2011-01-01

Altered Leptin Signaling On Dendritic Cells As A Potential Mechanism For Cancer Immunotherapy

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ALTERED LEPTIN SIGNALING ON DENDRITIC CELLS AS A POTENTIAL MECHANISM FOR CANCER IMMUNOTHERAPY

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Dedication

This thesis is dedicated to my family.

My parents, Miguel Angel and Patricia, have always supported my decisions in life and it's because of them that I have furthered my education. From childhood, my parents always instilled in my sisters and me the value of an education and the importance of being able to stand on our own two feet. I thank them for their support, their love, and their guidance.

My sisters, Karla Sarahi and Claudia Samirah, have always been there for me. I am blessed to have them in my life and I am thankful for their support, their guidance, and the arguments that have only brought us closer as the years go by. I am thankful of them because they have served me as role models, two beautiful and bright women whom I look up to and admire.

Most importantly, I dedicate this thesis to **my boys**, Nathan Patrick and Ian Shawn. It has been them who have motivated me with their love, their smiles, their laughter, and their tender kisses to continue to move forward, keeping my head held high, and with pride. I thank them for being with me during those long hours in lab and supporting me with tender words. I hope this serves them as inspiration to always move forward, to never stop learning and to further their education as much as possible, and to never forget to stop and smell the roses along the way. I will not lie, it has been challenging at times but at the end of it all everything was very well worth it.

To my family: I love you.

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By:

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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

May 2011

Acknowledgements

Many people have helped me accomplish this goal and without them none of this would have been possible.

Special thanks go to Oscar Ramirez PhD, Christine Delgado MS, Jeff Sivils PhD, Adrian Avila BS, and Yadira Arellano. I thank them greatly for all their support, guidance, and endless talks both in a professional and personal manner. I have learned much from them and they have made my journey worthwhile.

I would like to thank my committee Manuel Miranda PhD, Renato Aguilera PhD, Laura O'Dell PhD, and Jerry Johnson PhD for being a part of this process. Thank you to Dr. Kristine Garza for allowing me to be a member of her lab and for introducing me to the world of research. Thank you for allowing me to grow and become independent in my research.

This research project was funded in part by NIH/RISE Program (2R25GM069621-05), NIH-SCORE Grant (S06-GM08012) and NSF/AGEP (0302788) and was made possible with the use of Border Biomedical Research Center's (G12RR008124) Molecular and Tissue Culture Core Facility & technicians.

Abstract

Leptin is a pleiotropic hormone synthesized primarily by white adipocytes and its receptors are expressed in a variety of tissues and cells such as in the hypothalamus and cells of the immune system. Multiple cell types can produce a considerable amount of leptin such as skeletal muscle, placenta, and osteoblasts to name a few and its synthesis has been shown to be regulated by sex hormones and a broad range of inflammatory mediators. Although leptin has been shown to directly affect immune response, we are interested in how leptin affects dendritic cell function and their ability to induce a proper and effective anti-tumor response.

The leptin receptor primarily activates the Janus kinase/Signal Transducer and Activators of Transcription (Jak/STAT) pathway with its own negative feedback loop in the production of suppressor of cytokine signaling 3 (SOCS3). Leptin resistance and leptin sensitivity have been suggested to be due to increased SOCS3 expression. Although this pathway is the target pathway, the MAPK/ERK and PI3K pathways are also known to be activated and exert different functions within the cells. Due to the presence of the long isoform of the receptor, Ob-Rb, on dendritic cells and the known effects of leptin on both branches of the immune system, we hypothesized that all three of the signaling pathways activated by leptin will be activated in both bone marrow-derived dendritic cells (BM-DC) and splenic dendritic cells (sDC) making them susceptible to leptin resistance/sensitivity.

In determining leptin's signaling pathways in BM-DCs and sDCs, the cells were exposed to obese concentrations of leptin for 0-30min or 0-45mins, then fixed, permeabilized, and intracellularly stained for activation levels of pSTAT3, p-p38 MAPK, and p-AKT. Initial studies assessed by flow cytometry have shown significant increases in all 3 signaling pathways upon leptin stimulation indicating sensitivity of these cells to leptin and the potential for leptin resistance.

Several studies have indicated that tumor derived factors (TDF), which hyperactivate STAT3 in both cancerous cells and cells of the immune system, reduced the ability of immune cells to differentiate and function properly allowing the cancer to evade immune surveillance and at the same time maintain an unregulated cellular proliferation. Nefedova and colleagues have suggested the use of a novel inhibitor, JSI-124 (curcubitacin I), as an immunotherapeutic agent to increase DC maturation and immunogenicity. Obese individuals have an increased risk of developing certain cancers, some of which are leptin-dependent. Leptin signaling, in this case, may be a possible target in cancer immunotherapies for these individuals.

Taking the Nefedova and colleagues studies into consideration we proposed to determine if, in obese individuals, the inhibition of STAT3 activation via the leptin receptor but still allowing MAPK & PI3K signaling pathways to continue their role, may lead to enhanced immune functions. The Jak2/STAT3 signaling

pathway is essential in almost all cellular functions and development and we sought to determine the effects of JSI-124 on dendritic cells in a pharmaceutical attempt to inhibit STAT3 phosphorylation and cellular function. We were able to determine that the compound reduced viability of the dendritic cells, both BM- and sDCs, by 50% at 0.5uM concentrations and it was also at this concentration where a significant reduction in phosphorylated STAT3 was observed. Because dendritic cells are the link to between both branches of the immune system with the ability to activate naïve T cells, we sought to determine the effects of acute inhibitor exposure on DCs and their ability to activate T cells with the use of T cell hybridomas (TCH). BM- and sDCs were pretreated for 30 minutes with the inhibitor prior to coculturing with TCH for a 72 hour period. The inhibitor reduced both the ability of the DCs to activate TCH through the production of IL-12 and IL-2 production by the TCH.

Although these studies are in the preliminary stages, much is yet to be understood regarding leptin's role in these interactions and the ability to specifically target leptin signaling in an attempt to improve cancer immunotherapies in overweight and obese individuals.

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Chapter 1

Overall Introduction

Obesity

Obesity has grown to become an epidemic in the United States and is of great concern to our society (Zyromski et al 2009). From the 2007-2008 National Health and Nutrition Examination Survey (NHANES), an estimated 68.3% of U.S. adults are overweight (body mass index (BMI) 25-29.9) and 34.3% are obese (BMI greater than 30) as measured by BMI (CDC, Flegal 2010). The increase in obesity is mostly seen in men and children and the prevalence to becoming obese or overweight continues to rise (Ogden et al 2007). Although this health concern seems to affect only developed nations, studies have shown that regardless of economic status the issue of obesity is predominant in urban areas of all nations (WHO, Low et al 2009). This condition predisposes these individuals to other diseases, and as a result, has also been associated with increased healthcare expenditures doubling costs in persons with a BMI over 40 (Sturm 2007). In 2008, obesity related medical costs were shown to have been over $147 billion dollars, which $127 million are due to the individual care of obese individuals (CDC). Increase in healthcare expenditures is due to the association of obesity and an increased risk of cardiovascular disease, osteoarthritis, respiratory problems,

diabetes, and cancer (Garofalo et al 2006, Trevisan et al 1998, Zyromski et al 2009, CDC). Obesity has been associated with Type 2 diabetes worldwide and affects both children and adults. Studies have shown an association between increased weight gain, increased body mass index (BMI), and waist-to-hip ratios with type 2 diabetes and the reduction of these has been shown to reduce the risk of progression from impaired glucose tolerance (Tuomilehto et al 2001, Steyn et al 2004, WHO). In cancer patients, especially those that are hormone-dependant, excess fat tissue may cause enhanced tumor growth and metastasis (Garofalo et al 2006).

It is important to understand and try to find new methods and specific targets for the development of a healthier society. Despite the increasing problem with obesity, little is known as to its resulting in the development of other serious diseases mainly due to the inability of the immune system to function properly and protect the body. Although the immune system's purpose to protect the body is inhibited, leptin may be one of the many sources for this decrease in immune function.

Leptin

A potential target to increase immunity in obese individuals is leptinbecause it has been shown to affect a variety of physiological processes including immunity. Leptin is a 16kDa hormone synthesized primarily by white adipocytes

(Loffreda et al 1998). Leptin is a product of the obese (*ob*) gene, and leptin serum levels positively related to body mass and body fat stores (Loffreda et al 1998). Although adipocytes are the primary producers of leptin, they are not the only source since other cell types can produce a considerable amount. For example skeletal muscle, placenta, and osteoblasts all produce significant levels of leptin (Sanchez-Margalet et al 2003).

