and negative relationship with atherosclerosis. The phosphorylation of CSF-1R stimulates several pathways within macrophages that lead to chemotaxis, macropinocytosis, survival, and proliferation (Pixley and Stanley, 2004). In a mouse model for atherosclerosis the knockout of CSF-1R caused a significant decrease in atherosclerotic plaque area in the aortic root (Smith et al., 1995). Interestingly, the administration of CSF-1 to hyperlipidemic rabbits suppressed atherosclerotic lesions, indicating that there

is not a simple linear relationship between CSF-1 and atherosclerosis (Chitu and Stanley, 2006).

Macrophages respond to CSF-1 by activating and propagating signaling cascades within the cell. CSF-1 binds to the colony stimulating factor one receptor (CSF-1R), CSF-1R then forms a dimer and auto-phosphorylates, before phosphorylating other signaling molecules leading to Akt and ERK phosphorylation (Pixley and Stanley, 2004). CSF-1R is subsequently internalized into early endosomes where it presumably continues signaling (Huynh et al., 2012). The early endosomes then fuse with the macropinosomes before degradation (Lou et al.,2014). The activation of different signaling pathways dictates the macrophages response to CSF-1.

Cell culture allows for a pointed study of CSF-1R in lipid-laden macrophages, like those found in atherosclerotic lesions. Modified LDL is taken up by scavenger receptors and non-modified LDL is taken up by the LDL receptor or macropinocytosis to form lipid-laden macrophages (Kruth, 2011; Kruth et al., 2005). Cultured lipid-laden macrophages can be used to investigate cellular response to CSF-1 and changes in receptor expression. Removal of supplemental CSF-1 from culture media causes an upregulation of the CSF-1R on the cell surface. Saturating this receptor by adding back CSF-1 to the macrophages causes a synchronized signaling and internalization of the receptor. By investigating how lipid-laden macrophages differ from normal macrophages in their cellular response to CSF-1, we can better understand the pathogenesis of atherosclerosis.

Materials and Methods

Media types and preparation

Bone marrow media (BMM) was used to differentiate and grow bone marrow derived macrophages. BMM consisted of 20% premium heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 30% L-cell conditioned media, with Penicillin and Streptomycin (Pen Strep) (30-002-Cl, Corning, Manassas, VA), and 2-mercaptoethanol (ThermoFisher Scientific, Waltham, MA) in Dulbecco's modification of Eagles Media containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (DMEM) (Corning, Manassas, VA). Before use these components are filtered with a 0.46 μm vacuum filter (EMD Millipore, Darmstadt, Germany).

L-cell conditioned media was grown in the lab. L-cells were seeded into 75 cm2 tissue culture treated flasks (Corning, Manassas, VA) with 10% FBS and Pen Strep in DMEM. L-cell growth was observed daily. The L-cells became confluent approximately 4 days' post plating. They were allowed to grow for 3 additional days before harvesting supernatant. The supernatant was removed from the T75 flasks and frozen in 50 mL tubes until it was needed to make BMM. After harvesting media, flasks were washed with 5 mL of sterile PBS (GE Healthcare Life Sciences, Pittsburgh, PA) before the addition of 5 mL of 0.05% trypsin (Corning, Manassas, VA). Trypsin was added the plates were incubated at 37°C until the L-cells were easily be detached by pipetting. The L-cells were then centrifuged at 300 g (Allegra X-14R centrifuge, SX4750A rotor, Beckman Coulter, Brea, CA) for 5 min and suspended in 10% FBS in DMEM. The cell suspension was then used to seed the next round of flasks. The L-cells express the growth factor CSF-1 that is needed to support the differentiation, growth, and proliferation of macrophages.

Live cell imaging buffer (LCIB) was used for imaging and time course experiments outside of the CO2 buffered incubator. LCIB consists of DPBS 0.9 mM Ca 0.49 mM Mg (GE Healthcare Life Sciences, Pittsburgh, PA), with 0.45% glucose (Corning, Manassas, VA), and 10 mM HEPES (GE Healthcare Life Sciences, Pittsburgh, PA).

Bone Marrow Macrophage isolation

Adult black 6 (C57BLJ) mice were acquired (Jackson Laboratories, Bar Harbor, ME) and allowed to equilibrate to their new environment after transport before euthanasia with CO2 (Swanson, 1989). Femurs were harvested from the mice with care being taken to maintain the integrity of the bone. After harvesting the ends of the femurs were cut off and the bone marrow flushed from the bones with the use of DPBS -Ca -Mg (GE Healthcare Life Sciences, Pittsburgh, PA) delivered through a 0.5 inch, 26-gauge needle and 5 ml sterile Luer Lock syringe. The isolated bone marrow was centrifuged at 500 g for 5 minutes. The pellet of cell was then suspended in BMM. Cells where plated at a density of 10 million cells per non-tissue culture treated, sterile, 10 cm petri dish with 10 ml of BMM. Plates were placed in an incubator at 5% CO2 and 37°C. Plates were allowed to grow for 2 days before the addition of an additional 10 mL of fresh pre-warmed BMM to the plates. After an additional two days the macrophages stick to the bottom of the plate and the media can be removed and replaced with 10 ml of fresh BMM. To maintain an optimal level of growth factors and nutrients in the media the media was removed and replaced with 10 ml of BMM every two days. The nomenclature used for these cells starts with D0 macrophages on the day of isolation and plating. Cells on the following day are referred to as day 1 (D1) macrophages and that pattern continues throughout the lifespan of the cells with the day 4 (D4) macrophages adhering to the

plate. To maintain macrophages in growth phase, cells were observed and re-plated when the plates approached confluency. Macrophages from these isolations were either re-plated for experiments or frozen down for later use.

Detaching cells in culture

Detaching macrophages from the petri dishes allowed for re-plating for experiments, maintenance of cultured cells density, and freezing of cells for later use. Cells were detached from plates with cold, sterile, DPBS -Ca -Mg. Bone marrow media is first removed and the plates washed with 5ml of 4°C DPBS -Ca -Mg to remove the remaining media. Then 10ml of 4°C DPBS -Ca -mg was added to the plates and the plates were placed in the refrigerator for 20 minutes. After sitting in the refrigerator (4°C) macrophages rounded up and detached from the plate. Macrophages were further detached by pipetting. Cells were spun at 300 g for 5 min in 15 ml falcon tubes and re-suspended in selected media.

Freezing Cells

Freezing down of cells was done on the desired day after bone marrow isolation for primary bone marrow macrophages. Cells were detached from culture dishes and suspended in BMM. Concentration was determined with the use of a hemocytometer and trypan blue (GE Healthcare Life Sciences, Pittsburgh, PA). A 1:1 ratio of cells containing media and trypan blue allow for differential identification of live and dead cells. After counting cells were diluted with additional BMM to a final concentration of 1.1 million cells per ml before the addition of 10% DMSO (Corning, Manassas, VA) to the media. Immediately after addition of DMSO, 1 ml of cells was aliquoted into 1.5ml cryo-vials and put into CoolCell cell freezing containers for at least 4 h in the -80° freezer. After this time the cells are transferred to a liquid nitrogen tank for long term storage.

Plating cells

Macrophages grown in culture and from liquid nitrogen were plated in to petri dishes, 24 well plates, 6 well plates, and 96 well plates. Cells from liquid nitrogen were thawed quickly in a 37°C water bath and suspended in 10 ml of BMM. After suspension cells were spun at 300 g for 5 min and suspended in BMM before being counted with trypan blue and a hemocytometer. Cells were then plated at a density of 10,000-20,000 cell per well in 100µl for a 96well plate, 50,000 cells per well in 0.5 ml media for 24 well plate, 300,000 cells per well in 3 ml media for a 6 well plate, and 1,000,0000 cells per 10 ml of media in a 10 cm dish.

Macrophages that were plated into 24-well and 6-well plates were put directly onto glass coverslips. The coverslips used were 12 mm for the 24 well plates and 25 mm for the 6 well plates, #1.5 circular glass (ThermoFisher Scientific, Waltham, MA). Before putting coverslips into the empty plate, they were dipped in 95% ethanol and flamed, to sterilize them. Macrophages were plated onto glass to improve image quality for immunofluorescent and live cell experiments.

LDL Exposure/loading

Macrophages were grown to the desired day post isolation. Before the addition of LDL to the culture media macrophages were plated onto the glass or optical bottom 96-well plates, unless the macrophages were to be used in a flow cytometry experiment. Oxidized, acetylated, and native (non-modified LDL) were used (Alfa Aesar, Ward Hill, MA). Concentrations of LDL were varied in experiments. A majority of the experimental results presented here were generated with 100 µg/mL acetylated LDL. LDL was added in either BMM or DMEM+10%FBS and loaded overnight. Figures for experiments contain notes of LDL type and concentration used.

CSF-1 activation time course

To better visualize trafficking of CSF-1R and phosphorylation of pAkt, pERK, and pCSF-1R, cells were deprived of CSF-1 to upregulate expression of the receptor. This is done so that upregulated expression will allow for the best visualization of the receptor throughout the attenuation and signaling process. CSF-1R expression after CSF-1 ligand removal was also observed. BMM was removed from cells 24 h prior to the experiments and DMEM high glucose plus 10% FBS was added so that macrophages would upregulate CSF-1R expression. Recombinant murine CSF-1 (BioLegend, San Diego, CA) was then added to cells at a concentration of 0.2µg/mL in LCIB and cells were fixed at time points past this initial stimulation with 4% paraformaldehyde (PFA) (ThermoFisher Scientific, Waltham, MA).

Immunofluorescence cell staining

Immunofluorescent (IF) staining was used to visualize and quantify CSF-1R, pCSF-1R, pERK, pAkt, and Ki67. IF staining was done on both glass coverslips and in 96 plates. Cell were fixed with 4% PFA in PBS for 10 minutes. After fixation cells were permeabilized to allow antibodies access to the inside of the cells, some coverslips were not permeabilized in order to only see surface expression. Cells were permeabilized for 30 minutes with 0.1% Triton-X 100 (ThermoFisher Scientific, Waltham, MA) in PBS. Blocking for non-specific binding with 2.5% BSA in PBS was done for at least an hour. Primary antibodies were added to the cells in 2.5% BSA (ThermoFisher Scientific, Waltham, MA) for at least one hour at room temperature. Antibodies for phosphorylated proteins were kept on cells overnight at 4°C. After staining with primary antibody the species specific secondary antibody was added a 1:500 dilutions for one hour to two hours.

Table 1. Primary antibodies used for immunofluorescent staining coverslips

Primary Antibody to	Clone	Type	Supplier	Species
Mouse CS115 (CSF1R)	AFS98	Monoclonal	eBioscience	Rat
Human Ki67 Antibody	SP6	Monoclonal	abcam	Rabbit
p-CSF1R Receptor (Y723)	49C10	Monoclonal	Cell Signaling	Rabbit
(pERK) p-p44/42 MAPK (T202/y204)	D13.14.4E	Monoclonal	Cell Signaling	Rabbit
pAkt S473	D9E	Monoclonal	Cell Signaling	Rabbit

Cell and nuclear masks were stained to facilitate automated image analysis. Nucblue (ThermoFisher Scientific, Waltham, MA) was used for nuclear staining, which was added to cells at the recommended 2 drops per mL, for 5 minutes. Dye 647-I phalloidin (Abnova, Jhongli, Taiwan) was used to mask cells. The phalloidin was added for 20 minutes at a 1:100 dilution in BSA.

