The Effect Of Exogenous Leptin On Murine Dendritic Cell Morphology And Function

Christine Delgado
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THE EFFECT OF EXOGENOUS LEPTIN
ON MURINE DENDRITIC CELL MORPHOLOGY AND FUNCTION

CHRISTINE DELGADO

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DEDICATION

This thesis is dedicated to my mother and father, Rosa and Raul Delgado, who have always believed in me and encouraged me to reach for the stars. They taught me the value of a good education and hard work. I would also like to dedicate this thesis to my sisters Claudia Diaz, Annette Delgado, and Hazel Delgado for all their love, support and understanding. My family has never doubted me or my abilities; but most importantly they respected and accepted my hectic schedule and random thoughts.
THE EFFECT OF EXOGENOUS LEPTIN
ON MURINE DENDRITIC CELL MORPHOLOGY AND FUNCTION

By:

CHRISTINE DELGADO

THESIS

Presented to the Faculty of the College of Science of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER IN SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

August 2009
ACKNOWLEDGMENTS

Many people have contributed to the completion of this thesis. I first would like to thank my mentor, Dr. Kristine M. Garza, for introducing me into the world of research, pushing me forward, and constantly teaching me new techniques. With her guidance, patience and enthusiasm for teaching I have learned the ropes of research. I truly believe she has deeply impacted my life and redirected my future; without her this thesis would not be possible.

I would also like to thank Dr. Jerry Johnson for the advising and guidance during my master’s degree. Also, my sincere thanks to the members of my committee: Dr. Sukla Rowchowdhury, Dr. Malcom Cooke and Dr. Laura O’Dell for agreeing to be on my committee and for their help in trouble shooting certain aspects of my project.

Additionally, I would like to thank all the past and present Garza Lab members, in particular Oscar Ramirez, the laboratory technician and senior graduate student, for his help, training, and tips. Thanks to both Oscar and Lorena De Los Santos for long discussions and countless hours of debates and conversations. Thank you to John Reyna and Alonso Andrade for the previous work done on this project. Finally, to Amanda Cordova, Raquel Suro, Jeff Sivils, Yadira Arellano, Leo Estrada, Brenda Rodriguez and Rosario Arroyo for their companionship and laboratory assistance.

This study was funded and supported by NIH-SCORE (Grant S06-GM08012), NIH-RCMI (Grant 5G12RR008142), and the Kaufmann Foundation.
ABSTRACT

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. In this study we assessed the effect of leptin on bone marrow derived dendritic cell (BM-DC) cytoskeletal structure and functionality. Our preliminary data demonstrates that the addition of physiological concentrations of exogenous leptin to BM-DC causes cytoskeletal rearrangement specifically that of actin as evidenced by increased number of lamelopodia/dendrites and increased staining for actin within the lamelopodia/dendrite region. This suggests that leptin is a necessary component of DC – T cell interaction in that leptin may be responsible for enhanced migration of the DC to draining lymph nodes and potentiating the extent of the physical contact between these cells. We therefore hypothesized that leptin increases the number and length of dendrites in DCs by causing a re-arrangement of the cytoskeleton, specifically actin, which in turn leads to an increased capacity to migrate and to activate T cells. To test this hypothesis we used BM-DCs from C57Bl/6 (B6) mice enriched by magnetic bead cell sorting. The effect of leptin on BM-DC cytoskeletal re-arrangement was assessed by light and confocal microscopy; actin polymerization was determined by Western blot analysis for cell content of F-actin versus G-actin in fractioned cellular lysates of treated cells. The functional effects of leptin on BM-DC were evaluated in-vitro by assessing DC migration and DC ability to active antigen-specific T cells. The differentially treated BM-DC were evaluated in transwell migration assays to assess their ability to migrate towards a DC-specific chemokine CCL19. Finally, treated BM-DC ability to activate T cells was assessed in-vitro by measuring activation of an antigen-specific T cell hybridoma or of antigen-specific primary T cells (both specific to ovalbumin) as a function of IL-2 and INF-γ.
production or proliferation, respectively. We found that there is only a slight increase in actin content within the cell post treatment of leptin, however this increase is not significant. The addition of an exogenous physiological concentration of leptin to BM-DC enhanced polymerization of actin and therefore the formation of dendrites, both in number and length and thickness. This effect was particularly evident in the leptin and LPS duo treatment. Functionally the presence of leptin appears to enhance migration and promote T cell activation. This is likely due to the increased surface area, as well as dendrite number length and thickness which increase the likelihood of DC-T cell interactions, therefore potentiating the extent of the physical contact between these cells. This is further supported by enhanced T cell activation by leptin and LPS treated cells as measured by incorporation of tritiated thymidine, IL-2 and INF-γ production. The data obtained from this study suggest leptin plays an important role in immunological responses, and may specifically contribute to the pro-inflammatory state and increased susceptibility of autoimmune disorders found in obesity.
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<tr>
<td>1ºAb</td>
<td>primary antibody</td>
</tr>
<tr>
<td>2ºAb</td>
<td>secondary antibody</td>
</tr>
<tr>
<td>^3^H</td>
<td>Tritiated</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>APC</td>
<td>antigen presenting cells</td>
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<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CCR5</td>
<td>Chemokine C-C motif receptor 5</td>
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<tr>
<td>CCR7</td>
<td>Chemokine C-C Motif Receptor 7</td>
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<td>CCL19</td>
<td>Chemokine C-C Motif Ligand 19</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>Con A</td>
<td>concanavilin A</td>
</tr>
<tr>
<td>CO_2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DAPI</td>
<td>diamidino-2 penylinrole dihydrochloride</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell (s)</td>
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<tr>
<td>DIC</td>
<td>Differential Interference Contrast (microscopy)</td>
</tr>
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<td>EDTA</td>
<td>ethleendiamine-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoabsorbent assay</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>gm</td>
<td>Gram</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
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<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>hr(s)</td>
<td>hour (s)</td>
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<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
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<td>IL-2</td>
<td>interleukin-2</td>
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<tr>
<td>IL-4</td>
<td>interleukin-4</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>IMEM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
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<td>LN</td>
<td>lymph nodes</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein kinase pathway</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>min(s)</td>
<td>minute (s)</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MIP-1a</td>
<td>Macrophage Inflammatory Protein-1a</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>Macrophage Inflammatory Protein -1b</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MSB</td>
<td>Microtubule Stabilization Buffer</td>
</tr>
<tr>
<td>MΦ</td>
<td>macrophage(s)</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>ND</td>
<td>not detectible</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
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<td>NMS</td>
<td>normal mouse serum</td>
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<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>Pen/Strep</td>
<td>penicillin and streptomycin</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<td>RBC</td>
<td>red blood cells</td>
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<td>RCF</td>
<td>Relative Centrifugal Force</td>
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<td>Description</td>
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<tr>
<td>RPM</td>
<td>rounds per minute</td>
</tr>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of Cytokine Signaling 3</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
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<td>T&lt;sub&gt;H1&lt;/sub&gt;</td>
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</tr>
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<td>T&lt;sub&gt;H2&lt;/sub&gt;</td>
<td>T helper 2</td>
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<td>toll like receptor</td>
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<td>Western Blot</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>times</td>
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CHAPTER 1

INTRODUCTION

Obesity

Obesity is of immense concern for today’s health care system. Webster’s Dictionary defines obesity as “a condition characterized by the excessive accumulation and storage of fat in the body” (Webster’s Dictionary 2009). The cause for obesity is a greater caloric intake than that required for an individual’s day to day activities. The World Health Organization (WHO 2006) marks a clear difference between overweight and obesity status in adults. Overweight individuals are identified as having a body mass index [(BMI) measured Kilograms/meter$^2$] of equal to or greater than 25, while obese individuals have a body mass index of 30 or higher. It appears that these limits are not set in stone; recent studies by Dr. Chizuru Nishida (Nishida 2004) have raised concerns due to an increase in risk of chronic obesity-related disease that has been noted in individuals with BMIs as low as 21(Nishida 2004).

Initially, this increasing health concern primarily affected developed nations; but recent evidence has demonstrated that this condition is predominant in urban settings of all nations regardless of economic status (WHO 2006, Low 2009). In 2005, the World Health Organization revealed that world-wide approximately 1.6 billion individuals over the age of fifteen were overweight; and over 65% of the US population is overweight. Furthermore 400 million adults world wide have been diagnosed as obese, this includes 35% of the US population. Even more staggering, 20 million children under the age of five were declared obese in 2005 (WHO 2006, Low 2009). As time passes these numbers are expected to continue rising.
Obesity is affected by factors such as metabolic status, food intake, and energy expenditure (Freidman 1998, Matarese 2000). It carries with it a large array of risk factors and all organ systems within an organism are highly affected by excess body mass. One critical aspect of the body negatively affected by obesity is the immune system. Obesity can lead to pathological conditions such as, diabetes mellitus (type II), cardiovascular diseases and even some cancers (WHO 2006). High amounts of leptin cause blood to become highly viscous and therefore increase likelihood for strokes, aneurisms and heart attacks. Circulatory problems kill over 17 million people a year making them the number one killer for adults (WHO 2006). Diabetes (type II) was recently declared world wide epidemic. It has been linked to obesity and is expected to grow up to 50% from 2000 to 2010. Furthermore autoimmune and inflammatory diseases such as rheumatoid arthritis, and colitis, have been found to be more prevalent in patients with high circulating leptin levels. This is significant since obesity continues to be on the rise. It is estimated that 700 million adults will be obese by 2015.

Obesity is not typically diagnosed suddenly since weight gain is usually gradual. Some individuals will struggle with weight gain and loss throughout life, exponetiating the degree of difficulty in treating this disease and those it is associated with. Obesity related conditions may be manageable, studies have demonstrated a decrease in symptoms when leptin levels are lowered by fasting (Fraser 1999, Bernotiene 2004, Otero 2005). Combinations of the conditions in addition to obesity may be deadly, but luckily obesity effects are reversible by simply altering lifestyle ensuring to increase energy output and decrease food intake. However, there are a variety of factors that contribute to body composition including environmental and genetic characteristics. To add to the complexity of obesity is the various adipokines that are produced by the resulting adipose tissue that is in turn affected by the very same products (Tilg 2006,
Fat/lipid was recently controversially categorized in the endocrine system since it produces and secretes hormones that function locally and over long distances. There are a variety of hormones with varying functions that are secreted by fat cells, they include but are not limited to adiponectin, resistin, and leptin (Tilg 2006, Moschen 2006).

**Leptin**

Leptin is a hormone product of the OB gene found on human chromosome 7 and mouse chromosome 6. Although leptin is produced by the placenta, gastric mucosa, bone marrow, mammary epithelium, skeletal muscle, pituitary, hypothalamus and bones in small quantities, it is primarily produced by the white adipose tissue and is secreted in direct correlation with the content of adipose tissue (Friedman 1998, Frühbeck 2001). It is mainly found circulating in the blood, either bound to serum or freely circulating. Protein bound leptin may increase in function because of its superior ability to bind to its corresponding ligand (Sinha 1996). Concentrations of leptin vary by individual based on metabolic and nutritional state, but higher concentrations of freely circulating leptin have been observed in obese individuals (Maffei 1995). The average human leptin concentration ranges from 5 ng/ml in lean individuals to 200 ng/ml in obese individuals (Sinha 1996 and Peltz 2007). In 1997 Halas et al demonstrated that obesity produces consistently elevated leptin serum levels. He also established an association between obesity and leptin resistance (a decrease in leptin sensitivity) (Halas 1997). Leptin resistance is described as having high circulating serum leptin levels but low leptin effects (Maffei 1995, Banks 2004). One very common side effect to leptin resistance is hyperphagia, which results an increase in body size/weight gain (Maffei 1995, Banks 2004). Additionally leptin resistance causes a decrease in blood insulin levels and produces a pro-inflammatory environment.

The hormone leptin has many similarities with cytokines, it is therefore known as an
adipokine. This adipokine is a 16 kDA protein; structurally it resembles the long-chain helical class I cytokine family, but folds like the short helix cytokine family (Madej 1995). It is the product of the ob gene found by Zhang and Friedman in 1994. Leptin recognizes and ligates to the OB-R receptor (Madej 1995). This receptor resembles those of the class I cytokine family and has six different isoforms (Madej 1995). These isoforms are the product of alternative mRNA splicing of the db gene (Ghilardi, 1994, Baumann 1996 Wang 1996, Bendinelli 2000) and are short, long or secreted. Short receptor isoforms OB-Ra, OB-Rc, OB-Rd, and OB-Rf contain 30-40 cytoplasmic amino acids (aa) (Wang 1996). All short isoforms mentioned are thought to be capable of producing intracellular signaling through the MAPK pathway. The long isoform OB-Rb contains an intracellular domain of 300 aa with the necessary motifs to activate the intercellular JAK/STAT pathway (Wang 1996). Diagram 1.1 illustrates the six different receptor isoforms described above.