Synthesis of leptin has been shown to be regulated by sex hormones and a broad range of inflammatory mediators (Sarraf et al 1997, Gualillo et al 2000). Testosterone has been shown to inhibit the expression of leptin while ovarian steroids increase it resulting in women tending to have higher levels of leptin when compared to males (Blum et al 1997, Teichtahl et al 2005).

Leptin exerts its effects via the leptin receptor (Ob-R), a product of the *db* gene and member of the class I cytokine receptor family (Madej et al 1995, Mattioli et al 2009). There are six isoforms of the leptin receptor but only one is suggested to have full signaling capabilities (Figure 1.1). All six leptin isoforms, Ob-Rb, Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf, have a conserved extracellular ligand-binding domain with a single transmembrane domain and a cytoplasmic signaling domain with the exception of Ob-Re, which is the only soluble isoform.

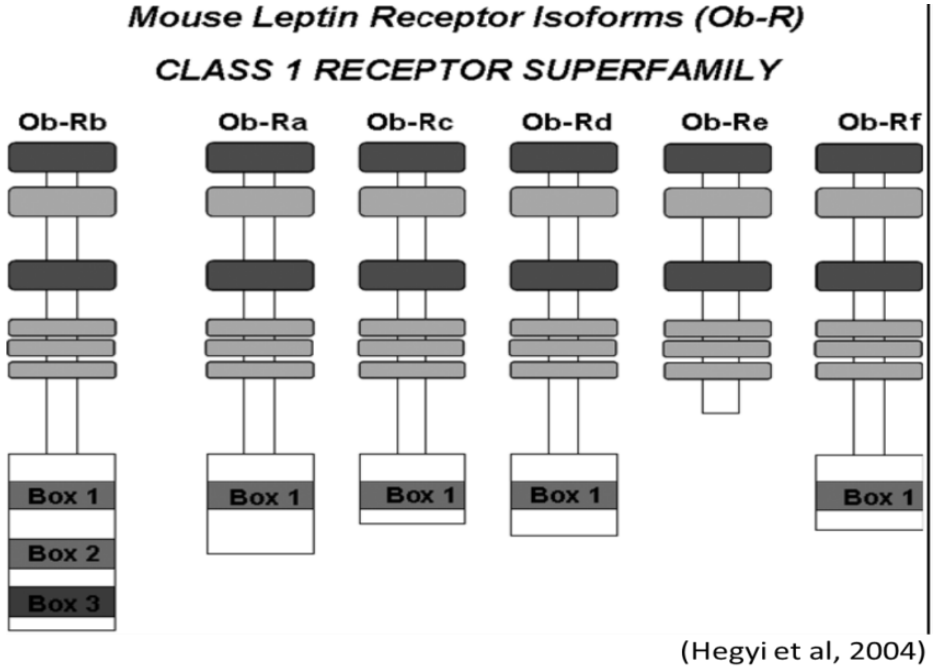


Figure 1.1. **<u>Leptin Receptor Isoforms</u>**. Depicted here are the six different isoforms of the class I cytokine leptin receptor. The extracellular ligand-binding domain is conserved throughout all the isoforms. With the exception of Ob-Re, which is the only soluble isoform, all isoforms have a single transmembrane domain and a cytoplasmic signaling domain. Box 1 is also conserved within these isoforms but Ob-Rb is the only isoform with full signaling capabilities. Box 2 is suggested to mediate Jaks association to the receptor and Box 3 contains the binding site for STAT3, key for full signaling capabilities.

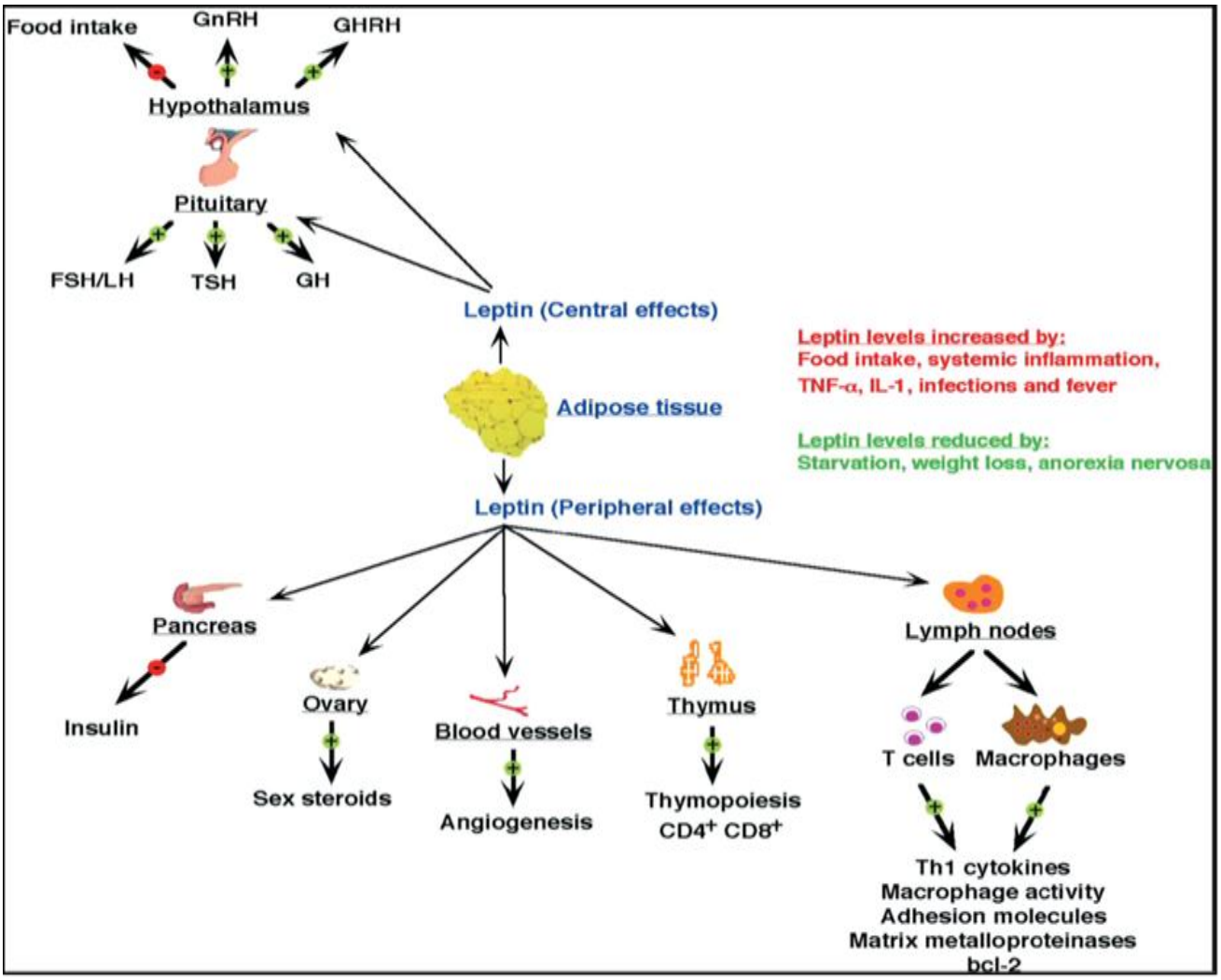
All the isoforms, with the exception of Ob-Re, have an extracellular ligand domain, a single transmembrane domain, and a cytoplasmic signaling domain. The leptin receptor does not have intrinsic kinase activity and therefore relies on Janus kinases (Jak) for downstream signaling (Bates and Myers 2004). Ob-Re is a soluble isoform containing only the ligand domain, which may be responsible for the transportation of leptin to specific targets in the brain (Busso et al 2002). The other four isoforms (Ob-Ra-f) still signal through the receptor but fail to activate the Janus kinase/Signal Transducers and Activators of Transcription (Jak/STAT) signaling pathway.

There are three boxes within the receptor's cytoplasmic signaling domain on Ob-Rb. Box 1 is a proline-rich motif essential for the activation and interaction of Jak kinases and is conserved in all isoforms with the exception of Ob-Re. The cross phosphorylation of these kinases causes receptor phosphorylation and activates the entire intracellular domain. Box 2 is a less conserved sequence suggested to mediate the association of Jaks to the receptor. Box 3 is the binding site for STAT3 and mediates transcriptional regulation upon phosphorylation and translocation of STAT3 into the nucleus (Bates and Myers 2004, Lago et al 2008).

