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Identification of Kinase Signaling Pathways Inducing Morphological Changes in Dendritic Cells upon Leptin Treatment

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IDENTIFICATION OF KINASE SIGNALING PATHWAYS INDUCING MORPHOLOGICAL CHANGES IN DENDRITIC CELLS UPON LEPTIN TREATMENT

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Dedication

This Thesis is dedicated to my mother, Marisol Wesoloski, and all of my past lab mates.

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By

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Abstract

Leptin is a pleiotropic hormone secreted by white adipose tissue. Obesity leads to overexpression of leptin as a result of leptin receptor desensitization. Obese leptin serum levels (160ng/ml) have been found to enhance immune functions in macrophages, natural killer cells, B cells, T cells, and dendritic cells. Previous data from our lab suggests that obese concentrations of leptin cause morphological changes in DCs, although further studies are required to verify these results. We hypothesized that leptin promotes DC cytoskeletal changes though activation of PI3K signaling. Morphological and functional changes were evaluated in JAWS II immortalized DCs though microscopy, transwell migration, assessment of cytokine production, and T cell activation. The results obtained, however, did not correlate with those observed in previous studies using primary DCs. The lack of changes in JAWS cells upon leptin treatment was accompanied by a lack of change in PI3K signaling.

Further studies were conducted to evaluate PI3K signaling and morphological changes in primary DCs upon leptin treatment. Primary DCs from Balb/c mice were observed by light and confocal microscopy. Analysis of cell morphology by light microscopy did not show any significant changes in cell morphology upon leptin treatment. There were, however, morphological changes observed by confocal microscopy in expression and localization of F-actin. Treating cells with Leptin and LPS leads to an increase in F-actin staining compared to media and LPS or leptin alone. Furthermore this increase was lost when cells were treated with a PI3K inhibitor and F-actin levels reverted to those observed in LPS treated cells. F-actin was also found to localize to the borders of cells treated with LPS, leptin, or Leptin and LPS with a high amount of actin visible in dendrites of L+L treated cells.

We proposed that the changes observed in DC morphology were being induced by increased signaling in the PI3K pathway. Leptin has been found to activate PI3K, a pathway important for cell viability and growth. PI3K is able to signal through Rac1, leading to F-actin polymerization. PI3K signaling was assessed by ELISA and flow cytometry for the phosphorylation of Akt, but there was not a significant change in either total Akt expressed or phosphorylation of Akt.

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Chapter 1: Introduction

1.1 Obesity

Obesity describes a weight range that consists of high body fat and is associated with several health issues. Obesity is most often caused by a caloric intake higher than what is required to perform one's daily activities (WHO). Obesity can be defined as having a body mass index (BMI) of 30 or higher with a BMI being the relation between one's height and weight: BMI= Kg/M^2. The prevalence of obesity in both adults and children has increased significantly in recent years. As of 2012, 149.3 million Americans were ranked as overweight or obese and 1 in 6 American children were ranked as obese (55). This is of particular concern due to the many health problems associated with obesity and the high cost of their treatment. Obesity related morbidity and mortality rates cost an estimated 147 billion dollars in the US each year (51). Some of the health risks associated with obesity include coronary heart disease, Type-2 diabetes, osteoarthritis, breast, colon, and endometrial cancers, hypertension, stroke, abnormal menses and infertility, and respiratory problems (55). The increase in childhood obesity has led to many of these adult diseases to be observed in children (51).

The many health problems associated with obesity have led to an increase in obesity related research; a large amount of that has been interest in obese immune function. Adipose tissue has recently been categorized as an endocrine organ due to its ability to secrete peptide hormones and bioactive molecules capable of signaling in auto-, para-, and endocrine fashions (52). Many of these signaling molecules have been identified as adipokines. Adipokines are adipose produced hormones with the ability to act as cytokines. Among these adipokines are leptin, which regulates satiety; adiponectin, which regulates energy homeostasis; and visfatin, which mimics insulin leading to insulin resistance

(52). A summary of adipokine functions can be found in table 1. These adipokines play critical roles in obesity related metabolic and immune dysfunctions (52).

Table 1. List of adipokines. Adapted from Piya et. Al (2012)

Adipokine	Function	Change in Obesity
Leptin	Satiety, increased energy expenditure	↑ In obesity correlates with BMI ↓after fasting or weight loss
Adiponectin	Regulates energy homeostasis, insulin sensitivity and glucose uptake. anti-inflammatory	↓ in obesity and insulin resistance. ↑after weight loss
Resistin	Affects glucose metabolism and causes insulin resistance in rodents	↑ in human obesity, metabolic syndrome, T2DM, and CVD.
PAI-1	Inhibitor of fibrinolytic pathway	↑ in human obesity, metabolic syndrome, and T2DM.
RBP4	Insulin resistance, increased hepatic glucose output, impaired muscle insulin signaling	↑ in obesity and insulin resistance
MCP1	Increases insulin resistance, macrophage infiltration in adipose tissue and hepatic steatosis	↑ ob/ob and db/db mice. ↑ in obesity, T2DM and CVD.
Visfatin	Pro-inflammatory and insulin mimic	↑ in obesity Insulin resistance
Vaspin	Improves insulin sensitivity	↑ in obesity, insulin resistance and T2DM
Nesfatin	Reduces appetite	↓ In obesity, T2DM and PCOS
Omentin	Increase insulin sensitive	↓ In obesity
Apelin	Improves insulin sensitivity	↑ in obesity, impaired glucose tolerance and T2DM. ↓ after weight loss

Not only is adipose tissue capable of producing adipocytes, but it is also able to lead to higher pro-inflammatory cytokine production due to the large number of immune cells which can be found in adipose tissue. Macrophages make up the largest portion of immune cells in adipose tissue (53), but B2 cells, T cells, mast cells, eosinophils, neutrophils, and dendritic cells have been found in adipose tissue as well (53). The roles of neutrophils and dendritic cells have not been well studied. Obesity has also been found to cause a shift in immune cell populations (53) (Figure 1). This causes a shift towards a Th1 response characterized by the high levels of TNF-α produced.

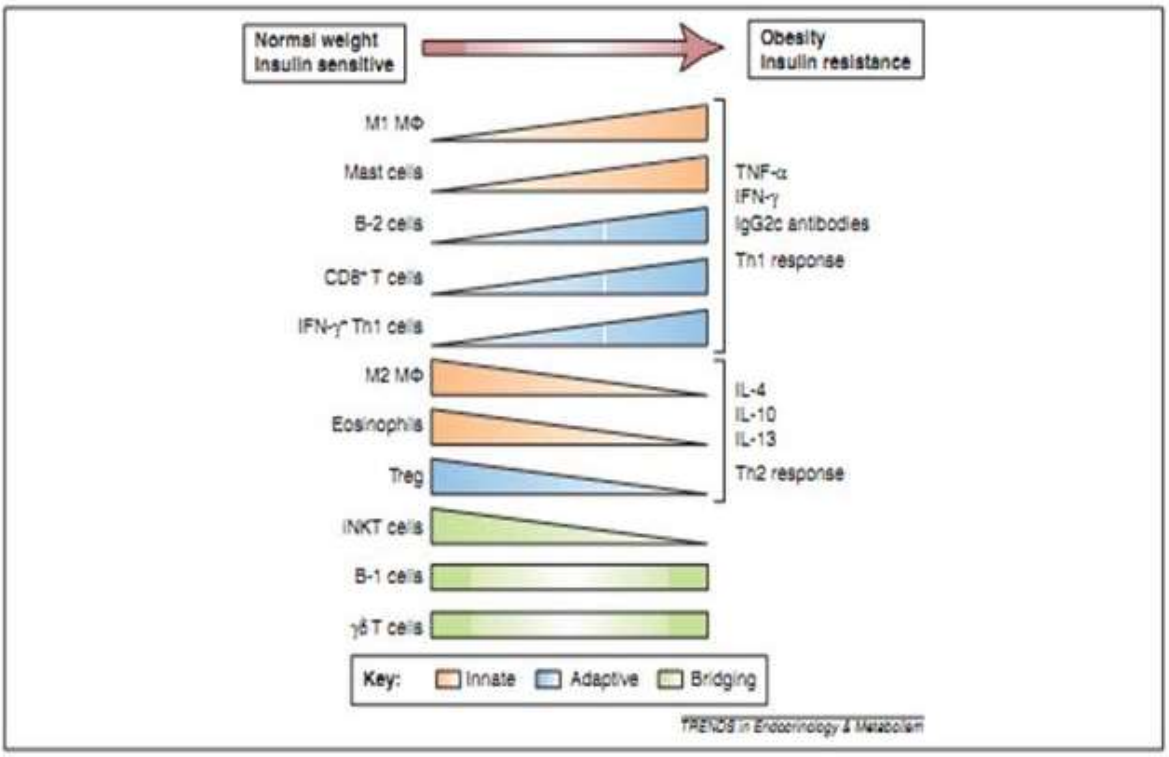

Figure 1. Summary of immune cells population changes that occur in obesity. (Schipper, 2012) Obesity causes changes in immune cell populations that push towards a Th1 response whereas immune cell populations in a lean person push towards a Th2 response.

1.2 Leptin

Leptin is an adipocyte-derived hormone/cytokine that links nutrition, metabolism, and immune homeostasis and is endowed to modulate several immune responses [4]. Leptin is produced mainly by white adipose tissue but it is also produced by placenta, gastric mucosa, bone marrow, mammary

epithelium, skeletal muscle, pituitary, hypothalamus and bone in small quantities [5, Frühbeck, 2001 #73, 6]. This hormone is produced by the Ob gene which is found on human chromosome 7 and mouse chromosome 6. Leptin is mainly found circulating in blood in either a free circulating or serum bound state. Leptin levels correlate to metabolic and nutritional state with higher leptin levels observed in obese persons[7] . One of leptin's main functions is satiety; therefore leptin production can be induced though food intake as a result of changes in insulin levels. A decrease in leptin promotes hunger; once an individual eats, their insulin levels increase leading to increased leptin production. Leptin is then able to send signals of satiety to the hypothalamus causing one to stop eating [8-10] . Leptin also plays roles outside of appetite control by signaling to various endocrine organs (Figure 2).

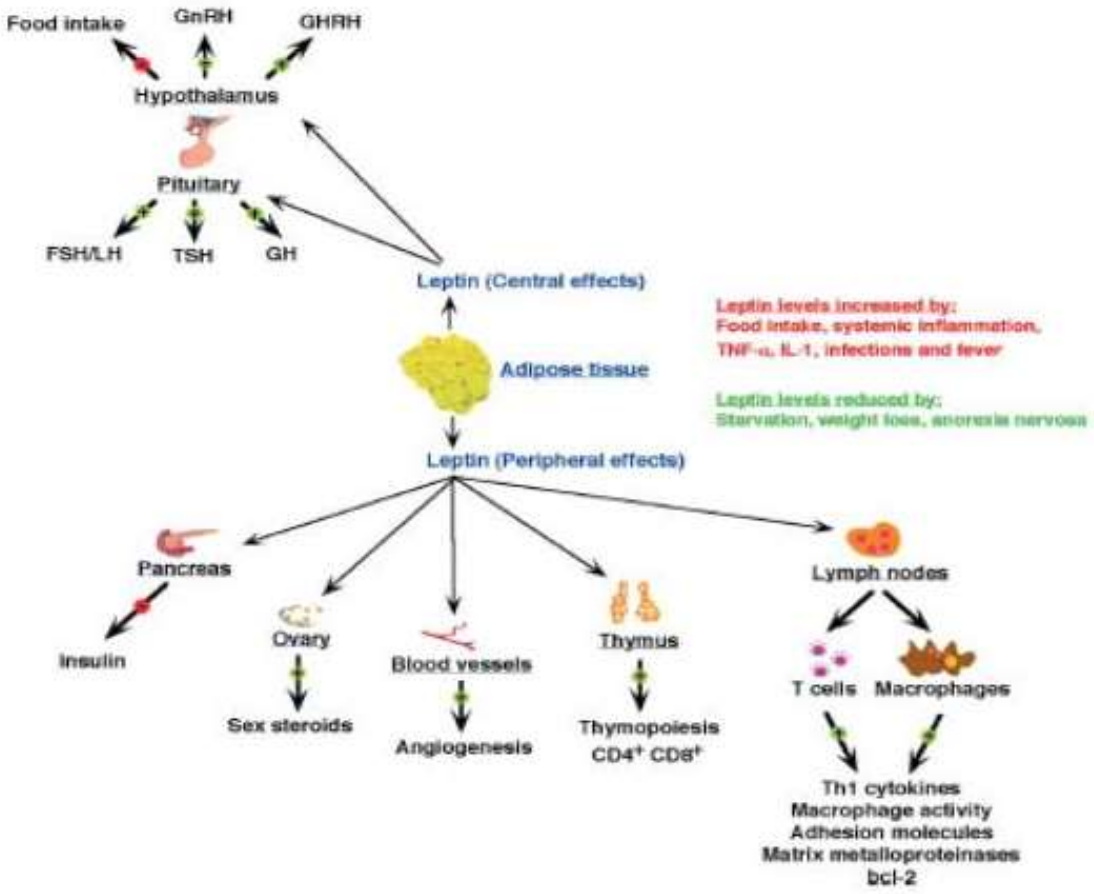

Figure 2 . Effects of Leptin on Endocrine System (La Cava, 2003) Leptin has the ability to signal to several endocrine organs leading to a variety of effects including cytokine production, hormone changes, and angiogenesis.

One such role is leptin's role as an adipokine. Leptin is an adipokine because it possesses many similarities to cytokines but is produced by adipose tissue. Many cells of the immune system, both innate and adaptive, have been found to express the signaling isoform of the leptin receptor [11]. One such immune cell is the macrophage; increased phagocytosis capability by macrophages has been found in the presence of leptin due to a dramatic increase in production of oxidative species [12-16]. Leptin has also been found to promote proliferation, differentiation, activation, and cytotoxicity of NK cells [17] and to promote production and maintenance of B cells[18]. T cells have also been found to be affected by Leptin; Leptin is able to promote T cell activation, inhibit production of T regs [19] and push for a Th1 response by promoting the production of pro-inflammatory cytokines[20].

1.3 Leptin Signaling

Leptin's structure is a 16kDA protein that resembles a long-chain helical class I cytokine but folds like the short helix cytokine family[21]. Leptin recognizes and binds to the OB-R receptor which resembles those of the class I cytokine family and has 6 isoforms (Figure 3) as a result of alternative splicing of the DB gene [1, 21-24] . The six different isoforms can be categorized as short, long, or secreted. The short receptor isoforms are OB-Ra, OB-Rc, OB-Rd, and OB-Rf; they contain 30-40 cytoplasmic amino acids[24]. The secreted isoform OB-Re and the short isoforms are not believed to be capable of producing intracellular signaling. The long isoform OB-Rb contains an intracellular domain of 300 amino acids with the necessary motifs to activate intercellular pathways[24].