The MAPK signaling pathway may be used by leptin for signaling, although, the major signaling pathways used is the Janus kinases 2 (JAK2) and signal transducers and activators of transcription 3 (STAT3) (Frühbeck 2006, Lago 2008, Robertson 2008). Initially circulating leptin recognizes and binds to the leptin receptor on the cells surface causing a homodimer to form. This homodimerization of the two receptors leads to phosphorylation that causes conformational changes. The conformational change reveals the cytosolic domain of the receptor, this activates the JAK2 pathway which undergoes autophosphorylation and continues to phosphorylate the intracellular tail of the long isoform receptor at Tyrosine (Y) 985, Y1077 and Y1138. The phosphorylation of these tyrosines facilitates the recruitment of a variety of STATS, in particular the phosphorylation of Y1138 attracts STAT3. Once the STATS are activated they homodimerize and translocates to the nucleus where they induces transcription. The activation of
STAT3 leads to an increase in Suppressor of Cytokine Signaling 3 (SOCS3) expression. SOCS3 blocks leptin signaling by binding to Y985 and preventing STAT activation. Since SOCS3 is increased as STAT3 becomes activated, STAT3 controls its own negative feedback mechanism. (Frühbeck 2006, Lago 2008, Robertson 2008). Diagram 1.2 summarizes the leptin intracellular signaling process.

Leptin production is controlled by many means but one of the most significant is the presence or absence of insulin. During fasting or starvation, insulin levels decrease causing a decrease in leptin levels. Although leptin does affect a variety of cells throughout the body it exerts its main affects on the brain because it contains the largest population of LepRb expressing neurons, found in the hypothalamic nuclei. Within this population there are two sub-types of neurons, one that makes orexigenes neuropeptides agouti-related peptide and neuropeptide and the other which other makes anorexigenic pro-hormones pro-opiomelanocortin [Elmquist 1999, Schwartz 2000]. The decrease in leptin promotes hunger; once an individual eats, insulin levels increases. The marked increase in insulin concentration in the blood results in leptin production. The presence of leptin causes depolarization of the neurons and results in an increase in pro-hormone pro-opiomelanocortin. The increase in hormone production causes a signal of satiety; appetite is therefore controlled by concentrations of leptin in the blood stream and hormone production (Ganong 2005, Elmquist 1999, Schwartz 2000, Cowley 2001).

It was originally thought that leptin only controlled appetite and energy expenditure (Peltz 2007) but recent studies have shown that it affects many systems; of particular interest is the endocrine system. Leptin alters hormone production and in turn affects multiple systems such as the reproductive (Aydin 2008, Hill 2008, Schubring 1999, Cioffi 1998, Holness 1999), digestive (Friedman 1998, Frühbeck 2001, Mizuta 2008), nervous (Nogueiras 2008, Gao 2008),
circulatory (Sabatier 2008, Fouillioux 2008, Sierra-Honigmann 1998, Correia 2004) and immune systems (La Cava 2004, La Cava 2003, Hukshorn 2004, Seufert 2004, Fantuzzi 2006, Lam 2006, Mattioli 2005, De Rosa 2007, Dellas 2007, Mattioli 2007, Lago 2008). Diagram 1.3 from La Cava 2003 review, demonstrates leptin’s effect on the endocrine system as well as altered function of various systems. Leptin’s effect can be seen throughout the body; its receptors have been identified in many cell types including immune cells (Tartaglia 1994). Table 1.1 summarizes leptin’s wide reaching effect on a number of body systems.
Diagram 1.1 Leptin Receptor Isoforms - This diagram illustrates the 6 different known isoforms of the murine leptin receptor. All receptors have identical extracellular ligand-binding domains but they each contain different C-terminus ends. Only the soluble receptor (Ob-Re) does not have a transmembrane domain. The long Isoform Ob-Rb is the only receptor capable of activating the JAK2-STAT3 pathway. Within the cytoplasmic domain are Box 1, 2 and 3. The box 1 motif is required for JAK2 activation, Box 2 activity has not been verified but it is thought to be required for JAK2 maximal activation. Finally the long isoform (Ob-Rb) contains three conserved tyrosines (Y985, Y1077, and Y1138) which function as docking sites for STAT3. (Lago, 2008)
Diagram 1.2 Leptin Signaling Pathway - Leptin binds to its receptor causing a conformational change that reveals binding sites for JAK2 on box 1 and 2. JAK2 then phosphorylates itself and the receptor particularly on Y985, Y1077 and Y1138. Phosphorylation of Y1077 and 1138 recruits STAT 5 & 3, phosphorylation of Y985 activates SHP2 and ERK MAPK pathway. Both MAPK and STAT then regulate gene expression. The activation of STAT3 leads to an increase in SOCS, its negative feedback mechanism, therefore STAT3 and SOCS regulate leptin signaling. (Robertson 2008)
Diagram 1.3 Leptin Effects on the Endocrine System - The effect of Lepton on the endocrine system leads to alterations in function and protein production of multiple systems. (La Cava 2003).

<table>
<thead>
<tr>
<th>Organ System</th>
<th>High Amounts of Circulating Leptin</th>
<th>Low Amounts of Circulating Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestive</td>
<td>In Food Intake In Insulin Production</td>
<td>In Food Intake In Insulin Production</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Early Onset of Puberty (Menstruation in Adolescent Females)</td>
<td>Possibility of a Miscarriage</td>
</tr>
<tr>
<td>Circulatory</td>
<td>Likelihood for Blood Clots, Aneurisms, Heart Attacks &amp; Other Circ. Problems Hematopoiesis Hypertension</td>
<td>Blood Viscosity Angiogenesis</td>
</tr>
<tr>
<td>Endocrine</td>
<td>Synthesis of Various Hormones Including Thyroid, Growth and Sex Hormones</td>
<td>Production of Sex Hormones</td>
</tr>
<tr>
<td>Nervous</td>
<td>Disturbed Sleep Patterns Altered Homeostasis</td>
<td>Disturbed Sleep Patterns Altered Homeostasis</td>
</tr>
<tr>
<td>Immune</td>
<td>Promotes Th1 Responses, Enhances Activation of Many Immune Cells &amp; Promotes Autoimmunity</td>
<td>Thymopoiesis</td>
</tr>
</tbody>
</table>

**The Immune System**

The function of the immune system is to protect the host from microbial invasion and from tumorigenesis. To accomplish this, the immune system utilizes a bipartite approach: a fast responding and non-specific response (innate immunity) and a slow and extremely specific response (adaptive immunity). The innate branch is the first responder to an insult on the organism; it includes phagocytic cells such as macrophages (MΦ), neutrophils, and dendritic cells (DC) plus product-secreting cells such as mast cells, basophils, eosinophils, and granulocytes (Cruse 1999). These cells do not have the capacity to specifically recognize a pathogen but do however, recognize specific components of the pathogen through the use of Toll-like receptors (TLR) (Watts 2007). The main function of these cells is to contain the microbe or tumor until adaptive immunity is developed or recalled, responding immediately to the insult (Janeway 2005). Adaptive immunity is composed of lymphocytes known as T and B cells. This branch of the immune system has the capacity to recognize a specific pathogen and mount a response uniquely targeted to the invader. Unlike innate immunity, adaptive immune cells respond slowly taking days or weeks to mount an effective attack. To activate adaptive immunity, innate immune cells must inform adaptive immune cells of the invasion. This is done via a series of chemical protein signals called cytokines and through direct interactions between innate and adaptive immune cells.

DCs are exclusively unique to the immune system. At first glance they seem to merely mimic macrophage activity by sampling their environment and presenting peptide to T cells; but they alone hold the key to link the innate and adaptive branches of the immune system. DCs, phagocytose material, process it and migrate from tissue to draining lymph nodes to present
peptide fragments of ingested material to T cells. They are the only cells capable of directly activating naïve T cells and are therefore required to develop immunity and clear infections or tumors. Functionality of DC and expression of cell surface molecules varies depending on maturity status (Alberts 2002, Kindt 2007, Cruse 1999). Immature DCs are primarily phagocytic, expressing relatively low levels of cell surface activation markers needed for interactions with T cells. Mature DCs possess antigen processing and presentation capabilities and express high levels of cell surface activation markers. A significant difference between immature and mature DC is a change in morphology, caused by re-organization of the cytoskeleton. The morphological change greatly affects DC-T cell interaction. It is during these interactions, that T cells ultimately decide whether to become activated or not. Exactly how this decision is made is still not fully understood, although it is known that cytokines, DC maturation status, and DC-T cell interactions play a major role (Kindt 2007).

DCs induce activation of both CD4+ T cells, which are characterized as helper T cells since they secrete cytokines that coordinate the activities of all responding immune cells, and CD8+ T cells, which are cytotoxic T cells. For the helper T cells, there are two potential types of responses, T helper 1 (T\textsubscript{H1}) or T helper 2 (T\textsubscript{H2}). The type of response is dependent on the pathogen as well as T cell exposure to cytokines before and during peptide presentation and is uniquely designed for clearance of the specific invader. Bacterial and viral infections typically promote a pro-inflammatory response known as T\textsubscript{H1}, which is marked by the production of interleukin (IL)-12 (IL-12) and interferon gamma (IFN\textgamma). Parasitic infections and allergens tend to promote production of IL-4, IL-5, IL-9, IL-10, and IL-13, which is generally described as an anti-inflammatory response. The different types of cytokines are secreted by both adaptive and innate cells but secretion of cytokines from innate cells appear to determine the response type.
The purpose of the immune system is to protect the host organism against disease or infection but it can however, occasionally become auto-reactive, meaning self tissues are targeted for destruction. This is known as autoimmunity and occurs because the system is recognizing self antigen and is becoming activated in response to these self components. Autoimmune diseases include type 1 diabetes mellitus (destruction of the beta islet cells of the pancreas) (Havari 2004), Systemic Lupus Erythematosus (SLE) (Lopez 2003), Multiple Sclerosis (destruction of the myelin sheaths of the central nervous system) (Williams 1994) or Rheumatoid Arthritis (destruction of joint collagen) (Rannou 2005).

The actual cause of autoimmune disease is unknown and is particularly difficult to study since the clinical manifestation of the disease occurs when approximately 80% of the tissue has been affected (Havari 2004). However, pathogenic autoimmunity has been strongly linked to genetic characteristics as well as environmental factors (Janeway 2005). To limit the possibility of destructive autoimmunity, the immune system has developed a series of checkpoint processes to induce a status known as immune tolerance, which is characterized by the lack of pathogenic immunity (Janeway 2005). If any of the checkpoints fail and autoimmunity occurs, treatments are available but vary depending on the target tissue (whether it is systemic or organ-specific); however, treatments typically include global immunosuppressants with supplementation of the lost protein if possible (insulin for example) (Bruce 2002, Janeway 2005, National Center for Chronic Disease Prevention and Health Promotion 2005).

**Obesity and Inflammation**

Obesity plays a role in an assortment of diseases affecting any/or all organ systems of the
body. High concentrations of leptin in the serum seem to increase susceptibility to autoimmune and infectious diseases. This may be because leptin seems to act as a pro-inflammatory cytokine (Faggioni 2001). Not only is it produced by inflammatory regulatory cells but it is also produced and secreted into the serum in increased amounts in the presence of other inflammatory cytokines (Sanna 2003, Faggioni 2001). Due to leptin’s inflammatory nature it is thought that high amounts of circulating leptin promotes a rich environment for autoimmune disease. Bokarewa found an apparent increase in leptin levels in most patients with rheumatoid arthritis (RA). In this disease highly associated with inflammation, it was shown that patients with lower leptin serum levels exhibited a decrease in disease symptoms. More importantly they produced a lower amount of T\textsubscript{H}1 cytokines with an increase in T\textsubscript{H}2 cytokines that lead to a decrease in CD4+ T cell activity (Fraser 1999). In both an Antigen Induced Arthritis (AIA) and ZIA mouse model of immune-mediated joint inflammation, a decrease in inflammation, antibodies and pro-inflammatory cytokines in mice with dysfunctional leptin receptors (Lep\textsuperscript{db}) or leptin deficiency (Lep\textsuperscript{ob}) was observed in comparison with B6 mice. In addition an increase in anti-inflammatory cytokines was detected (Fraser 1999, Bernotiene 2004). Similar results were found using experimentally induced colitis, experimental autoimmune encephalomyelitis, type I diabetes and experimentally induced hepatitis (Otero 2005). These studies all strongly infer that leptin and its signaling is crucial for inflammation and immune responses.