Leptin receptors are expressed in a variety of tissues and cells, primarily in the hypothalamus but also in ovarian follicular cells, bone marrow precursors, as well as in cells of the immune system (La Cava et al 2003, 2004, Hukshorn et al

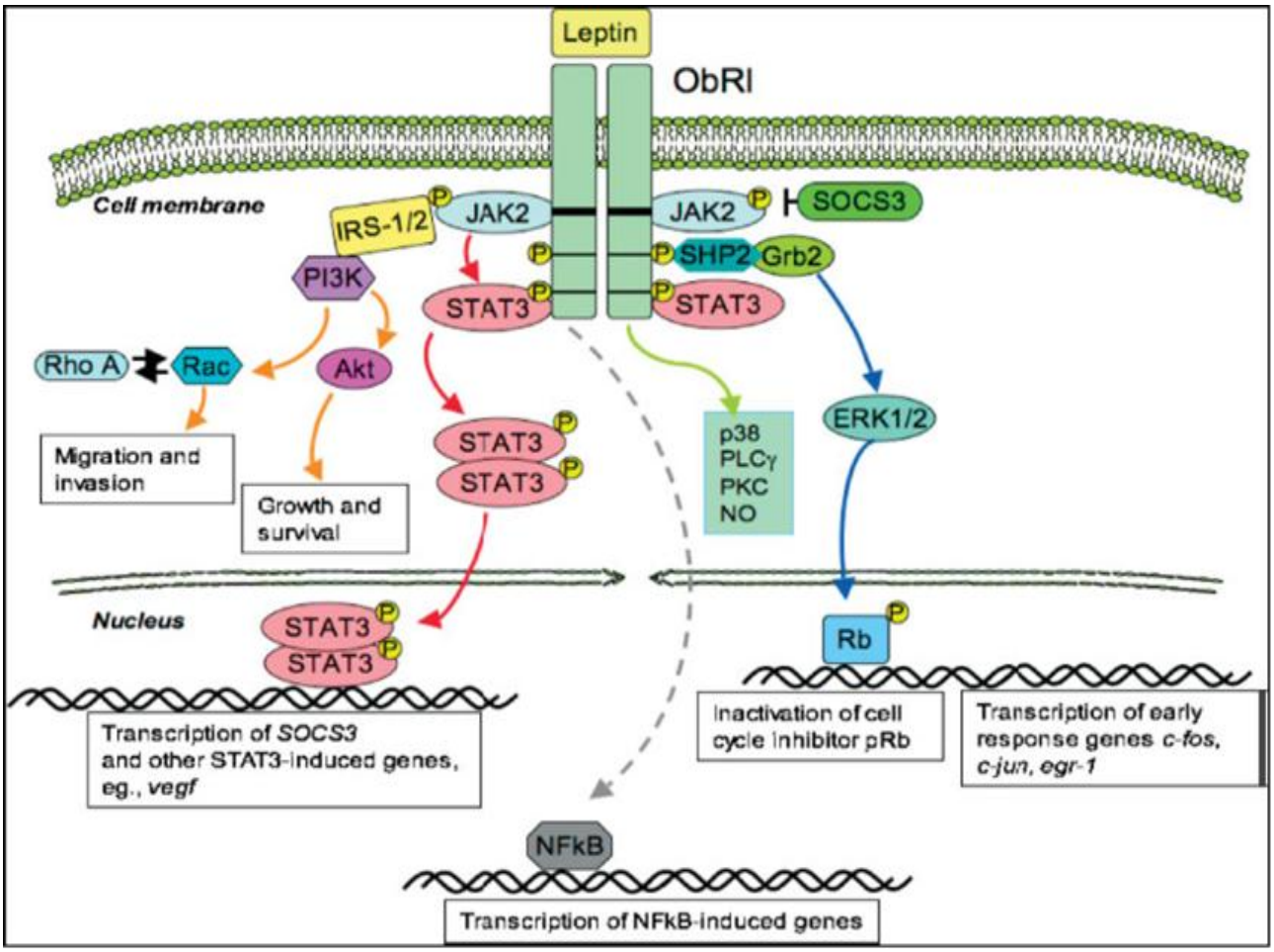
2004, Seufert 2004, Fantuzzi 2006, Lam et al 2006, Mattioli et al 2005, 2008, 2009, De Rosa et al 2007, Dellas et al 2007, Lago et al 2008). Due to the different sources of leptin and the different expression profiles of the receptor, leptin is a well-established pleiotropic hormone which affects multiple systems (Figure 1.2). Although the primary signaling pathway of Ob-Rb is the Jak2/STAT3 signaling pathway, other pathways that are activated include mitogen-activated protein kinase (MAPK) cascade, and phosphoinositide 3-kinase (PI3K) pathway, and Nuclear Factor-kappa B (NFkB) pathway (Figure 1.3) (Garofalo et al 2006).

All of these pathways, when activated, exert a different cellular response. For example, the classical JAK/STAT pathway induces transcription of not only suppressor of cytokine signaling 3 (SOCS3) but other STAT induced genes such as vascular endothelial growth factor (vegf) responsible in blood vessel development and suggested to negatively affect DC differentiation (Gabrilovich et al 1996). The MAPK pathway leads to the transcription of early response genes such as c-fos and c-jun that upregulate transcription of genes responsible for proliferation and/or differentiation. The PI3K pathway is involved in growth, survival, migration and invasion (Garofalo et al 2006, Mattioli et al 2009). The signaling pathways activated by leptin a variety of physiological processes of importance to us, immune function.



(La Cava et al 2003)

Figure 1.2. **<u>Leptin's Pleiotropic Effects</u>**. Produced predominantly by adipose tissue, leptin exerts its effects not only on the central nervous system (CNS) but also on the peripheral (PNS). Leptin in the CNS affects/controls primarily food intake and hormone release. Leptin in the PNS has a much broader effect including sex steroids produced by sex organs, angiogenesis, and immune functions.



(Garofalo and Surmacz, 2006)

Figure 1.3. **Signaling Pathways Activated by Ob-Rb**. Depicted here are the different pathways activated by the leptin receptor upon the recruitment of JAK2 and consequently the phosphorylation of the entire receptor. The main signaling pathway of leptin is through the Jak2/STAT3 pathway resulting in the receptor's own negative feedback loop by the production of SOCS3. The cross-phosphorylation of Jak2 and the phosphorylation of the receptor by Jak 2, lead to the exposure of a series of sites (Y985, Y1077, and Y1138) which recruit other molecules necessary for ERK, MAPK, PI3K, and STAT3 signaling cascades.

Immune System

The immune system has evolved to protect the organism from disease-causing pathogens. It protects the body from a variety of pathogens including viruses, parasites, and have the ability to distinguish between self and nonself antigens (Kindt 2007). The immune system is composed of two branches: innate and adaptive. The innate branch is defined by its lack of specificity and involves several barriers to prevent and/or eliminate the invasion of pathogens. Some of these barriers include anatomical, physiological, endocytic and phagocytic, and inflammatory mechanisms. The adaptive immune response is mediated by B and T cells following exposure to an antigen with the following criteria: specificity, diversity, memory, and self-nonself recognition (Janeway 2005).

Innate immunity is the first line of defense against pathogens. This response helps prevent most infections and can even clear the host of infections within hours of exposure. This response is non-specific; instead it uses a "pattern recognition" system which recognizes certain types of molecules exclusive to microbes, activating a response against them (Netea 2011). Cells of the innate immune response include macrophages, dendritic cells, neutrophils, and natural killer cells. All these cells share similar functions within the host, but at the same time differ in some ways. For example, neutrophils are essential for responding to bacteria and fungi; macrophages are essential for recognizing microbial components; natural

killer cells are critical in the defense against viral infections; and dendritic cells directly link the innate and adaptive immune responses (La Cava and Matarese 2004).

Dendritic cells (DC) are the most potent antigen presenting cells (APC) and the only APCs capable of activating naïve T cells (Schuurhuis et al 2006). Due to this ability of dendritic cells, they are the focus of our studies and the target for immunotherapies. There are at least 4 major categories of DCs: Langerhans, interstitial, monocyte-derived, and plasmacytoid DCs; all derived from hematopoietic stem cells but localize to different regions. DCs play several roles. Their primary role is to induce naïve T cell immune responses but they are also very critical in the induction of T cell tolerance and the regulation of T cell responses (Schuurhuis et al 2006, Lin et al 2010). Immature DCs (iDC) have the purpose of antigen uptake and are very efficient phagocytes. They are identified by having low expression levels of their clusters of differentiation (CD) surface markers such as CD40, CD54, CD80, CD86, and major histocompatibility complex class II (MHC). Once iDC have phagocytosed an antigen they begin their migration to draining lymph nodes where they present antigens to T cells and their maturation process. During their migration, DCs process antigens into peptides that are then loaded onto MHC class II molecules for presentation to T cells. The maturation process is characterized by decreasing phagocytic abilities and

increasing antigen presentation capabilities, which include increased levels of surface marker expression of co-stimulatory molecules necessary for naïve T cell activation and differentiation into T helper cells (Janeway 2005, Kindt 2007). iDCs express chemokine receptors that direct them to the periphery whereas mDCs express chemokine receptors that direct them to T cells within the lymph nodes where they present antigen peptides (Figure 1.4).