Several lipid stains were trialed with Oil Red O being used for a majority of experiments. A stock solution of Oil Red O (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 0.4% Oil Red O in 100% isopropanol (ThermoFisher Scientific, Waltham, MA). The same day of use, Oil Red O stock was diluted to 60% isopropanol with deionized water. This 60% working solution was filtered to remove undissolved particles. The Oil Red O stain was added to the cells for 30 sec before removal and copious washes with PBS. Care was taken to not allow the Oil Red O to dry out during the washing steps as this leads to high background fluorescence.

After staining, cells fixed for microscopy were mounted or put in PBS with sodium azide (Santa Cruz Biotechnology, Dallas, TX). Stained glass coverslips were picked up with tweezers and the water wicked away with contact on the edge with a paper towel. After the removal of water, coverslips were inverted onto a droplet of

Flouromount-G (Southern Biotech, Birmingham, AL) on a glass slide. Flouromount-G was allowed to dry for at least half an hour before the edges of the coverslip were tacked down with clear nail polish. The nail polish is to prevent the coverslip from coming off of the slide or moving. Cells in 96 well plates had the media replaced with PBS plus sodium azide to prevent any growth of bacteria or fungus in the plate before imaging.

To facilitate washing of the coverslips between the different staining steps a vacuum pump was used to remove liquid from coverslips. The pump was attached to a 1000ml Erlenmeyer flask via the flasks side arm. A shortened 10 mL serological pipette was inserted into the central in the rubber stopper, a good seal was achieved by wrapping the pipette with parafilm, and glass wool was removed. The pipette tip was connected to the serological pipette and a pipette tip via latex tubing. A secondary pipette tip was used to remove the stain and wash solutions from the coverslips.

Imaging

Fixed coverslips were imaged with the use of fluorescent microscopes. An EVOS FL imaging system was use with GFP (Alexa 488 secondary antibody), TxRed (Oil Red O or LipidTOX), DAPI, and Cy5 (647 phalloidin) filter cubes (ThermoFisher Scientific, Waltham, MA). An Andromeda spinning disk confocal microscope was used for the acquisition of live cell images. The 561nm laser with Zet 442/514/561x excitation wheel and 605/64 emission wheel were used for “red”. The 445 laser with Zet 442/514/561x excitation wheel and 537/26 emission wheel were used for “green”.

Dextran uptake in live cells

Bone marrow derived macrophages were grown on 25 mm coverslips in 6-well plates. These cells were plated at a density of 3×10^5 cells per well. Coverslips were taken and put into imaging chambers with live cell imaging buffer (LCIB). Large

fluorescent dextrans were used to image macropinocytosis. Texas Red tagged to 70 kDa dextran was used as it is size excluded from most forms of internalization such as endocytosis and can be taken up with macropinocytosis. Lucifer yellow was used in addition to recombinant CSF-1 to visualize stimulated macropinocytosis. The Lucifer yellow in LCIB with CSF-1 was added to the cells for 2 minutes to load macropinosomes. The cells were then washed to remove non-internalized Lucifer yellow.

Nile red (ThermoFisher Scientific, Waltham, MA) was used to stain lipid droplets in live cells. A 1 mM solution of Nile red in DMSO was diluted to a 1 µM working solution in LCIB. This 1 µM solution was used to stain cells for 5 minutes before cells washing with LCIB and imaging.

Flow cytometry

Macrophages were grown in culture to the desired day from stock D4 macrophages in liquid nitrogen. These cells were grown in 10 cm petri dishes, with one dish of control cells and another dish of cells treated with 100 µg/mL acetylated LDL overnight. Cells were detached from plates with cold DPBS without calcium or magnesium, then fixed with 4% PFA for 10 min. Staining was done directly with conjugated antibodies and Nile red to quantify lipid content (Greenspan et al, 1985).

Table 2. Antibodies used in flow cytometry

Primary antibody	Clone	Fluorophore	Manufacturer
CD68	FA-11	Alexa Fluor 488	BioLegend
IA/I-E (MHCII)	M5/114.15.2	Alexa Fluor 647	BioLegend
CD80	16-10A1	PE	BioLegend
CD11B	M1/70	Alexa Fluor 488	BioLegend
CD14	Sa14-2	APC	BioLegend
CD115	Afs98	PE	BioLegend

Image analysis

Image analysis and quantification was done with the use of open source software packages ImageJ and CellProfiler (Carpenter et al., 2006; Rasband, 1997-2016). ImageJ was used to visualize raw images and process the images into montages (Rasband, 1997-2016). Quantification of image data was done through the use of the program CellProfiler (Carpenter et al., 2006). Images were quantified using cell masks constructed from F-actin staining, assisting this process was the requirement that each cell mask contain one nuclear mask. The image analysis pipelines along with the images used have been retained for reference and adjustment of analysis parameters.

Statistics

The open source statistical package R version 3.3.0, 64 bit, for Windows was used for the analysis of the output from CellProfiler (R Team, 2008). Graphical representation of data utilized the R package "ggplot2" (Wickham, 2009).

Results

Experiments began by establishing lipid droplet loading and staining then moved to looking at signaling and outcomes. Lipid droplet loading looked at media type, and LDL modification. Staining looked into the use of different stains and protocols. Signaling investigated the expression of CSF-1R and then at the phosphorylation of signaling intermediates post CSF-1 stimulation. Ki67 was used to look at cell proliferation and live cell imaging with fluorescence to look at macropinocytosis.

Macrophages exposure to acetylated LDL produced lipid droplets that readily stained with Oil Red O

Dose response experiments determined optimal conditions for loading primary bone marrow derived macrophages with lipid droplets and procedures for staining them.

The first experiment assessed formation of lipid droplets in macrophages exposed to 50 µg/ml, 100 µg/ml, and 150 µg/ml acetylated LDL for 48 h (Figure 1). Some control cells (<10%) showed small lipid droplets. Subsequent experiments demonstrated this population of lipid droplet containing control cells persisted and that 24 h of exposure to 100 µg/ml acetylated LDL loaded the macrophages with lipid droplets as effectively as the 48 h exposures (Figure 1).

A concentration of 100 µg/ml acetylated LDL was used for subsequent experiments, because it showed more consistent loading than non-modified LDL. Even at 500 µg/ml and 48 h exposure times, the non-modified LDL loaded a small percentage of cultured macrophages (Figure 2). The loading of macrophages with acetylated LDL appeared to be independent of the whether LDL was delivered in BMM or DMEM+10%FBS (Figure 2). This independence from media type allowed for the combination of CSF-1 starvation and lipid loading in some experiments. Macrophages were also exposed to oxidized LDL, which showed levels of lipid droplet formation in between levels seen in cells exposed to acetylated and non-modified forms of LDL (data not shown).

Different lipid stains were tested and Oil Red O was found to be the most consistent, fastest, and cheapest method to visualize lipid droplets. The fixed cell stain, LipidTox, was much slower and dimmer than Oil Red O. The live cells-stain monodansylpentane (MDH) worked well to visualize lipid droplets in the DAPI channel and was also effective at high concentrations at staining fixed cells. Nile Red initially worked well as a live cell lipid droplet stain however its results have been inconsistent, possibly due to its storage (data not shown).

CSF-1R expressed more slowly, degradation at the same rate, and was less phosphorylated in LDL-exposed cells

Macrophages exposed to acetylated LDL have a decreased rate of CSF-1R expression compared to non-exposed macrophages (Figure 3). Average values for total stained CSF-1R remains higher in the control cells up until 24 h post supplemental CSF-1 removal. Looking only at surface CSF-1R, a clear trend is not apparent, however capturing surface CSF-1R can be fickle due to rapid internalization of receptor (Figure 4). After stimulation with CSF-1, the internalization and degradation CSF-1R appears to occur at the same rate in LDL exposed and non-LDL exposed macrophages (Figure 5). Phosphorylated CSF-1R was stained at lower concentrations in LDL exposed macrophages from when they came out of CSF-1 starvation media to the last time point recorded at half an hour after initial stimulation (Figure 6).

Phosphorylation of ERK fluctuates but appears similar between LDL exposed cells and non-LDL exposed

The staining of pERK after CSF-1 ligand addition showed two significantly different time points at 7 min and 10 min, indicating a spike in the activation of non-LDL exposed macrophages at 7 min that rapidly decreases by 10 min (Figure 7). Previous experiment done with pERK showed no visible difference between the LDL exposed and non-LDL exposed macrophages.

Phosphorylation of Akt remains similar between LDL exposed and non-exposed macrophages 3-60 min post CSF-1 but increases 10 h post CSF-1 exposure

The level and localization of phosphorylated Akt was investigated with a series of experiments. An initial CSF-1 stimulation time courses indicated that LDL treated macrophages had a higher level of nuclear phosphorylated Akt. However, this result was not duplicated in subsequent experiments. From repeated experiments it would appear that there is no difference in the level of cellular Akt (Figure 8). Though some individual

points are significantly different with Tukey's test they do not hold up in replicates and do not seem to follow an obvious pattern. If the nuclear masks are used to make to graph the intensity of pAkt there is little difference observed from that quantification of the cellular masks. Looking past the timeline of initial experiments the phosphorylated level of cellular Akt is higher in non-LDL exposed macrophages from for the tested 10 h to 26 h post CSF-1 stimulation (Figure 9).

Proliferation is increased in LDL-exposed macrophages 24 h post LDL removal, but LDL exposure does not affect long term proliferation

Ki67 was stained in macrophages after they had been exposed to a 24 h pulse LDL. One-day post removal of LDL the LDL exposed cells saw a significantly higher expression of Ki67. After the initial spike in Ki67 expression, there does not appear to be a significant difference in the expression of Ki67. From this Ki67 staining experiment with a pulse of LDL it is apparent the lipid droplet filled morphology of the macrophages disappears almost entirely two days after the removal of the LDL from the cells (Figure 10).

Flow cytometry of LDL exposed macrophages

Surface staining and flow cytometry was done to characterize the macrophages. The lipid stain Nile red capture the change in lipid content with the use of flow cytometry. LDL exposed cells expressed higher of CD68. While non-LDL exposed cells expressed higher levels of CD11B and CD80. Expression of MHCII and CD14 appears to be similar (Figure 11).

The rate of CSF-1 stimulated macropinocytosis is similar in LDL-exposed and control cells

Live cells were imaged to investigate if being laden with lipid droplets had an effect on internalization of dye from the media via macropinocytosis. Initial experiments indicated a decrease in the ability of cells to take up dextran in a resting state; however, this result was not duplicated in subsequent experiments. Both treated and control cells were observed macropinocytosis dextran at the same rate and trafficking macropinocytosis dye from the media to lysosomes in a similar time frame (Figure 12).

Discussion

CSF-1R signaling and degradation in lipid-laden macrophages is remarkably similar to non-LDL-exposed cells but CSF-1R expression and phosphorylation of CSF-1R appear to be different. Even subtle changes in CSF-1R may shed light on what is happening in lipid-laden macrophages as CSF-1 activates chemotaxis, macropinocytosis, survival, and proliferation (Pixley and Stanley, 2004). The expression rate of CSF-1R is slowed in lipid-laden macrophages indicating that they may not be as responsive to CSF-1 as non-exposed cells in a resting state. If the macrophages are deprived of the CSF-1 to upregulate CSF-1R and then stimulated with CSF-1, the receptor is degraded at the same rate. The degradation timeline for the receptor is significant as the receptor continues to phosphorylate Akt and ERK from the early endosome (Huynh et al., 2012). The same rate of receptor degradation between LDL-exposed and non-exposed macrophages indicates the time CSF-1R spends from CSF-1 binding to degradation is not altered by the presence of lipid droplets in the cell.