Leptin Immunity and Dendritic Cells

Several studies have demonstrated that various cells of the immune system, both innate and adaptive, express the signaling isoform of the leptin receptor (Tartaglia 1995). Moreover, the same studies have demonstrated a direct affect of leptin on these cells. M\textsuperscript{Φ} in the presence
of leptin have been found to increase phagocytosis and processing capability due to a dramatic increase in production of oxidative species (H. Zarkesh-Esfahani 2004, P. Mancuso 2004, G.M. Raso 2002, F. Caldefie-Chezet 2001, F. Caldefie-Chezet 2003). This therefore enhances function and ability to remove debris, dead and dying cells and pathogens. In 2007 Dellas noted thrombocytes expressing leptin receptors appear to enhance agonist-induced platelet aggregation (Dellas 2007). This effect is both beneficial and counterproductive since clotting prevents excessive bleeding but can also cause cardiovascular problems including blood clots and aneurisms. The presence of leptin promotes production and maintenance of B cells (Kate Claycombe 2006) as well as promote proliferation, differentiation, activation and cytotoxicity of natural killer (NK) cells (Z. Tian, R. Sun 2002) and. For T cells, leptin has been proven to promote T cell activation, inhibit production of T\textsubscript{reg} cells (De Rosa 2007) and push for T\textsubscript{H}1 responses by promoting production of pro-inflammatory cytokines (Lam 2006). However, leptin’s effect on all immune cells is still not fully understood. Its affect on DCs remain largely elusive, recent findings suggest it may play a role in DC activation (Lam 2006, Mattioli 2005).

In this study, we focus on the effect of leptin on DC because of their critical role in linking the two branches of the immune system together. In 2005, Mattioli et al demonstrated that human DC express all six isoforms of the leptin receptor, including the long receptor OB-Rb which has the ability to signal intracellularly when bound by its ligand. Recent work on human DC by Mattioli (Mattioli 2008) demonstrated that leptin induces morphological changes such as actin microfilament rearrangement leading to uropod and ruffle formation. Mattioli also demonstrated that leptin enhances human DC activation in the presence of lipopolysaccharide (LPS). We believe that the morphological and functional changes induced in DC by leptin promotes the pro-inflammatory state observed in obesity due to the enhanced interaction with T
cells (as a direct result of increased surface area) and promotion of a Th1 response. We therefore proposed to verify these findings in a murine system to ultimately assess whether these findings have any physiological significance in vivo. We hypothesized that leptin increases the number and length of dendrites in DCs by causing a re-arrangement of the cytoskeleton, which leads to an increased capacity to migrate and to activate T cells. In this study, we aimed to determine the effects of leptin on DC cytoskeleton re-arrangements, on DC migration ability, and on DC ability to activate T cells. This study will help to elucidate leptin’s affect on DC morphology and functionality specifically in regards to a high concentration of exogenous leptin, which will mimic the condition of obesity and could help to elucidate its potential role obesity-associated inflammation.

Overall the presence or absence of leptin can enhance immune responses; Since leptin has been shown to promote a pro-inflammatory environment, enhance MΦ and T cell activity as well as suppress Treg production it is evident that obesity increases the probability of an individual to develop autoimmune disorders. In this study we proposed that leptin also potentiates DC function, therefore increasing the likelihood for an autoimmune disease to develop. It is critical to understand the effect leptin has on DCs since they are the link between the innate and adaptive branches. Although MΦ are able to prime memory and activated T cells, their main purpose is to maintain T cells activated and direct them to the site of infection. DC however, are the only cell type known to be capable of activating naïve T cells to produce a robust immune response. Therefore, activation of the adaptive immune system is primarily orchestrated through DC-T cell interactions (Cruse 1999). Diagram 1.4 summarizes known effects of leptin on immune cells.
CHAPTER 2

ASSESSMENT OF THE EFFECT OF LEPTIN ON CYTOSKELETAL RE-ARRANGEMENT OF DENDRITIC CELLS

Dendritic cells received their name because of the protrusions that extend from their bodies, which resemble neuronal dendrites. Immature DCs have a limited number of dendrites and are much more circular in shape while activated/mature DCs have numerous dendrites that vary in length and width. The protrusions are formed by the cytoskeleton (Dancker 1975, and Vandekerckhove 1985).

The cytoskeleton is vital for growth and division of all cells. It provides shape, resistance/rigidity, and allows for locomotion and proper internal structure. It is composed of three basic filaments, each with a different function. Intermediate filaments are ropelike and give the cell strength. Microtubules direct intracellular transport and aid in cellular division. Structurally they are long hollow cylinders made of protein tubulin. Actin filaments are highly flexible, they are commonly known as microfilaments. Usually these microfilaments are distributed throughout the cell but are concentrated just below the plasma membrane an area called the cortex. Actins’ main function is to provide a shape for the cell surface, and to promote migration. Accessory proteins, including motor proteins, are necessary to assemble the different components of the cytoskeleton. They regulate distribution and dynamic behavior of the three filaments by binding to them and “directing traffic”, taking into consideration extracellular and intracellular signals. Mature DCs contain increased projections and length of projects known as lamellipodia, which also increases DC cell surface area, potentially augmenting DC ability to
migrate and increasing their ability to interact with multiple T cells at the same time (Alberts 2002).

There are two basic forms of actin, G- and F-actin. Its size ranges from 42 - 45 kDa (Pollard, T.D. 1998, & Kuhn J. R, 2005). G-actin is the free globular monomer form of actin found throughout the cytoplasm. This monomer can dimerize and trimerize to form polymers, known as filamentous actin (F-actin). The purpose of creating F-actin is to aid in many cellular processes such as compartmental movements, internal support, cell shape and size. The initiation to convert the monomer to dimers or trimers is slow but once begun elongation occurs rapidly (Pollard, T.D. 1998, & Kuhn J. R, 2005). This process requires energy in the form of Adenosine Triphosphate (ATP). G-actin therefore binds to ATP and hydrolyzes the nucleotide to produce a weak non-covalent bond to another actin molecule. Additionally it is then bound to Adenosine Diphosphate (ADP) (Susan Nicholson-Dykstra, 2005). At this point the addition of more monomers occurs in a polar direction where the faster growing end is known as the plus end and the slower growing end is the minus end (Pollard, T.D. 1998, & Kuhn J. R, 2005).

Total cellular actin exists in flux between the monomer and polymer form. Actin filaments are composed of the globular actin subunits; these subunits polymerize to produce a two-stranded helix. They are associated in linear bundles that can produce a two dimensional network or a three-dimensional gel. Since filamentous actin subunits are held together by non-covalent interactions they are extremely unstable. This facilitates movement and rearrangement of the actin filaments, thus, easily manipulating cellular morphology as necessary.

Distinct morphological differences are observed between immature and mature DC. As stated previously, resting/immature DCs possess limited numbers of dendrites and are more rounded in shape; activated/mature DCs possess numerous dendrites that vary in length and
Immature and mature DCs also differ functionally. Immature DCs excel at acquiring antigen but have limited presentation capabilities. Mature DCs have limited antigen acquisition but are far superior at presenting and activating T cells. Changes in DC morphology are directly linked to changes in DC function. Differentiation from a resting/immature state to an activated/mature state is accompanied by an increase in dendrite numbers and length. The morphological change is thought to enhance the ability of DC to migrate towards T cells in the secondary lymphoid organs and to enhance DC – T cell interactions. Our preliminary data demonstrated that the addition of exogenous leptin to bone marrow (BM)-derived DC promoted the formation of dendrites (increase in number or an increase in length). For DC derived from human peripheral blood mononuclear cells (PBMCs), leptin was shown to enhance uropod formation (Mattioli 2008). We therefore hypothesized that one potential effect of leptin receptor ligation on DC is to increase the DC surface area by promoting dendrite formation and thus enhancing DC migration and interactions with T cells. We further hypothesized that the changes in DC morphology visualized upon treatment with exogenous leptin resulted directly from changes in actin architecture.

**Results**

To visualize actin re-arrangement and morphological change of DC upon treatment with leptin, we used two types of microscopy: a) light microscopy, and b) confocal microscopy. Light microscopy was used to assess gross morphological changes and confocal microscopy was utilized to assess the localization of actin filaments. DCs were differentiated from bone marrow (BM) stem cells of C57BL/6 mice in the presence of granulocyte monocyte/macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). On day 6, the DCs were enriched by
positive selection for CD11c-positive cells and were allowed to rest for 24 hours; the purified BM-DC were then treated with one of the following conditions:

Treatments were as follows:
1.) 1 µg/mL of Ob-R-Fc
2.) Non-treated DC in RPMI 1640 media
3.) 10 µg/mL LPS
4.) 160 ng/mL recombinant murine leptin
5.) 10 µg/mL LPS + 160 ng/mL recombinant murine leptin
6.) 16 ng/mL recombinant murine leptin
7.) 10 µg/mL LPS + 16 ng/mL recombinant murine leptin.

Non-treated cells functioned as negative controls. To demonstrate that potential changes observed were directly due to addition of exogenous leptin, the Ob-R-Fc chimera was used. The chimera is a fusion between the extracellular domain of the leptin receptor and the Fc portion of an immunoglobulin (Ig). The chimeric protein is soluble and binds to leptin, preventing it from ligating cell surface-bound leptin receptors. Thus, Ob-R-Fc treatment blocks bovine leptin found in the media from the serum supplement (which can bind to murine leptin receptors) and served as an additional negative control. Lipopolysaccharide (LPS) functioned as a positive control for DC maturation. A low (10 ng/ml) and high (160 ng/ml) concentration of leptin was chosen based on circulating levels of leptin found in studies of Mexican Americans by Peltz (Peltz 2007).

Following a 24 hr treatment period, light microscopy images were obtained from the
treated BM-DC with a magnification of 40X and are pictured in Figure 2.1. As was expected, there is no noticeable change in cell morphology between the Ob-R-Fc-treated and media treated cells (Figure 2.1A and 2.1B), suggesting that the leptin concentration in the bovine serum concentration in the media is not significant enough to cause any detectable morphological changes. In contrast, the positive control, LPS-treated cells presented with distinct morphological changes in the form of increased dendrite length, number, and thickness (Figure 2.1C). Interestingly, a similar change was induced with leptin treatment alone [Figure 2.1D (160 ng/ml) and 2.1F (16 ng/ml)]. Of particular note is the combination of leptin and LPS treatment, which produced the most significant observable changes [Figure 2.1E (160 ng/ml leptin + 10µg/ml LPS) and 2.1G (16 ng/ml leptin + 10µg/ml LPS)]. The leptin plus LPS treatment increased the total number of DC with observable dendrites and induced a marked increase in the number of dendrites per DC. Moreover, the higher concentration of leptin (160 ng/ml), induced the most pronounced morphological change.

For confocal microscopy, BM-DC were generated, purified, and treated as previously described. Following the 24 hour treatment, the cells were fixed, permeabilized, and then stained with rhodamine-labeled phalloidin to visualize filamentous actin (F-actin) and were counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) to facilitate cell orientation. Due to the required centrifugation of the purified BM-DC onto the microscope slides, dendrite morphology is lost, although not entirely. Thus, the formation of ruffles or lamellopodia is used as a signature of morphological changes. As shown in Figure 2.2, the control samples [Ob-R:Fc-treated (Figure 2.2A) and non-treated (Figure 2.2B)] presented with little to no membrane ruffles, with the Ob-R:Fc-treated BM-DCs appearing slightly rounder than that of the media treated DC. In addition, the intensity of rhodamine staining for these two
treatments is quite dim, demonstrating weak levels of F-actin. For the positive control (LPS-treated DC figure 2.2C), spikes of dendrites are visible and F-actin levels are increased above that of both negative controls [Ob-R:Fc-treated (Figure 2.2A) and media treated cells (Figure 2.2B)]. Leptin treatment alone markedly enhanced the formation of lamellipodia [Figure 2.2D (160 ng/ml leptin + 10µg/ml LPS) and 2.2F (16 ng/ml leptin + 10µg/ml LPS)] and caused an intense localization of F-actin at the peripheral edges of the cell. Moreover, the extent of morphology change and F-actin intensity is proportional to leptin concentration. Leptin plus LPS appears to greatly elongate and define the lamellipodium and dendrite spikes much more so than the LPS alone [Figure 2.2E (160 ng/ml leptin + 10µg/ml LPS) and 2.2G (16 ng/ml leptin + 10µg/ml LPS)] and is also proportional to leptin concentration. However, the intensity of F-actin is decreased with the combination treatment.