A specific subset of DCs, CD8+DCs, also have the ability to effectively stimulate cytotoxic CD8+ T lymphocytes (CTL) response by directly cross-presenting CTLs with extracellular antigens on Class I MHC molecules (Heath and Carboni 2001, Janeway 2005, Kindt 2007). This process has two outcomes; cross- tolerance and/or cross-priming. Cross-tolerance is the ability of DCs to induce tolerance in CTLs against self-tissues that could otherwise lead to the activation of autoreactive T cells and ultimately an autoimmune disease. Cross-priming is the result of activating pathogen-specific CTLs and generating an immune response (Heath and Carboni 2001, Janeway 2005, Kindt 2007). This process suggests that DCs can effectively prime CTLs against tumor-associated antigens. However, the role of DCs in tumor immunity is controversial. Therefore, the manipulation of DCs *ex vivo* or genetically may be the key to enhanced immunotherapeutic treatments for many diseases.

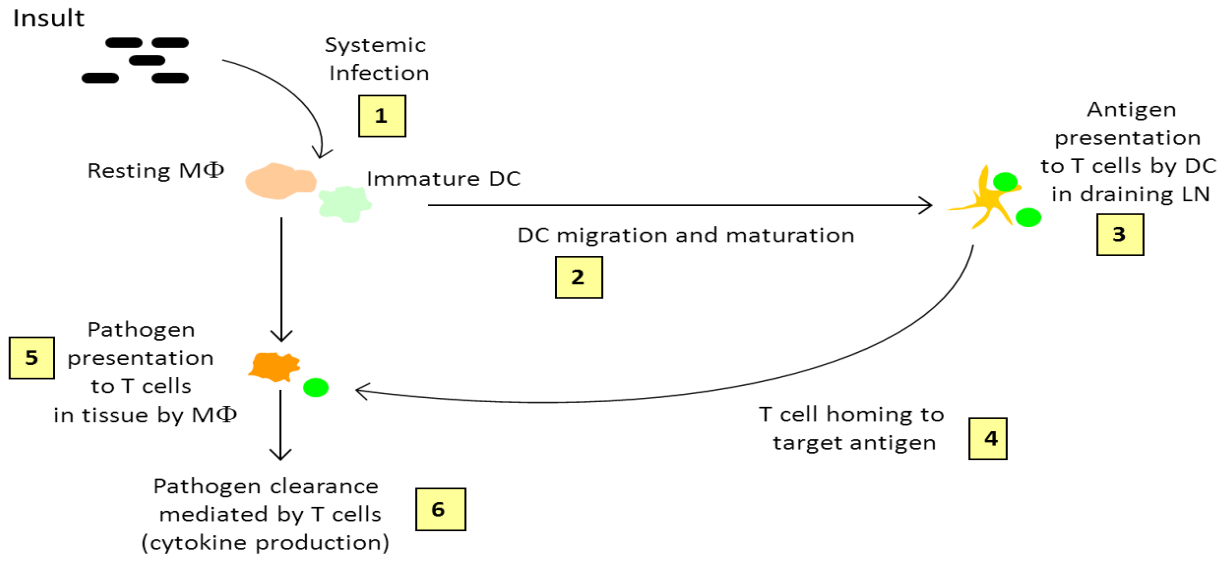


Figure 1.4. Induction of an Adaptive Immune Response. Depicted here is the process by which an adaptive immune response is initiated. Due to DCs being the only APC with the ability to activate naïve T cells, they are the link between the innate and adaptive immune response. Immature DCs survey the environment and upon antigen uptake (1) they begin their migration to draining lymph nodes and become mature DCs in the process (2). During the maturation process, DC reduce phagocytic abilities and increase expression of costimulatory molecules and MHC complexes. In the draining lymph nodes, DCs present antigens to naïve T cells (3). If the proper stimulation is present (cytokines, costimulatory molecules, etc), T cells then migrate out of the lymph nodes and into the site of insult (4). At the site of insult, macrophages aid in the induction of the immune response by presenting antigen to antigen-specific T cells (5). Pathogen Clearance is then mediated by cytokine production by T cells (6).

Leptin and the Immune System

Cells of both the innate and adaptive immune system are altered by leptin exposure due to the structural similarities between leptin and cytokines (Fantuzzi 2009, La Cava and Matarese 1998, Lam et al 2007). A decrease in plasma leptin levels has been shown to reduce and impair immune function (Lord 1998). Researchers have reported that macrophages from leptin-deficient mice have some abnormalities in expression of antimicrobial enzymes as well as pro-inflammatory cytokines (Fung-Yee et al 1999). Also, macrophages of wild type strains that have been exposed to leptin appear to increase cellular processes such as phagocytosis and antigen processing (Zarkesh-Esfahani et al 2004, Mancuso et al 2004, Caldefie-Chezet et al 2003, Raso et al 2002, Caldefie-Chezet et al 2001). Natural killer cell functions have also been shown to be altered in the presence of leptin resulting in the increased activation and cytotoxicity of these cells (Tian et al 2002).

Within the adaptive immune system, it has been reported that leptin also regulates T cell activity (De Rosa et al 2007, Lam et al 2006, Loffreda et al 1998). Leptin has been found to promote T cell activation and polarization to a Th1 response and suppressing production of Th2 cytokines (Lord et al 1998, Farooki et al 2002). Leptin deficiency or leptin receptor mutations have also been shown to increase susceptibility of the individual to bacterial infections. However, the

administration of exogenous leptin has been shown to induce significant immunogenic increase of CD4+ and CD8+ T cells, NK cells, and cytokine production (Ikejima et al 2005, Mancuso et al 2002, Wieland et al 2005). Data suggests that leptin plays a major role in modulating an immune response but the mechanism behind this process still remains unclear.

Obesity and Cancer

Cancer causes over half a million deaths per year in the United States alone and it has become the second leading cause of death (CDC). Cancer, as with obesity, has a large toll on the overall healthcare costs in the United States. In 2007, the cost for cancer treatment was estimated to be as high as $219 billion. Several different types of cancers occur in higher frequency in obese individuals (Garfinkel 1985, Engel et al 2003). For example, obesity has been recognized as a risk factor for the development of adenocarcinoma, breast, prostate, and colon cancers (Zyromski et al 2009). Data also strongly suggests that obesity directly contributes to the development of pancreatic adenocarcinoma (Zyromski et al 2009). Researchers have been able to identify that increased BMI on patients worsen the outcome of the cancer when compared to lean patients (Fleming et al 2009, House et al 2008). Although the exact link between obesity and cancers remains unclear, the adipokine milieu of obesity serves as a striking possibility to the increase in tumor growth and dissemination.

Leptin, a key molecular marker of obesity, is found in high levels of obese individuals and has been shown to enhance tumor growth in an array of cancers including breast, gastric and even lung (Garofalo et al 2006). Insulin may also play a role in tumor growth. Obese murine strains that express high levels of leptin and high levels of circulating insulin levels have been shown to display higher rates of tumor growth (Zyromski et al 2009). Several authors have also suggested that increased insulin is an important factor in the regulation of pancreatic cancer growth (Pollack 2007, Stolzenberg et al 2005). Accountability for the development of all these different types of cancers may not fall on leptin alone but its signaling cascade may serve as a potential target for the development of cancer immunotherapies.

Cancer and the Immune System

In the past ten years many advances have been made in the field of translational immunology. It has been suggested that cancer exploits signals used by the immune system to evade their detection and/or elimination. An important intrinsic oncogenic signal in tumors is the hyperactivation of STAT3s, a key culprit in the secretion and regulation of *vegf,* a soluble tumor-derived factor (TDF) (Niu et al 2002). ***TDFs have been shown to specifically inhibit DC maturation*** (Niu et al 2002, Nefedova et al 2004). Studies by Grabrilovich and colleagues, as well as by Yang and colleagues, suggest that these factors negatively affect DC function

by hyperactivating STAT3 and inhibiting NF-kB activation (Gabrilovich et al 1998, Yang et al 2009, Nefedova et al 2005). Tumor cells have also been found to secrete interleukin (IL)-10, which negatively regulates DC maturation and their ability to induce CD4 anti-tumor T cell responses (Steinbrink et al 1997). Bringing together obesity, leptin, cancer, and the immune system, the increased serum leptin levels with the decrease in immune function and an increase in hormone-dependent cancer diagnoses, leptin resistance may play an even bigger role behind immune evasion by cancer cells.