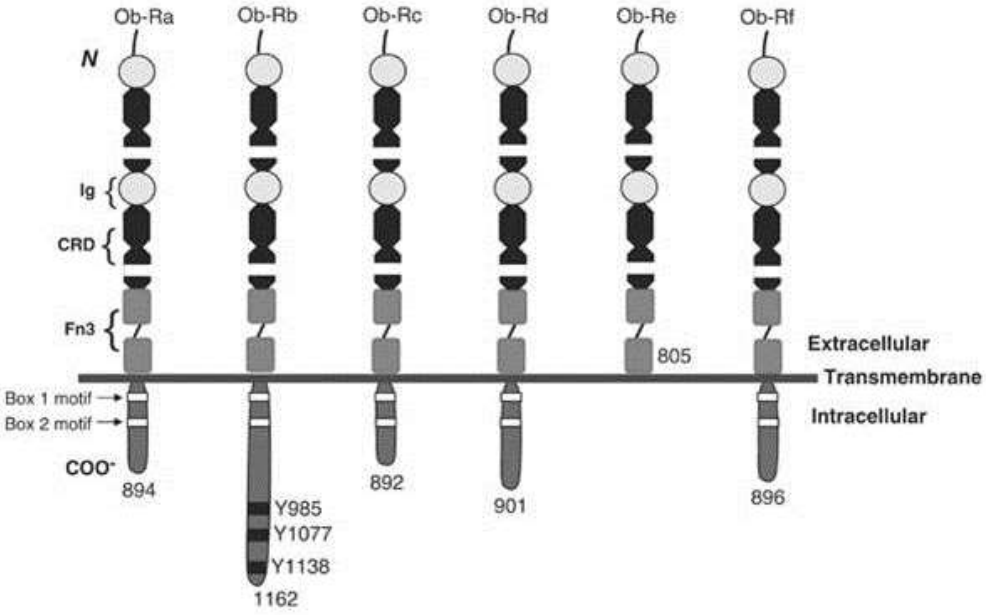

Figure 3: Leptin receptor isoforms (Ghilardi et. al., 1996). The Leptin receptor has 6 different isoforms produced by alternative splicing. Leptin signals intracellularly through the long isoform Ob-Rb.

Circulating leptin binds to the leptin receptor on a cell's surface causing the formation of a homo-dimer. When two receptors become homo-dimerized it leads to phosphorylation and a conformational change. The conformational change exposes the cytosolic domain of the receptor activating the JAK 2 pathway. JAK 2 autophosphorylates and phosphorylates tyrosine 985, y1077 and y11138 on the intracellular tail of the long isoform. This leads to recruitment of various STAT proteins. Y1138 phosphorylation leads to STAT3 homo-dimerization; the STAT3 homo-dimer then translocates to the nucleus where it induces transcription. Stat3 activation leads to an increase in suppressor of cytokine signaling 3 (socs3) expression. SOCS 3 is able to block leptin since it acts as a cytokine by binding to y985. Socs3 increases as STAT3 becomes activated leading to a negative feedback loop [25-27].

All the intracellular domains of the membrane bound isoforms contain the same 29 amino acid sequence which codes for Box1, including the Janus family tyrosine kinase (JAK) binding domain. Only the Ob-Rb isoform contains a motif for signal transducer and activators of transcription (stat) binding site [28] . Ob-Rb is a class 1 receptor and uses the JAK2/STAT3 pathway. This pathway is used for most of leptin's known functions. Leptin stimulation leads to its dimerization with its receptor; this allows intracellular communication with JAK proteins (figure 4). The JAKS juxtaposition and become activated by trans-phosphorylation of tyrosine 1138[29]. These phosphorylated tyrosine residues, found on the long isoform of the leptin receptor signal to STAT proteins which are subsequently phosphorylated[30] . Upon their phosphorylation, STATS will disassociate from the complex, homo-dimerize, and translocate into the nucleus, initiating transcription[30]. Once inside the nucleus, STATs are able to interact with specific DNA elements in the promoters of target genes to regulate SOCS 3, NFκB, POMC, and other genes' expression [31, 32]. SOCS protein is able to regulate cytokine signaling, thereby creating a negative feedback loop for leptin[33]. SOCS contains a central SH2 domain which enables it to inhibit signaling by binding to phosphorylated JAKs.

Another pathway stimulated by leptin is the mitogen-activated protein kinase (MAPK) pathway (MAPK). MAPK activation can result in the activation of several pathways leading to cell proliferation (figure 4). One such pathways activates growth factor receptor bound protein 2 (grb2) as a result of SH2 activation. Another pathway leads to proliferation by activating early growth response protein 1 (EGR1) which then activates TIMP1, halting matrix metalloproteinases (MMP) causing extracellular matrix degradation and promoting cell growth. This pathway can also lead to angiogenesis by activating vascular endothelial growth factor A (VEGFA) [34, 35]. MAPK signaling can also occur downstream of JAK2 phosphorylation. JAK2 phosphorylating can activate the c-Jun N-terminal kinase (JNK) stimulating the transcription of cFOS, cJUN, and tumor necrosis factor alpha (TNF-a).

Phosphatidylinositol 3 kinase (PI3k) can be regulated by a broad range of ligands, including leptin. PI3k becomes activate when its regulatory subunit binds to tyrosine phosphorylated proteins causing a conformational change allowing the catalytic subunit to become activated. One such activation occurs when the phosphorylated JAK activates insulin receptor substrate which activates PI3k. Phosphoinositides are phosphorylated on 3 positions of the inositol ring by PI3K [36-40]. The resulting products, phosphatidylinositol(3)monophosphate(PI(3)P), phosphatidylinositol(3,4) biphosphate (PI(3,4)P2) and phosphatidylinositol(3,4,5,)triphosphate (PI(3,4,5)Ps), play roles in cell growth, cell survival, cytoskeletal remodeling, and trafficking of intracellular organelles[36] .There are four classes of PI3Ks, IA, IB, II and III; the class activated depends on the type of cell and/or receptor of the immune system [36-40].. Cytokines such as IL-2, IL-3, IL-6, IL-7, IL-15, GMCSF, erythropoietin, oncostatin M and interferons activate class IA PI3Ks in many immune cells, including T cells and DCs[36].

PI3k can activate protein kinase B (Akt) which activates phosphodiesterase. Downstream of PI3K and AKT is the mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase important for cell development and growth [41] . OB-Rb is required for mTOR activation. PI3K-AKT-mTOR signaling is important for the formation of neuronal dendrite spines and branching [42].

Akt can also initiate the RhoA/Rock pathway (figure 4) which plays a critical role in actin-cytoskeletal rearrangement [32, 43] . RhoA/Rock activation can occur though the short forms of the receptor since the SH2 box is not required; it is, however, dependent on JAK2 phosphorylation [32] . RhoA/Rock signaling leads to phosphorylation of cofilin. Cofilin is an actin-depolymerizing factor that causes changes in the ration of g to f actin [44] . Inactivation of cofilin by phosphorylation leads to f-actin polymerization thereby promoting formation of hypertrophy [44-46].

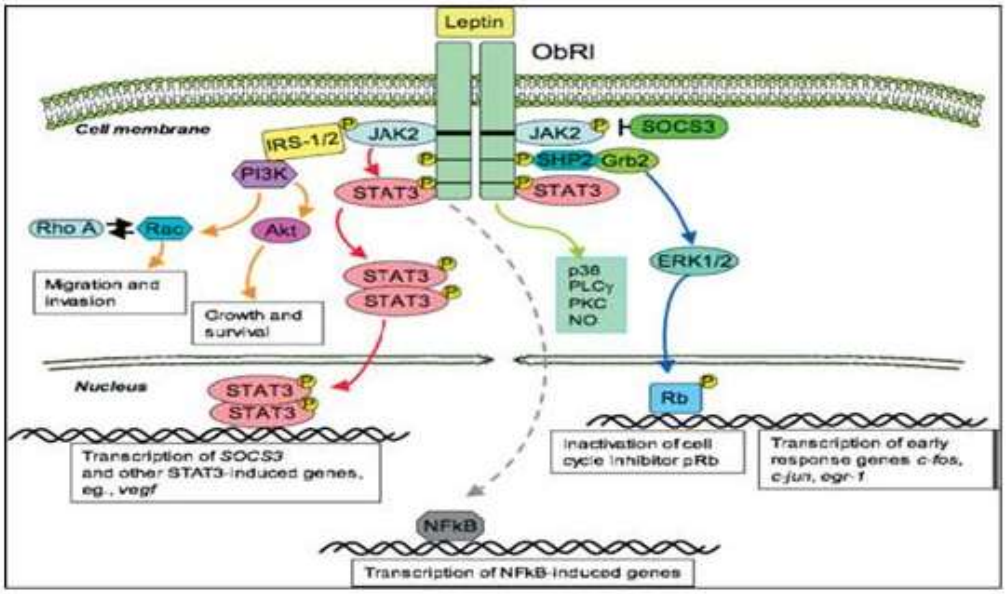

Figure 4: Leptin Signaling Pathways (Garofalo and Surmacz, 2006). Leptin can signal intracellularly through JAK2/STAT3, PI3K, MAPK, and NFκB signaling pathways.

1.4 Dendritic cells

Dendritic cells are antigen presenting cells. Antigen presenting cells serve as initiators and modulators of immune response (4). DCs phagocytose material, process it and migrate from tissue to draining lymph nodes to present peptide fragments of ingested material to T cells. Antigen presenting cells do as their name implies and present antigens to T cells. Dendritic cells are the only cells capable of activating naïve T cells and therefore are required to develop immunity and clear infections. T cells require an antigen which lets them know what they are up against but before the antigen can be recognized by a T cell it must be processed by an APC. Instead of recognizing the entire antigen, T cells will recognize fragments of the antigen bound to multi histocompatibility complexes on the antigen presenting cells. Peptides from infected cells anywhere in the body must be recognized by T cells that

circulate in the bloodstream. T cell receptors often are unable to recognize the small quantities of MHC on tumors and infected cells due to low affinity and lack of co-stimulatory molecules. This renders the cells incapable of producing cytokines and developing into killer cells. Since dendritic cells are located on most tissues, they are able to capture and process antigens in order to display a large amount of MHC peptide complexes at their surface (4).

There are two classes of MHC: MHC I and MHC II. MHC I is recognized by cytotoxic T cells and MHC II is recognized by helper T cells. Therefore, the class of MHC on which the antigen is presented contributes to the type of immune response. Intracellular antigens cut into peptides in the cytosol of APC bind to MHC I, once recognized by the CTLs, the target cells are directly killed by CTLS (4). Extracellular antigens that have entered the endocytic pathway of the APC are processed there and generally presented by MHC II to Th cells. Th cells do not directly kill their target but instead interact with CTLs and B cells to direct an immune response.

Once activated, DCs travel to lymphoid tissues such as the spleen and lymph nodes where they can mature. In spleen and lymph nodes DCs are able to attract T and B cells by releasing chemokines and maintain the viability of recirculating T cells. DCs are a heterogeneous population of cells that can be divided into three populations: non-lymphoid tissue migratory, lymphoid tissue resident DCs and plasmacytoid DCs (5). Splenic DCs constitutively express MHC class II and the integrin CD11c. MHC class I is present on almost every cell in the body, whereas MHC class II is present only on antigen presenting cells.

DCs have large projections that bend, retract, and re-extend in a non-polarized fashion (4). These projections give DCs their name since they look similar to dendrites on neurons. DCs' shape and motility make them unique and help them to capture antigens and select antigen specific T cells (4). Antigen processing, capturing, and presentation to T cells are functions available to DCs at two different stages in their lives. Terminally differentiated DCs can readily prime T cells. These T cells, once

activated, are able to complete the immune response by interacting with other cells (4). These cells include B cells which form antibodies against the targeted pathogen, macrophages which release cytokines, and targets for lysis (4). Immature DCs are much less potent as initiators of immunity but are instead specialized in capturing and processing antigen to MHC peptide complexes (4). As DCs mature, their morphology also changes due to reorganizations of the cytoskeleton. These morphological changes allow DCs to interact with T cells and decide whether or not to activate them.

DCs are the most potent APCs because they express MHC peptide complexes 10-100 times higher than other APCs (4). Aside from presenting antigens, DCs are also capable of producing high levels of IL-12 (4), a pro-inflammatory cytokine which helps in both acquired and innate immunity. DCs also express many accessory molecules, including CD58, CD54 and CD86 (4), that are able to interact with receptors on T cells to enhance adhesion and signaling. DCs can stimulate a variety of T cells thus affecting the type of immune response created. This is not only done by the MHC class on which the antigen is presented but also by the types of cytokines present. In the presence of mature DCs and IL12, T helper cells produce IFN-γ by inducing a TH1 response which activates antimicrobial activities of macrophages and together with IL-12 can promote the differentiation of T cells into cytotoxic T cells (4). A different response occurs in the presence of IL-4, this leads to a Th2 response which activates B cells leading to the production of appropriate antibodies (4).

1.5 Purpose

Leptin is a pleiotropic hormone primarily secreted by white adipose tissue. Although originally found to regulate food intake and energy expenditure, it is now known to also significantly affect the immune system. The current study was conducted to determine the signaling mechanism responsible for changes in dendritic cell (DC) cytoskeletal structure and functionality caused by leptin. Our previous data demonstrates that the addition of obese concentrations of leptin to DC causes an increased number

of lamellipodia/dendrites, an increase in cell surface area, and an increased staining for actin within these areas. The morphological changes were accompanied with enhanced DC functionality as well, including increased migration and activation of T cells. The data suggests that leptin is a necessary component of DC – T cell interaction in that leptin promotes migration of DC to draining lymph nodes and potentiates the extent of the physical contact between these cells. Our preliminary data demonstrates that four different signaling pathways are activated upon ligation of the leptin receptor (ObRb): Jak2/STAT3, PI3K, MAPK, and NFκB. We hypothesized that leptin promotes DC cytoskeletal changes though the activation of the PI3K pathway. To test this hypothesis we characterized the changes occurring in JAWS II cells upon leptin treatment, verified the morphological changes occurring in BMDCs upon leptin treatment and characterized the loss of these changes upon PI3K inhibition, and identified changes in PI3K signaling upon leptin treatment.

Chapter 2:

Assessment of the Effects of Leptin on JAWS II cells

2.1 Background and significance

DCs are a key member of the immune system due to their ability to link innate and adaptive immune responses. Primary DCs can be cultured from the bone marrow of mice but this procedure requires a lengthy process with a low cell turnover. Primary DCs have a short lifespan in vitro, do not reproduce, and are very sensitive. Immortalized cell lines provide a low maintenance, continuous source of available cells. A DC cell line that reacts to leptin treatment in the same way as a primary cell line would be beneficial for in vitro alterations of the cells.

JAWS II cells are an immortalized immature DC line created from bone marrow derived DC precursors obtained from p53-/-C57BL/6 mice [47, 48]. JAWS II exhibit immature BM-DC characteristics with low expression of CD80, CD86, and MHC class I and II (Rossowska). This cell line has been used in anti-tumor and pathogen specific studies (jiang) however their response to leptin has not been studied.

Previous data from Dr. Garza's lab shows that obese concentrations of leptin induce morphological and functional changes in primary BMDCs from C57/bl6 mice. These changes occur when the DCs are treated with both leptin and a TLR stimulant. Due to the large number of cells required for signaling studies, and the low turnover rate of primary DCs, leptin treatment of JAWS II cells will be characterized to identify a possible alternate source of DCs. The cells will be characterized for their expression of DC cell surface markers, morphology, viability, ability to activate T-cells, and ability to migrate.

2.2 Methods

Cell culture

JAWS II cells were grown in MEM-α containing 20% FBS in the presence of granulocyte monocyte colony stimulating factor (GM-CSF). When cells became confluent, they were treated for 24 hours with one of the following treatments:

Treatments

1. No treatment in MEM-α
2. 10 μg/mL LPS
3. 160 ng/mL Leptin
4. 10 μg/mL LPS and 160 ng/mL leptin
5. 10 μg/mL Poly IC
6. 10 μg/mL Poly IC and 160 ng/mL Leptin
7. 75 μg/mL Zymosan
8. 75 μg/mL Zymosan and 160 ng/mL Leptin

Non treated cells serve as a negative control, LPS serves as a bacterial infection mimic stimulating TLR 4, Poly IC serves as a viral mimic stimulating TLR 3, and Zymosan serves as a fungal mimic stimulating TLR 2.