To better assess the relative amounts of filamentous actin present in the differentially treated DC, microscope software was used to quantitate the intensity of fluorescence of rhodamine-phalloidin (Figure 2.3). The data was collected as surface area, mean intensity (MI) and MI/µm². Analysis of 30 cells at each treatment was performed. Table 3 contains a summary of the results obtained. Quantification of confocal studies further supports leptin’s ability to rearrange the cytoskeleton of DC as evidenced by an increase of overall surface area increase in dendrite number and length as well as an increase in F-actin intensity when comparing the leptin-treated DC to the non-treated control. The addition of LPS to the leptin treatment did not augment the morphological changes promoted by leptin; in fact, the combination treatment was less effective than either treatment alone. Of interest are the changes induced by the low versus high concentrations of leptin. The low concentration (16 ng/ml) of leptin almost triples the relative amount of F-actin and increases the total surface area by a little more than double. The
high concentration of leptin (160 ng/ml) induces a five-fold increase in F-actin but does not alter surface area. Despite the different results, both concentrations of leptin induced significant morphological changes in DC.
Figure 2.1 Leptin Enhances Dendrite Formation in BM-DC: Light microscopy of DC enriched on day 6 and treated for 24 hours as indicated. Magnification at 40X Data is representative of three separate experiments. (A.) chimera, (B.) media, (C.) LPS, (D.) leptin 160 ng/ml (E.) L+L 160 ng/ml, (F.) Leptin 16 ng/ml, (G.) L+L 16 ng/ml
Figure 2.2. Leptin Enhances F-Actin Polymerization. Day six BM-DC were enriched and then treated for 24 hours with the indicated reagents. The cells were then harvested, fixed onto microscope slides, stained with rhodamin-phaloidin (red), counterstained with DAPI, and were then analyzed by confocal microscopy. Panel A: Ob-R:Fc; Panel B: non-treated; Panel C: LPS-treated; Panel D: 160 ng/ml leptin; Panel E: 160 ng/ml leptin + LPS; Panel F: 16 ng/ml leptin; and Panel G: 16 ng/ml leptin + LPS. All images were acquired at the same gain, pinhole size 156 µm and magnification of 63X (oil emersion).
Figure 2.3. Surface Area and Mean Intensity Analysis of Leptin-Treated DC: Day six BM-DC were enriched and then treated for 24 hours with the indicated reagents. The cells were then harvested, fixed onto microscope slides, stained with rhodamin-phaloidin (red) and then analyzed by confocal microscopy. Panel A: Ob-R:Fc; Panel B: non-treated; Panel C: LPS-treated; Panel D: 160 ng/ml leptin; Panel E: 160 ng/ml leptin + 10 µg/ml LPS; Panel F: 16 ng/ml leptin; and Panel G: 16 ng/ml leptin + 10 µg/ml LPS. Data is representative of 30 cells per treatment.
Table 2.1. Leptin Induces Marked Changes in DC Morphology - The following parameters were assessed using Zeiss Scanning Confocal Microscope with Pascal Deconvolution Time-Lapse Temp Control software.: Mean Intensity (MI) Surface Area (SA) and MI/μm², Number of Dendrites and Length of Dendrites. Data presented on this table is the mean of 30 cells per treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Avg. # of Dendrites</th>
<th>Avg. Dendrite Length (μm)</th>
<th>Surface Area (μm²)</th>
<th>Avg Mean Intensity (MI)</th>
<th>MI per μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ob:Rt:Fc</td>
<td>2</td>
<td>1.2</td>
<td>344.74</td>
<td>1147.23</td>
<td>3.33</td>
</tr>
<tr>
<td>Media</td>
<td>2.7</td>
<td>1.3</td>
<td>279.11</td>
<td>3111.51</td>
<td>11.15</td>
</tr>
<tr>
<td>LPS (10 μg/ml)</td>
<td>13.3</td>
<td>4</td>
<td>423.12</td>
<td>1191.32</td>
<td>3.15</td>
</tr>
<tr>
<td>Leptin (160 ng/ml)</td>
<td>13</td>
<td>5.5</td>
<td>381.47</td>
<td>2081.02</td>
<td>5.46</td>
</tr>
<tr>
<td>Leptin (160ng/ml) + LPS (10μg/ml)</td>
<td>23.8</td>
<td>5.8</td>
<td>587.69</td>
<td>2506.98</td>
<td>4.42</td>
</tr>
<tr>
<td>Leptin (16 ng/ml)</td>
<td>9</td>
<td>3.8</td>
<td>475.48</td>
<td>1503</td>
<td>3.23</td>
</tr>
<tr>
<td>Leptin (16ng/ml) + LPS (10 μg/ml)</td>
<td>16.7</td>
<td>7.3</td>
<td>409.07</td>
<td>1058.87</td>
<td>2.59</td>
</tr>
</tbody>
</table>
The microscopy data demonstrated that treatment of DC with a low and high, yet physiological, concentration of leptin induces morphological changes similar to those observed in the human system. Maximal effects were observed at 160 ng/ml of leptin and minimal changes were observed at 16 ng/ml of leptin; therefore further studies were conducted at 160 ng/ml of leptin to determine actin content and form.

To quantify changes induced by leptin to actin, whole cell lysates as well as cytoskeletal and soluble protein fractions were prepared. Quantified by Western blot (WB) analysis with densitometry and actin indirect-ELISA. The purpose of fractional cell lysates was to separate the polymer form of actin (F-actin), found in the cytoskeleton, from the monomeric form (G-actin) found in the cytoplasm. Western blots and densitometry analysis of total cell lysates illustrated that in comparison to the negative controls, leptin causes little to no increase in overall actin (Figure 2.4). In comparison to media treatment, G actin WB and densitometry showed a decrease in actin in chimera treatment and an increase in LPS, Leptin and L+L (in increasing order) (Figure 2.5). Furthermore, F actin WB and densitometry showed a slight increase in Leptin, LPS, and L+L treated DC (from smallest increase to largest increase) (Figure 2.6B). Ratios of treated DC in G vs F actin were as follows: Media = 7:5, Chimera = 26:25, LPS = 11:10, Leptin = 161:125, L+L = 103:100. This demonstrated that leptin induces an increase in both G and F-actin.

Indirect actin Enzyme Linked Immunosorbent Assays (ELISA) was performed because it is a far more sensitive technique that may detect minute differences; additionally it is much more quantifiable (Figure 2.4). To some extent results from ELISA mirrored those from WB analysis (Figure 2.2 and Table 2.1). Using whole cell lysates, a slight increase in total actin expression was detected in leptin and LPS treated cells however this was not significant (Figure 2.7A). This
strongly somewhat implies that the addition of leptin in combination with LPS causes an increase in actin production in comparison to media, OB-R:Fc or LPS, but the reason for this is unclear. Indirect ELISA also demonstrated differences in DC content of F and G actin caused by the distinct treatments. An increase in G-actin in LPS and L+L treated DCs was observed however, leptin, media and OB-R:Fc remained unchanged and equivalent to one another (Figure 2.7B). Furthermore, ELISA test revealed that leptin, LPS and leptin + LPS express an increase in F-actin with L+L showing the largest increase followed by LPS then leptin, once again non-treated and OB-R:Fc treated cells remained unaltered and equivalent (Figure 2.7C).

**Conclusion**

Analysis of confocal data demonstrated an increase in surface area, number of dendrites and length of dendrites in leptin, LPS and leptin + LPS treatments; therefore their F-actin MI (rhodamine) was lower than that of non-treated media cells. However this does not signify a decrease in F-actin in comparison to OB-R-Fc, rather a dispersal of F-actin throughout the cell to cover a larger area.

Leptin, LPS and L+L cause little to no increase in actin production. G-actin and F-actin levels also increase in all 3 treatments. Leptin treatment increases amount of F actin but LPS is more efficient at this, the duo leptin and LPS treatment is even more successful this is possibly due to the increase in available G-actin within the treated cells. We believe that there is a rapid shift from G to F-actin and F to G-actin that may be due to effects on actin-modulating proteins that allow for rapid assembly and disassembly of actin. Therefore F actin may be increasing in treated DC because there is more G actin available at a given time. The ratios of G vs F-actin may therefore not be changing a great deal between treatments but the amount of F actin is
increasing, this change may ultimately lead to altered cell morphology.
Figure 2.4. Leptin Causes an Increase in Overall Actin. Whole cell lysates were obtained from DC treated for 24 hours with the indicated reagents. 5 µg/lane of protein were loaded from each treatment onto lanes of an 8% SDS Gel (A). Densitometry was conducted on the bands obtained (B). Data is one of three representative experiments.
Figure 2.5. Leptin Treatment Increases the Amount of Soluble Actin (G-Actin)  Monomeric fractioned lysates were obtained from DC treated for 24 hours with the indicated reagents. 5 µg/ml were loaded from each treatment onto lanes of an 8% SDS Gel (A). Densitometry was conducted on the bands obtained (B). Data is one of three representative experiments.
Figure 2.6: Leptin-Treatment Increases the Amount of F-Actin. Polymeric actin (Fractioned lysates) was obtained from DC treated for 24 hours with the indicated reagents. 5 µg/ml were loaded from each treatment onto lanes of an 8% SDS Gel (A). Densitometry was obtained on the bands obtained (B). Data is one of three representative experiments.
Figure 2.7. Measurement of Total, G-, and F-Actin by Indirect ELISA - Whole cell lysates (A) or fractioned lysates (B. Monomeric actin and C. Polymeric actin) were obtained from the different populations of treated DC and were plated onto ELISA plates overnight at 500 ng/ml of total protein. The plates were later blocked with 5% non-fat dry milk, then incubated with a primary anti-actin antibody followed by a second incubation with horse radish peroxidase-conjugated secondary antibody. Chromogen was then added and the plate was read on a spectrophotometer at 450nm. Data is presented as the mean ± SEM of duplicate wells and is one of two representative experiments.
CHAPTER 3

ASSESSMENT OF THE EFFECT OF LEPTIN ON DENDRITIC CELL FUNCTION IN TERMS OF MIGRATION

Dendritic cells are naturally mobile cells that sample their environment and report their finding to T cells in the form of peptide antigen. They are located in all tissues throughout the body and form part of the innate immune system. Their role within the system as a whole is crucial because they alone hold the key to link the innate and adaptive immune systems. DC phagocytes material, process it and migrate from tissue to draining lymph nodes to present peptide to T cells. They are the only cells capable of directly activating naïve T cells and are therefore required to clear infections and develop immunity. While in situ, they phagocytosis and pinocytosis their surroundings. The ingested material is placed in phagosomes or endosomes which fuse with lysosomes to create phagolysosomes. The pH within these phagolysosomes is very low and enzymes within the compartment break the antigen down into peptides of 8-30 amino acids in length. The newly formed peptides are placed on MHC II to be presented to T cells. If the ingested material is an infected cell or a pathogen, the DC receives a signal to become activated. The signal is received through the Toll-like receptors binding to unique pathogen-derived ligands (Abbas 2006).

These APCs contain unique characteristics; because they are phagocytic cells they express a MHC class II molecule to present processed material to T cells. Other cell surface molecules commonly found on DCs, include CD11c (most frequently used to purify cells) as well as CCR7 (chemokine receptor for CCL19 ), CCR5 (chemokine receptor for CCL4 ), CD40
(DC activation marker), CD54 (Adhesion molecule to stabilize DC-T cell interaction), CD80 (DC and T co-stimulatory molecule), and CD86 (DC and T co-stimulatory molecule). Functionality of DC and expression of these cell surface molecules varies depending on maturity status (Alberts 2002, Kindt 2007, Cruse 1999). Immature DCs phagocytose very well and have lower concentrations of MHC II CD40, CD54, CD80, and CD86 than mature DC. The presence of a high concentration of the chemokine receptor, chemokine C-C motif receptor 5 (CCR5) is indicative of immature DC. Upon activation by a pathogen or exposure to foreign material their phagocytic ability and CCR5 receptor diminishes as chemokine C-C motif receptor 7 (CCR7) appears. This receptor allows for the migration of DC to the draining lymph nodes and is indicative of a mature or maturing DC (Alberts 2002). During the maturation process MHC class II, CD40, 54, 80, and 86 are all up-regulated to enhance the DCs ability to present peptide to T cells (Alberts 2002, Kindt 2007, Cruse 1999). One significant difference between immature and mature DC is the obvious change in morphology, caused by re-organization of the cytoskeleton. The morphological change greatly affects DC-T cell interaction (Kindt 2007). Diagram 3.1 summarizes the stated differences between mature and immature DC.