Leptin resistance (SOCS3)

As mentioned earlier, of the six leptin receptor isoforms only the Ob-Rb receptor has full signaling capabilities that lead to the receptor's negative feedback loop. None of the receptor isoforms have intrinsic kinase activity though and therefore rely on cytoplasmic kinases for its signaling events. The primary signaling pathway of Ob-Rb is the Jak/STAT signaling pathway downstream effects result in several gene transcripts that include a negative regulator of its own signaling cascade (a negative feedback loop mediated by suppressor of cytokine signaling 3 (SOCS3)). Other pathways activated by Ob-Rb include MAPK cascade, PI3K pathway, and AMPK which play roles in various cellular functional activities depending on the cell type as previously described (Fantuzzi 2009, Garofalo et al 2006, Mattioli et al 2009).

High circulating levels of leptin are present in obese people and this causes leptin resistance (Kalra 2001). Leptin resistance is the result of the body's ineffective response to endogenous and exogenous leptin (Munzberg and Myers, 2005). Much work has shown that leptin induces expression of SOCS3 and its induction specifically blocks leptin receptor signaling (Bjorbaek et al 1999, Dunn et al 2005). Münzberg and colleagues have made the suggestion that overexpression of this protein may be a possible mechanism for leptin resistance (Munzberg and Myers 2005). The expression of this protein has also been observed in obese mouse strains, further supporting its role in leptin resistance (Zabeau et al 03). Dunn and colleagues also investigated the process by which signaling through the receptor is controlled during chronic leptin stimulation. They were able to determine that although SOCS3 has multiple binding sites on the leptin receptor (Box 1, Y985, and Y1138), SOCS3 inhibits the receptor from re-stimulation during chronic leptin exposure which mimics the effects in obese states (Figure 1.5) (Dunn et al 2005). Thus, much like TDFs, chronic leptin exposure also induces hyperactivation of STAT3.

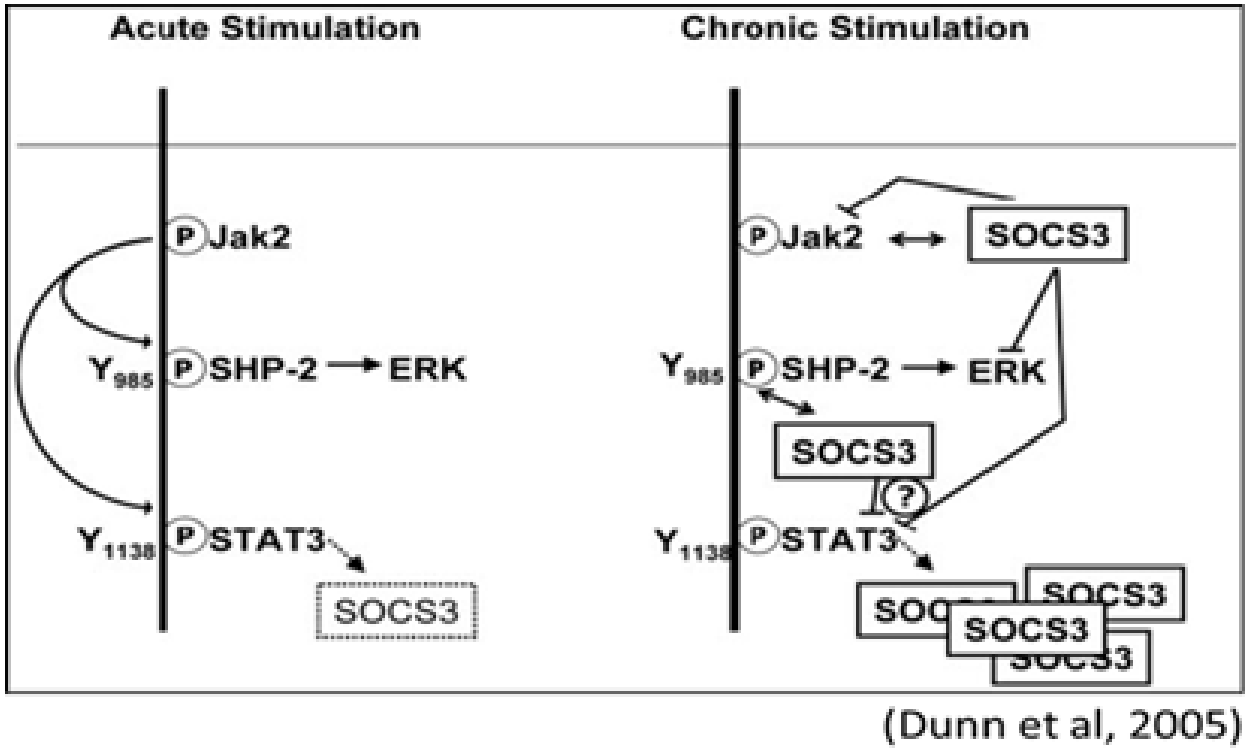


Figure 1.5. **<u>Acute/Chronic Leptin Signaling Cascades</u>.** Depiction of the pathways activated during an acute or chronic stimulation by leptin. Although the pathways are the same, the major difference is the overexpression of SOCS3 induced by chronic STAT3 activation. This causes an interference with all of the leptin signaling pathways and their downstream functional activity.

Overall Significance of Study and Impact

Obese patients not only express extremely high levels of leptin but also display leptin resistance and desensitization of the leptin receptors (Bjorbaek et al 1999). It is well accepted that obesity is factor in the development of many diseases some of which include the development of hormone-specific cancers (Garofalo et al 2006); moreover, a direct correlation between leptin levels and tumor growth has been demonstrated (Zyromski et al 2009). As previously mentioned, DCs are the key to an adaptive immune response. In order to activate/induce a proper anti-tumor T cell response, DC must be able to present tumor-specific antigens. Nefedova and colleagues have found that the hyperactivation of STAT3 in DCs caused by TDF induce abnormal differentiation of these cells as well as the accumulation of immature and immunosuppressive myeloid cells weakening the immune system (Niu et al 2002, Nefedova et al 2004). They too have shown that the inhibition of the Jak2/STAT3 pathway in DCs results in a dramatic activation not only of these cells but also in their ability to stimulate an adaptive immune response in the presence of TDF (Nefedova et al 2005).

Study Aims

We wanted to assess the different signaling pathways activated by leptin in BM- and sDCs and assess their immunological function when leptin-specific STAT3 activation is inhibited. **We hypothesized** that leptin will activate multiple

signaling pathways of the receptor in DCs leading to an array of cellular responses. We also hypothesized that specific inhibition of STAT3, responsible for the expression of the receptor's own negative feedback loop (SOCS3), will allow for increased activation of the MAPK and/or PI3K pathways promoting DC immunogenicity, potentially contributing to the advancement of anti-cancer therapies in overweight and obese patients.

Although leptin has been shown to directly affect aspects of immunity, my project focused on determining whether altered leptin signaling directly affects dendritic cell function, more specifically, DC ability to activate an effective anti-tumor T cell response. Leptin has been associated with cancer in many ways. In cancer, leptin expression is induced under hypoxic conditions (Ambrosini et al 2002). It has also been demonstrated that leptin vascularizes tumors and regulates neoangiogenesis either by itself or in conjunction with vascular endothelial growth factor (*vegf*) and fibroblast growth factor 2 (FGF) (Bouloumie et al 1998). In addition, it has been shown that constitutively active STAT3 activated by oncoproteins directly contributes to oncogenesis. Constitutively active STAT3 contributes to oncogenesis by increasing proliferation of T cells and myeloid cells among others cell types and also by inhibiting cell apoptosis (Bowman et al 2000). Contributing to this, one of the hallmark characteristics of cancer is the abnormality caused in myeloid cell differentiation. Studies by Nefedova and

colleagues have shown the effects of Jak2/STAT3 on dendritic cell function in the presence of tumor derived factors (TDF). The presence of these factors causes a hyperactivation of STAT3 which prevents monocyte differentiation into DC affecting proper immune response against the specific tumor (Nefedova et al 2004). Nefedova and colleagues have also shown that specific inhibition of the Jak2/STAT3 pathways only and no other pathways increase DC function and immunogenicity in the presence of these factors (Nefedova et al 2005).