Although the degradation of CSF-1R appears to be the same and the amount of the pCSF-1R level in LDL exposed cells is lower. This is true for phosphorylated CSF-1R at Y723 throughout the observed 30 minutes after stimulation. There is not a clear explanation as to why there is a higher level of phosphorylated CSF-1R. CSF-1R expression in other experiments with the same experimental design appear to be similar indicating the increase in phosphorylated CSF-1R is not simply due to more CSF-1.

While the phosphorylation of CSF-1R after CSF-1 stimulation is depressed in LDL exposed cells the, phosphorylation of Akt at S473 is not different in the first hour but increases 10-26 h post CSF-1 exposure. The S473 phosphorylation of Akt is required for the maximum activation of the serine/threonine Kinase (Hart and Vogt, 2011). Ten hours past the initial signaling that occurs at CSF-1 addition, the level of cellular pAkt is increased in non-LDL exposed cells. The decreased level of pAkt in exposed cells could indicate that they are less prone to proliferation and more prone to apoptosis (Hart and Vogt, 2011).

ERK is another significant signaling kinase how's phosphorylation was at quantified at the Thr202/Tyr204 site. The spike in the phosphorylation of ERK in the non-LDL exposed cells at 7 min demonstrated that ERK'S phosphorylation is more sensitive to CSF-1 in these cells. Based solely on this result it would appear that lipid droplets suppress ERK phosphorylation which would lead to decreased cell proliferation.

Ki67 staining indicated a spike in macrophage proliferation after removal of LDL that quickly rejoined the non-LDL exposed cells. It does not appear that a 24 h pulse of LDL has an effect on the long term proliferation of the macrophages, but having lipid droplets increases proliferation with fresh CSF-1 addition. Ki67 is a protein found in cells

as they are actively growing but disappears when the cells enter a resting state (Scholzen and Gerdes, 2000). As Ki67 is only present in proliferating cells we can use it to determine what portion of the population is proliferating. Besides the Ki67 staining it is apparent from the lipid staining that the cells are able to clear the lipid droplets within 48 h of LDL removal. From this experiment we are unable to tell if the disappearance of lipid laden macrophages is due to clearance of the lipid droplets from the macrophages or the death of the macrophages themselves. In future experiments it would be interesting to look at continuous LDL exposure throughout the course of the experiment rather than removing LDL from the culture medium at the start of the experiment.

Looking at the expression of different surface markers can give us insight into the activation of our LDL exposed and non-exposed macrophages. Through the use of Nile Red the increased lipid content of the LDL-exposed cells is confirmed, as Nile Red is an effective lipid stain for use in flow cytometry (Greenspan et al., 1985). CD11b is a commonly used marker that is found on all cells of myeloid lineage (Murray and Wynn, 2011). The integrin CD11b forms a heterodimer with CD18 forming complement receptor 3 (CR3, MAC-1) (Wagner et al., 2001). On macrophages active CD11b is associated with an inhibitory signal to T-cells, making it appear that the LDL-exposed cells are less inhibitory to T-cell than the non-exposed cells (Hume, 2008). CD68 is a scavenger receptor commonly used to identify macrophages as it is expressed by all macrophages. LDL exposed macrophages have higher expression of CD68 but lower expression of CD11b and CD80. CD80 is a costimulatory receptor needed for T-cell activation. The expression of MHCII and CD14 does not appear to be different between treated and non-treated macrophages.

Macropinocytosis is a cellular function stimulated by CSF-1 (Pixley and Stanley, 2004). Macropinocytosis does not appear to differ between LDL-exposed and non-LDL exposed cells. This similarity appears to hold true for stimulated macropinocytosis, unstimulated macropinocytosis, and the traffic of macropinocytosis dye. It does not appear that being full of lipid droplets limits macropinocytosis in macrophages. This is in contrast phagocytosis where macrophages are limited in the number of particles that they can phagocytose due the limited membrane (Cannon and Swanson, 1992). It is possible that this difference occurs due in part to the lipid droplets being monolayer and the cell having time to synthesis more membrane.

In conclusion, lipid-laden macrophages have some signaling differences that may lead to successful future treatments. Research in treatment strategies has begun to shift from only decreasing LDL and to keeping macrophages from entering and proliferating in plaques. By knowing which pathways are active in lipid laden macrophages and how these cells respond to growth factors we can make intelligent choices on how to target and influence them.

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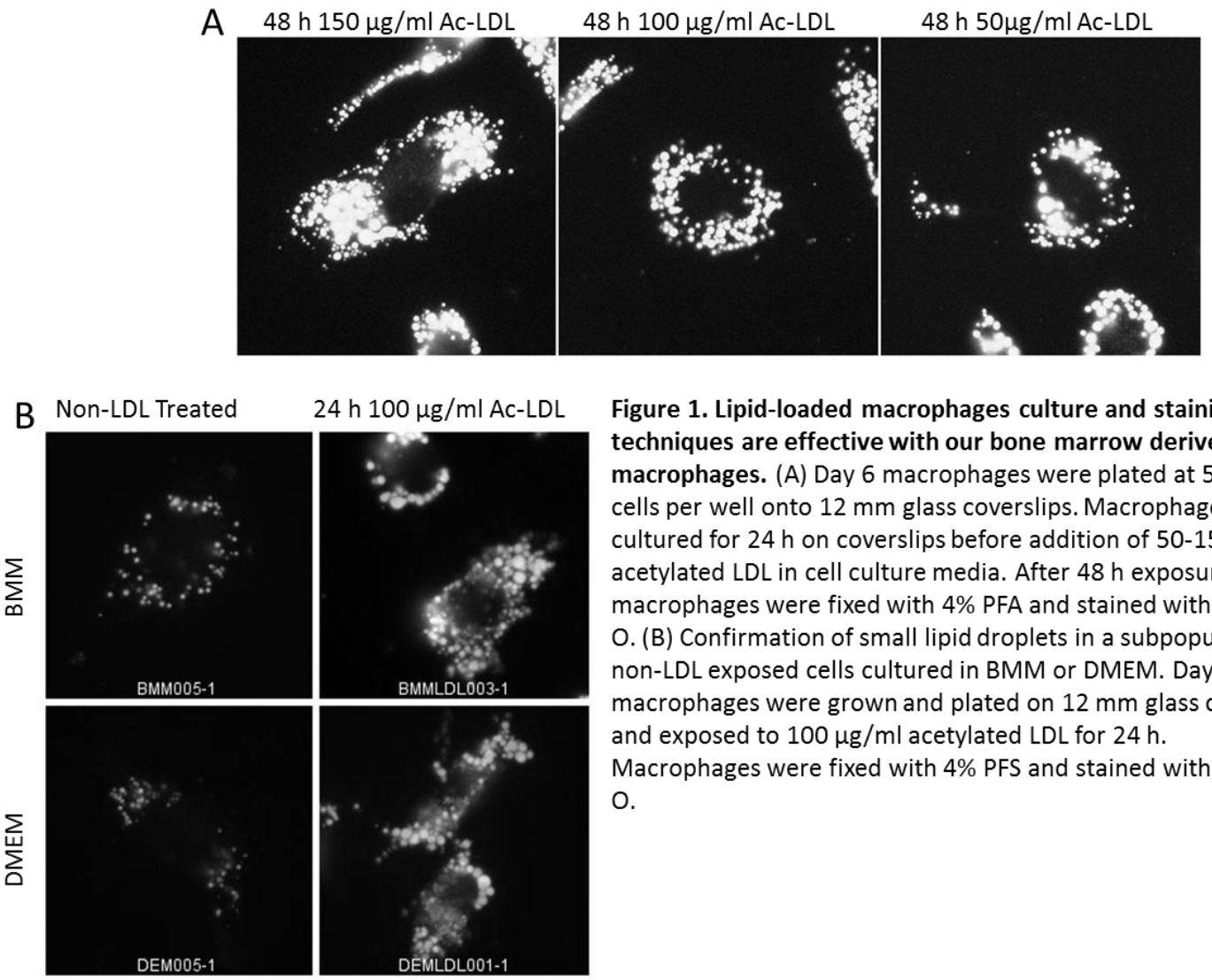


Figure 1. Lipid-loaded macrophages culture and staining techniques are effective with our bone marrow derived mouse macrophages. (A) Day 6 macrophages were plated at 50,000 cells per well onto 12 mm glass coverslips. Macrophages were cultured for 24 h on coverslips before addition of 50-150 µg/ml acetylated LDL in cell culture media. After 48 h exposure to LDL, macrophages were fixed with 4% PFA and stained with Oil Red O. (B) Confirmation of small lipid droplets in a subpopulation of non-LDL exposed cells cultured in BMM or DMEM. Day 6 macrophages were grown and plated on 12 mm glass coverslips, and exposed to 100 µg/ml acetylated LDL for 24 h. Macrophages were fixed with 4% PFS and stained with Oil Red O.