One of the most important differences between mature and immature DC is the change in chemokine receptors on the cell surface. This up-regulation of CCR7 and down-regulation of CCR5 facilitates the migration of the DC to the draining lymph node (where T cells are located). As described DC mobility is required for adequate presentation capabilities of these APCs; therefore migration is a crucial process for capacity to present antigen and likelihood to activate both naïve and primed T cells (Butcher 1996, Ansel, 2001and Von Andrian 2000).

The process of migration is complex and requires a number of different cellular components, signals, and attractants that vary depending on cells status (immature or mature);
this is true for most leukocytes. The process of migration is usually initiated by the presence of a chemoattractant such as a chemokine. Cells migrate towards these 8-10 kDa proteins by following the concentration gradient towards the higher concentrations. As described, DC express a variety of cell surface markers that vary by maturity status. As immature, DC they express high levels of a CCR5 whose ligand is Macrophage Inflammatory Protein-1α (MIP-1α) or Macrophage Inflammatory Protein-1β (MIP-1β). However, as mature DC they decrease expression of CCR5 to increase expression of CCR7. This receptor responds to ligand chemokine C-C motif ligand 19 (CCL19) which recruits the mature DC toward the secondary lymphoid organs (Muller 2003). Migration towards the lymphoid organs increases the probability of DC to encounter T cells specific for the peptide they care and therefore provoke an immune response.

One key component to successful DC migration is morphological changes that ultimately result in cytoskeletal plasticity. This allows DC to navigate through interstitial tissues, blood and lymph (Worthylake 2001). The cytoskeleton is composed of microfilaments, intermediate filaments, and microtubules. Migration is characterized by polymerization of actin, one of the key components of the cytoskeleton (microfilaments) who is primarily responsible for cellular morphology and movement. Thus, migration is typically due to altered cellular morphology caused by changes in the ratios between the monomer globular and the polymer filamentous forms of actin and the rearrangement of the actin filaments. Actin dynamics is therefore the primary cause of membrane protrusions and cell matrix adhesion that result in cell migration.

Enhanced migration could possibly increase the chance of DCs activating T cells specific for the peptide they are carrying by limiting the time spent migrating and maximizing the time DCs spend interacting with T cells. This could be crucial, because the amount of time a DC and
T cell interact may affect the efficiency and efficacy of the resulting response. We therefore hypothesize that leptin promotes DC migration towards draining lymph nodes due to increased lamellipodia formation.

Results

DC migration was assessed by placing enriched murine bone marrow derived DC into 24 well 10 µm transwells. DC grown in culture in the presence of GM-CSF and IL-4 as indicated in materials and methods. For all experiments conducted purity of enrichment was greater than 90 percent as demonstrated by flow cytometry (data not shown). DC were treated as described in chapter 2 for either 24 or 48 hours [Treatments were as follows: 1.) Non-treated DC in RPMI 1640 media 2.) 1 µg/mL of Ob-R-Fc 3.) 10 µg/mL LPS 4.) 160 ng/mL recombinant murine leptin 5.) 10 µg/mL LPS & 160 ng/mL recombinant murine leptin. Cells were washed to remove traces of treatment and placed in RPMI 1640 in the upper chamber of a transwell at 400,000 cells/well. DC-specific chemokine (CCL19) was placed at 200 ng/ml, 50 ng/ml and no CCL19 in the lower chamber. The plates were then incubated at 37°C for 4 and 24 hours. Cells in the lower chamber were digitally photographed to assess morphology using a magnification of 40X. Cells were finally harvested and counted by trypan blue exclusion to determine the percentage of migrated DC.

Leptin appears to enhance DC migration as demonstrated in the 24 (Figure 3.1) and 48 hour treatments (Figure 3.2) at both 4 and 24 hour migration time-points. The X-axis of the each graph illustrates the percentage of migrated cells, while the Y-axis of the graph indicates the amount of CCL19, each sample was run in duplicates and the graphs represent the data of one of three experiments but similar results were obtained in all experiments. Migration appears to
occur in a CCL19 dose dependent manner where the higher the concentration of CCL19 the higher the number of cells that migrated. Additionally, treatment of DCs treated with leptin, LPS or the duo combination of leptin and LPS significantly migrated much more efficiently than the non-treated or OB-R-Fc treated cells. However the duo treatment of leptin and LPS caused a much greater increase in migration than leptin or LPS alone. Leptin and LPS only treatments were comparable, lower than the duo combination but still greatly increased from the media and OB-R-Fc treatment.

We were curious whether the migrated DC maintained the previous cellular morphology determined in chapter 2, therefore prior to harvesting the 24 hour treated 4 hour migrated cells digital light microscopy images at a magnification of 40X were obtained (Figure 3.3). This data verified that the migrated cells are indeed DC, and that the successfully migrated DC were able to maintain the cytoskeletal rearrangement caused by the treatments. Although images for the 24 hr migrations time-point and 48 hour treatment were obtained the data is not shown but similar results were obtained.

**Conclusion**

Leptin may possibly play a vital role in DC migration both in immature and mature DC as observed by the presence or absence of LPS. Additionally percent of migrated DC increased in a dose dependent manner as the chemokine concentration increased. Digital images of migrated DC demonstrated that DC retained their morphology post migration. It should be noted that although DC migration was enhanced in the leptin treatment it was greatly increased in the duo combination of leptin and LPS. Therefore leptin alone or together with LPS promotes DC migration which is dependent on cytoskeletal rearrangements. We hypothesize that the rapid
turnover between G and F-actin is what allows for the enhanced migration. A second possibility is an up-regulation of the chemokine receptor CCR7. Moreover it seems that the concentration of leptin present may affect the migrational outcome since the 24 hour treatments migrated more efficiently than 48 hour treatments. This is noteworthy since the 48 hour treatments were not given fresh treatment post 24 hours; therefore the concentration of the treatment decreased over time. This may indicate that leptin’s effect is nearly immediate and that migration is dependent on leptin serum/media levels where the higher concentrations exert a more potent effect.
Figure 3.1. Leptin Enhances DC Migration Post 24 Hour Treatment: Murine bone marrow derived DC were enriched and treated as indicated for 24 hours. 400,000 cells were placed in each transwell, CCL19 was added to the lower chamber at 0, 50 and 200 ng/ml. Migrated DC were harvested at 4 hours and 24 hours post incubation and counted by trypan blue exclusion. The percentage of migrated DC was determined and graphed.
Figure 3.2. Leptin Enhances DC Migration Post 48 Hour Treatment: Murine bone marrow derived DC were enriched and treated as indicated for 48 hours. 400,000 cells were placed in each transwell, CCL19 was added to the lower chamber at 0, 50 and 200 ng/ml. Migrated DC were harvested at 4 hours and 24 hours post incubation and counted by trypan blue exclusion. The percentage of migrated DC was determined and graphed.
Figure 3.3 Migrated DC Maintained Morphology: Prior to harvesting of migrated cells digital images were obtained to verify cellular morphology was maintained post migration. These are images obtained from 24 hour treated 4 hour migrated cells. Although images for 48 hour treatment and 24 hour migrations are not shown a similar trend was observed.
CHAPTER 4

ASSESSMENT OF THE EFFECT OF LEPTIN ON DENDRITIC CELL ABILITY TO ACTIVATE NAÏVE T CELLS

Adaptive immunity is composed of lymphocytes known as T cells and B cells. This branch of the immune system has the capacity to recognize a specific pathogen and mount an immune response directed specifically towards it. Adaptive response is delayed in comparison to the response by the innate branch. A complete response by the adaptive immune branch requires approximately 2 weeks time (Kindt 2007)

T cells responses are cell mediated. These cells mature in the thymus, where they undergo a process known as VDJ recombination which determines T cell receptor (TCR) specificity. This random process produces a vast amount of diversity, up to $10^{18}$ different possibilities of TCRs (Janeway 2005). There are a variety of sub-populations within T cells each with a distinct function, these include memory T cells, cytotoxic T cells, Helper T cells and regulatory T cell. Memory T cells allow for a rapid response; they are long lived T cells that remain in the LN to facilitate the activation process when the host encounters a pathogen a second time. Cytotoxic T cells are CD8+, they target and kill infected cells to prevent further infection. Helper T cells are CD4+; their main function is to provide support in the form of cytokines, to both the innate and adaptive immune response. This action augments and facilitates the effectiveness of all responding cells. Finally, the relatively new sub-population of T cells are known as regulatory T cell (T_reg), they function as an “off” switch to stop an immune response. This is accomplished by producing T_H1 cytokines during a T_H2 immune response and T_H2
cytokines during a T\textsubscript{H1} response to suppress the currently active immune response. One commonly identifiable characteristic of T\textsubscript{reg} is the expression of both CD4 and CD25 on the cell surface (Janeway 2005).

Naïve T cells living in the LN and thymus encounter many DC to scan the peptide carried on their MHC class II molecules. Recognition of a peptide by T cells causes a complex prolonged interaction that ultimately leads to T cell activation (Shaw 2008). For this to occur, a multiple number of interactions must take place simultaneously. It is currently thought that T cells require an interaction with APCs that is 4-16 hours long to actually become activated, proliferate and secrete cytokines such as IFN-\(\gamma\) and IL-2 and migrate to affected areas (Huppa J.B. 2003 and Lezzi, G. 1998).

A response to a signal requires three elements: a signal, a receptor and a signal transducer. For a signal, matured APCs, particularly mature DC, migrate toward the concentration gradient of CCL19 (found in high concentrations in the lymphoid organs) and encounter many T cells. They present processed antigen in the form of peptide on their MHC II cells surface marker to T cells’ TCR receptors. Additionally the DC B7 family molecules (CD80 and CD86 in mice) bind to T cell CD28 molecules to co-stimulate activation and signaling in both DC and T cells (signal 2) CD40L (CD40 Ligand) on T cells interacts with DC CD40 receptor to further activate DCs. A CD4 molecule on the T cells surface then binds to this complex to stabilize the immunological synapse and therefore enhance adherence. This prolonged interaction stimulates and pushes T cells into the G1 phase and induces transcription (signal 3) of the IL-2 gene to produce CD25 (IL-2 receptor), and promotes IL-2 production 100 fold. Once the secreted IL-2 binds to surrounding T cell receptors they too begin to proliferate and differentiate to the different sub-types of T cells. However it is unclear how the T cell
actually commits to one particular sub-population, but it is thought that cytokines play a critical role (Faggioni 2001). Activated T cells then secrete IFN\(\gamma\) to further enhance and facilitate removal of a pathogen by activating M\(\Phi\) and increasing the expression of MHC II on APCs. The newly formed population of CD8\(^+\) T cells then migrates toward the inflamed/affected area and attacks their targets cells. Overall T cells mediate clearance of pathogens or destruction of self tissue by enhancing APC function or puncturing infected or self tissue in the case of auto-reactive T cells (Kindt 2007). A brief summary of DC-T cell interactions is illustrated in Diagram 4.1.

Obesity and leptin appear to create a rich environment for autoimmune disorders to develop as previously discussed. Consistently high levels of insulin and leptin cause cells to become leptin resistant provoking an increase in circulating leptin which results a pro-inflammatory environment. We propose that leptin also potentiates DC function, therefore increasing the likelihood for an autoimmune disease to develop. With regards to T cell activation we hypothesized that increased lamellipodia will increase the DC ability to activate T cells because the total surface area to interact with T cells is increased. This will therefore enable the DC to present to a larger number of T cells at one time. To determine if our hypothesis was correct we assessed the interactions of leptin-treated DC with antigen specific T cells in vitro.

**Results**

Antigen-specific T cell hybridoma, an established T cell line, and primary antigen-specific T cells were presented with peptide by leptin enriched (>90\%) treated or untreated DC. The T cell hybridoma is a CD4\(^+\) T cell line specific to ovalbumin peptide. It was generously donated by Dr. Philippa Marrack from the National Jewish Medical and Research Center in
Denver, CO. Primary T cells were obtained from TCR transgenic mice with a C57BL6 background also specific to ovalbumin. They were enriched by positive selection for CD4+ T cells as indicated in the materials and methods. It was determined by flow cytometry that the purity of the cells obtained was greater than 90 percent (data not shown). The use of these two different systems provide complimentary information about T cell activation where activation of the T cell hybridoma was measured as a function of IL-2 production (Figure 4.1) and TCR transgenic T cell activation was measured as a function of IFNγ production (Figure 4.2) and proliferation (Figure 4.3).