Light Microscopy

JAWS II cells were plated in 12 well plates. The cells were treated for 48 hours with either LPS (10 μg/ml), Leptin (160ng/ml), Zymosan (75 μg/ml), Poly IC (10 μg/ml), Leptin and LPS, Leptin and zymosan, Leptin and Poly IC or left untreated. The cells were then visualized using a Leica light microscope at 40X magnification

Confocal Microscopy

Cells were treated for 24 hours in 96 well plates with LPS (10 µg/ml), Leptin (160 ng/mL), Leptin and LPS, or left untreated. After 24 hour treatments, cells were centrifuged for 5 min at 500 rpm to attach the cells to the plate. Supernatants were removed and cells were washed in 10% PBS. Cells were then fixed in 1% paraformaldehyde (PFA) for 10 min. Cells were once again washed in PBS then permeabilized in .5% triton X-100 in PBS for 5 min. Once cells were permeabilized, they were rewashed in PBS. 100nM Rhodamine labeled phalloidin (Cytoskeleton) in PBS was then used to stain F-actin for 2 hours in the dark. Cells were rewashed in PBS then counterstained with Diamidino-2 penylindole dihydrochloride (DAPI) at 10nM in PBS for 15 min in the Dark. Cells were washed one final time then 200 µL of PBS were added to each well and the pate was stored at 4° C. Cells were visualized using a LSM 700 Confocal Microscope (Zeiss) and images were analyzed using Zen 2009 software.

MTS Assay

Cells were grown to confluency as previously described. When cells became confluent they were plated onto 96 well plates and treated with Leptin, LPS, LPS and Leptin, or left untreated as previously described for a time course of 24, 48, 72, and 96 hours. Cells were co-incubated with MTS for 1 hour at which point fluorescence was measured.

Trypan Blue Exclusion

Cells were grown to confluency as previously described and which point they were plated into 6 well plates and treated with LPS, Leptin, Leptin and LPS, or left untreated, as previously described, for 24 hours. Cells were then trypsinized, and resuspended in MEM-α. A sample of cells from each treatment was removed and mixed with trypan blue. The cells were then counted with a Nexelom cell counter and the viability was determined.

Measurement of Cytokine Production using ELISA

JAWS II cells were plated in 6 well plates at a concentration of 1 million cells/ well. The cells were treated with LPS (10µg/ml), Leptin (160ng/ml), LPS and Leptin, or left untreated for 48 hours at which point supernatants were collected. ELISAs were also performed for IL-12 and TNF-α according to the manufacturer's protocol.

IFN-γ production was also measured by ELISA. T cells were co-incubated with JAWS II cells for 72 hours at which point supernatants were collected. The supernatants were then assessed for IFN-γ production according to the manufacturer's protocol. IFN-γ production was used as a measure of T cell activation.

Transwell Migration

JAWS II cells were plated in a 12 well plate 5 x 10^5 cells / well. The cells were treated for 24 hours with either LPS, Leptin, Leptin and LPS or left untreated as previously described. After 24 hours, the cells were counted and resuspended at 500,000 cells/ 400 µl. 400 µls of each treatment were placed in the apical chamber of the transwell migration chamber with an 8 µM filter. The bottom portion of the chamber was filled with 1mL of media containing 5ng/mL of CCL 19. Cells were allowed to migrate for 48 hours at which point the cells able to migrate were removed trypsinized and counted using a Nexcelon Cellometer.

T-cell Isolation

T-cells were obtained from the lymph nodes of OT-2 TCR transgenic mice. Mice were anesthetized with Isofluorane and sacrificed by cervical dislocation. Lymph nodes were placed in 10

mL of HPMI and crushed though mesh until they were in a single cell suspension. The mesh was washed with 5 more mL of HPMI.

Cell Enrichment using an Automacs Cell Purifier.

CD4+ T cells were isolated using an Automacs Cell Purifier. The Lymph cells which were previously isolated from OT-2 mice were centrifuged at 1200 rpm for 10 min and the supernatant was removed. The cells were resuspended in Automacs buffer at a concentration of 10^7 cells/ 4 mL containing 20µL of CD4 Automacs beads. The cells were incubated on ice for 15 minutes. Cells were then washed in Automacs buffer and sorted by the Automacs cell purifier.

T-cell Proliferation Assays

JAWS II cells were treated with LPS, Leptin, Leptin and LPS or left untreated as previously described for 24 hours. Purified T cells were added to 96 well plates at a concentration of 80,000 cells per well. The T cells were co-incubated with 20,000 treated JAWS cells in triplicates and 48 µg/mL of ova peptide. Con A was added at 10 µg/mL as a positive control. The cells were incubated for 72 hours at which point supernatants were collected and the cells were pulsed with tritium. At 96 hours the cells were harvested and tritium incorporation was measured using liquid scintillation. An ELISA was performed for IFNγ using the supernatants which had been collected.

Cell Lysates

JAWS II cells were plated at a concentration of 1 million cells per well, then treated as previously described for 30 min. Cells were trypsinized, harvested, and centrifuged for 10 min at 1200 rpm. The cells were then washed in PBS. The cells were then lysed using 1 mL of MPER lysis buffer consisting of a complete protease inhibitor tablet at 4°C with continuous shaking for 10 minutes.

Lysates were stored at 20° C until ready to be used. Protein concentrations were quantified using BCA kit.

Quantification of Protein using a BCA Kit

/ Whole cell lysates were prepared as previously described and their concentration was determined using a BCA kit (Pierce). 25µL of each protein standard and cell lysate were placed into wells of 96 well plates in duplicates. 200 µL of working reagent, prepared according to the manufacturer's protocol, were added to each well. The plate was incubated for 30 min at 37° C. The plate was then read using a spectrophotometer with an absorbance of 562 nm.

Western Blots for AKT signaling

Western blot analysis of PI3K signaling was performed on JAWS II cells treated with Leptin, LPS and Leptin and LPS. Whole cell lysates were separated using sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a PVDF membrane. The membrane was incubated with an antibody for p-T308 AKT (cell signaling) at 1:1000 in 5% milk in T-TBS overnight. The membrane was then washed 6 times for 5 minutes in T-TBS. A secondary anti-rabbit fluorescent antibody was used to label the protein. Anti-rabbit IgG was added to the membrane at a concentration of 1:5000 in 5% milk in T-TBS for 1 hour at room temperature with continuous shaking. A Licor imaging system was then used to detect the presence of phosphorylated AKT. Total AKT was also measured by western blot. The PVDF membrane was incubated overnight with an antibody for total AKT (cell signaling) at 1:1000 in 5% milk. A secondary fluorescent anti-rabbit antibody was once again used to detect the presence of the protein using the Licor imaging system. Densitometry was performed using the Licor software.

2.3 Results

JAWS II Cells Do Not Exhibit Morphological Changes Upon Leptin Treatment.

Prior data from Dr. Kristine Garza's lab shows that treatment of primary DCs with Leptin (160ng/ml),LPS (10µg/ml) or Leptin and LPS is able to induce morphological changes in the treated cells. These changes are found in the form of increased projection number and length on the cells. We wanted to verify whether these same changes were occurring in JAWS II cells. JAWS II cells were visualized using light microscopy in order to visualize morphological changes and confocal microscopy: to visualize localization of actin filaments.

Following treatment, JAWS II cells were visualized using 40X magnification. JAWS II cells were found to not typically contain dendrites. There were no significant changes in the shape or size in JAWS II cells upon any of the treatments.

Treated JAWS II cells were observed for morphological changes and changes in amount of F-actin using confocal microscopy. Confocal Images of primary BMDCs taken in the lab showed changes in number and length of dendrites on cells as well as an increase in staining for F-actin when treated with Leptin and LPS. Morphological changes correlating to those observed in primary BMDCs were not observed in JAWS II cells and no significant changes in F-actin staining were observed.

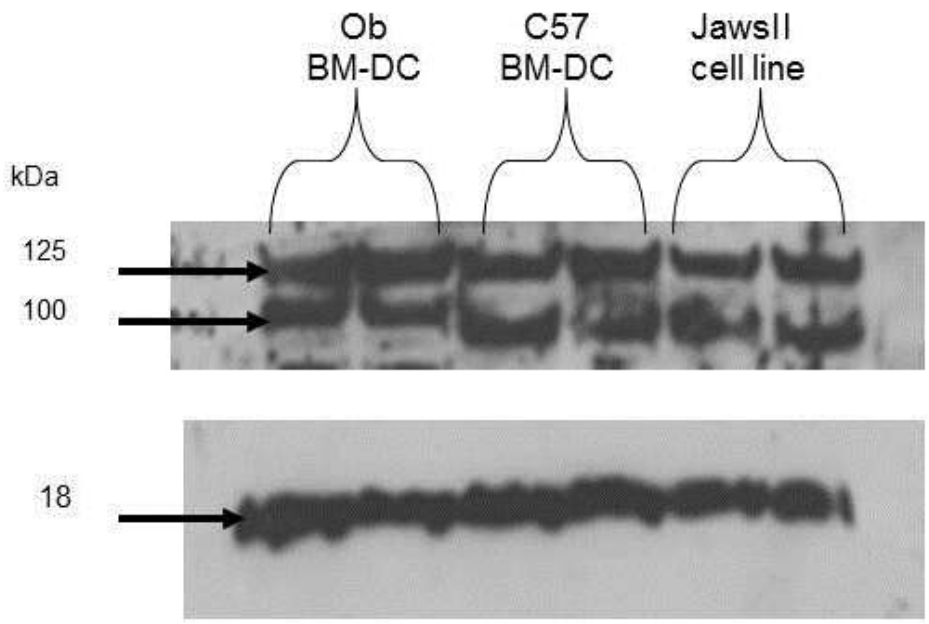

Figure 5: JAWS II Cells Express the Ob-Rb Leptin Receptor. Western blot analysis show that JAWS II cell line expresses the Ob-Rb long isoform of the leptin receptor at similar concentrations as primary DCs. Western blot by Oscar Ramirez

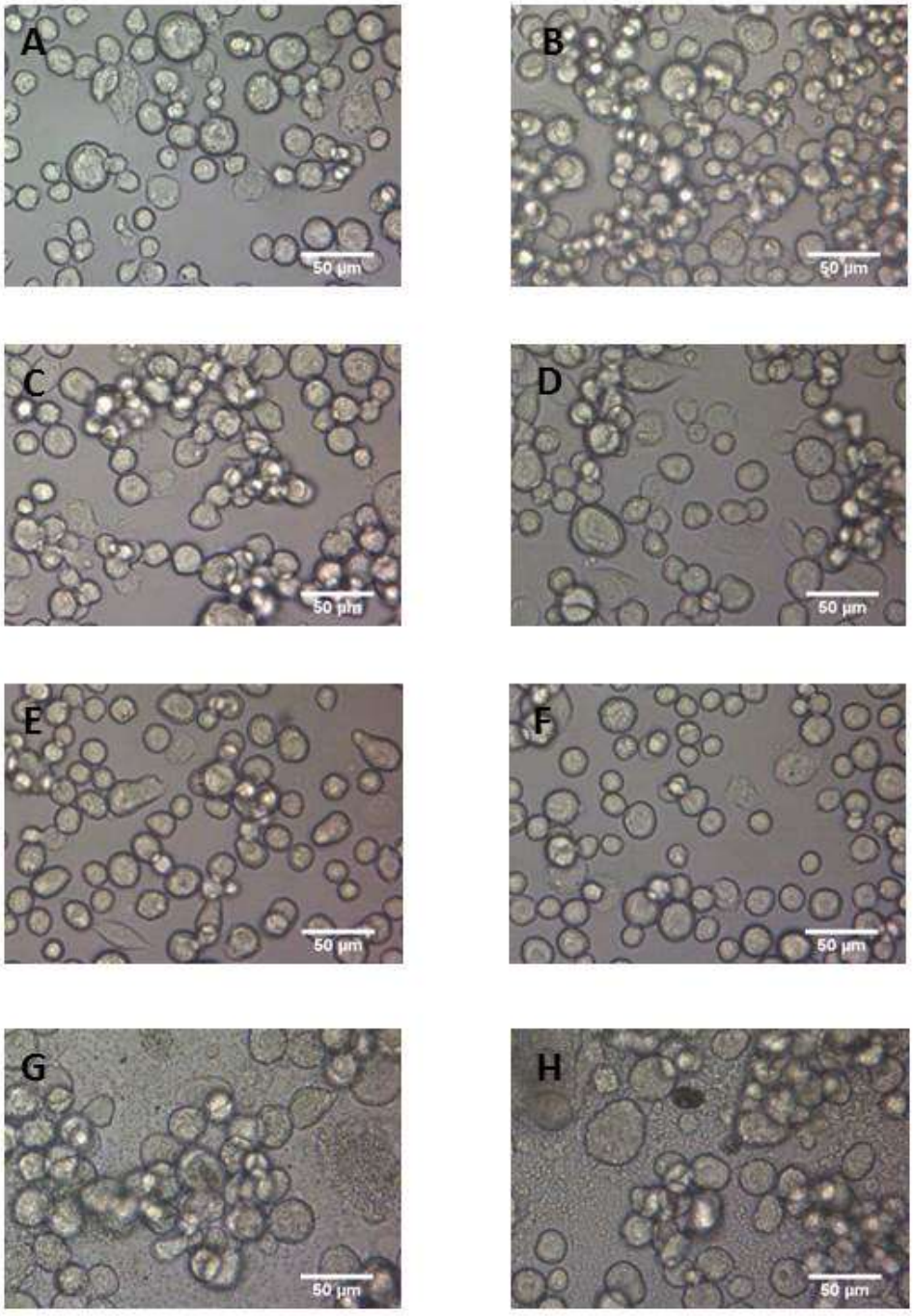

Figure 6: Light microscopy images of JAWS II cells . JAWS II cells were treated for 48 hours with A) Media, B) Leptin (160ng/ml), C) LPS (10 µg/ml) D) Leptin and LPS, E) Poly IC (10 µg/ml), F) Poly IC and Leptin, G) Zymosan (75 µg/ml), H) Zymosan and Leptin. Images show no significant changes in morphology. JAWS II cells do not contain dendrites.

Leptin Treatment Does Not Induce Changes in Viability of JAWS II Cells.

Previous data from Dr. Garza's lab shows that co-treatment of BMDCs with Leptin and LPS is able to enhance viability of the cells. We wanted to know whether these effects would also occur in JAWS II cells. Viability was tested in JAWS II cells using an MTS assay and Trypan blue exclusion. There were no significant changes in the viability of JAWS II cells upon treatment with Leptin and LPS.