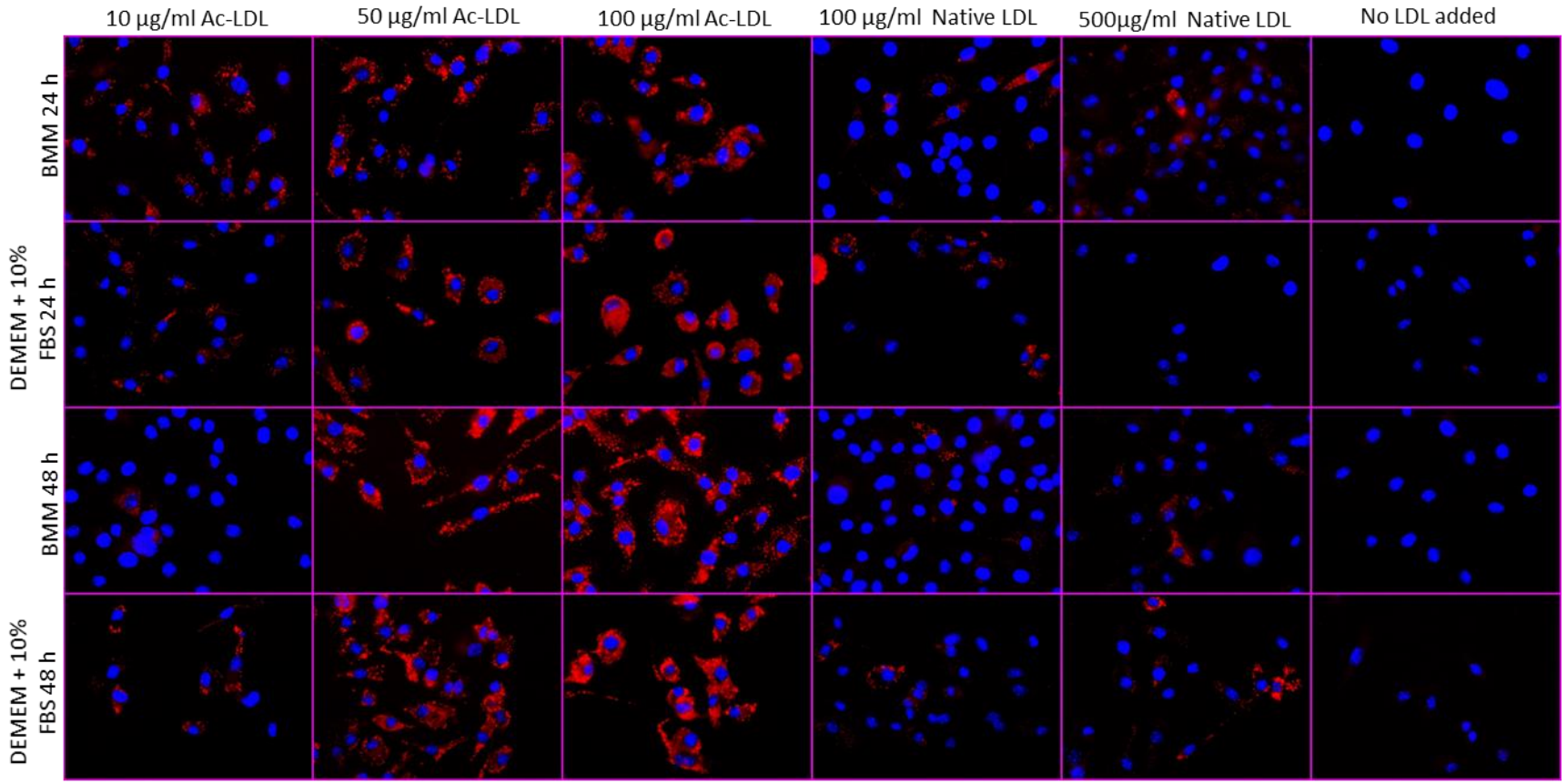
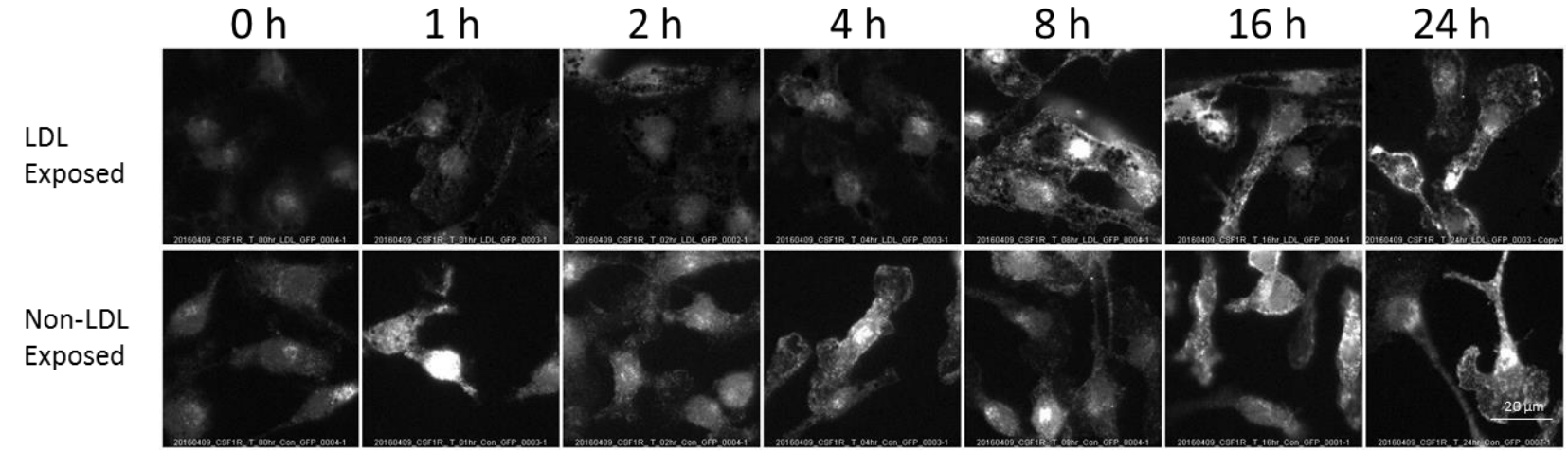


Figure 2. Macrophages responded to acylated LDL in a dose dependent manner. Neutral lipids increased with increasing Ac-LDL exposure with optimum loading at 100 µg/ml Acylated LDL at 24 hrs. Even high concentrations of native LDL (500 ug/ml) did not effectively load macrophages. Day 5 macrophages were plated at 50,000 cells per well in a 24 well plate onto 12 mm glass coverslips, and allowed to attach overnight. LDL was added in the conditions indicated above. Cells were fixed with 4% PFA for 10 min, permeabilized with Trition X-100 for 30 min, and stained with DAPI (nucleus) and Oil Red O (neutral lipid).



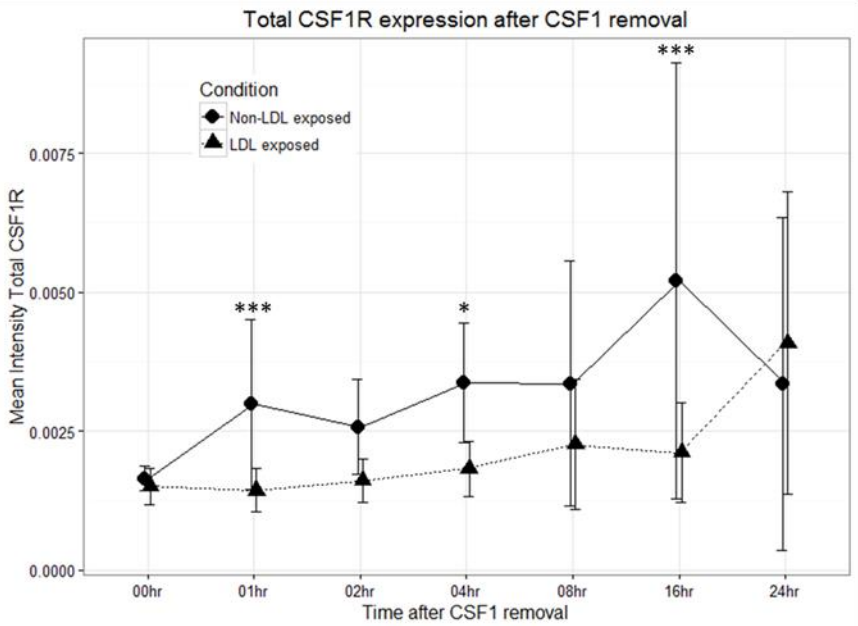
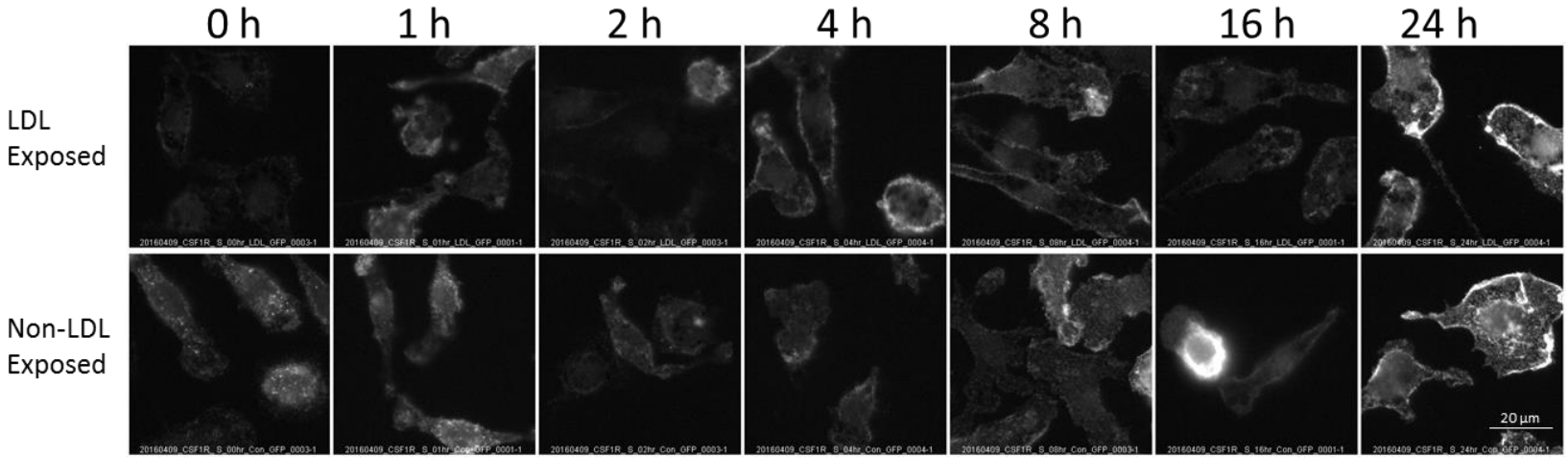


Figure 3. CSF-1R production is slowed in LDL exposed macrophages after CSF-1 ligand removal. Day 4 bone marrow macrophages were cultured in BMM for 24 h before the addition of 100 µg/ml acylated LDL in BMM overnight. BMM and LDL were removed and cells were cultured in CSF-1 free media (DMEM + 10% FBS) for the indicated times. Cells were fixed with 4% PFA for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 30 min and immunostained with anti-CSF1R (AFS98 clone), Oil Red O for lipid droplets, phalloidin for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean CSF-1R intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



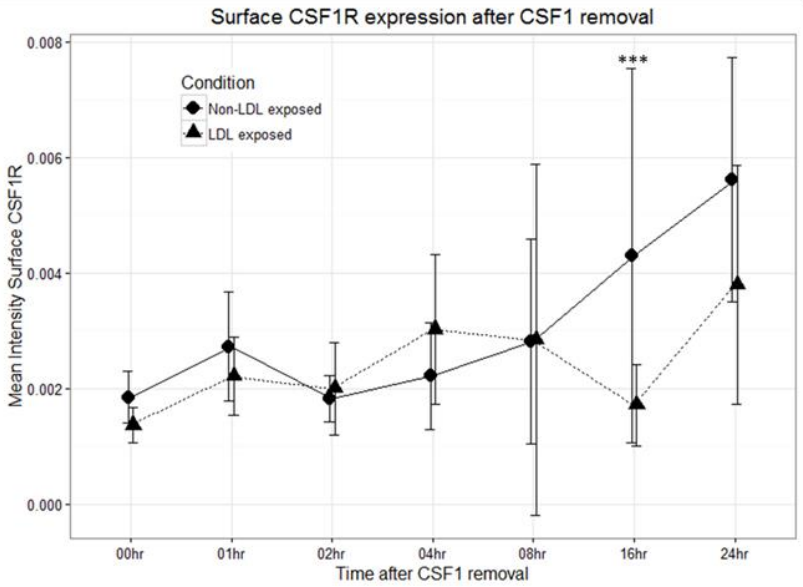
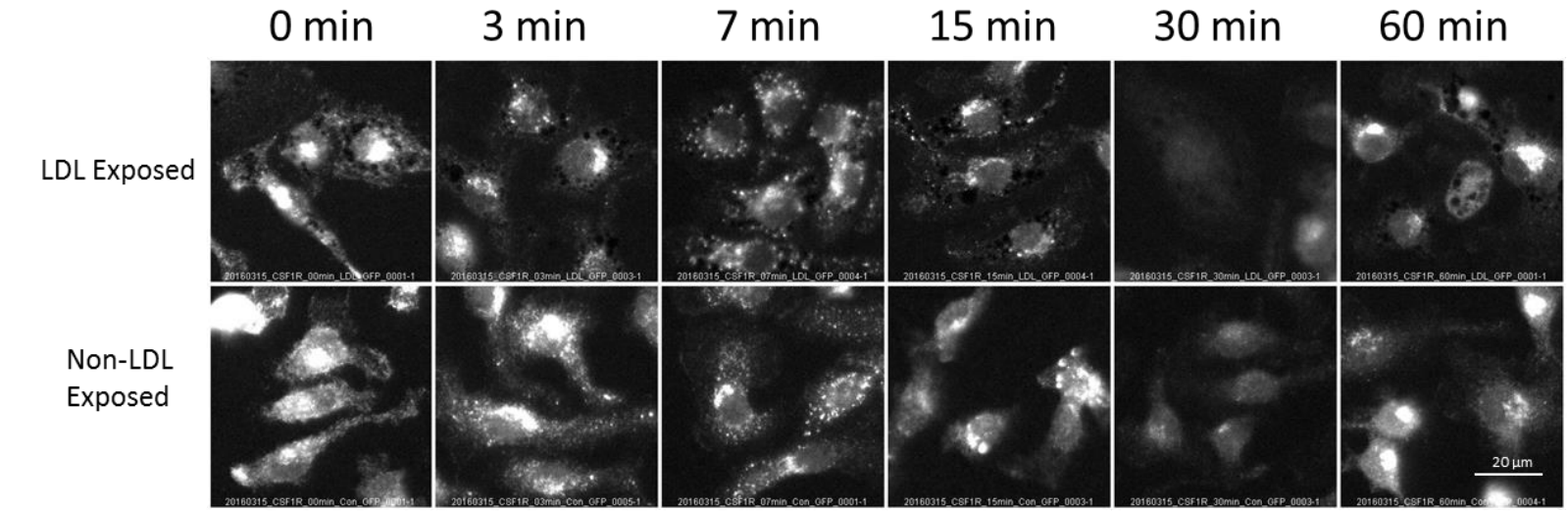