The five different DC populations/treatments were pulsed with ovalbumin peptide at 500 mg/ml, this is a simple exchange at the cell surface and does not require DC processing. The antigen-pulsed DC were then co-cultured with the T cell hybridoma or the TCR transgenic T cells at a ratio of 1:1, 1:5, 1:10, and 1:20 for a 72 hr incubation period. An additional group without ovalbumin peptide at a ratio of 1:5 was added to account for background. As a positive control DC + T cells at a ratio of 1:5 were co-cultured in the presence of 10 µg/ml of Concanavalin A (Con A) because this is a chemical well known to activate T cells in the presence of DC by forcing TCR MHC interactions. At the completion of the 72 hr. incubation supernatants were removed for further analysis by ELISA for cytokine production, IL-2 (Figure 4.1) in the case of the hybridomas, and IFNγ production (Figure 4.2) in the case of the TCR transgenic T cells. TCR transgenic co-cultures were further analyzed for proliferative capabilities. A radio-actively labeled nucleotide ([^3]H]-thymidine) was added post 72 hours initial co-culture for an additional 19 hrs. Proliferation was assessed by the incorporation of the tritiated nucleotide and thus radioactivity was measured, where the amount of radioactivity directly correlates with DNA replication/cellular division and therefore proliferation (Figure 4.3).
IL-2 production (Figure 4.1) in the supernatant of the hybridoma-DC co-culture as measured by sandwich Elisa demonstrated a significant increase in the ability of leptin and LPS treated cells to activate T cells. This effect is more evident in the duo treatment of leptin and LPS. A similar trend was noted on DC: T cell ratios of 1:20, 1:10 and 1:5, where duo treatment leptin and LPS was most efficient at activating T cells, followed by LPS treated DC then leptin treated DCs while media and chimera were nearly equivalently inefficient at activating T cells. As expected the addition of Con A to non-treated DC also enhanced T cell activation although not as efficiently as T cells in the presence of the duo leptin and LPS treated cells at a DC:T cell ratio of 1:20. Minimal amounts of IL-2 were detected in 1:1 DC:T cell ratios (all treatments) possibly because of low T cell numbers. A barely detectible or not detectible (ND) levels of IL-2 was found in the non-peptide containing wells with both treated and non-treated DC.

IFN-γ production also detected by Sandwich ELISA revealed a very similar trend to IL-2 results (Figure 4.2). Again leptin and LPS treated DC were much more efficient at activating T cells in terms of IFN-γ production with the duo treatment the most efficient. However the difference between the duo leptin and LPS treatment and LPS alone treatment was much more marked in IFN-γ than it was in IL-2. Additionally the trend observed in IL-2 held true for IFN-γ production for all DC:T cell ratios including 1:1 This means that the duo treatment was most efficient at activating T cells followed by the LPS treatment, then the leptin treatment and finally non-treated and OB-R-Fc treated. Once more Con A stimulation of T cells (positive control) produced a robust INF-γ production but the duo leptin and LPS treatment was still approximately 1.5 times higher.

The final parameter assessed to analyze T cell activation was proliferation (figure 4.3). At
the conclusion of the 72 hour co-culture 0.5 µCi tritiated thymidine was added overnight). The following morning cells were harvested and analyzed on a scintillation counter to assess radioactivity. The results obtained for this study further supported cytokine data. Leptin treatment significantly enhanced T cell activation in terms of proliferation in comparison to both media and OB-R-Fc treatments. However it was not as efficient at activating T cells as the LPS treated cells, but the most efficient DC at activating T cells were the duo treated cells. The difference between the LPS and duo treated DCs was not as high as predicted but the trend observed in both IL-2 and IFN-g remained. These effects were primarily observable in the DC:T cell ratio of 1:20. All negative controls (media DC, DC + Ova, T cells and T cells + Ova) had limited proliferation, while positive control Con A yielded highly proliferative T cells.

Conclusion

In conclusion exposure to leptin by DC prior to contact with T cells and a peptide visibly enhance T cell activation. The results obtained are not a manifestation of the leptin acting directly on the T cells to stimulate them and promote their activation since the treatments are removed prior to co-culture. Additionally low detection of cytokines and radioactivity in negative controls demonstrates that T cell activation was caused by DC and results obtained were not due to DC proliferation or random T cell activation. It is clear that T cell are more activated by DC treated with either leptin, LPS, or the duo leptin and LPS treatment as indicated by proliferation, IL-2 and INF-γ production. Enhanced T cell activation by leptin treated cells with and without LPS stimulus indicates that leptin treatment causes DC to become more potent antigen presenting cells. We hypothesize that the increase in DC cell surface area and increased
number/length/thickness of dendrites increases the number of T cells interacting with an individual DC promoting an increased T cell response.
Diagram 4.1 Summary of DC-T Cell Interactions – This diagram illustrates the basic interactions required for T cells to become activated. **Signal 1:** MHC II DC present peptide to T cells TCR. **Signal 2:** DC co-stimulatory receptors (CD80 and CD86) interact with their ligand CD 28 on T cells to stimulate both DC and T cells, also DC CD40 receptor is simultaneously stimulated by its ligand CD40L on T cells enhance DC activation. These interactions stabilize the two cells and aid in cell signaling. **Signal 3:** A prolonged interaction occurs and that induces transcription of the IL-2 gene to produce CD25 (IL-2 receptor), and promotes IL-2 production 100 fold (Immunology and immunotherapy approaches for prostate cancer E Elkord)
Figure 4.1. Leptin Enhances DC Ability to Activate T Cell Hybridomas: DC were treated for 24 hours as indicated then co-cultured with T cell Hybridomas specific to ovalbumin. Peptide was added at 500 µg/ml, co-culture was incubated at 37°C for 72 hours with 5% CO₂. Supernatants were collected and IL-2 production was determined by sandwich ELISA.
Figure 4.2. Leptin Enhances DC Ability to Activate Primary T Cells in Terms of IFN-γ Production - DC were treated for 24 hours as indicated then incubated for 4 hrs with ovalbumin peptide at a concentration of 500 µg/ml. Primary CD4 enriched T cell specific to ovalbumin were added at the indicated concentrations; co-culture was incubated at 37°C for 72 hours with 5% CO₂. At the conclusion of this incubation supernatants were collected and INF-γ production was determined by sandwich ELISA.
Figure 4.3 Leptin Enhances DC Ability to Activate Primary T Cells in Terms of Proliferation - DC were treated for 24 hours as indicated then incubated for 4 hrs with ovalbumin peptide at a concentration of 500 μg/ml. Primary CD4 enriched T cell specific to ovalbumin were added at the indicated concentrations; co-culture was incubated at 37°C for 72 hours with 5% CO₂. At the 72 hr time point tritiated (³H) thymidine (0.5 μCi) was added to co-culture overnight for 18 hours and proliferation was measured by incorporation of ³H thymidine/radioactivity on a scintillation counter.
DISCUSSION

Leptin is secreted in direct correlation with the content of adipose tissue. Additionally, leptin levels are not constant; rather, they range by sex and throughout the day with nutritional state and activity level. Individuals who are overweight have a higher concentration of serum bound and free circulating leptin in their blood stream (Sinha 1996). Leptin, the product of the OB gene responsible for energy expenditure and appetite control, appears to affect the immune system and increase the likelihood to develop autoimmune diseases as well as circulatory, renal, and other chronic obesity related disease. The role that obesity and leptin play on autoimmunity is unclear, little is known on their mechanisms of action and how leptin is involved. Unfortunately autoimmune disorders have gradually increased over time, and there are limited treatment options with high risks of infection or side effects. Chronic obesity related diseases are also highly complicated to treat. The identification of the rapid rise of obesity has lead to it being referred to as a worldwide epidemic, it is therefore necessary to understand the role of both obesity and leptin on the immune system. DC play a critical role in the immune system because activation of the adaptive immune system is primarily orchestrated through DC-T cell interactions (Janeway 2005), it is therefore critical to understand the effect leptin has on DC. The adipokine leptin may increase incidences of autoimmune diseases, by promoting a consistent pro-inflammatory environment and enhancing macrophage, NK cells, B cells and T cell activity as well as preventing production of regulatory T cells (Lam2006, Mattioli, 2005, De Rosa 2007). This study seems to indicate that leptin plays an essential role in DC physiology and function. Table 5.1 summarizes the results obtained in comparison to the non-treated (media) cells. In this study we have determined three things. First, we have demonstrated that leptin
promotes morphological changes and enhances pseudopodia formation particularly in the presence of a maturation stimulator such as LPS. It also appears leptin induces an increase in actin expression as well as promotes polymerization of actin, again particularly in the presence of a stimulus. The increase in F actin may be due to effects on actin-modulating proteins that allow for rapid assembly and disassembly of actin. It is therefore likely that the altered cellular morphology is due to the shift from G-actin to F-actin and vice versa. Therefore the morphological changes observed appear to be due to the actin re-organization as observed in confocal studies, Western Blot analysis and indirect ELISA. In the absence/blocking of leptin there is an obvious decrease in actin content as well as a decrease in F-actin. This may further support that leptin is necessary for adequate DC morphology. A physiologically low and high concentration of leptin were initially chosen for this study; however only the higher concentration (160 ng/ml) was further studied due to its observed dramatic dendrite formation and relevance to obesity (Figures 1.1, 1.2, 1.3 and Table 2.1).

Secondly, leptin alone or together with LPS promotes DC migration which is dependent on cytoskeletal rearrangements. Leptin may possibly play a vital role in DC migration both in immature and mature DC as observed by the presence or absence of LPS. It is apparent that leptin plays a critical role in DC functionality, by promoting DC migration towards a chemoattractant such as CCL19. This again is particularly true in the presence of LPS (Figure 3.1 and 3.2 Migration Assays). Leptin signaling is accomplished primarily by the JAK/STAT 3 pathway. Effects are transcriptionally observed within minutes of leptin addition (Data not shown). Physiological/functional effects are observed within 24 hours, however as leptin serum/media levels decrease the functionality also seems to decrease. This was clearly evident in the migration assay, where the 24 hour treated DC migrated much more efficiently than the 48
hour treated DC. Leptin may therefore affect migration in a dose dependent manner. CCL19 also
effected migration in a dose dependent manner with higher concentrations inducing greater
migration and lower concentrations producing limited migration. Similar trends were observed in
both 24 and 48 hour treatments but I believe that the decrease in migration at all concentrations
of CCL19 of the 48 hour treated DC is due to the decrease in available leptin in the media.
Further studies must be conducted to verify this theory. Leptin alone or together with LPS
promotes DC migration which is dependent on cytoskeletal rearrangements. We hypothesize
that the rapid turnover between G and F-actin is what allows for the enhanced migration. A
second possibility is an up-regulation of the chemokine receptor CCR7 and cell adhesion
molecules such as integrins. Further studies must be conducted to verify either of these theories.

The third and final observation established in this study is exposure to leptin by DC prior
to encountering peptide and T cells visibly enhances T cell activation as measured by T cell
proliferation, and cytokine (IL-2 and INF-γ) production. It is not clear however, how leptin
promotes DC to activate T cell. It may be due to increased surface area and lamellipodia
formations in the presence of leptin, allowing for an increase in the number of simultaneous T
cells interactions that therefore increase the likelihood of encountering a T cells specific to the
peptide it is carrying. Further studies must also be conducted to determine what exactly causes
this phenomenon.

The results obtained in this study demonstrate that leptin, particularly in the presence of a
stimulus such as LPS, enhances DC morphological changes and enhances functionality.
Furthermore this data suggest that leptin’s effect on DC is nearly immediate and that migration
and ability to activate T cells is in part dependent on leptin serum/media levels where the higher
concentrations exert a more potent effect. The combination of the described possible factors
leptin plays in autoimmunity and the findings described in this study further support obesities’ link to autoimmune disorders. In these studies however, leptin was added or blocked post DC differentiation and enrichment, not immediately upon initial bone marrow extraction; therefore it is unclear what role leptin definitively plays on monocyte derived DC. In a physiological setting cells are exposed to varying circulating leptin levels throughout the differentiation process, not only post-differentiation; therefore a future study in which DC are provided leptin from start to finish will be necessary to fully grasp leptin’s affect and role in the DC life cycles. In conclusion, leptin is important in differentiated murine DC morphology and functionality because a sudden increase in leptin levels in addition to a stimulus enhances DC function and induces morphological changes. This leads us to conclude that leptin may play a role as an adjuvant, this newly obtained knowledge could possibly aid in vaccine development targeting DCs and may lead to newer, improved vaccines.