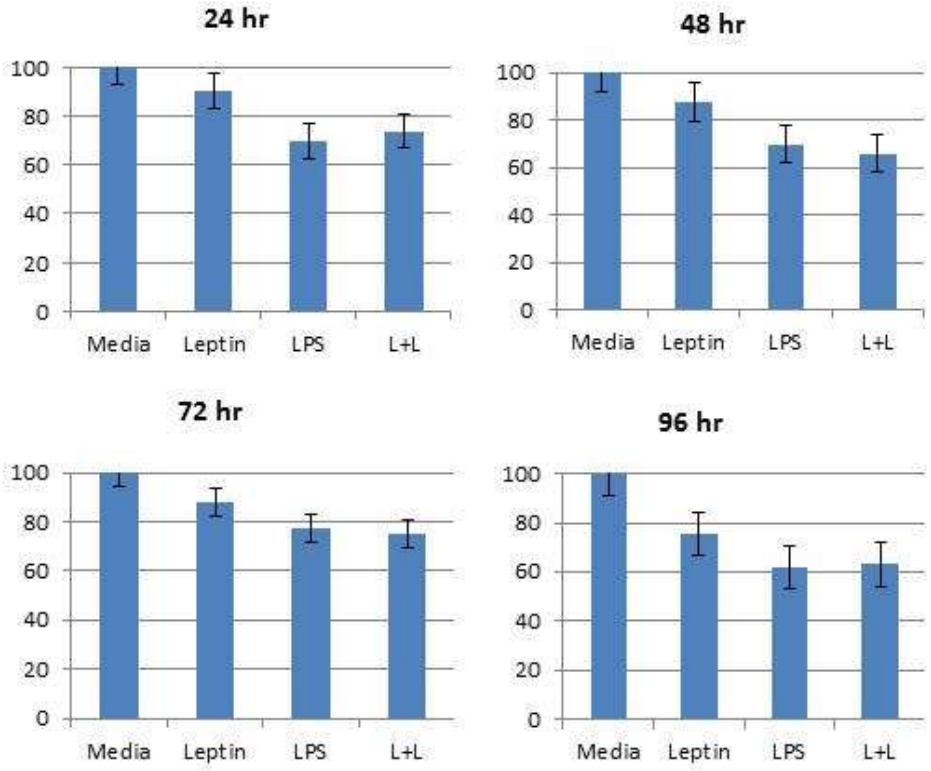

Figure 7. Leptin does not enhance survival of JAWS II cells. JAWS II cells were treated with LPS (10μg/ml), Leptin (160 ng/ml), or leptin and LPS in 96 well plates. The cells were coincubated with MTS for 24, 48, 72, and 96 hours. Treatment with Leptin did not significantly enhance survival (n= 4).

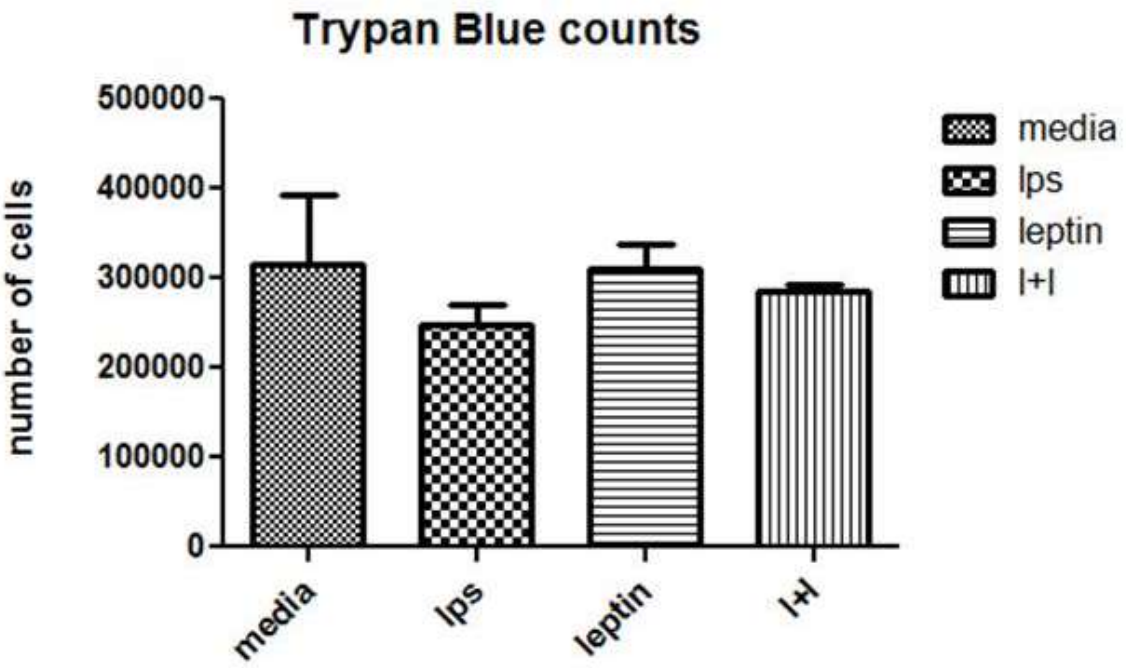

Figure 8. Leptin does not enhance JAWS II survival. JAWS II cells were treated with LPS (10 µg/ml), Leptin (160ng/ml), or leptin and LPS. After 24 hours of treatment, trypan blue was added to the cells and the cells were counted. No significant changes in cell numbers were observed (n = 2).

Leptin Treatment Does Not Lead To Changes In Cytokine Production In JAWS II Cells.

Enzyme-linked immunosorbent assays (ELISAs) provide a method for quantifying proteins with a colorimetric assay. A capture antibody is added to a 96 well plate; the capture antibody will remain

attached to plate enabling it to bind the protein of interest. Samples containing the protein of interest are added to the plate; the protein will bind to the capture antibody. A second detection antibody is added to the plate. This antibody will stick to the protein of interest creating an antibody sandwich. The detection antibody is enzyme linked enabling it to change colors upon substrate addition. This will then allow one to quantify the amount of a given protein based on the amount of color change that occurs. Treatment with Leptin did not lead to any significant changes and results did not correlate with those observed in primary DCs.

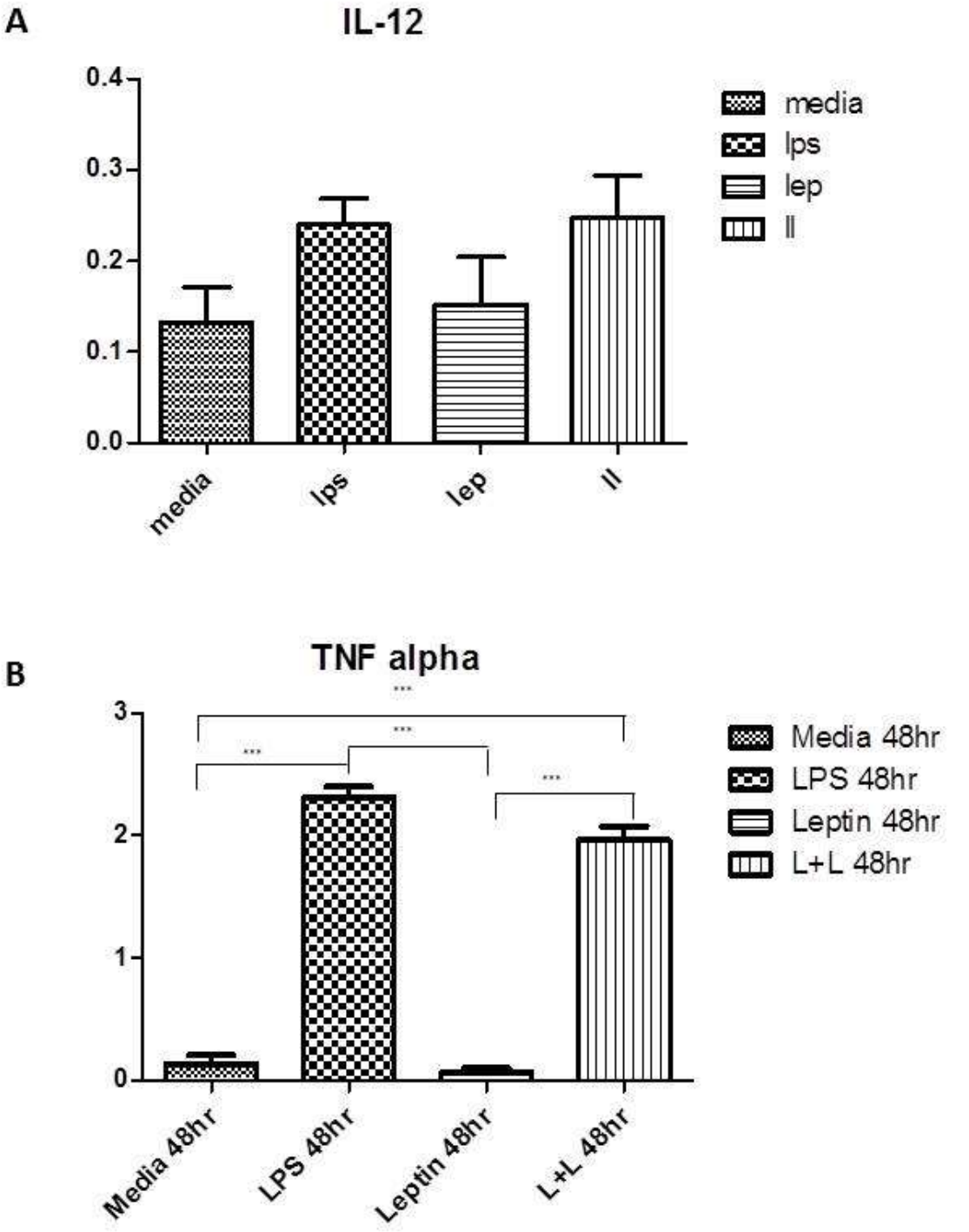

Figure 9. Leptin does not cause increased proinflamattory cytokine production in JAWS II cells. JAWS II cells were treated with LPS (10μg/ml), Leptin (160ng/ml), LPS and Leptin, or left untreated. Supernatants were collected after 48 hours. ELISAs were performed for A) IL-12 and B) TNfα. Leptin did not cause any significant changes in cytokine production (n = 3) ($p < 0.0001$).

JAWS II Cells Do Not Migrate Towards CCL5.

Previous studies in Dr. Kristine Garza's lab have shown that primary DCs treated with leptin and leptin and LPS have enhanced migration.

JAWS II cells were treated as previously described and allowed to migrate in a transwell migration system. The cells did not seem to migrate towards CCL 19; there were large variations in the number of cells that migrated in the duplicates and no significant differences between treatments. The same results were observed when the experiment was replicated. Furthermore, during a time course observing cells at 4 hours, 24 hours, and 48 hours, cells were not observed in the bottom chamber until 48 hours.

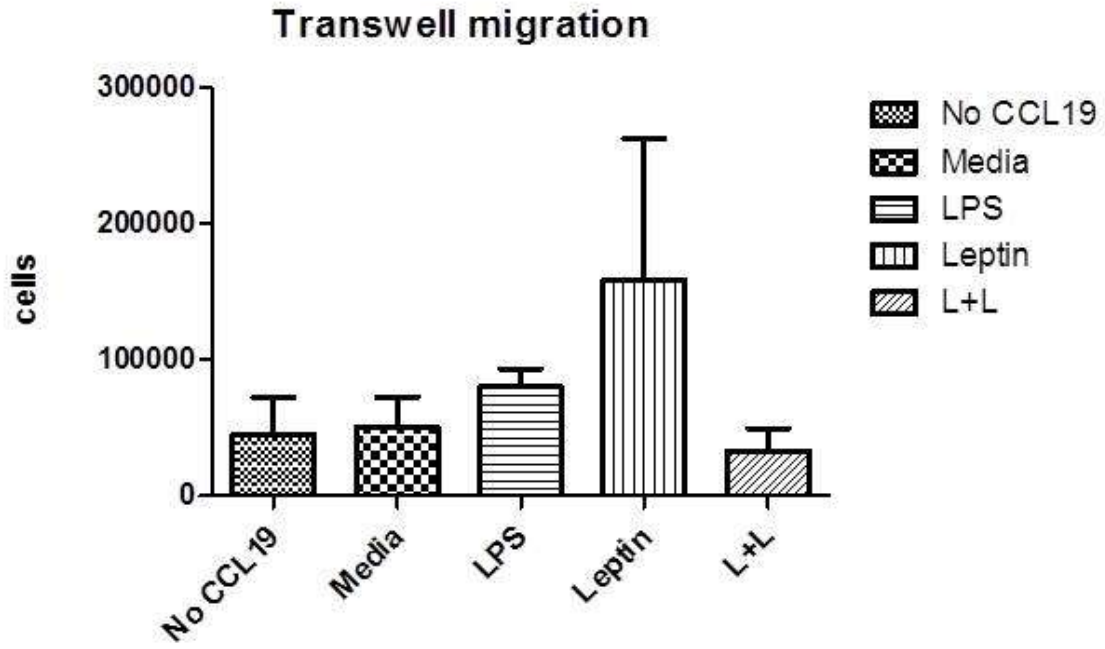

Figure 10. JAWS II cells are unable to migrate. JAWS II cells were treated with Leptin, LPS, or leptin and LPS for 24 hours at which point they were plated on the insert of an 8.0 um transwell system at a concentration of 3.0×10^5 cells. Chemokine CCL19 was added to the bottom chamber. Cells were counted at 48 hours; no significant changes were observed (n = 2).

Leptin Does Not Enhance the Ability of JAWS II Cells to Activate T-cells.

Tritium (H3) is a radioactive isotope of Hydrogen. The incorporation of tritium into thymidine provides a method of measuring proliferation. Thymidine is a deoxyribonucleaside known as deoxythymidine (T) which pairs with deoxyandenosine (A) in the double stranded DNA helix[49]. By treating proliferating cells with tritium we will be able to measure the amount of proliferation by measuring the amount of tritium that has incorporated into the cells by liquid scintillation. Treated, irradiated JAWS II cells were co-incubated with T-cells for 72 hours then pulsed with tritium. Tritium was allowed to incorporate for 24 hours. The cells were then harvested onto fiberglass paper using an automatic cell harvester. The incorporation of tritium into the DNA of the T cells was measured by liquid scintillation using the LS5801 Beckman Coulter.

The results of the tritium incorporation did not show an increase in proliferation upon treatment with Leptin and LPS as had been observed in primary BMDCs. The results of the IFN-γ production did show a trend of increasing production with Leptin and LPS but the change was not statistically significant.