Obesity negatively impacts cancer development as a result of excess leptin/leptin resistance rendering dendritic cells less effecting resulting in a poor anti-tumor T cell immunity. This phenomena therefore provides a potential target for immunotherapy in obese cancer patients by preventing the overexpression of SOCS3 on dendritic cells upon leptin stimulation while still maintaining all other signaling pathways active and functional.

Chapter 2a

Assessment of Leptin Signaling in Murine Bone Marrow-Derived and Splenic Dendritic Cells

Leptin signaling through the different cascades have the ability to direct several different cellular functions. In this chapter, we sought to determine the three main signaling pathways activated by leptin in murine BM- and sDCs. As mentioned before, the primary signaling cascade for leptin is through the Jak2/STAT3 pathway ultimately leading to the receptor's own negative feedback loop with the production of SOCS3 among other responses. The Jak family members are responsible for the activation of DNA binding transcription factors, STATS, which occur through tyrosine phosphorylation either receptor mediated or via intrinsic pathways (Leaman et al 1995). These phosphorylated STATS dimerize, translocate into the nucleus, and bind to specific DNA elements to elicit transcription and thus a cellular response.

For immune cells, cytokine production is a major role for STATS ranging from IL-2 and IFNγ in T cells to IL-10 and IL-12 in dendritic cells. STAT3 specifically plays an important and crucial role in dendritic cells for the induction of antigen specific T cell tolerance (Wardle 2009).

At the end of the Jak2/STAT3 signaling cascade SOCS proteins are produced, which directly inhibit the pathway. SOCS can inhibit the Jak/STAT pathway by inhibiting Jak kinases from binding to the receptor or by inhibiting its activation loop through competition with domain molecules or even targeting the receptor for proteosomal degradation (Yoshimura 2007). SOCS proteins regulate the production of different cytokines in all cells of the immune system as well as regulate the development of these cells (Wardle 2009).

With the activation of the Jak/STAT pathway by leptin, PI3K and MAPK/ERK pathways are also activated. The PI3K signaling pathway has different isoforms and several downstream targets that regulate an array of cellular functions. The PI3K isoforms regulate cell survival, growth and/or proliferation, glucose metabolism (Van Haesebroech 1999), and cytoskeletal rearrangement/organization (Coffer 1998) to name a few. One downstream target which plays a role in the majority of cascade's regulatory effects is Akt, a cell survival kinase. The activation of Akt leads to the activation of the NFkB pathway (Tanaka 2005) but also monitors cellular growth and survival and can counteract potential cellular apoptosis through glucose metabolism promoting its uptake and glycolysis (Van Haesebroech 1999).

The MAPK/ERK pathway is, in essence, involved in cell survival but, depending on environmental signals, it can also assist in apoptosis (Wardle 2009).

Activation of this pathway, either transient or sustained, is necessary for mitogenesis and cellular differentiation, respectively. p38 MAPK, a downstream activation molecule within the MAPK/ERK pathway responds to environmental stressors such as lipopolysaccharide (LPS), osmotic stress, and pro-inflammatory cytokines to name a few and also control an inflammatory response and cell proliferation (Varghese 2001, Han 2007). p38 MAPK, as Akt, leads to the activation of NFkB as part of the anti-inflammatory response (Saccani 2002). Other important roles of this molecule include its implication in monocyte differentiation to dendritic cells (Puig-Kroger 2001) and the cellular response to chemokines (Cora 2001).

Nuclear Factor kappa B (NFkB) is a family of transcription factors that play a vital role in immune and inflammatory response as well as cell survival (Kumar 2004, Barnes 1997, and Bonizzi 2004). The activation of this transcription factor is also a downstream effect of all the signaling pathways activated by leptin. NFkB regulates an array of genes significant in inflammation and innate immunity some of which involve cytokine and chemokine production, regulation of cell cycle and cell survival, regulation of cellular adhesion and invasiveness, as well as pro- and anti-apoptotic responses (Wardle 2009).

The NFkB can be divided into its classical canonical pathway and its alternative noncanonical pathway with differences in their regulatory mechanisms

(Keats 2007). Briefly, the classical canonical pathway affects the immune system and inflammation (Wietek 2007). NFkB interacts with different transcription factors such as regulatory factors, STATs, and even p53 which allows for its many roles in the regulation of cellular functions. The alternative noncanonical pathway of NFkB is essentially responsible for providing cytokine and chemokines responsible for the development of lymphoid tissue and the adaptive immune response.

Results

Our lab had previously shown expression of Ob-Rb, leptin's long isoform receptor, on both BM-DC and sDC and has also determined Jak2/STAT3 signaling on BM-DCs via Western blot upon leptin treatment measured as phosphorylated STAT3 and SOCS3 expression (data not shown) (Ramirez and Garza 2010 (manuscript in progress)). ***We sought to determine the activation of all three major signaling pathways activated by leptin in both bone marrow-derived and splenic DCs.*** Our experiments have demonstrated signaling of all three major leptin signaling pathways through Ob-Rb with the novel finding of these pathways on sDCs upon leptin treatment. Flow cytometry was used to detect the following phosphorylated proteins: STAT3, Akt, and p38 MAPK. BM-DCs were differentiated from bone marrow of C57BL/6 mice in the presence of GM-CSF and interleukin-4 (IL-4) for eight days replenishing the media every two day. On day

eight, the cells were collected, enriched through positive selection using anti-CD11c antibodies and were left to rest with HBSS or PBS for 24 hours at 4°C. For splenic DCs, spleens of two C57BL/6 mice were obtained and treated with Collagenase II in order to separate the cells from the tissue. Red blood cells were lysed before enrichment of sDC via positive selection using anti-CD11c antibodies and were left to rest with HBSS or PBS for 24 hours at 4°C. Enrichment of both types of cells was done with the use of the AutoMacs system.

After the 24 hour resting period, the enriched DCs were treated with 160ng/ml of recombinant murine leptin at different time points in 5 minute increments from 15 to 30 minutes or 15 to 45 minutes. Untreated/Media samples functioned as negative controls in order to compare background levels of the phosphorylated proteins against phosphorylation of these proteins upon leptin treatment alone. All samples were then washed, fixed with 4% PFA, permeabilized with 90% methanol, and stained for flow cytometry. The samples were surface stained with anti-CD11c antibodies and intracellular stained with the above mentioned antibodies.

Through the use of flow cytometry we were able to determine the activation of Jak2/STAT3 signaling pathway, as measured by phosphorylated STAT3 (Y705) on BM-DCs (supporting previous findings) and in sDCs (Figure 2a.1). We also determine the activation of the PI3K signaling pathway on both BM-DCs and

sDCs as measured by phosphorylated Akt (T308) (Figure 2a.2). Observed PI3K signaling by leptin in BM-DCs resembles previously published data (Lam et al 2006, Mattioli et al 2008) but activation of this pathway within sDCs is a novel finding. Finally, we were also able to determine the activation of p38 MAPK signaling pathway in sDCs (Figure 2a.3).