Although much more work is needed to completely understand leptin’s effect on the immune system particularly DC, this study has slightly elucidated leptin’s affect on DC morphology and functionality. Moreover, the addition of a high concentration of exogenous leptin mimicked obesity and further studies may eventually help to elucidate its role on autoimmune mechanisms. The results in this study now lead us to question how leptin effects monocyte differentiation into DC. Future studies also include the assessment of the effect of leptin on the molecular pathways of cytoskeletal re-arrangement, further analysis of DC-T cell interaction to include interaction time, DC cytokine production and T cell and DC survival. Ideally leptin’s affect should be monitored in-vivo, possibly in C57BL/6, Lep DB and Lep OB mice and Diet Induced Obesity (DIO) mice.
Table 5.1: Result Summary - This table is a summary of the results obtained in this study. It is represented in comparison to media (non-treated cells).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Actin</th>
<th>F-Actin</th>
<th>#Dendrites</th>
<th>Length of dendrites</th>
<th>Migration</th>
<th>T cell Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>=</td>
<td>=/-</td>
</tr>
<tr>
<td>LPS (10 µg/ml)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Leplin (16 ng/ml)</td>
<td>X</td>
<td>X</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Leplin (16ng/ml) + LPS (10 µg/ml)</td>
<td>X</td>
<td>X</td>
<td>+++</td>
<td>+++</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Leplin (160 ng/ml)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leplin (160ng/ml) + LPS (10µg/ml)</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

**Key**

+ Means Increase, - Means Decrease, = Means Comparable, X Means Not Studied
Materials and Methods

Mice

Bone marrow stem cells were obtained from the long bones of C57Bl/6 mice upon reaching adulthood (6-8 weeks of age). Primary CD4+ T cells were obtained from ovalbumin specific TCR transgenic mice (strain C57BL/6-Tg425CBM/J). All strains of mice were either ordered from Jackson Laboratory or bred on site at the University of Texas at El Paso Animal facility from mice originating from Jackson Laboratory.

Medias

- **RPMI 1640** – RPMI 1640 (Invitrogen Rockville, MD) was used for Bone marrow (BM) DC. It was supplemented with 1% Penicillin Streptomycin (pen/strep) (Invitrogen Rockville, MD) and 10% Fetal Calf Serum (FCS) (Invitrogen Rockville, MD) and cytokines IL-4, and GM-CSF (10ng/ml) (Peprotech Rocky Hill, NJ).

- **Iscove’s complete media** - Iscove's complete media was made for T cells from DMEM or Iscove’s media (Invitrogen Rockville, MD) supplemented with 10% FBS, 1% pen/strep, 1% sodium pyruvate (Gibco Langley, OK), 1% Glutamax (Gibco Langley OK) and 0.4% β-mercaptoethanol (EM Science Lawrence, KS).

- **Minimal Esential Media** - S-MEM media was used for T cell hybridomas (Invitrogen Rockville, MD). It was enhanced with 1% Gentamycin, Pen/Strep, Sulfate (Gibco Langley, OK) and β-Mercaptoethanol and 10% FBS 6% tumor cocktail

- **Tumor cocktail** - Tumor Cocktail was made of Dextrose (Fisher Houston, TX),
Glutamine Essential and Non-Essential Amino Acids (VWR Brisbane, CA). The final product was set at a pH of 7.0 with 10N NaOH, Sodium Pyruvate, Sodium Bicarbonate (Sigma St. Louis, MO).

**Cytokines, Peptides and Proteins**

- **Interleukin-4** - Lyophilized Interleukin-4 (IL-4) (Peprotech Inc. Rocky Hill, NJ) was reconstituted in Hanks Buffered Salt Solution (HBSS) (Thermo Scientific Logan, UT) for a final concentration of 10µg/ml. Stock was then aliquoted out stored at -80 °C.

- **Granulocyte Colony Stimulating Factor** - Lyophilized Granulocyte Colony Stimulating Factor GM-CSF (Peprotech Inc. Rocky Hill, NJ) was reconstituted in Hanks Buffered Salt Solution (HBSS) for a final concentration of 10µg/ml. Stock was then aliquoted out stored at -80 °C.

- **Leptin** - Murine recombinant leptin purchased from Alpha Diagnostic (San Antonio, TX) was dissolved in 15 mM HCl and mixed with 7.5 mM NaOH. Stock solution was then diluted in PBS, aliquoted out and stored at -20°C.

- **Recombinant Mouse MIP-3 β/CCL19** - Lyophilized Recombinant Mouse MIP-3β/CCL19 was purchased from R & D Systems (Minneapolis, MN) and reconstituted in sterile PBS at a final concentration of 50 µg/ml. CCL19 was then aliquoted out and stored at -20°C.

- **OB-R-Fc** – Lyophilized Recombinant Mouse Leptin R/Fc Chimera (R&D, Minneapolis, MN) was reconstituted in sterile PBS to 200 µg/ml. It was aliquoted out and stored at -20°C.

- **Ovalbumin Peptide (257-264)** - Chicken ovalbumin fragment/Ovalbumin Peptide was
purchased from Peptides International (Louisville, Kentucky) and diluted in Phosphate Buffer System (PBS). It was then aliquoted out and stored at -20°C.

- **Lipopolysaccharide** – Lipopolysaccharide (LPS) was purchased from Sigma Aldrich (St. Louis MO); it was diluted in PBS, aliquoted out and stored at -20°C.

- **Concanavalin A** - Concanavalin A (Con A) was purchased from Sigma Aldrich St. Louis MO). It was dissolved in PBS and aliquoted out at a concentration of 1 mg/ml.

**Antibodies**

- **Anti-Actin, Clone C4** - Anti Actin Ab (Millipore, Temecula, CA) was diluted (1:500 for Western blot analysis or 0.1 µg/ml for indirect ELISA) in TBST with 5% Bovine Serum Albumine (BSA) (Fisher, Fairly Lawn, NJ).

- **Goat anti-mouse IgG (H+L)** – Goat anti- mouse IgG (KPL Baltimore, MD ) was diluted (1:4000 For Western blot analysis or 5 µg/ml for Indirect Elisa) in 5% Non-Fat Dry milk in TBS.

- **Rhodamine Phalloidin** – Rhodamine Phalloidin (Cytoskeleton Inc. Denver, CO) was reconstituted in 100% methanol to a stock solution of 14 µM. It was then aliquoted out and stored in the dark at -20°C

- **Diamidino-2 penyindole dihydrochloride** - Lyophilized Diamidino-2 penyindole dihydrochloride (DAPI) (Fluka, Sigma-Aldrich, St. Louis, MO) was reconstituted in 10mMtris and 1 mM EDTA in milliQ at a pH of 8. It was stored at -20°C

**Primary T Cells**
TCR transgenic mice were sacrificed at about 10 weeks of age. Mice were anesthetized with Isoflurane USP (VWR Brisbane, CA) and sacrificed by cervical dislocation. The spleen and all lymph nodes were isolated and placed in single cell suspension. T cells were enriched by positive selection for CD4+ using IMAG purification system. Cell concentration were be determined by trypan (Invitrogen, Rockville, MD) blue exclusion on a hemocytometer.

**T cell hybridomas**

T cell hybridomas specific for ovalbumin were generously donated by Dr. Philippa Marrack from the National Jewish Medical and Research Center in Denver, CO and are currently maintained in S-MEM media enhanced with tumor cocktail at 37°C with 5% CO₂.

**Bone Marrow Isolation to Generate DC**

C57BL/6 mice were anesthetized with Isoflurane USP and sacrificed by cervical dislocation. Skin and tissue on hind legs was removed and long bones placed in HBSS, they were then placed in 70% EtOH (Fisher Houston, TX) to prevent contamination. Bone marrow was then flushed from the bones and filtered through an autoclaved nylon mesh. RBCs were lysed using lysis buffer and removed by centrifugation. Remaining cells were resuspended in complete RPMI 1640 with cytokines (GM-CSF, and IL-4) and incubated at 37°C with 5% CO₂. On day 2 supernatant were removed and replaced with fresh complete media containing cytokines. On the 4th day, the cells were resuspended and split. Cells were enriched by positive selection for CD11c using IMAG purification System on day 6. Finally cells were counted using trypan blue exclusion on a hemocytometer and were ready for use.
**DC & T cell Enrichment**

Purification was performed by positive selection using BD IMAG cell separation system (BD Biosciences Pharminogen San Diego, CA). Cells were harvested, counted and placed in FACs tubes at $10^7$ cells per tube in 20% normal mouse serum (NMS) (Sigma. St Louis MO) and IMAG buffer (composed of 0.5% BSA (Sigma. St Louis MO) 2mM EDTA, 0.1% Sodium azide (Fisher Houston, TX) in PBS) then incubated on ice for 15 min. 1 µl of CD11c biotin antibody (Dendritic Cells) or CD4 biotin antibody (CD4+ T cells) (Ab from BD Biosciences Pharminogen San Diego, CA) was added and cells were again incubated on ice for 15 minutes. Contents were washed with IMAG buffer then resuspended with streptavidin particles (20 µl for every $10^7$ cells), tubes were then incubated for 30 minutes at 4°C. Upon completion of incubation cells were added 450 µl of IMAG buffer and placed on the magnet for 6-8 minutes. Supernatants were removed while on magnet and cells resuspended in 500 µl of IMAG buffer. They were then placed on the magnet for 3 minutes and supernatants removed, this process was repeated 3 times to ensure purity. Final population of cells was washed in IMAG buffer and resuspended in RPMI media (DC) or Iscove’s Complete Media (T cells). Concentration of cells was finally determined by trypan blue exclusion on a hemocytometer.

**Flow Cytometry**

Enrichment of cells was confirmed by flow cytometry to be >90%. Cells were placed in Flow cytometry vials and analyzed by FC500 flow cytometer (Beckman Coulter, Miami, FL) using CXP software. 10,000 viable cells per sample, were gated for lymphocytes (CD4+) or for DC (CD11c) by a combination of forward and side scatter.

**Treatment of Cells**
Enriched DC were placed in one the following treatment for 24 or 48 hours.

1.) 10 µg/mL LPS
2.) 1 mg/mL of OB-R-Fc
3.) Non-treated DC in RPMI 1640 media
4.) 16 ng/mL recombinant murine leptin
5.) 10 µg/mL LPS & 16 ng/mL recombinant murine leptin.
6.) 160 ng/mL recombinant murine leptin
7.) 10 µg/mL LPS & 160 ng/mL recombinant murine leptin

Non-treated cells functioned as a negative control. Bovine leptin in the media can bind to murine leptin receptor therefore Ob-R chimera (Ob-R:Fc) treatment was added as a second control to block bovine leptin found in the FBS of the media. This control will ensure that results obtained are not due to leptin found in the media. Lipopolysaccharide (LPS) will function as a positive control for DC maturation. A low (16ng/ml) and mid range (160 ng/ml) concentration of leptin were chosen based on studies of Mexican Americans by Peltz (2007).

**Light Microscopy**

Enriched cells were placed in 4 or 8 chamber slides and allowed to rest for 24 hours. Treatments were then added and cells were incubated for 24 hours with treatments as described above. Images of cells were obtained with Micron 2004 software (by Westover Scientific ) using a Fisher Scientific inverted light microscope at a magnification of 40x.

**Confocal Microscopy**
Cells were treated as in light microscopy; but after 24 hour treatment slides were centrifuged at 500 rpm for 5 min and supernatant removed and replaced with 1% Paraformaldehyde (PFA) (Fisher, Fair Lawn, NJ) for 10 minutes at room temperature. PFA was removed and slides were washed out with PBS at room temperature, then permeabilization buffer was added (composed of 0.5% Triton X-100 in PBS From Sigma Aldrich, St. Louis, MO) for 5 minutes at room temperature to allow staining of the cytoskeleton. Buffer was removed and slide re-washed with PBS. F-actin was then stained with Rhodamine labeled Phalloidin (Cytoskeleton Inc Denver, CO) 200 µl/chamber at 100 nM diluted in RPMI 1640 for 2 1/2 hrs at room temperature in the dark. Slides were then washed with PBS at room temperature three times and the nucleus was counterstained with 4’,6-diamidino-2-phenylindole (DAPI) at 100nM in RPMI 1640 for 30 seconds at room temperature in the dark. Slides were washed one final time and Anti-fade mounting medium (Fluka Biochemika Sigma Aldrich, St. Louis, MO) was applied to the slide to prevent loss of fluorescence. Cover slips were placed on slides and sealed with clear nail polish. Images were analyzed with a Zeiss Scanning Confocal Microscope with Pascal Deconvolution Time-Lapse Temp Control software.