Figure 4. Surface expression of CSF-1R is slowed in LDL-exposed macrophages after CSF-1 ligand removal. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) were added to the plate for the times indicated. Cells were fixed with 4% PFA for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 30 min and immunostained with anti-CSF1R (AFS98 clone), Oil Red O for lipid droplets, phalloidin for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean CSF-1R intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



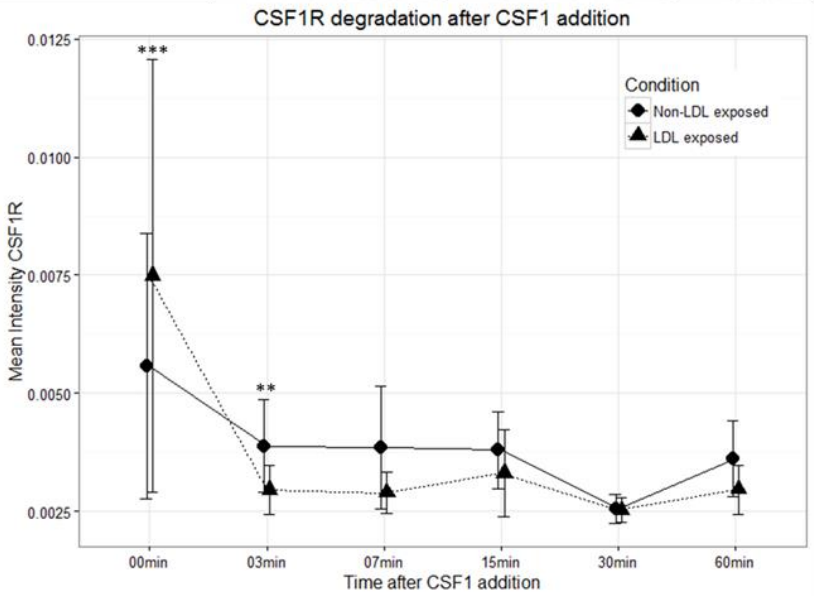
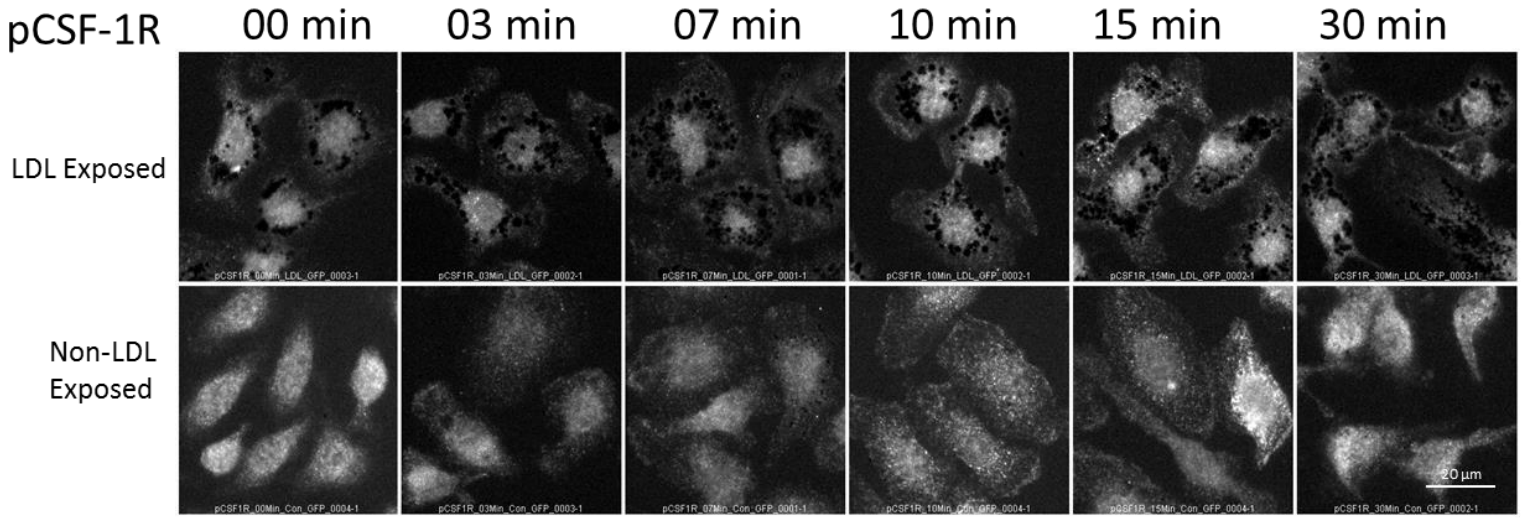


Figure 5. Internalization and degradation of CSF-1R is the same with LDL exposure and non-LDL exposed macrophages. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) was added for another 24 h. CSF-1 free media was removed and LCIB with CSF-1 was added for indicated time before fixation with 4% PFA for 10 min and permeabilized with Triton X-100. Cells were immunostained with anti-CSF1R (AFS98 clone), Oil Red O (neutral lipids), phalloidin (f-actin) for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean CSF-1R intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



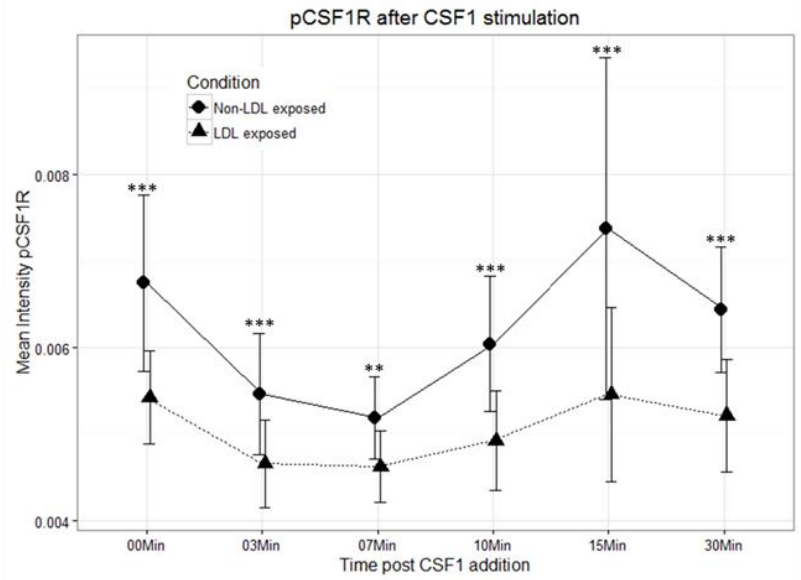
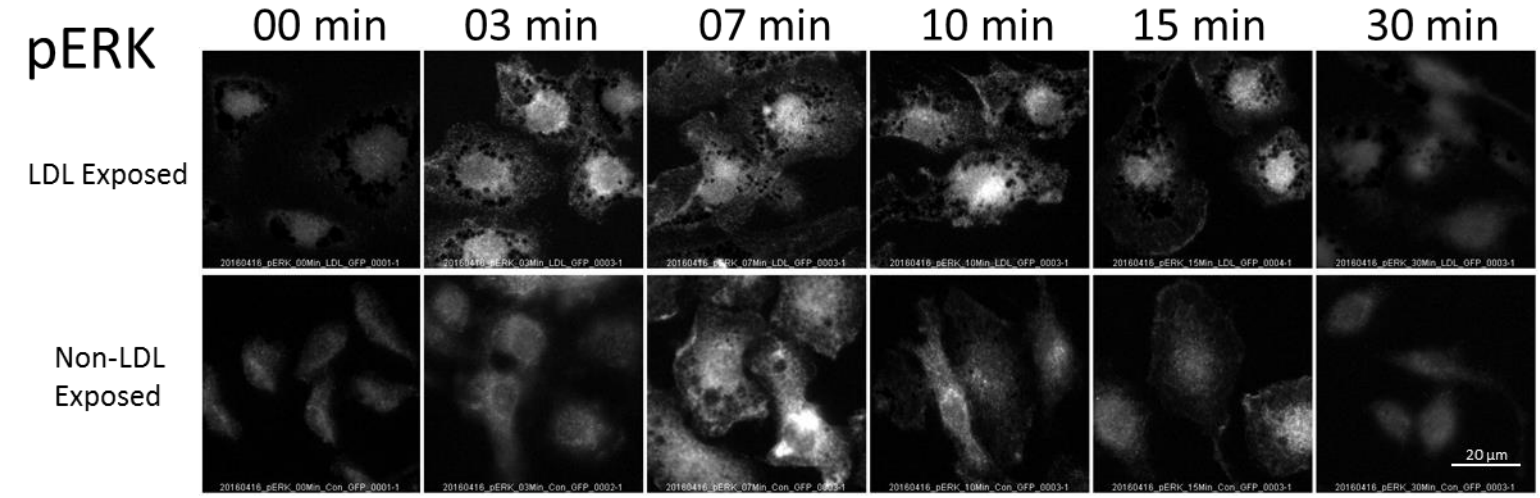


Figure 6. Phosphorylation of CSF-1R is decreased in LDL exposed macrophages. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) was added for another 24 h. CSF-1 free media was removed and LCIB with CSF-1 was added for indicated time before fixation with 4% PFA for 10 min and permeabilized with Triton X-100. Cells were immunostained with anti-pCSF1R (49C10 clone), Oil Red O (neutral lipids), phalloidin (f-actin) for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean pCSF-1R intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