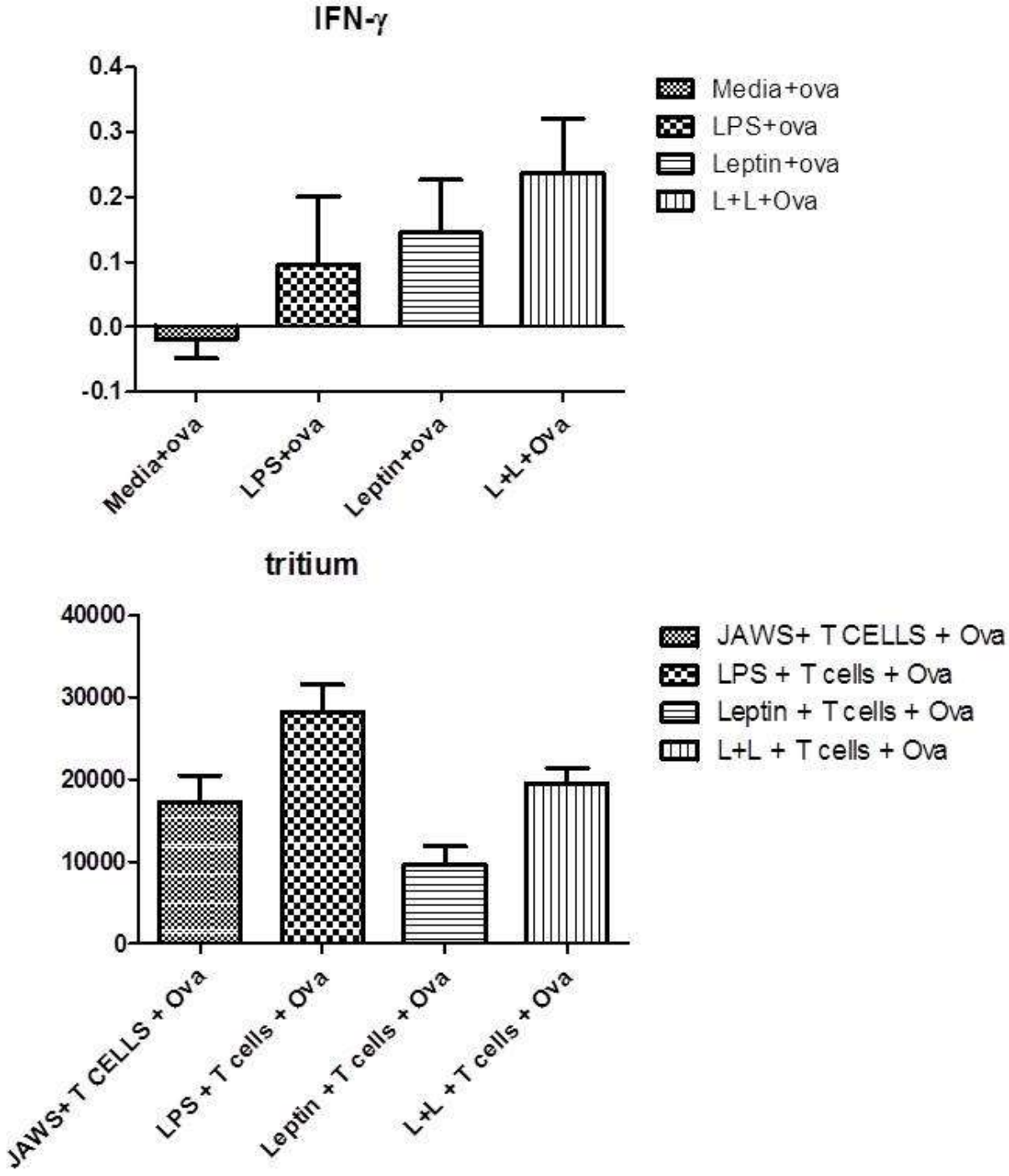

Figure 11: Leptin treatment of JAWS II cells does not significantly enhance T cell activation. JAWS II cell were coincubated with naïve T cells and activated with LPS (10 µg/ml), Leptin (160 ng/ml), leptin and LPS, or left untreated. Cells were pulsed with 32 µg/ml of ovalbumin peptide. After 72 hours, supernatants were collected for IFN-γ ELISAs and tritiated-thymidine was added to the cells. The cells were incubated overnight then harvested. The incorporation of tritium was measured by liquid scintillation. IFN-γ ELISA results show a trend of increased cytokine production but results are not significant (n=3). Tritium incorporation results do not show any enhanced T cell activation upon leptin treatment (n= 3).

PI3K signaling

The PI3K signaling pathway is important for cell survival and growth. It has also been identified as an important pathway in the growth of dendrites on neural cells. Leptin has been found to activate the PI3K pathway downstream of JAK2. We have therefore hypothesized PI3K is inducing the morphological changes which had been identified in primary BMDCs. PI3K activity was measured in JAWS II cells through the phosphorylation of Akt. There were no changes in the phosphorylation of Akt observed upon Leptin and LPS treatment.

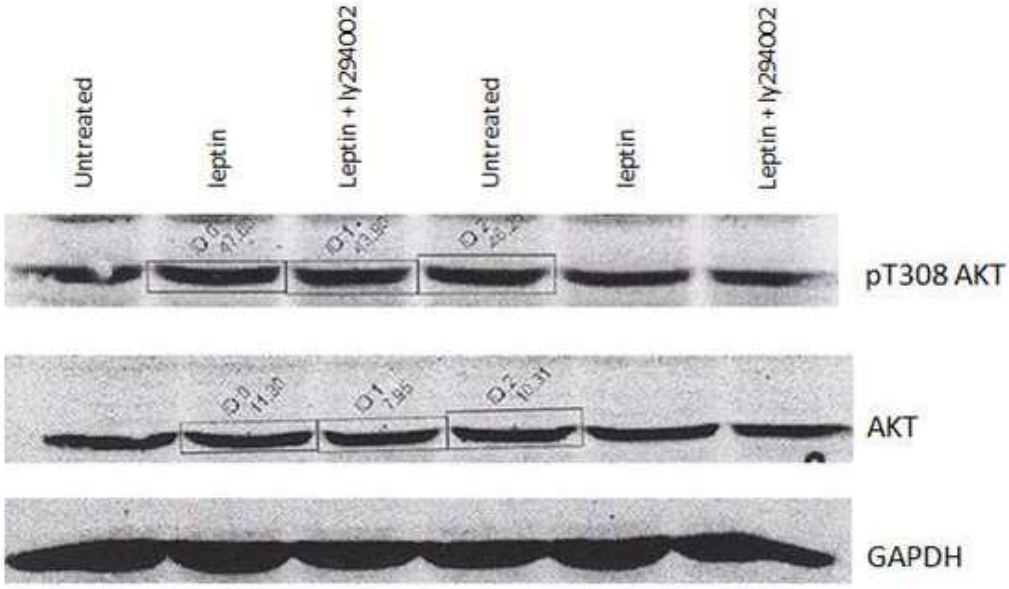

Figure 12: Leptin treatment does not cause PI3K signaling changes in JAWS II cells. JAWS II cells were treated with Leptin (160ng/ml) or leptin and Ly294002 (50 µmol) for 30 min. Phosphorylation of AKT was analyzed by western blot using antibodies from cell signaling. No significant changes in AKT phosphorylation were observed (n=2)

2.4 Conclusion

Although JAWS II cells express a Leptin receptor, they are unable to display the changes observed in primary BMDCs. JAWS II cells did not contain dendrites and therefore were unable to express the morphological changes that had been observed in primary BMDCs upon leptin and LPS treatment. Treatment with Leptin and LPS did not enhance the viability of JAWS II cells. JAWS II cells were unable to migrate. They were also unable to produce the same cytokine signature that had been observed in primary BMDCS upon leptin and LPS treatment. Leptin and LPS treatment of the cells did not enhance the ability of JAWS II cells to activate T-cells. JAWS II cells also did not express a change in phosphorylation of AKT indicating that there was not a significant change in PI3K signaling. Primary BMDCs will therefore be used for all future studies.

Chapter 3:

Morphological Changes Produced in Primary DCs upon Leptin Treatment

3.1 Introduction

Preliminary data suggests that treating primary DCs with leptin causes changes in DC morphology. These changes occur in the length and amount of lamellipodia on the DCs as well as actin staining. Leptin is able to signal through several kinases that are able to induce growth and changes in actin polymerization that could be leading to these morphological changes. Further studies are needed to verify and quantify the morphological changes occurring upon leptin treatment.

3.2 Methods

Cell culture

Balb/c primary bone marrow cells were obtained from Astarte. The cells thawed, washed in RPMI and resuspended at 1 vial/4 mL of RPMI containing 10% FBS and 10ng/mL of GMCSF and IL-4. A vial contains 5-10 million cells but a large amount of these cells are lost during freezing and thawing. The cells are split every two days for 6 days. On day 7, the cells were purified for CD11c using an Automacs cell purifier. The cells were centrifuged at 1200 rpm for 10 min. The supernant was replaced with Automacs running buffer at a concentration of 10^8 cells/ 4 mL. 100 µL of CD11c Automacs beads were added to the cells. The cells were incubate with the beads for 20 min at 4° C. The cells were washed then passed through the Automacs cell purifier.

Flow Cytometry

Flow cytometry was used to measure the percentage of cells expressing CD11c and MHC class II. Cells were collected just after being thawed, on day 7 before being purified for CD11c, and after being purified for CD11c. The cells were washed in FACS buffer, and blocked in mouse serum for 20 minutes. Biotin conjugated antibodies for CD11c and MHC Class II were added to the cells for 1 hour at 4° C. Cells were then washed in FACs buffer and stained with PE-Cy5 conjugated streptavidin for 20 minutes in the dark, at 4° C. The cells were washed once again in FACs buffer then preserved using 1% PFA. Fluorescence was measured using Cytomic FC 500 System.

Light Microscopy

Balb/c BMDCs were grown as previously described for 7 days. Once cells were purified for CD11c, they were treated for 24 hours under the following conditions:

1) Untreated
2) LPS (10 µg/mL)
3) Leptin (160 ng/mL)
4) LPS (10 µg/mL)+ Leptin (160 ng/mL)
5) Untreated + Ly294002 (10 µM)
6) LPS (10 µg/mL) + Ly294002 (10 µM)
7) Leptin (160 ng/mL) + Ly294002 (10 µM)
8) LPS (10 µg/mL) + Leptin (160 ng/mL) + ly294002 (10 µM)

Cells were then visualized under 40X magnification using a Leica microscope with a filter.

Confocal Microscopy

Balb/c DCs were cultured and for 7 days as previously described. The cells were then treated for 24 hours as previously described. After the cells were treated for 24 hours, they were placed into a 96 well imaging plate (BD) in quadruplicates. The plate was centrifuged for 5 min at 400 rpm to adhere cells to the plate. The cells were then fixed, permeabilized, and stained with rhodamine-labelled phalloidin and DAPI as previously described. Cells were visualized using an LSM 700 Confocal Microscope (Zeiss) at 20X magnification and analyzed with Zen 2009 software.

3.3 Results

Expression of MHC II and CD11c on Balb/c DCs

Expression of MHC II and CD11c of Balb/c DCs were analyzed via flow cytometry. The cells were analyzed before being pushed to DCs, prior to purification and after purification. Prior to being pushed, the cells are considered bone marrow cells (BMCs); BMCs were found to express MHC II on 29.5% of cells and CD11c on 22.2% of cells. The cells were analyzed before and after the purification for CD11c was performed. Prior to purification the cells expressed MHC II on 21.9% of cells and CD11c on 55.5% of cells, but once purified, the cells only expressed MHC II on 15.9% of cells and CD11c on 34.9% of cells. A lower percentage of cells were found to express CD11c post purification even though the cells were being purified for CD11c. This may be due to the cells being highly sensitive.

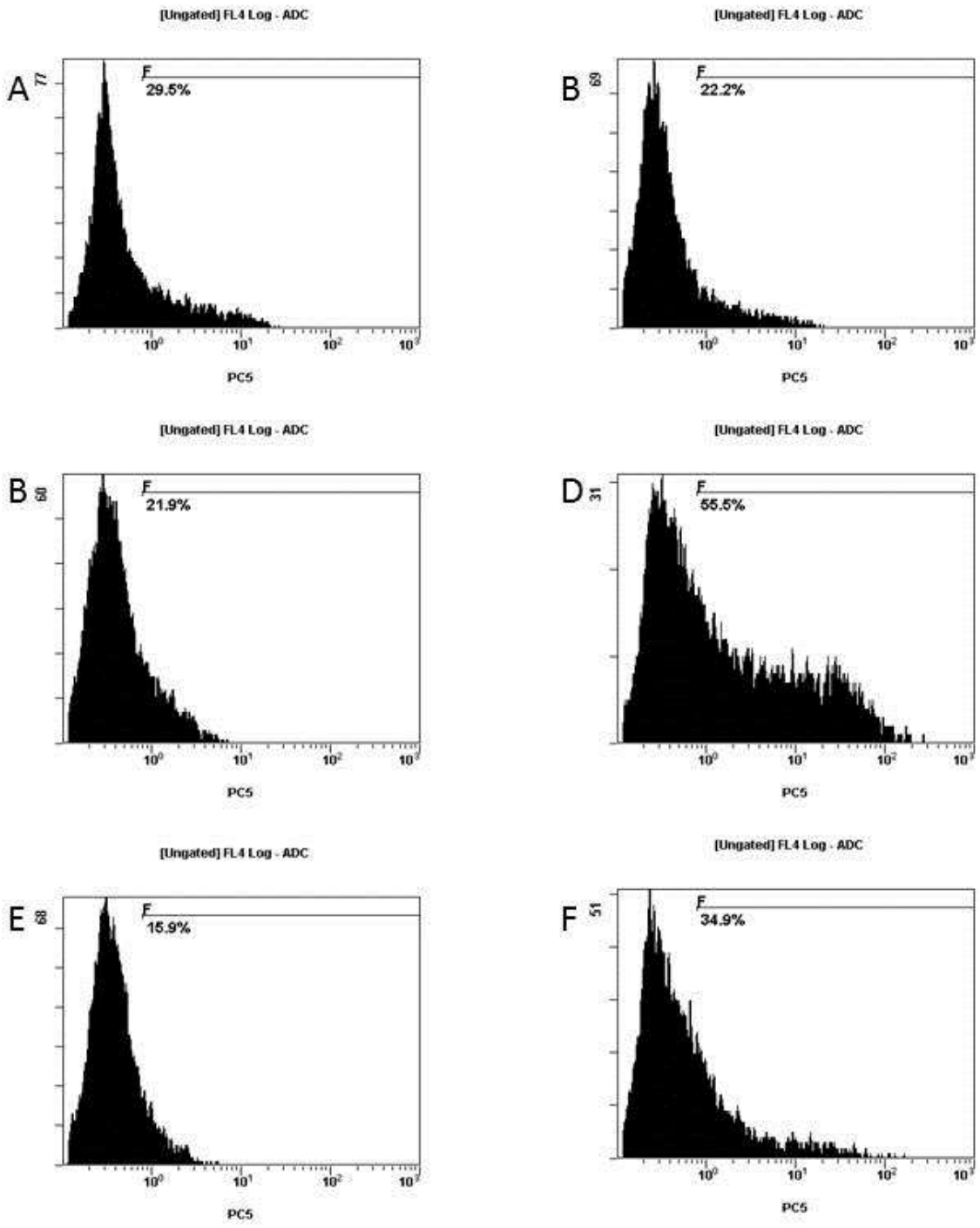

Figure 13: MHC II and CD11c expression in Balb/c bone marrow cells, unpurified DCs, and purified DCs. Panel A) shows the expression of MHC II in BMCs, B) CD11c in BMCs, C)MHC II in unpurified DCS, D) CD11c in unpurified DCs, E)MHC II in purified DCs, F) CD11c in Purified DCs. MHC II is expressed on a low percentage of these cells. CD11c is expressed on the highest percentage of cells before purification.

Balb/c DC morphology

Previous data from the lab shows that BM DCs obtained from C57/Bl6 mice undergo morphological changes upon treatment with leptin and LPS; these changes include longer dentrites and a greater number of dendrites per cell. DCs from Balb/c mice were visualized for similar changes upon leptin and LPS treatment. There did not appear to be a significant change in the length or number of dendrites in the DCs upon Leptin and LPS treatment compared to cells treated with Leptin or LPS alone. The morphological changes observed in C57/bl6 DCs were therefore not observed in Balb/C DCs.

PI3K is the pathway hypothesized to be responsible for leptin induced morphological changes; therefore, cells were observed in the presence of PI3K inhibitor Ly294002. When the inhibitor was added the cells, there was a loss of dendrites in all of the treatments. The inhibitor also seemed to induce cell death since a large number of apoptotic bodies were observed. To verify whether this loss of morphology is due to reduced PI3K signaling or whether it is being caused by the cells initiating apoptosis, actin localization was observed.