**Whole Cell Lysate Preparation**

DC were enriched and treated (4-8 million cells/treatment) as previously stated for 24 hours, they were then harvested and centrifuged at 1200 RPM for 5 minutes and supernatants were discarded. Cells were resuspended in 250 µl of 1x CPI (composed of 1 protease inhibitor cocktail tablet [Roche Applied Sciences Mannheim Germany] diluted in 1x PBS) and transferred to microcentrifuge tubes. Vials were continuously mixed for 15-20 minutes at 4ºC. Microcentrifuge tubes were then centrifuged in an ultracentrifuge at 12 RCF at room temp for 10 minutes,
supernatants were collected and transferred to new labeled centrifuge tubes and maintained at -80°C until quantification by BCA kit was performed.

**Quantification of Protein using BCA Protein Assay Kit**

Whole cell lysates were obtained as previously mentioned, their concentration was determined using a BCA protein Assay Kit from Pierce (Rockford, IL). Procedure was performed as recommended by manufacturer. BSA standards were provided with kit and 8 doubling dilutions were performed using PBS. Ten µl of each sample or standard were placed in duplicates on to a 96 well flat bottom tissue culture plate. 200 µl of working reagent was added to each well and plate was incubated for 30 minutes at 37°C. Plate was then removed from incubator and allowed to reach room temperature. Protein concentration was determined using a spectrophotometer at an absorbance of 562 nm.

**F & G Actin Cell Lysates using Extration of Cytoskeletal and Soluble Protein Fractions**

DC were treated for 24 hours as previously stated (4-8 million cells/treatment) and harvested into 15 ml conical tubes. They were then centrifuged at 1200 rpm for 5 min and supernatant was discarded. Cells were resuspended in microtubule stabilization buffer (MSB) generously donated by Dr. Sukla Roychowdhury (composed of 0.1M PIPES, 2M Glycerol, 5 mM MgCl₂, 2mM EGTA, 0.5% Triton X-100) to remove all traces of previous media. Tubes were then recentrifuged and resuspended in 200 µl of MSB with 0.1M DTT (Biosource, Camarillo, CA), 1mM of Adenosine-5'-triphosphate (ATP) (Cytoskeleton Inc. Denver, CO) and 1 protease inhibitor tablet. Tubes were incubated for 10 minutes at room temperature with periodic mixing, then placed in a microfuge and centrifuged for 10 minutes at 12000 rpm. The supernatants were
collected and placed into a clean tube labeled ST fraction and placed on ice. The remaining pellet was resuspended in 200 µl of complete MSB and placed on ice for 15 minutes. Each vial was then sonicated for 1 minute in intervals of three 20 seconds intervals. Finally pelleted samples were centrifuged again for 12000 RPM for 10 minutes and supernatant was collected and placed in a new centrifuge vial labeled MT fraction. ST and MT fractions were frozen at -80ºC until quantification by Bradford Assay was performed.

**Quantification of Protein using a Bradford Assay**

BSA standards were made in PBS starting at 2 mg/ml; seven doubling dilutions were performed to create a standard curve. 10 µl of standards or previously made samples were added to the bottom of a clean cuvette (Bio-Rad, Hercules, CA) and 1 ml of Bradford Reagent was to each sample adding one blank cuvette to account for background. Content of each vial was mixed by inverting tub a few times and avoiding bubbles. Results were read on a spectrophotometer at 595 nm.

**Western Blot**

- **Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)** – 1.5 mm thick glass plates were used to make 10% SDS gels, each composed of a separating and a stacking portion. Each stacking gel was made by adding 4 mls of Milli Q water, 3.3 mls of 30% Acrylamide, 2.5 mls of 1.5 M Tris in Milli Q at a pH of 8.8, 0.1 ml of 10% SDS, 0.1 ml of 10 % APS in Milli Q and 0.004 mls of TEMED (Sigma, St. Louis MO). The stacking gel was composed of 4.1 ml of Milli Q, 1 ml of 30% Acrylamide, 0.75 ml of 1.0 M Tris at a pH of 6.8, 0.06 mls of 10%SDS, 0.06 mls of 10% APS and 0.006 ml of
TEMED. SDS gel were placed in a running apparatus (Bio Trace, Pesacola, FL), the outer chamber was filled with lower buffer while the inner chamber was filled with upper buffer. Previously obtained quantified proteins were resuspended in 4X loading buffer at 3-10 µg/lane and loaded. One lane of the gel was reserved for Sigma’s ColorBurst Electrophoresis Marker (St. Louis, MO). Apparatus was connected to a Power Pac 200 (Bio-Rad, Hercules, CA) and run at 100 Volts for 60 minutes. The gel is then removed from the glass for transfer onto Polyvinylidene Fluoride (PVDF) (Bio Trace, Pesacola, FL) using a semidry technique with a Trans-Blot SD Semi-Dry transfer Cell (Bio-Rad, Hercules, CA). Two filter papers were soaked in transfer buffer (48mM Tris, 39 mM glycine, 20% methanol, 0.375mg/ml SDS in Milli Q) and placed on the anode of the transfer apparatus. The PVDF was soaked in 100% methanol, then rinsed in Milli Q and Transfer buffer, it is then placed on top of the filter paper and the gel is placed on top of it. Lastly a second set of filter paper is placed on top of the gel and the pile was pressed to ensure adequate transfer. The apparatus is closed and attached to the Power Pac 200 (Bio-Rad, Hercules, CA), it is run at 20 volts for 70 minutes.

- **Immunoblot** - Membrane was removed from the transfer apparatus and placed in a shaking container with 5% Carnation Non-Fat Dry Milk (Wal-Mart El Paso, TX) in TBS (10mM Tris, 150 mM of NaCl in Milli Q water) for 1 hour. Membrane was then washed three times by replacing milk with TBST (TBS + 0.05% Tween 20 [Fisher, Fair Lawn, NJ]). Primary Antibody (1ºAb) (Anti-Actin) was diluted 1:500 in 5% BSA in TBST, It was then placed in the container with the membrane shaking overnight at 4°C. The following morning the 1ºAb was removed and the membrane was washed 3 times in TBST (10 minutes each wash). The secondary Ab (2ºAb) (Goat anti- mouse IgG) was
diluted 1:4000 in 5% NF Dry Milk, it was incubated at room temperature for 1 hour with continuous shaking. The 2°Ab was discarded and the membrane was washed 3 times with TBST and one final time with TBS. The membrane was placed protein side up onto an X-Ray Cassette and Super Signal West Pico substrate (Pierce Rockford, IL) was added directly to the membrane. CL-XPosure file (Thermo Scientific Rockford, IL) was placed on top of the membrane for a varying amount of time and the image developed. Images were finally analyzed using densitometry.

**Densitometry**

Western blot images were scanned into a computer as a JPEG file and Intensity of bands was determined using Un-Scan-It Software and results were graphed on Prism Software.

**Measurement of Actin by Indirect ELISA**

Cell lysates were obtained as described; standards and lysates were diluted to 300 ng/ml or 500 ng/ml respectively in PBS. 50 µl were added to each well in a 96 well ELISA plate and incubated overnight at 4°C. Content was removed and plate was blocked with 200 µl/well of 5% Carnation Non-Fat Dry milk in TBS for 10 minutes at room temperature. Milk was removed and replaced with 100 µl/well of Anti-Actin, Clone C4 diluted to 0.1 µg/ml diluted in 5% BSA in TBST, it was then covered and incubated at room temperature for 1 hour. Contents were again removed and plate was washed 5 times with Elisa Wash Buffer. Secondary antibody (Goat antimouse IgG (H+L) was diluted in 5% Non-Fat Dry milk in TBS to 5 µg/ml (100 µl/well). The plate was covered and incubated at room temperature for 1 hour. At the conclusion of this incubation the plate was washed 7 times and 100 µl of chromogen was added to each well. The
plate was incubated in the dark for 30 minutes, then the reaction was stopped with Stop solution
and the plate read at 450 nm in a spectrophotometer. Results were analyzed and graphed on
Prism Software and.

**DC In-vitro Migration**

In-vitro migration was assessed using 24 well 10 µm transwells. DC were treated as previously
stated for 24 and 48 hours. Cells were washed to remove traces of treatment and placed in RPMI
1640 in the upper chamber of a transwell at 400,000 cells/well. DC-specific chemokine (CCL19)
was placed at 200 ng.ml, 50 ng/ml and no CCL19 in the lower chamber. The plates were
incubated at 37ºC for 4 and 24 hours. Cells in the lower chamber were digitally photograph to
assess morphology and then harvested and counted by trypan blue exclusion to determine the
percentage of migrated DC. Finally Migrated DC were verified to be DC by flow cytometry
(CD11c +).

**T Cell-DC Co-Cultures**

Treated enriched primary DC and T cells either primary (CD4+ TCR Transgenic) or CD4+
Hybridoma, were placed in a 96 well round bottom tissue culture plate at 100,000 cells/well at
varying DC: T cell ratios (1:1, 1:5, 1:10, or 1:20). Ovalbuimin peptide was added to all wells at
500 µg/ml except for negative controls of each treatment set up at a DC:T cell ratio of 1:5. The
plate was then incubated at 37ºC with 5% CO₂ for 72 hours. Supernatants were collected from
hybridoma plate for IL-2 production. Primary plate supernatants were analyzed for INF-γ
production and added [³H]-thymadine for future proliferation assay.

**In-Vitro T cell Activation**
T cell activation was assessed using co-cultures of antigen specific T cells and treated BM-DC. T cell hybridoma or primary CD4+ or CD8+ T cells from TCR transgenic mice were activated by ovalbumin peptide-pulsed treated BM-DC (100,000 T cells to 20,000 BM-DC in a U-bottom 96-well plate). After 72 hours supernatants will be removed and T cell activation will be measured by IL-2 or IFNγ production (to be assessed by Enzyme Linked Imunosorbent Assay (ELISA). An additional assay will be conducted for the primary T cells; the plates will be pulsed with 1 μCi of [3H]-thymadine for 18 hours (overnight pulse). Proliferation is determined as a function of cell-associated radioactivity, which is counted using a scintillation beta counter.

**Measurement of IFN-γ**

DC and Primary T cells were co-cultured as previously described, 72 hours after co-culture supernatants were collected. Production of Interferon-Gamma by T cells in co-culture was determined by R&D Systems QuantiKine Mouse IFN-α kit (Minneapolis, MN), Protocol was followed as recommended by manufacturer. Standards were diluted in Calibrator Diluent RD5Y starting at 6ng/ml with six doubling dilutions. 50 µl of Assay Diluent RD1-21 was added to all wells, then 50 µl of sample, standard or control were added to each well, the plate was covered and incubated for 2 hours at room temperature. Content was removed and plate was washed 5 times in wash buffer then 100 µl of conjugate was added to each well and plate was covered and incubated at room temperature for 2 hours again. Contents were again discarded and plate was washed 5 times. 100 µl of Substrate solution was added to each well and the plate was incubated at room temperature in the dark for 30 minutes. Finally reaction was stopped by adding 100 µl of stop solution to each well and results were read on a spectrophotometer at 450 nm with
correction wavelength at 540 nm. Data was graphed using Prism Software.

**IL-2 Detection**

DC and T cells hybridomas were co-cultured; post 24 hour DC treatment. Supernatants were collected 72 hours after co-culture and analyzed for IL-2 production by Biosource Invitrogen cytokine and signaling IL-2 Sandwich Elisa kit (Camarillo, CA). Procedure was performed as protocol indicated. Elisa plates were coated 100 µl/well with 1.25 µg/ml in coating buffer B and incubated overnight at 4°C. Plate was washed with washing buffer one time and then blocked for one hour at room temperature with 300 µl/well of assay buffer. Standards (100 or 50 µl/well at 1 ng/ml), samples (100 or 50 µl/well) and detection Ab (50 µl/well at 0.01 µg/ml in assay buffer) were added. Plate was covered and incubated for two hours at room temperature. The plate was then washed 5 times with was buffer, 100 µl/well of Streptavidn-HRP (0.05 µg/ml in assay buffer) was added to each well, it was then covered and incubated at room temperature for 30 min. At the conclusion of this incubation the plate was washed 5 times as before then 100 µl/well of 3,3’5,5’-tetramethylbenzidine (TMB) substrate solution was added. The Plate was finaly incubated for 30 min in the dark at room temperature and the reaction was stopped with 100 of stop solution. Results were obtained on a spectrophotometer at an absorbance of 450 nm. Data was then graphed on prism software and analyzed.

**Statistical Analysis**

Statistics were performed using Prism Graphing Software.

**CHAPTER 7**

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CURRICULUM VITAE

Christine Delgado was born in El Paso, Texas in 1983; she is the third daughter of Rosa
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