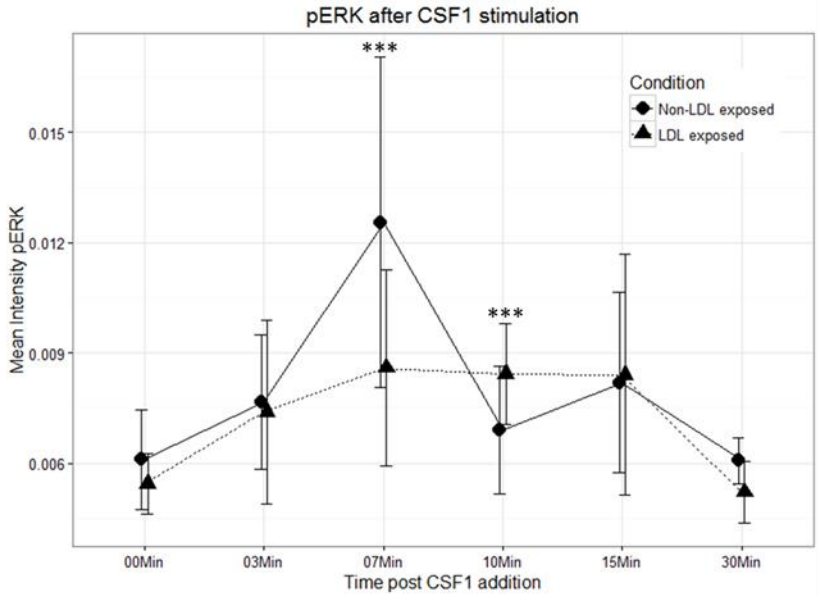
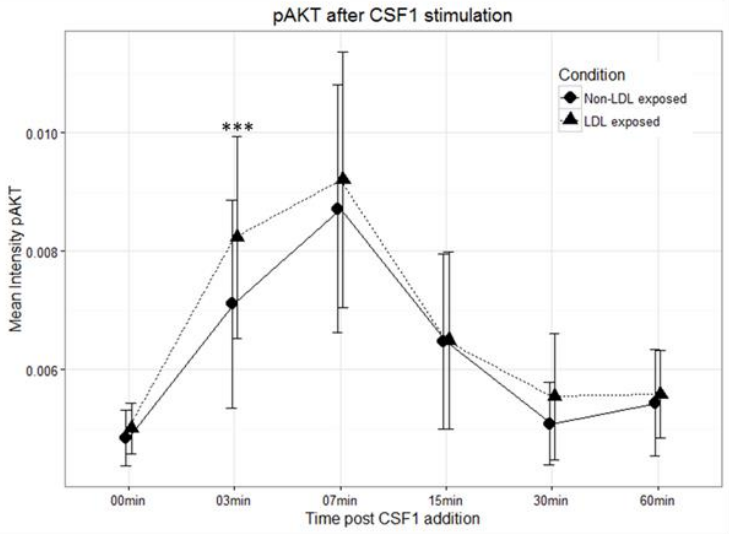
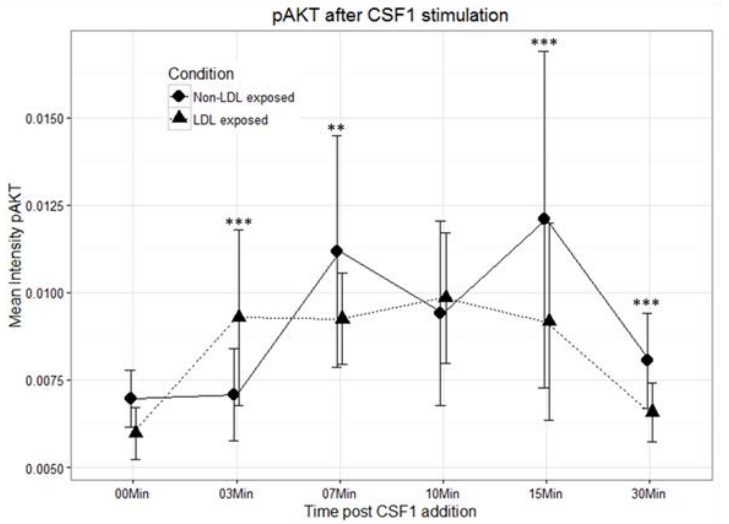


Figure 7. Phosphorylation of ERK fluctuates but appears similar with LDL exposed cells. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) was added for another 24 h. CSF-1 free media was removed and LCIB with CSF-1 was added for indicated time before fixation with 4% PFA for 10 min and permeabilized with Triton X-100. Cells were immunostained with anti-pERK (D13.14.4E), Oil Red O (neutral lipids), phalloidin (f-actin) for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean pERK intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$





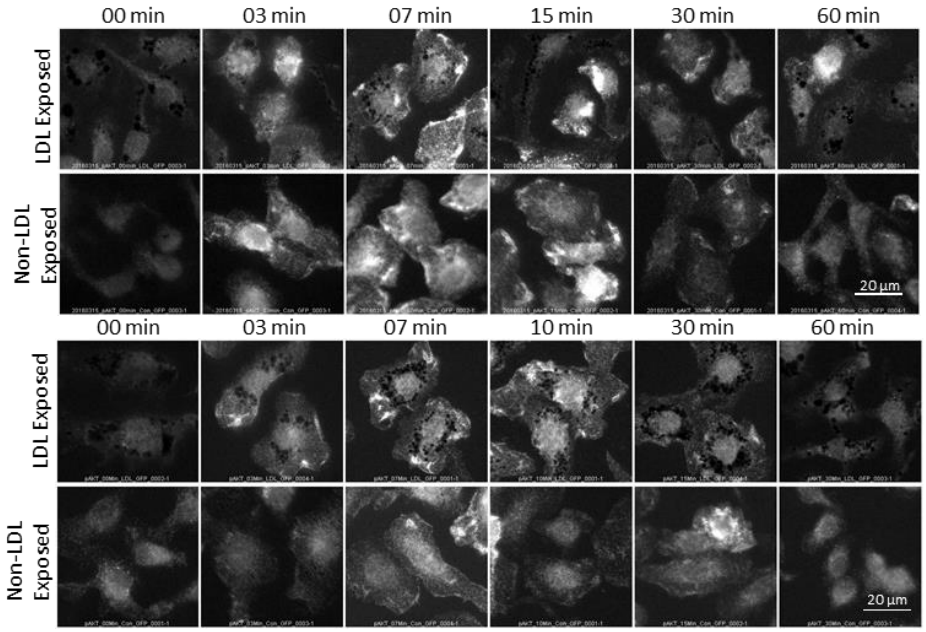
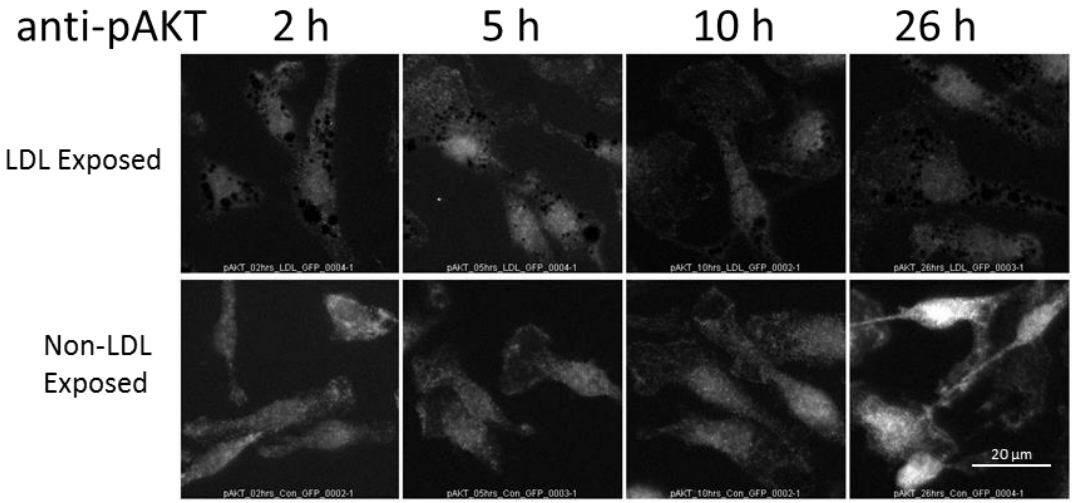


Figure 8. Phosphorylation of AKT remains similar between LDL exposed and non-exposed macrophages 3-60 min post CSF-1 stimulation. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) was added for another 24 h. CSF-1 free media was removed and LCIB with CSF-1 was added for indicated time before fixation with 4% PFA for 10 min and permeabilized with Triton X-100. Cells were immunostained with anti-pAkt (D9E clone), Oil Red O (neutral lipids), phalloidin (f-actin) for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean pAkt intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



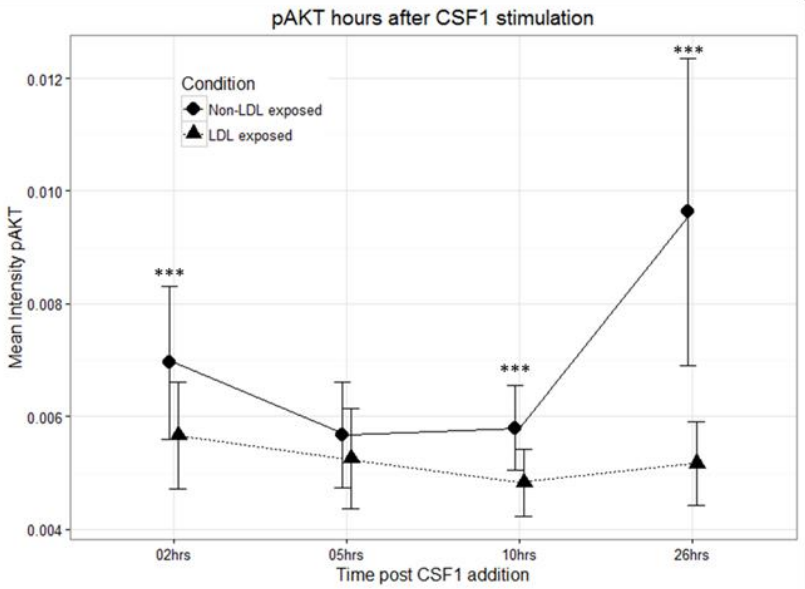
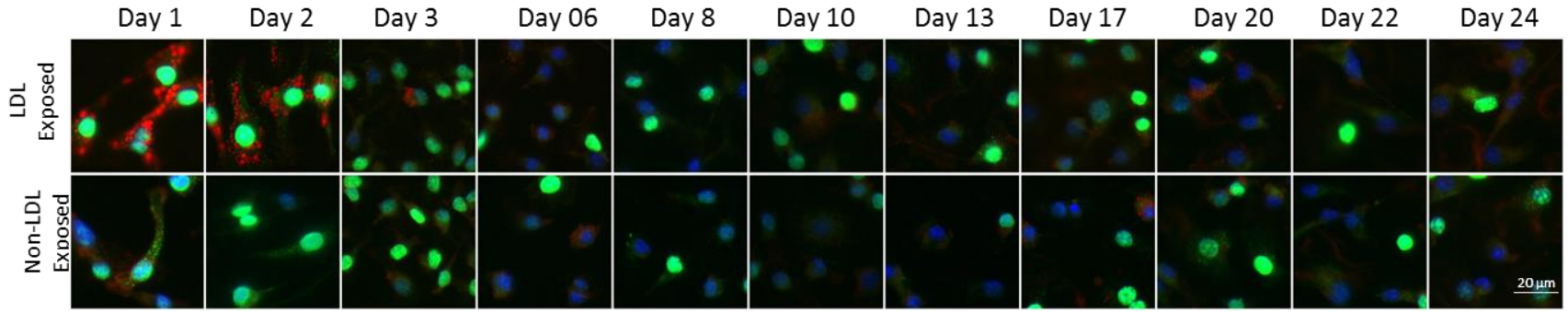