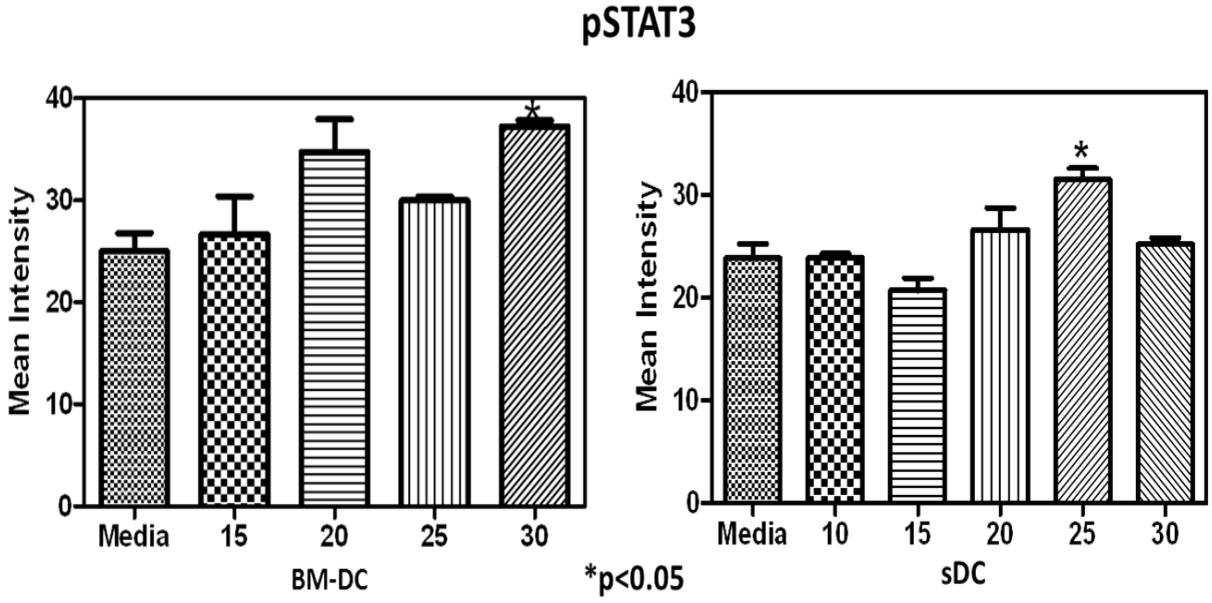


Figure 2a.1. **<u>Activation of Jak2/STAT3 Signaling Pathway in Dendritic Cells by Leptin</u>**. Enriched CD11c+ BM- and sDC were treated with 160 ng/ml of murine recombinant leptin. At the indicated time points, the cells were harvested, surface stained with anti-murine CD11C, fixed and permeabilized, and finally stained with anti-murine phospho-STAT3. The cells were evaluated by flow cytometry gating for CD11c+ cells. The data is presented as the mean ± SEM of duplicate samples and is one of two representative. Statistical analysis was performed using 1-way ANOVA with a Tukey Multiple comparison posttest; *$p<0.05$.

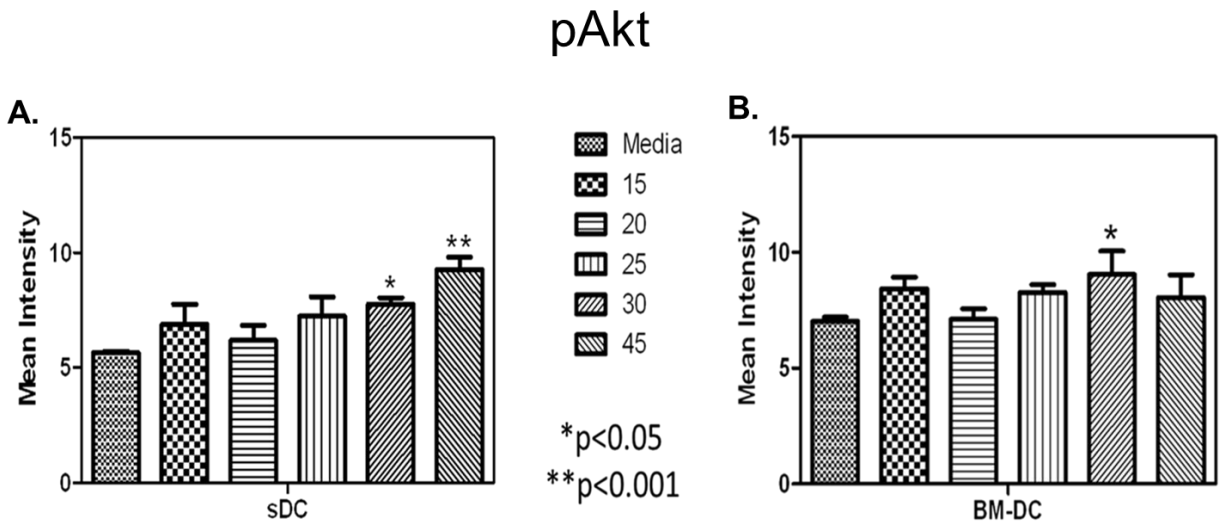


Figure 2a.2. **<u>Activation of p-Akt Signal Pathway in Dendritic Cells by Leptin</u>**. Day 6 enriched CD11c+ BM-DC and purified CD11c+ splenic DC were treated with 160 ng/ml of murine recombinant leptin. At the indicated time points, the cells were harvested, surface stained with anti-murine CD11C, fixed and permeabilized, and finally stained with anti-phospho Akt (T308) The cells were evaluated by flow cytometry gating for CD11c+ cells. The data is presented as the mean ± SEM of duplicate samples and is one of two representative, gated for surface stained CD11c marker. p-Akt peaks at 30min on BM-DCs and at 45min on sDC. Results are the mean ± SEM and representative of 2 individual experiments. Statistical analysis was using One-way ANOVA with a Tukey Multiple Comparison test; *$p<0.05$, **$p<0.001$.

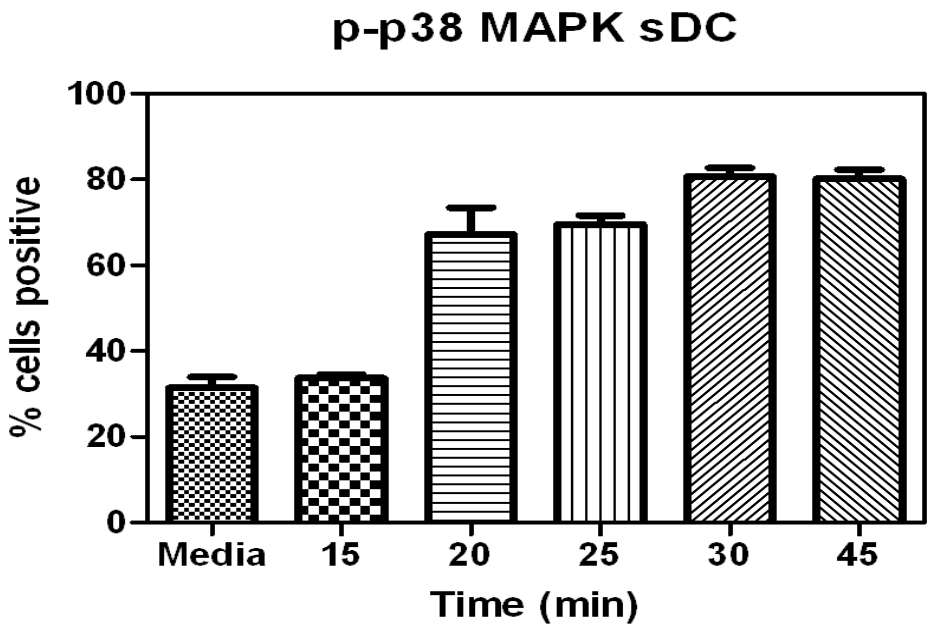


Figure 2a.3. <u>Activation of p38 MAPK Signaling Pathway in Dendritic Cells by Leptin</u>. Purified CD11c+ splenic DC were treated with 160 ng/ml of murine recombinant leptin. At the indicated time points, the cells were harvested, surface stained with anti-murine CD11C, fixed and permeabilized, and finally stained with anti-phospho-p38 MAPK (T180/Y182). The cells were evaluated by flow cytometry gating for CD11c+ cells. p-p38 MAPK peaks at 30min on BM-DCs. Results are the mean ± SEM and representative of 1 individual experiments. Statistical analysis was using One-way ANOVA with a Tukey Multiple Comparison test; *$p<0.05$, **$p<0.001$.

Chapter 2a Conclusion

We and others have shown that murine dendritic cells express the leptin receptor (Mattioli et al 2005, Ramirez et al 2010 (manuscript submitted)). Treatment of bone marrow-derived dendritic cells (BM-DC) with physiologically high levels of leptin (160ng/ml) induces enhanced dendritic cell survival and protection against induced apoptosis, and increased dendrite formation (Arellano et al, unpublished). Previous data from our lab that relates to the PI3K pathways shows that the addition of exogenous leptin to bone marrow-derived dendritic cells increases the formation of dendrite number and length (Delgado and Garza, unpublished). This increase in dendrite formation is associated with increased surface area of the cell suggesting potential increase in migration abilities and interactions with T cells. This is supported by *in vitro* and *in vivo* bone marrow-derived dendritic cell migration assays and by the enhancement of T cell activation by leptin treated BM-DCs as measured by tritiated-thymidine incorporation and the production of interferon-gamma (INF-γ) (Delgado and Garza, unpublished). In relation to the leptin receptor's Jak/STAT signaling pathway in dendritic cells, we were able to determine that in the presence of leptin, NF-kB translocates into the nucleus, which has been shown to be essential for DC survival, activation, and the polarization and activation of T cells upon interaction with DCs.

Our data suggest that all the signaling pathways activated by leptin through the leptin receptor are also activated in bone marrow-derived and splenic dendritic cells because of the different functional activities that are downstream of the leptin receptor signaling pathways.