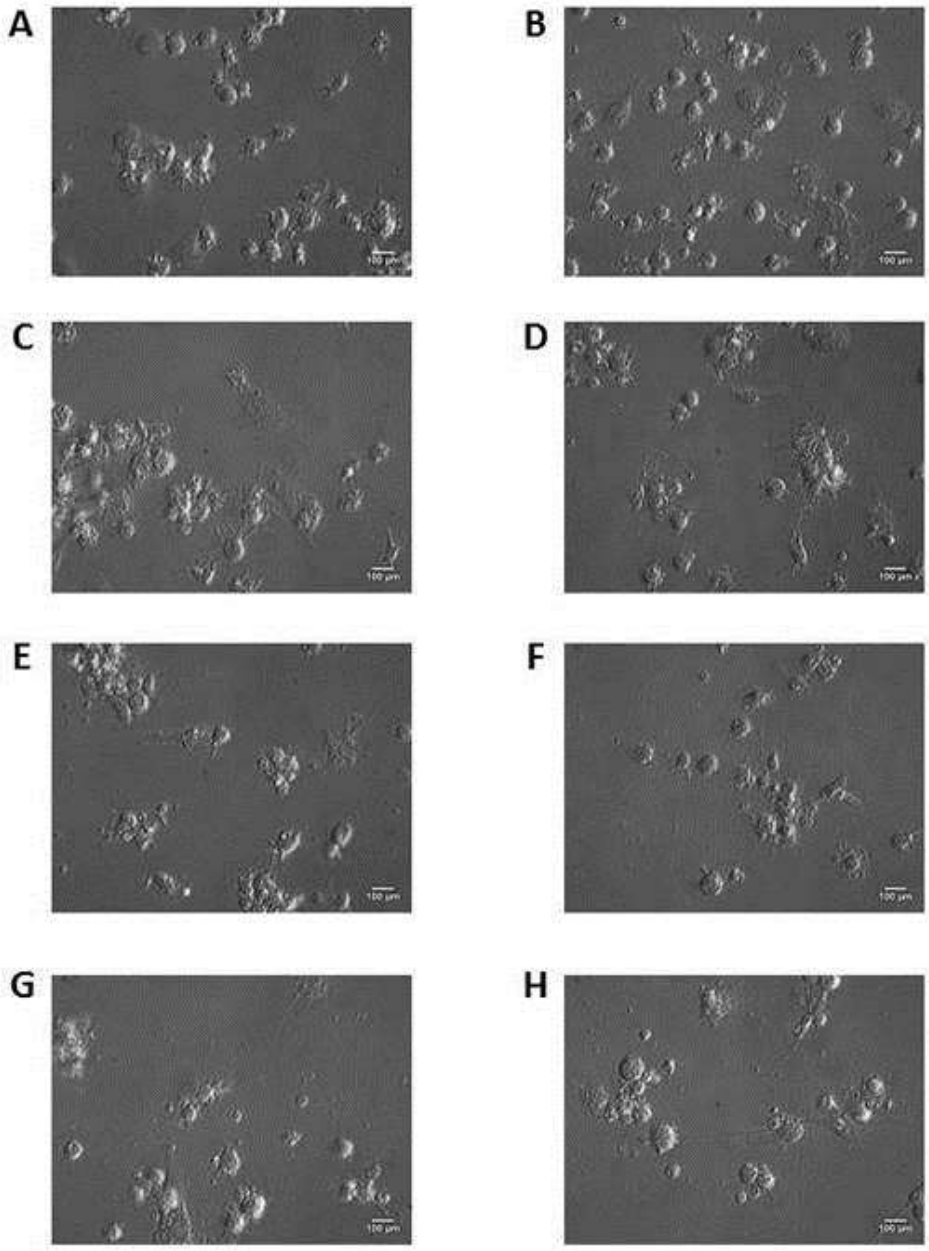

Figure 14: Balb/c DCs do not exhibit significant morphological changes upon Leptin treatment. Light microscopy images were taken of Balb/c DCs treated with A) media, B) Leptin, C) LPS, D) L+L, E) Media + Ly294002, F) Leptin + Ly294002, G) LPS + Ly294002, and H) L+L + Ly294002. Images A-D show the formation of dendrites under all treatments with no significant difference in length nor quantity. Images E-H show that PI3K inhibition is able to reduce the number of dendrites in all of the cell treatments.

Treatment with Leptin and LPS leads to increased staining for F-actin DCs

Primary BMDCs from Balb/c mice were used to visualize changes in F-actin staining upon Leptin treatment. Rhodamine labeled phalloidin was used to stain for F-actin. Mean intensities were measured for each 18 cells from each treatment. Treatment with LPS induces an increase in F actin staining compared to untreated cells. Leptin alone does not cause a significant change in F actin compared to media alone. When co-treatment of leptin with LPS was added to the cells, there was an increase in staining for F-actin. Leptin alone is therefore unable to enhance F-actin staining in DCs but when it is added in the presence of LPS it is able to significantly enhance the effects of LPS.

PI3K inhibition inhibits the observed morphological changes in DCs upon Leptin and LPS treatment

PI3K inhibitor Ly294002 was used to test our hypothesis that PI3K is responsible for the morphological changes occurring upon leptin and LPS treatment. As previously stated, when DCs are treated with Leptin and LPS Leptin there is an increase in staining for F-actin compared to untreated cells as well as cells treated with LPS or leptin individually. When PI3K was inhibited in these cells, the mean intensity of F actin staining reverted to the values observed in cells treated with LPS alone. Furthermore, there was no change in the F-actin staining for any of the other treatments.

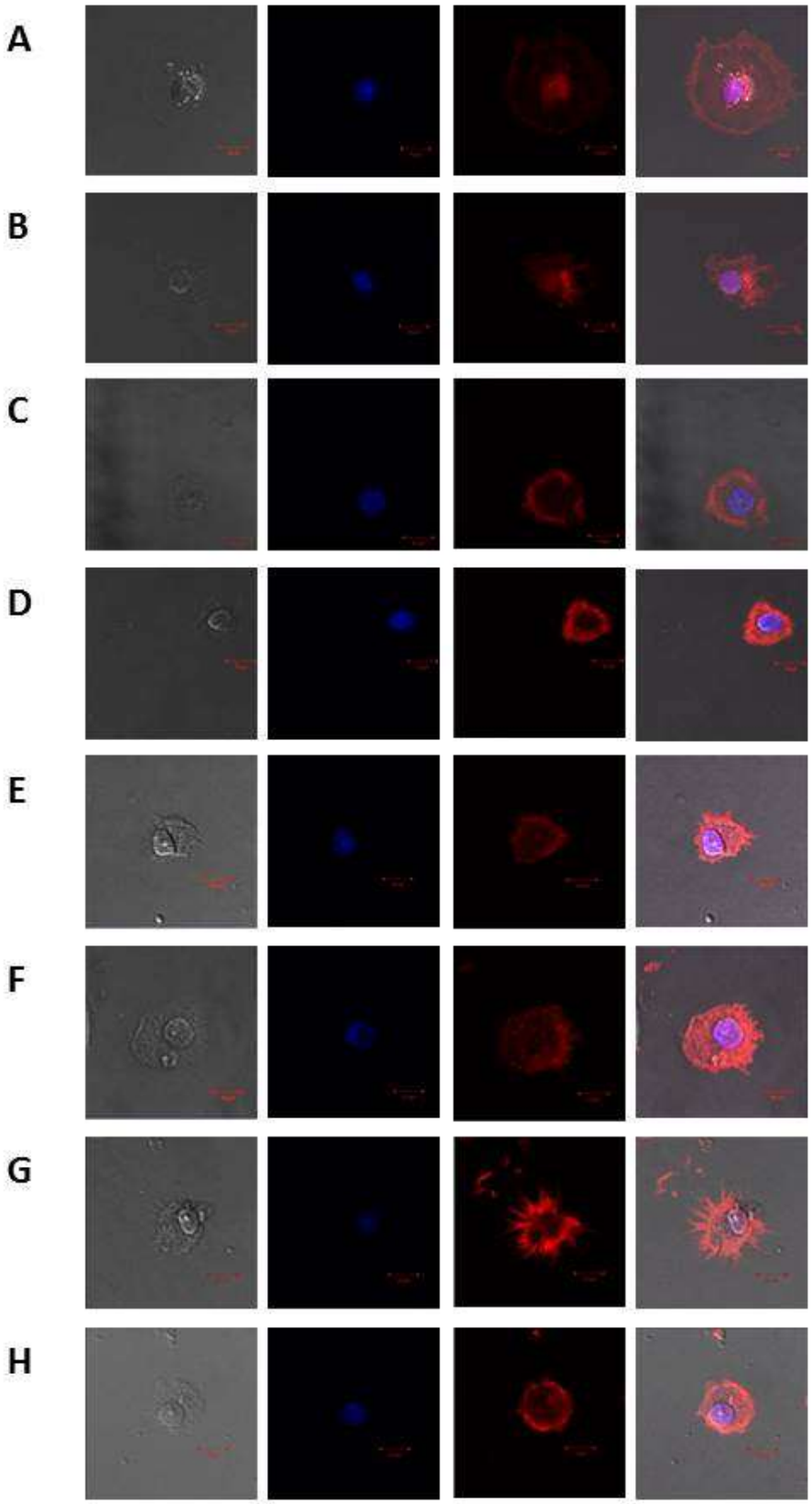

Figure 15: Treatment with Leptin and LPS leads to increased F-actin staining in DCs. Cells were treated with A) media, B) Media+ Ly294002 (10 μM), C) LPS (10 μg/mL) D) LPS (10 μg/mL) + Ly294002 (μM), E) Leptin (160 ng/mL), F) Leptin (160 ng/mL) + Ly294002 (μM), G) Leptin (160 ng/mL) + LPS (10 μg/mL), H) Leptin (160 ng/mL) + LPS (10 μg/mL) + Ly294002 (10 μM). Panel G shows that when cells are treated with L+L there is increased staining for F-actin. This increased staining is lost when PI3K is inhibited as shown in panel H. Treatment with LPS, leptin, and L+L also cause actin to become localized around the cell border (panels C-H). In untreated cells (panels A-B) higher staining of F-actin occurs near the cells' nucleus.

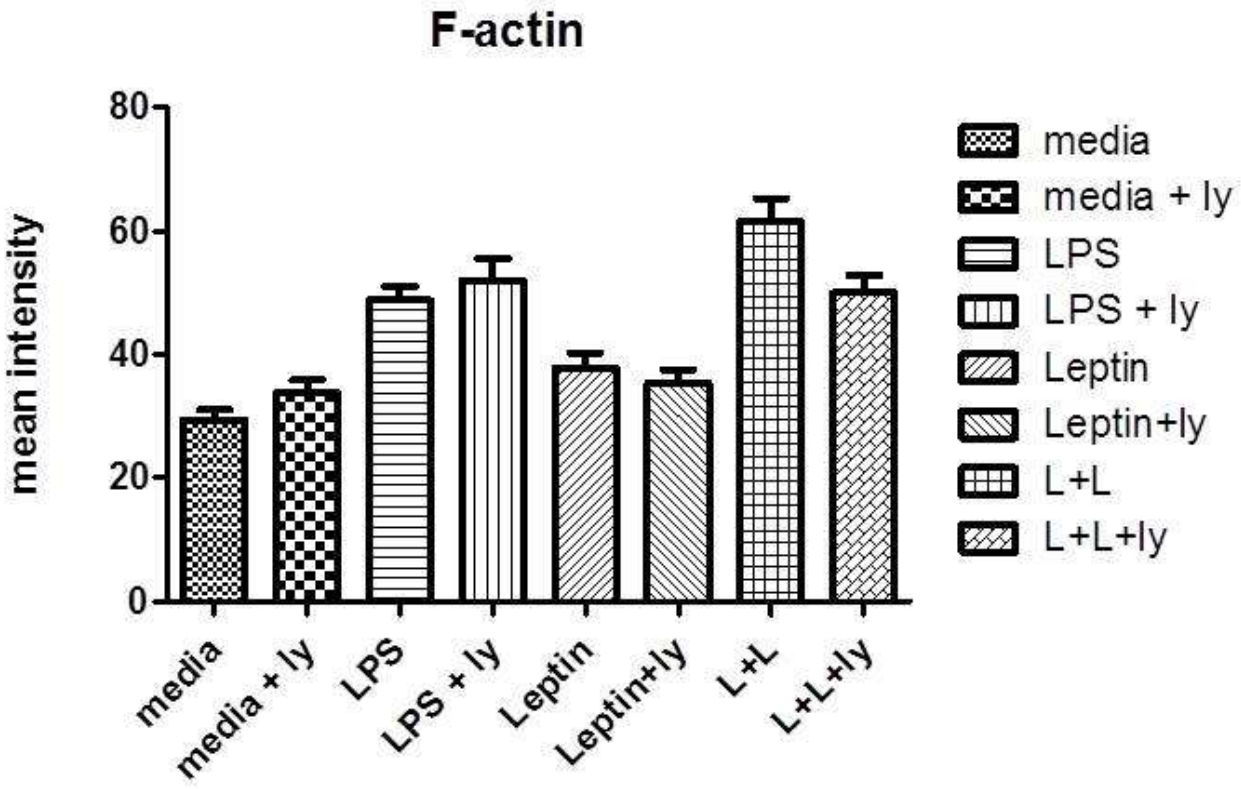

Figure 16: Inhibition of PI3K in L+L treated cells causes loss of increased staining. Treating cells with L+L leads to increased staining for F-actin compared to media (p= 8.42929E-09), LPS (p = 0.012610586), and leptin (p = 1.69897E-05). When PI3K inhibitor Ly294002 was added to L+L treated cells, increased staining was lost (p = 0.033082118).

3.4 Conclusion

Balb/c DCs are able to show increased staining for F-actin upon treatment with LPS and leptin. This increase is significant compared to untreated cells and cells treated with LPS or leptin alone. Leptin alone is unable to cause an increase in F-actin staining compared to untreated cells. Leptin may therefore only be able to enhance F-actin polymerization induced by TLR signaling but may not be capable of doing so on its own. We hypothesized that the observed changes were being induced by PI3K signaling through RAC1. We therefore observed cells treated with a PI3K inhibitor; enhanced F-actin staining in L+L treated cells was lost while none of the other treatments showed staining differences as a result of PI3K inhibition. The loss of enhanced staining supports our hypothesis that PI3K signaling is inducing these changes but further signaling analysis is required to verify this hypothesis.