Figure 9. LDL exposed macrophages had decreased Akt phosphorylation 10-26 h post CSF-1 stimulation. Day 5 macrophages were exposed to 100 µg/ml acylated LDL in BMM for 24 h. BMM and LDL were removed and CSF-1-free media (DMEM + 10% FBS) was added for another 24 h. CSF-1 free media was removed and DMEM + 10% FBS with CSF-1 was added for indicated time before fixation with 4% PFA for 10 min and permeabilized with Triton X-100. Cells were immunostained with anti-pAkt (D9E clone), Oil Red O (neutral lipids), phalloidin (f-actin) for cells masking, and NucBlue. Mean intensity values were calculated with cell masks made with phalloidin and NucBlue in CellProfiler. Mean pAkt intensity was graphed (left) and error bars are standard deviation. '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$



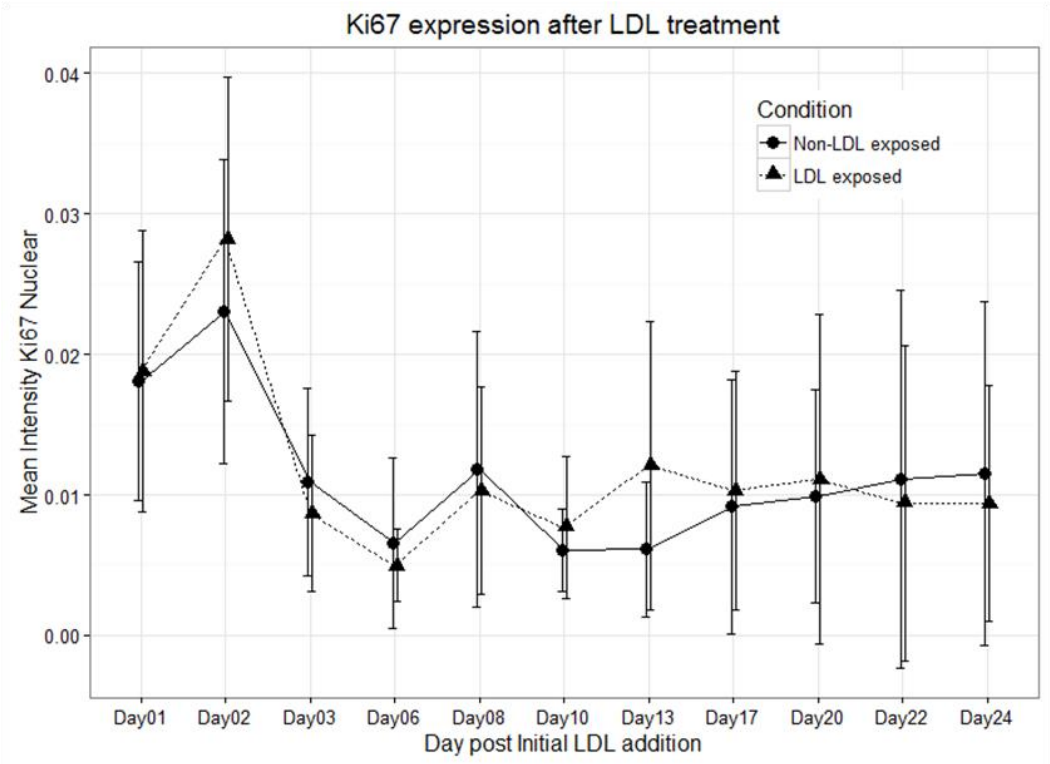


Figure 10. Proliferation is increased in LDL-exposed macrophages 24 h post LDL removal, but LDL exposure does not affect long term proliferation. Day 5 bone marrow macrophages plated onto 12mm glass coverslips in 24 well plates. Cells grew 24 h in BMM before the addition of 100 µg/ml acylated LDL in BMM overnight. BMM and LDL were removed and fresh BMM added on "Day 1". BMM was replaced every two days. Cells were permeabilized with Triton X-100 and stained with the SP6 Clone for Ki67, Oil Red O for lipid droplets, Phalloidin for cells masking, and NucBlue. Mean intensity values were calculated nuclear masks in CellProfiler.

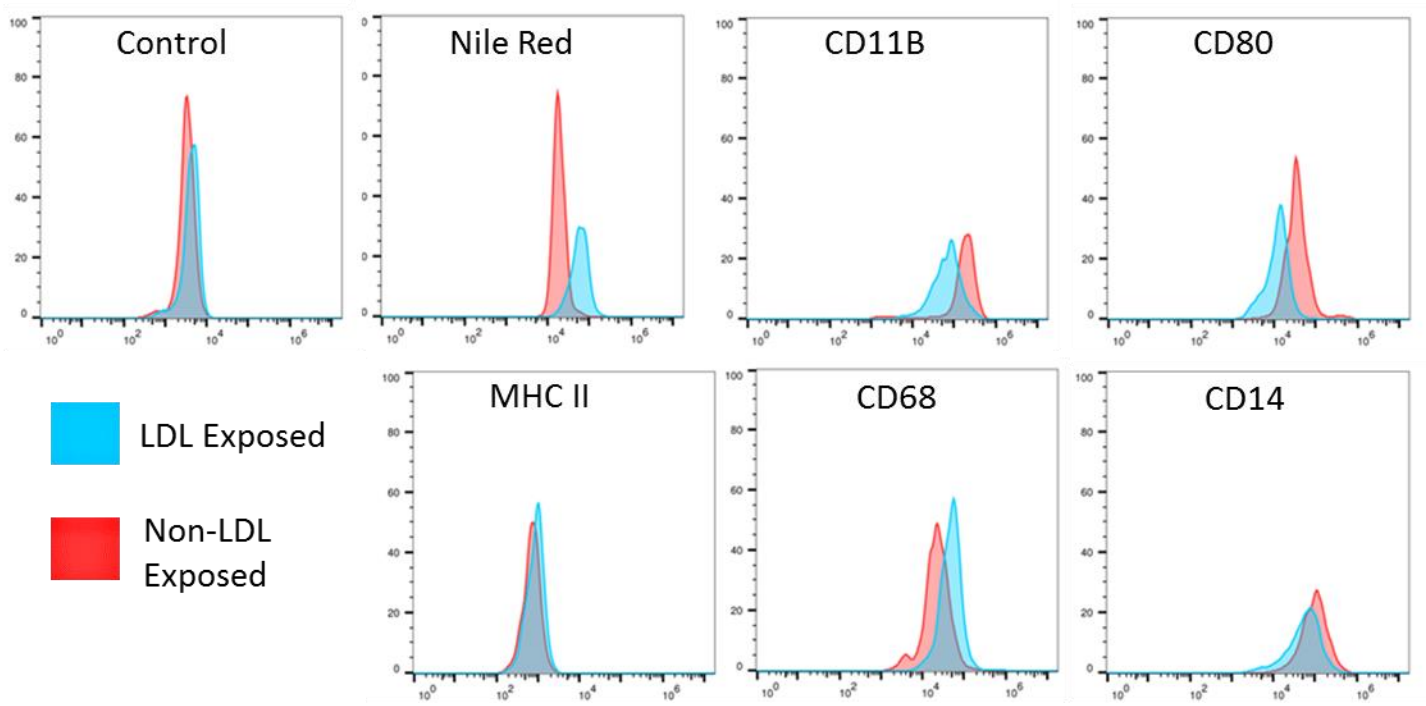


Figure 11. LDL exposed macrophages express some macrophage surface markers differently than non-LDL exposed cells. Day 4 bone marrow macrophages were taken from liquid nitrogen and plated onto a 10 cm petri dish. Macrophages were cultured for 24 h in BMM before the addition of 100 µg/ml acylated LDL in BMM for 24 h. Macrophages were detached from the plates with cold DPBS –Ca –Mg, fixed with 4% PFA for 10 minutes and stained with the respective antibody.

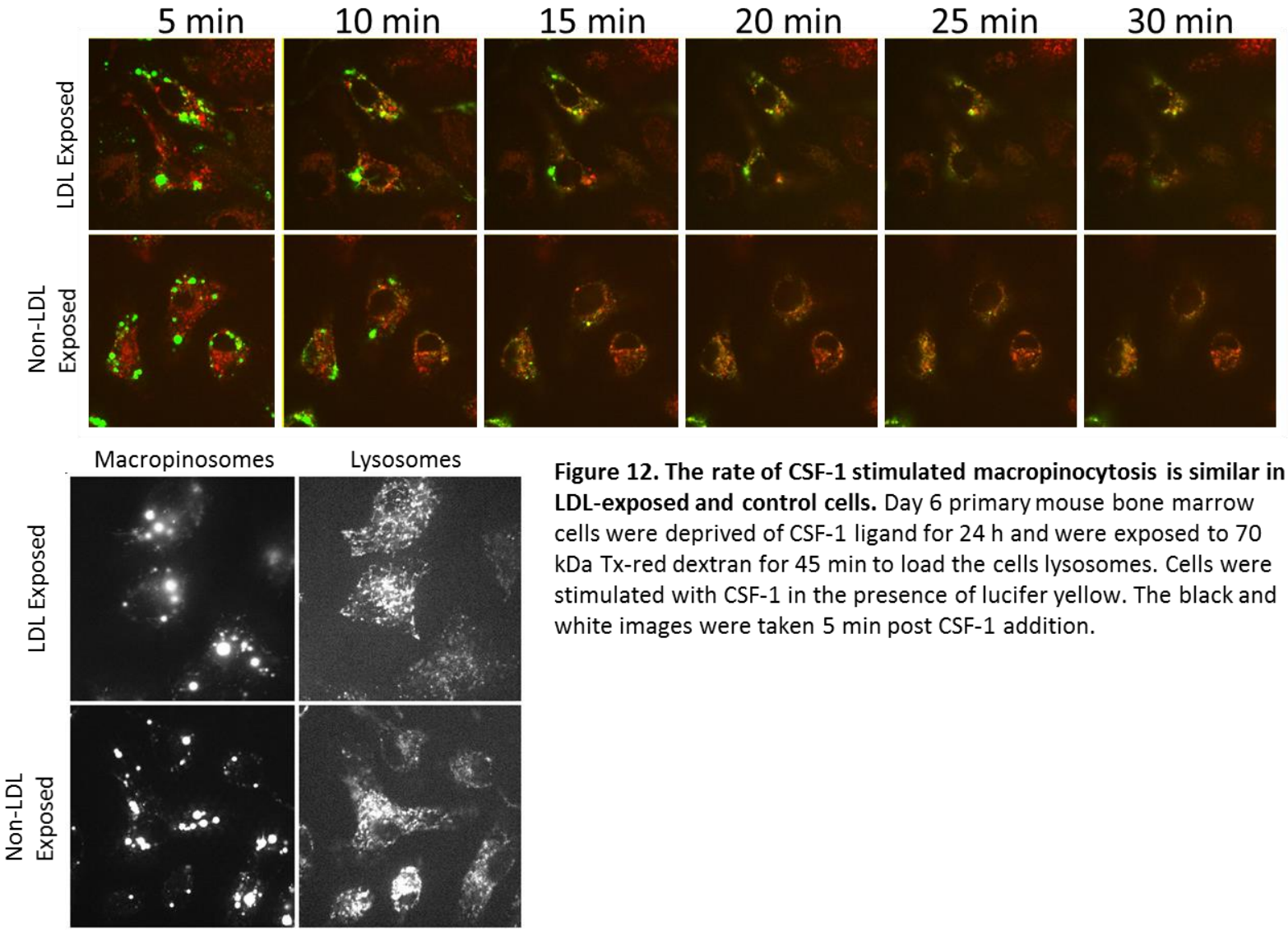


Figure 12. The rate of CSF-1 stimulated macropinocytosis is similar in LDL-exposed and control cells. Day 6 primary mouse bone marrow cells were deprived of CSF-1 ligand for 24 h and were exposed to 70 kDa Tx-red dextran for 45 min to load the cells lysosomes. Cells were stimulated with CSF-1 in the presence of lucifer yellow. The black and white images were taken 5 min post CSF-1 addition.

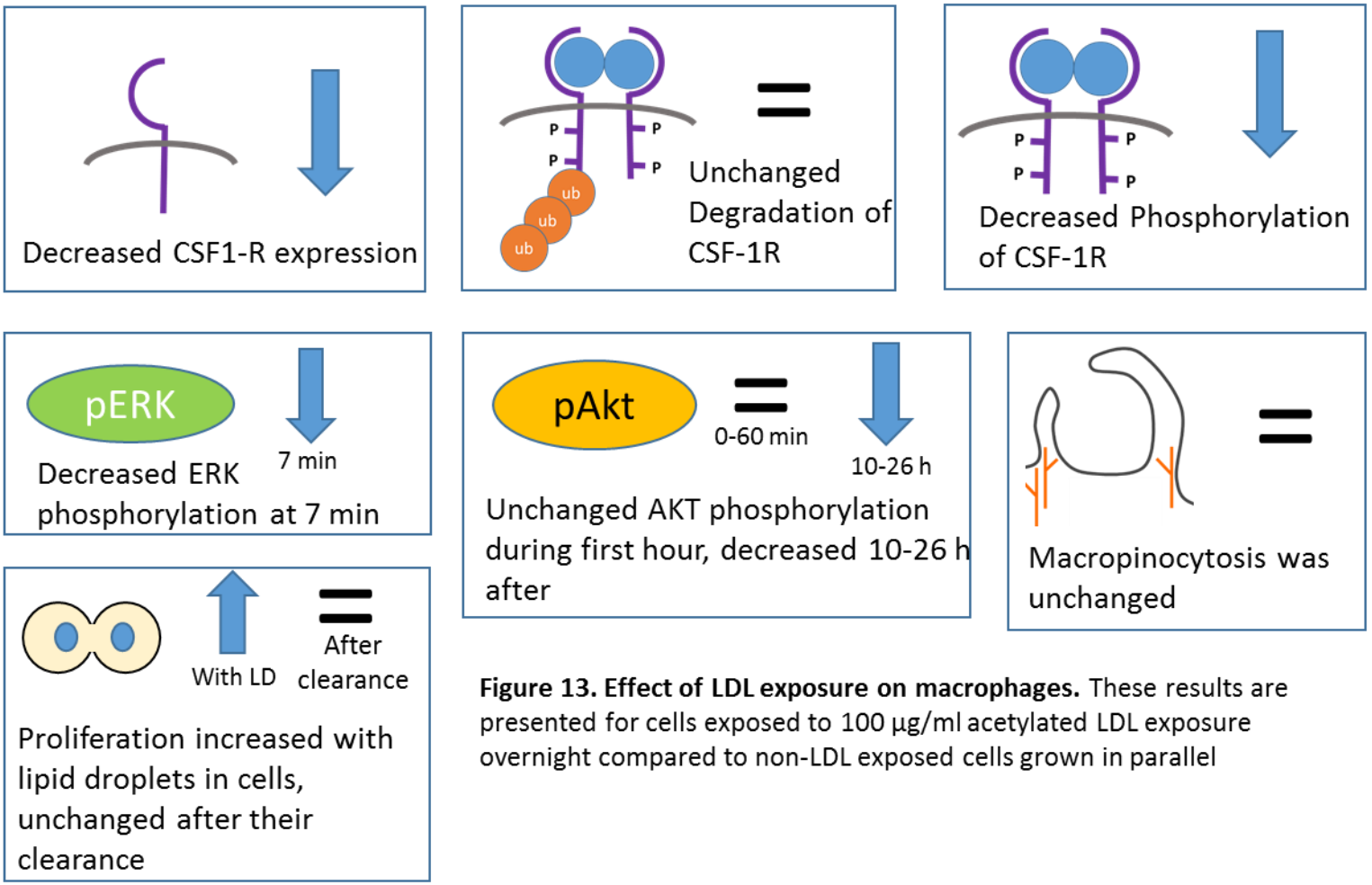


Figure 13. Effect of LDL exposure on macrophages. These results are presented for cells exposed to 100 µg/ml acetylated LDL exposure overnight compared to non-LDL exposed cells grown in parallel

Chapter 3

Conclusions

The goal of this project was to apply our laboratory expertise in macrophage cell biology toward understanding CSF-1R signaling and trafficking in lipid-laden macrophages. This project was a new emphasis for the laboratory, and I established protocols for culturing lipid-laden macrophages and staining lipid droplets. Further, I developed pipelines in CellProfiler and wrote R code for statistics and graphing to quantify imaging data.

I spent the bulk of my time developing methods to quantify immunofluorescent images of cell signaling, for example, phosphorylated Akt and phosphorylated ERK. These methods utilized four-color fluorescent images of which two colors were used to mask cells and nuclei, a third color was used to quantify a protein of interest and the fourth color was used to stain lipid droplets. In this approach both DAPI and phalloidin were used to create the cellular mask producing much more consistent masks and reproducible quantification of the fluorescence associated with the protein of interest across experimental conditions. Moving forward, it would be worth looking into the effects of focal plane on imaging results as the person imaging may shift planes slightly under different experimental conditions.

The current experimental protocols have been successful, but going forward a few protocol changes would streamline the process and provide better protein quantification. Current protocols utilize macrophages grown on glass coverslips that are fixed, stained, and imaged manually. Though growing the macrophages on glass is a great approach for

high quality images, it is slow and labor intensive. If only total or nuclear localization is of interest, then lower quality images would be sufficient. High content microscopy done in 96 well plates fills this niche, quickly providing a large number of mediocre images. Using 96 well plates with automated imaging has the advantage of:

- faster more consistent image acquisition,
- removal of potential bias from person taking images, and
- lower volumes of antibodies and cells needed for each experiment and condition,

Utilizing 96-well plates with automated imaging compared to glass coverslips and manual imaging has the shortcomings of:

- a steeper learning curve to set up experiments and optimize protocols,
- diminished picture quality with plastic and air lenses,
- "junk" in images that would not have been imaged by a user, and
- issues with uneven distribution of cells within the well (concentrated to the edges of the well).

If the lab decides that high content microscopy is going to be a major focus in future experiments, it may be beneficial to integrate robotic pipetting. The current high content microscopy system has a fluidics component that could be used to accurately reproduce time points, while at the same time keeping plates at a constant temperature and pH. A standalone pipetting robot with multiple pipette heads and wash capabilities could be beneficial, if a large volume of experiments were being conducted. Utilizing

such a robot would help to increase reproducibly and staining across all of the wells and with all users, while not using time on the high content microscope.

By incorporating use of the imaging software, CellProfiler, into the laboratory, I have turned the qualitative assessment of signaling in immunofluorescent images into a quantitative analysis (Carpenter et al., 2006). CellProfiler provides a user-friendly interface that has the flexibility needed to capture the information of interest from a microscopy image. When analyzing images with CellProfiler, the user constructs a pipeline to extract data from the images. This pipeline does not modify the images themselves and shows all of the steps that were used to collect the data. The data exported from CellProfiler is in long format that is compatible with graphing and statistical analysis using the open source statistics package R. Through the combination of the CellProfiler pipeline and an R script anyone can see the steps used and reproduce the same analysis from the raw images. This is hugely powerful for reproducibility and transparency.

Other methods besides the analysis of immunofluorescence images should be used to support and confirm findings. Flow cytometry could be used for markers such as Ki67 but would not be as effective for time sensitive protein phosphorylation as the cells must be detached from the plate. Western blots are the gold standard for quantifying proteins and post transcriptional changes, and any substantial claims from IF analysis will need to be backed by western blots (Mahmood and Yang, 2012). Western blots do require large numbers of cells and antibodies and would not be able to detect heterogeneous expression in the cell population.

The main focus of my experiments was to observe the effect of CSF-1 on macrophages stimulation. Macrophages were removed from CSF-1 supplemented media so that the CSF-1R signaling and traffic would be synchronized within the cell to the addition of CSF-1. In looking at this synchronized signaling pAkt, pERK, and the degradation of CSF-1R appeared the same while the phosphorylation of CSF-1R was decreased in LDL exposed cells. The difference in CSF-1R phosphorylation is rather curious and begs the question as to why CSF-1 phosphorylation is decreased and what the signaling outcomes are downstream of this.

Looking farther downstream from the initial CSF-1 stimulation, phosphorylation in LDL-treated macrophages appear to be much different. The phosphorylation of Akt was the only signaling molecule investigated hours after an initial CSF-1 stimulation; but, it is significantly lower in LDL exposed cells. Other signaling molecules such as PI3K would be worth investigating as basal levels of phosphorylation in signaling cascades appear to be much different from the synchronized stimulated response.

Macrophages in this thesis were primarily loaded with acetylated LDL. Acetylated LDL does an excellent job of forming lipid-laden macrophages; however, it is not found in vivo and there is some dispute how its metabolism in the cell differs. Compared to acetylated LDL the oxidized components can impair LDL degradation and stimulate inflammatory phenotype (Wang et al., 2007). It may be worth transitioning to utilizing oxidized of even non-modified LDL for experiments to keep the experimental model as close to in vivo conditions as possible. It is also important to keep native LDL from becoming oxidized which would involve controlling exposure to oxygen. It may be

worth establishing a protocol for the isolation of LDL to cut cost and supply the lab for experiments that utilize high concentrations over long periods of time.

One interesting observation from the Ki67 experiments was the disappearance of lipid-laden macrophages two days after removal of the LDL containing media. It is not exactly clear whether these cell died or if they effluxed the cholesterol. It would be possible to determine what happened to these cells by observing the same field of view overtime. Regardless of what happened to the macrophages, the swift disappearance of the lipid-laden phenotype indicated that it may be important to keep the loaded macrophages exposed to LDL for the entirety of an experiment, especially if that experiment is hours or days long.

The effect of lipid droplets on macrophage biology appears to offer many opportunities for discovery that the lab can investigate with the protocols I implemented and in directions that my experiments have indicated. CSF-1R signaling pathways appear similar when synchronized but appears to be different in LDL-exposed macrophages growth media. In the lab there are some exciting opportunities to further develop protocols for high content microscopy for investigating the timing and amplitude of different signaling events.

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