Chapter 4

Analysis of PI3K Signaling in DCs upon Leptin Treatment

4.1 Introduction

Preliminary data in the lab suggests that leptin can induce morphological changes in dendritic cells. These morphological changes lead to increased migration and activation of T cells by the DCs when the DCs are treated with leptin in the presence of a TLR ligand. We hypothesized that PI3K signaling is responsible for the changes observed. PI3K has been found to play a role in cell proliferation and motility in response to growth factors and chemotactic agents (52). PI3K products are essential for pseudopodia extensions and cell polarity allowing for cytoskeletal and membrane reorganization leading to cell migration (39). P110a subunit has specifically been found to play a role in membrane ruffle formation and vesicular trafficking (39). Production of PI3,4,5-P3 accompanies changes in actin polymerization in neutrophils and changes in actin cytoskeletal structure occur during mitogenesis (52). These cytoskeletal changes are mediated in part by PI-4,5-P2, which has been demonstrated to bind to profiling, gelsolin, and villin, and therefore promote actin polymerization (52). Actin, a major cytoskeletal protein can cause cell motility and projection formations though its rearrangement. T cell activation requires cell-cell interactions between T cells and DCs, structural reorganization, including membrane and cytoskeletal movement, the contact point between T cells and DCs has been found to have high concentrations of PI(3,4,5)P3. The PI3K signaling pathway has been identified as the pathway responsible for dendrite growth and formation in neuronal cells (42). We predicted that the actin rearrangements inducing dendrite grow in neuraonal cells could be similar to the changes that occur in dendritic cells. Furthermore, Leptin has been found to activate PI3K in neuronal cells when expressed in obese concentrations. Due to these abilities of PI3K to alter a cell's cytoskeleton, we believe the changes in dendritic cells observed by leptin exposure are being caused by PI3K signaling.

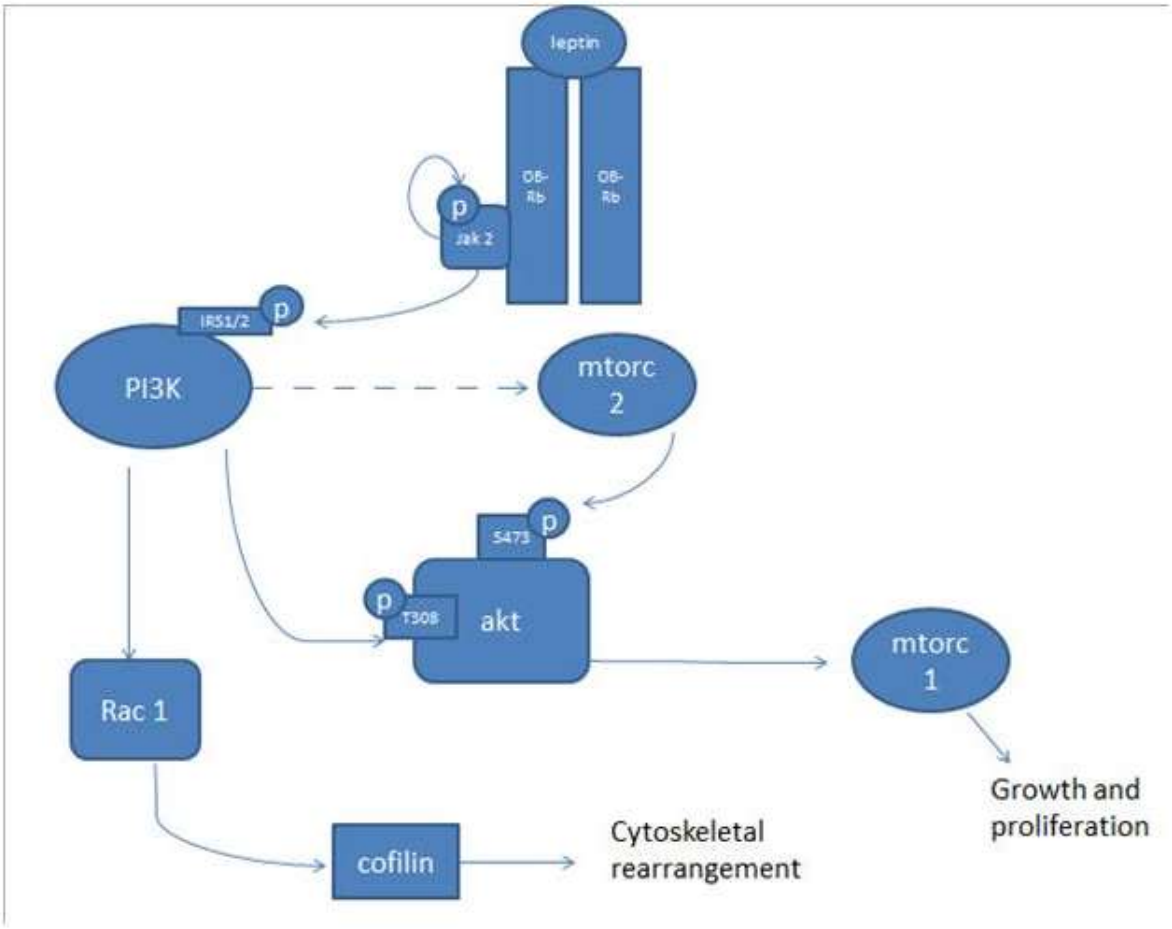

Figure 17. Pathway proposed to be inducing cytoskeletal changes observed upon treatment with leptin and LPS: DCs treated with leptin and LPS have shown increased staining for F-actin. We have proposed that these changes are occurring as a result of increased PI3K signaling. PI3K is able to signal through Rac 1, causing cofilin dephosphorylation, which leads to F-actin polymerization and cytoskeletal rearrangements.

PI3K and MAPK/ERK are both kinase signaling pathways that have been found to be activated upon leptin treatment. PI3K is able to induce cell growth through mTOR signaling and induce actin polymerization through Rhoa/Rock signaling. PI3K signaling through mTOR and MAPK/ERK have been found to induce growth and branching of dendrites in neuronal cells [42]. We believe that Leptin's ability to activate PI3K and MAPk/ERK is inducing the growth of lamellipodia in dendritic cells.

A PI3K inhibitor was used to verify that the morphological changes observed were being induced by increased PI3K signaling. Pharmacological inhibitor Ly294002, a morpholine derivative of quercetin[50, 51], is an inhibitor of PI3K. LY294002 inhibits PI3K via competitive inhibition of an ATP binding site on the p85a subunit[51]. By treating primary DCs with Ly294002 we will have an in vitro model of PI3K inhibition thereby allowing us to inhibit the effects caused by Leptin on PI3K signaling. Ly294002 has been found to effectively inhibit PI3K by competitively inhibition of an ATP binding site on PI3K p85 subunit. The p85 subunit is a regulatory subunit of PI3K and its inhibition should therefore inhibit PI3K function.

4.2 Methods

Cell Culture

Balb/C Bone marrow cells were obtained from Astarte at a concentration of 5-10 cells per vial. The vial was stored in liquid nitrogen until used. The cells were thawed in 9 mL of MEM-α and centrifuged at 1000 rpm for 10 min. The cells were then resuspended in 4 mL of MEM-α containing 10% FBS with 10ng/mL of GMCSF and 10 ng/mL of Il-4 and plated in 1 well of a 6 well plate. The cells were split every 2 days for 8 days. On Day 8, the cells were serum starved for 30 min in PBS at 37° C. The cells were then incubated for 10 minutes in the presence of AKT inhibitor Ly294002. After inhibition was allowed to occur, the cells were resuspended in media containing the following treatments for either either 10 or 20 min.

1) Untreated
2) LPS (10 µg/mL)
3) Leptin (160 ng/mL)
4) LPS (10 µg/mL)+ Leptin (160 ng/mL)
5) Untreated + Ly294002 (10 µM)
6) LPS (10 µg/mL) + Ly294002 (10 µM)
7) Leptin (160 ng/mL) + Ly294002 (10 µM)
8) LPS (10 µg/mL) + Leptin (160 ng/mL) + ly294002 (10 µM)

Cell Lysates

DCs were lysed using Cell lysis buffer (cell signaling). After cells were treated, they were collected and centrifuged at 1200 rpm for 10 min. The supernatants were discarded and the cells were washed in PBS. 400 µL of cell lysis buffer containing complete protease inhibitor tablet were added to each of the treatments. The cells were incubated on ice for 10 minutes with continuous shaking. The cells were then vortexed vigorously and stored at -20° c until needed. Protein was quantified using a BCA kit (Pierce) as previously described.

Detection of AKT and pT308 AKT by ELISA

A PathScan total Akt1 Sandwich ELISA kit and a p-S473 AKT ELISA were obtained from cell signaling. Cell lysates were diluted in the cell diluent and added to the precoated microplate wells. The lysates were incubated in the wells overnight at 4° C. The plates were then washed 4 times in wash buffer (1X PBS, .05% tween). The Detection Antibody was then added to the plate and the plate was incubated for 1 hour at 37° C. The plate was washed 4 times in wash buffer. The HRP-linked secondary antibody was then added to the plate and incubated for 30 min at 37°C. The plate was washed 4 more times with wash buffer. TMB substrate was added to each well and the plate was

incubated at 37° C for 10 min. Once a color change had occurred, a stop solution was added to the wells and the plate was read at 450 nM.

Detection of AKT and PT308 Akt by Flow-cytometry

Cells were cultured as previously described for 6 day. On day 6 the cells were enriched for CD11c using the Automax cell purifier. The cells were then serum starved overnight in RPMI. On day 7, the cells were treated as previously described. The cells were then centrifuged at 1200 rpm for 5 min and washed in FACS buffer. The cells were preserved in 1% PFA for 10 min at room temperature. Ice cold methanol was used to perforate the cells. The methanol was added to the cells until a concentration of 90% methanol was reach. The cells were allowed to perforate for 30 min at room temperature. The cells were then washed in FACS buffer and centrifuged. To prevent unspecific binding, the cells were blocked in 5% BSA in PBS. The cells were double stained using a PE-conjugate pT308 Akt (BD) antibody and a total Akt antibody (cell signaling) with a FITC anti-rabbit IgG secondary antibody (Santa Cruz). Cells were also double stained for p38 MAPK and p38 pMAPK using a Biotin conjugated p38 pMAPK (cell signaling) antibody with a PE-Cy5 conjugated Streptavidin antibody (BD) and a p38 MAPK (cell signaling) antibody with a FITC anti-rabbit IgG secondary antibody (Santa Cruz). Fluorescence was measured using Cytomic FC 500 System.

4.3 Results

Changes in Akt phosphorylation in Balb/c DCs are not detectable by ELISA

Cell signaling ELISA kits were used to measure changes in Akt phosphorylation upon treatment with leptin, LPS, L+L, and PI3K inhibitor Ly294002. There were no significant changes in the amount of total Akt or pT308 Akt among any of the treatments (figure 17). Cells were analyzed after 10, 20 and 30 minute treatments; figure 17 represents the results after 30 minutes of treatment. These ELISA kits had not been successfully used to detect Akt phosphorylation in leukocytes, and may therefore not be sufficiently sensitive to detect changes in Balb/c DCs.

Balb/c DCs do not express significant changes in Akt or MAPK phosphorylation upon Leptin treatment.

Balb/c DCs were serum starved overnight then treated with LPS, leptin, L+L, and Ly294002 for 30 minutes. The cells were then stained and analyzed by flow cytometry. Cells were analyzed for the percentage of cells expressing the protein and for the mean intensity of that expression. There were no significant changes among any of the treatments when Akt and pAkt expression were analyzed. Furthermore, Ly294002 did not successfully inhibit Akt phosphorylation. MAPK expression showed a significant increase in expression of total MAPK upon treatment with LPS, Leptin, and L+L compared to untreated cells. There was however not a significant change between Leptin or L+L vs. LPS treated cells.

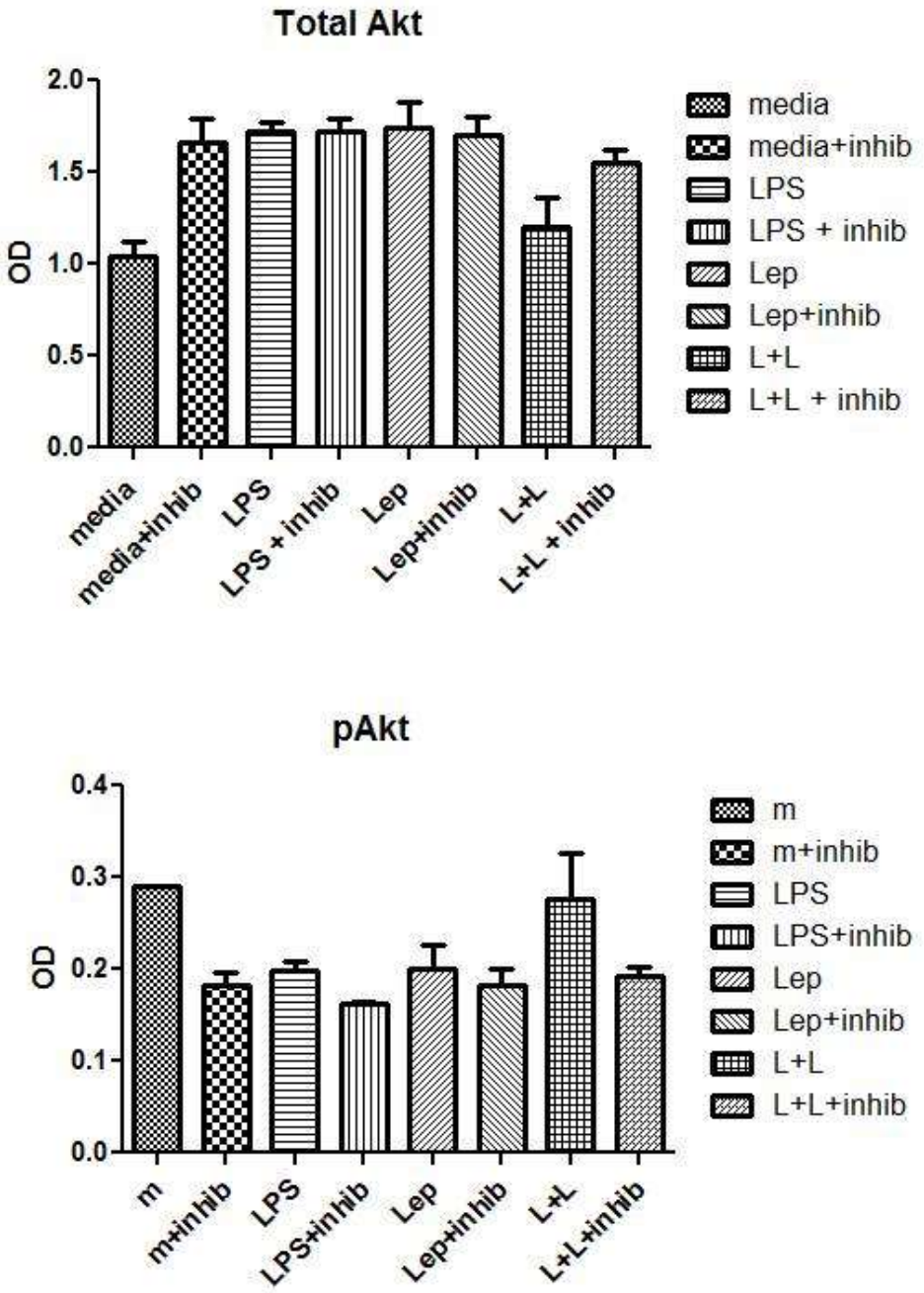

Figure 18: Changes in Akt phosphorylation in DCs are not detectable by ELISA: ELISA kits for Akt1 and pT308 Akt were used to measure changes in PI3K signaling upon treatment with LPS, Leptin, or L+L. There were no significant changes in phosphorylation in any of the treatments. Furthermore, there were no significant changes when PI3K inhibitor Ly294002 was added.

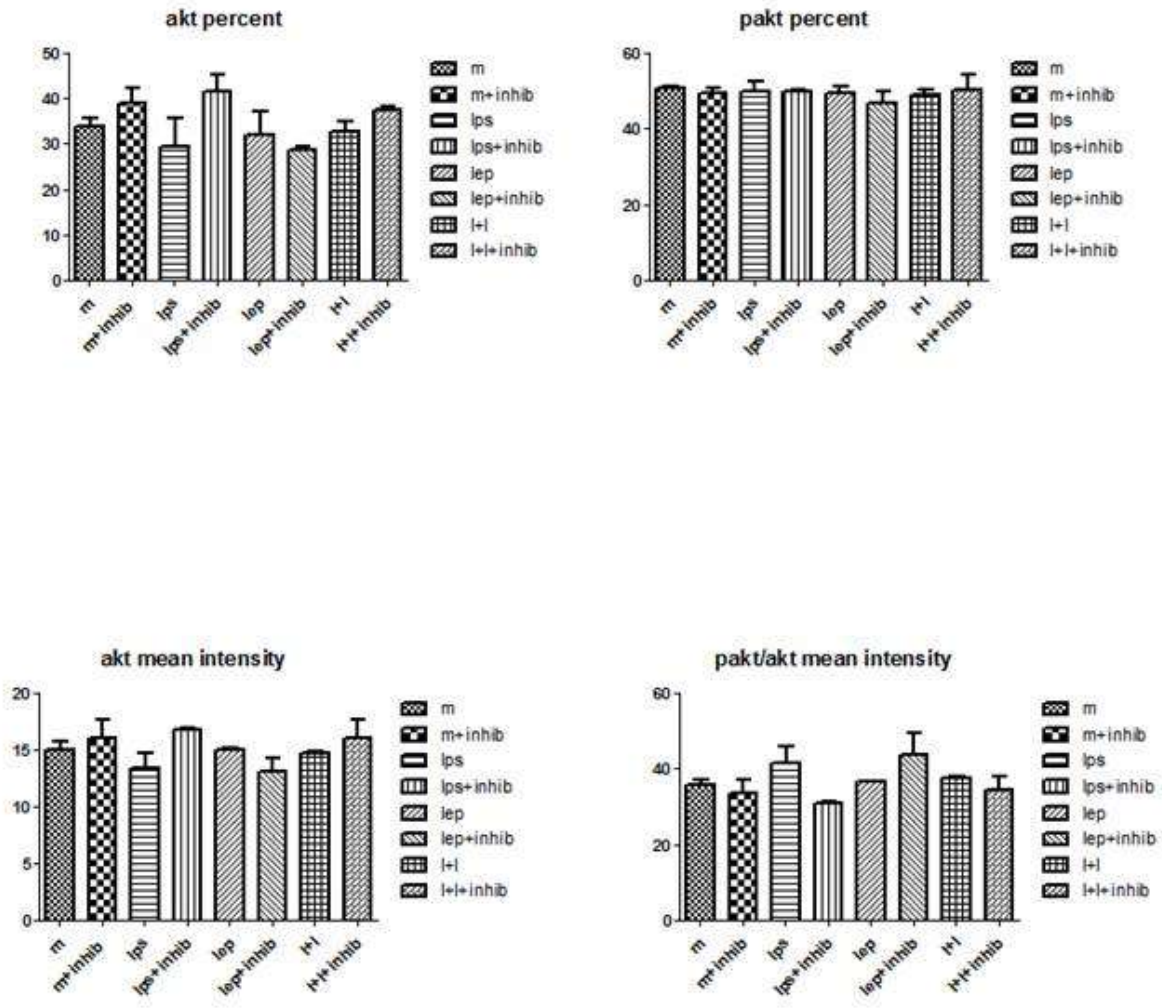

Figure 19: Balb/c DCs do not exhibit significant changes in Akt expression or Akt phosphorylation upon leptin treatment. Balb/c DCs were serum starved over night then treated with Leptin, LPS, L+L, and Ly294002 for 30 minutes. No significant changes were observed in total Akt expression, Akt phosphorylation, or percentage of cells containing Akt or pAkt.

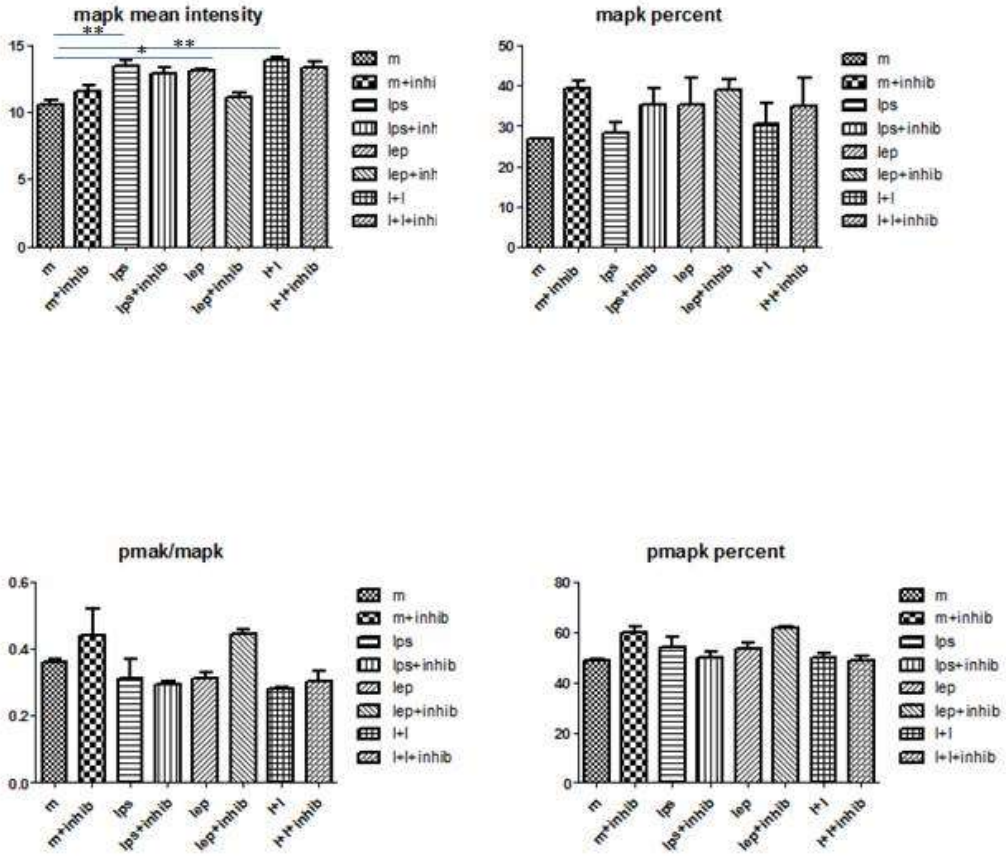

Figure 20: Balb/c DCs do not exhibit significant changes in MAPK expression or phosphorylation upon leptin treatment. DCs were treated serum starved over night then treated with LPS, Leptin, L+L, and Ly294002 for 30 minutes. While treatment with both leptin alone and L+L were able to increase the expression of total Akt compared to untreated cells, there was no significant difference compared to LPS treatment.

4.4 Conclusion

Although we were able to show that inhibiting PI3K causes loss of enhanced staining for actin in Balb/c DCs treated with Leptin and LPS, we were unable to show any changes in PI3K signaling via Akt phosphorylation. PI3K inhibitor Ly294002 was also unable to reduce the amount of Akt being phosphorylated. We can therefore conclude that there is not a significant change in PI3K signaling through Akt by 30 minutes, although changes may be observed at a later time point. Further analysis is required using a longer time course. We can also conclude that Ly294002 was not used at a sufficient quantity to inhibit PI3K in the time allotted and further work is required to determine an effective amount. Signaling changes were also observed in MAPK signaling; total MAPK expression was shown to increase upon treatment with LPS, leptin, or L+L compared to untreated cells but leptin was not able to enhance expression compared to LPS alone. Although there was an increase in total MAPK there was not an increase in the amount of MAPK being phosphorylated among any of the treatments.

Chapter 5

Discussion

Obesity is an increasing problem in this country with many associated health risks. Because obesity creates a state of chronic inflammation, it can induce immune dysfunction. Part of this immune dysfunction arises from the increased number of adipokines produced by adipose tissue. Leptin, an adipokine whose primary function is satiety, is one of such adipokines contributing to immune dysfunction in obesity. Leptin is secreted in direct correlation to the amount of one's adipose tissue leading to its overproduction in obesity. This excess has been found to enhance the function of macrophages, NK cells, b cells, and T cells. In the current study we evaluated the morphological changes induced by obese concentrations of leptin in DCs while and assessed the signaling pathways which may be involved in producing these changes.

DCs are critical members of the immune system due to their ability to bridge innate and adaptive immune functions. Research involving DCs in obesity, however, is limited. Part of the obstacle in assessing DCs involves difficulty in culturing DCs in-vivo. To attempt to overcome this obstacle, we characterized the effects of Leptin on JAWS II cells. JAWS II, an immortalized DC cell line from C57/Bl6 mice, has been used in tumor and pathogen studies (Jiang), but the effects of leptin treatment had not been characterized. We assessed the effects of Leptin on JAWS II cells through changes in morphology, viability, migration, cytokine production, and ability to activate T-cells. These results were compared to preliminary data collected from primary C57/Bl6 DCs. JAWS II cells did not demonstrate any of the changes observed in the primary cells and were therefore determined to not be a good model for Leptin studies.

Although JAWS II cells were shown to express a leptin receptor they were unable to reproduce any of the changes observed in primary cells. JAWS II is an immature DC cell line; although the cells

were pushed with GMCSF, they never showed a mature phenotype. This inability of the cells to mature could be responsible for the lack of changes. One major characteristic of mature DCs not observed in JAWS II cells was dendrites. JAWS II cells lacked dendrites, an important component of DCs. This lack of dendrites may be responsible for the cells inability to migrate. The morphological changes in dendrites observed in primary C57/Bl6 DCs could be responsible for the increased activation of T cells that was observed and therefore explain why this phenomenon was not observed in JAWS II cells.

Due to JAWS II not being a usable source of DCs, primary DCs from Balb/c mice were used for the remainder of these studies. While C57/Bl6 demonstrated more dendrites on Leptin and LPS treated cells, we were unable to reproduce this phenotype in Balb/c DCs. There were, however, morphological changes observed in the amount and localization of actin in these cells. When Balb/c DCs were treated with leptin and LPS, there was an increase in staining for F-actin. This actin was found to be localized in the dendrites of the cells so although there weren't an increased number of dendrites, there was a higher amount of F-actin in the dendrites of Leptin and LPS treated cells.

The pathway we were attributing to these changes is the PI3K pathway. Leptin has the ability to signal though the PI3K, MAPK, NFκB, and JAK2 pathways. Signaling in the PI3K pathway is important for cell growth and survival. We hypothesized that the morphological changes observed in primary DCs were occurring as a result of increased PI3K signaling upon treatment with leptin and LPS. We therefore treated cells with Ly294002, a PI3K inhibitor, and observed the changes that occurred in cell morphology. We found that when PI3K was inhibited in the cells, they lacked dendrites and there were a large percentage of apoptotic cells. This loss of dendrites could have been a result of the cells preparing for apoptosis. Since there was a loss of dendrites in all the cells, we also observed staining for F-actin in DCs in the presence of the PI3K inhibitor. We found that when DCs are treated with Leptin and LPS in the presence of a PI3K inhibitor, the increased staining for F-actin that was previously

observed was lost and the F-actin staining reverted to the level found in cells treated with LPS alone. Furthermore, there was not a change in actin staining in the presence of the inhibitor with any of the other treatments. These results indicate that leptin must be in the presence of a TLR ligand to induce morphological changes through PI3K signaling.

The enhanced morphology observed in DCS treated with Leptin and LPS can be contributing to the immune dysfunction observed in obesity since a characteristic of obesity is increased levels of circulating LPS (51). LPS has actually been found to increase sufficiently to activate monocytes and endothelial cells after a high fat meal. These increased levels of LPS can be attributed to gut flora which respond to diet and can contribute to weight and metabolic regulation (51).

Since inhibition of PI3K caused a loss of enhanced F-actin staining we wanted to see if there was an increase in PI3K signaling in Balb/c DCs. PI3K signaling was analyzed by ELISA and flow cytometry for Akt phosphorylation. No significant changes in phosphorylation were observed among any of the treatments and Ly294002 was unable to cause a decrease in phosphorylation. The level of phosphorylation was only measured up to 30 minutes after treatment and the images showing enhanced staining were taken after 24 hour treatment. The changes in phosphorylation may be occurring and a slower rate than anticipated which could attribute to the lack of increased signaling observed. The loss of F-actin staining which was observed upon PI3K inhibition may be attributed to the high percentage of apoptotic cells present after 24 hour treatment with Ly294002. Further studies including apoptosis assays are required to determine the why a loss of staining was observed. Further studies are also required to determine the time point at which Ly294002 is effective and its optimal concentration as well as the time point at which PI3K signaling is enhanced, if at all. There is also a possibility that PI3K signaling to Akt may not be enhanced. The proposed pathway for the morphological changes observed

involved signaling through RAC 1 which is in a pathway independent of Akt. Further studies are required to determine if there are any changes in RAC 1 signaling downstream of PI3K.

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Glossary

Ab	Antibody
Akt/PKB	Protein Kinase B
APC	Antigen presenting cells
BM	Bone marrow
BMC	Bone marrow cells
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CCR5	Chemokine C-C motif receptor 5
CCR7	Chemokine C-C Motif Receptor 7
CCL19	Chemokine C-C Motif Ligand 19
CD	Cluster of Differentiation
Con A	Concanavilin A
CO_2	Carbon dioxide
CPM	Counts per minute
DAPI	Diamidino-2 penylindole dihydrochloride
DC	Dendritic cell
EDTA	Ethleendiamine-tetraacetic acid
ELISA	Enzyme-linked immunoabsorbent assay
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
^3H	Tritium

HBSS	Hank's Balanced Salt Solution
HRP	Horse radish peroxidase
IFNγ	Interferon gamma
IL-12	Interleukin-12
JAK	Janus Kinase
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen-Activated Protein kinase
MHC	Major Histocompatability Complex
ml	Milliliter
mM	Millimolar
MPER	Mammalian protein extraction reagent
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MΦ	Macrophage
MEM-α	Minimum essential medium alpha
μg	Microgram
μl	Microliter
μM	Micromolar
ND	Not detectible
ng	Nanogram
nM	Nanomolar
NMS	Normal mouse serum

Ova	Ovalbumin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PE-Cy5	Phycoerythrin Cyanine 5
Pen/Strep	Penicillin and streptomycin
PFA	Paraformaldehyde
PVDF	Polyvinylidene Fluoride
RPM	Revolutions per Minute
RPMI	Roswell Park Memorial Institute Media
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS3	Suppressor of Cytokine Signaling 3
STAT	Signal Transducers and Activators of Transcription
T reg	Regulatory T cell
TCR	T cell receptor
T_H1	T helper 1
T_H2	T helper 2
TLR	Toll like receptor
TNFα	Tumor Necrosis Factor alpha

Vita

Marisol O'Neill was born in El Paso, Texas in 1988; she is the middle child of Marisol Pacheco and Peter O'Neill. Marisol received a Bachelor of Science in Biology with a Minor in Social Welfare from Syracuse University in 2011. While at Syracuse University, Marisol was part of the Distinction in Biology program and graduated Magna Cum Laude. Marisol entered the graduate program at the University of Texas at El Paso in 2011 where she conducted research in Dr. Kristine Garza's Lab. She will graduate with her Master of Science in the summer of 2013. Marisol has been accepted to the PhD program in Molecular and Cell Biology at Baylor College of Medicine in Houston, Texas.

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