Taken together, our data demonstrate that leptin activates all leptin receptor-mediated signaling pathways in both BM- and sDCs. The data suggests that DCs might therefore be susceptible to leptin resistance induced by an overactivation of STAT3 and its downstream gene product SOCS3 (which in turn blocks all leptin signaling). Thus, as we hypothesize, by specifically inhibiting Jak2/STAT3 signaling, responsible for the receptor's own negative feedback loop, but still allowing other signaling pathways to continue their role, may lead to enhanced immune functions which can translate to better immuno therapies for overweight and obese patients.

Chapter 2b

Assessment of the Effects JSI-124, a Novel Selective Inhibitor of Jak2/STAT3 Signaling Pathway, on Bone Marrow-Derived and Splenic Dendritic Cells

Dendritic cells are critical for the induction and maintenance of a proper immune response, more so for that of an antitumor immune response. In regards to the induction and maintenance of an antitumor immune response, this can be developed through the natural process of the immune system or as a result of immunotherapies. It has been well established in several laboratories that tumors evade the immune response due to a lack of functional dendritic cells and their differentiation (Nefedova et al 2005). The inability of the dendritic cells to function properly is due the inability of myeloid cells to differentiate to mature and functional dendritic cells and to the accumulation of myeloid and immature dendritic cells aiding in the induction of T cell tolerance and suppressive antigen-specific T cells contributing to tumor nonresponsiveness (Gabrilovich 2005).

An important element found to aid tumor malignancies is the constitutive activation of STAT3 within a variety of tumors that results in unregulated cell proliferation and anti-apoptotic effect of these tumors (Yu and Jove 2004). The hyperactivation of STAT3 in dendritic cells induced by tumor derived factors is also involved in the abnormal differentiation and function of dendritic cells

(Nefedova et al 2004, Wang et al 2004). Nefedova and colleagues have found that the inhibition of tumor derived factor-induced hyperactivation of STAT3 on myeloid cells improves myeloid/dendritic cell differentiation and function of mature dendritic cells that can potentially lead to an effective anti-tumor immune response (Nefedova 2005). It is known that STAT3 is an important factor as a negative regulator of tumor immune surveillance and its manipulation can indeed restore, induce, and increase immune response in the innate and adaptive immune system (Kortylewski et al 2005).

The Jak/STAT pathway, however, has a very critical role in development, especially important for normal cellular differentiation, and its inhibition can be deleterious. Inhibition of Jak1 results in neurological deficits and perinatal lethality (Rodig et al 1998), of Jak2 leads to defects in erythropoiesis (Neubauer et al 1998), and of Jak3 results in severe defects in the development of lymphoid organs and myelopoiesis (Grossman et al 1999). STAT3 is very significant, as well, for its inhibition results in severe embryonic development and fetal death (Takeda 1997). Although STAT3 inhibition can restore dendritic cell function in tumor-bearing hosts, studies have shown that congenital STAT3-/- in hematopoietic cell in mice does reduce dendritic cell number negatively affecting dendritic cell function and ultimately an anti-tumor response (Laouar et al 2004). Other studies have shown that complete ablation of STAT3 also leads to an

autoimmune response with a very short therapeutic window between the anti-tumor immune response and an autoimmune response (Stofega et al 1998).

Studies are being conducted where the Jak2/STAT3 pathway show promising results with the use of the pharmaceutical compound JSI-124 (curcubitacin I). JSI-124 is a novel selective inhibitor of the Jak2/STAT3 pathway inhibiting only Jak2 and STAT3 but not any other pathways. Although signaling pathways such as Src, Akt, and ERK/MAPK remain functional with the use of this inhibitor, the mechanism by which this is possible remains unclear (Blaskovich 2003). This compound, promotes protein phosphatase activation of SHP1 and SHP2 (Stofega et al 1998, Schaper et al 1998) and act on physiological inhibitors which decrease STAT3 activation (Turkson and Jove 2000). In tumor-bearing models, where JSI does not affect tumor growth, Nefedova and collegues have shown that JSI can significantly and dramatically reduce the accumulation of immunosuppressive myeloid cell and substantially increase dendritic cell presence and function in the lymph nodes (Nefedova 2005).

With what is currently known about the effects of this compound (JSI-124) and its potential use as an immunotherapeutic agent, ***we sought to determine the effects of JSI-124 exposure on normal dendritic cells***. Because of the influence it has on the improvement in immune response, we hypothesized that within leptin dependent tumors the ablation of STAT3 activation via the leptin receptor could

potentially lead to an increased immune response by dendritic cell and their ability to induce an antigen-specific T cell anti-tumor response. Cancerous cell, as mentioned previously, secreted tumor derived factors (TDF) that aid in their ability to evade immune surveillance and promote their cellular growth. In obese patients, the increased amount of circulating leptin has induced leptin resistance within the body, including the immune system, inhibiting even further immune function (Figure 2b.1) (refer to next page).

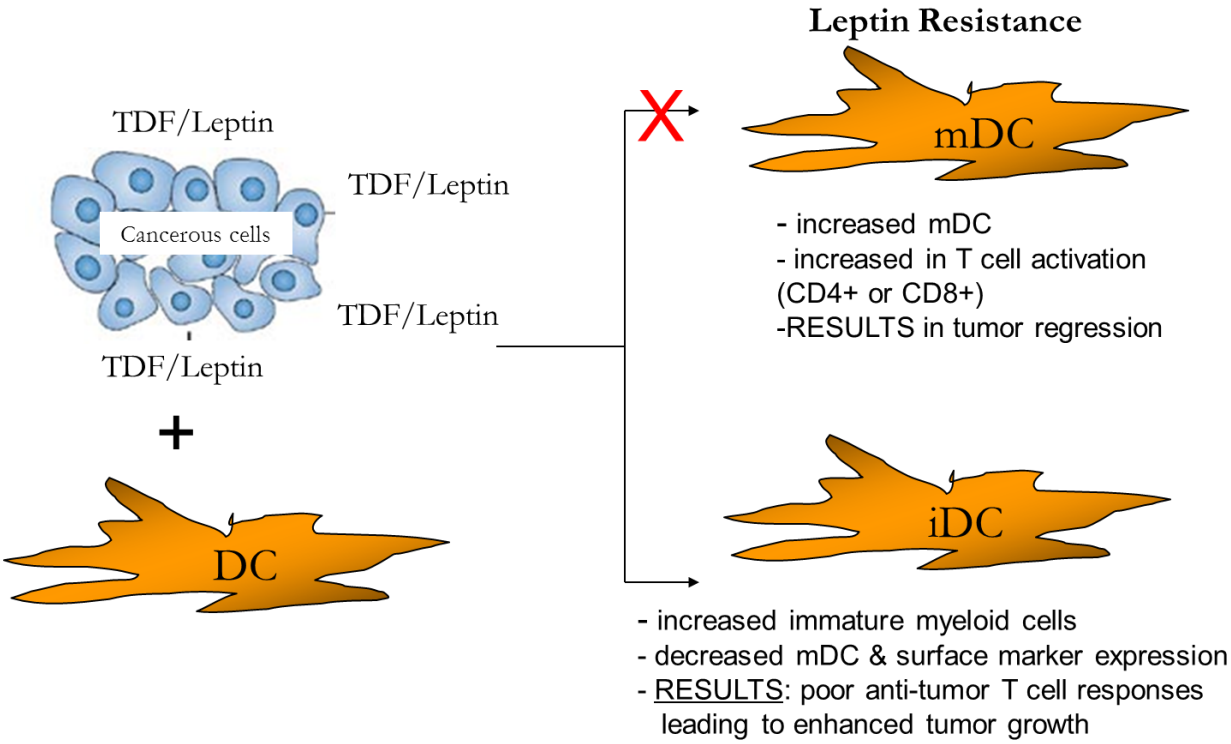


Figure2b.1. Leptin Resistance Aids Cancerous Cells in Evading Immune Surveillance. Leptin exposure positively regulates immune response both innate and adaptive. Increased or chronic exposure to leptin has the ability of inducing leptin resistance, as in obese individuals, reducing drastically an immune response. Obese individuals have an increased risk of developing certain cancers and, in some, leptin is the main culprit.

Results

Chemical inhibition of the Jak2/STAT3 pathway can result in adverse cellular responses due to the importance of this pathway within DCs. We first sought to determine what concentrations of the inhibitor to use that would maintain significant cellular viability. Using enriched bone marrow-derived or splenic DCs, we cultured the DCs in the presence of JSI-124 at different concentrations for 24 hours and measured viability of the cells by MTS, luminescence, or computerized trypan blue exclusion. BM-DCs were differentiated from bone marrow of C57BL/6 mice in the presence of GM-CSF and interleukin-4 (IL-4) for eight days replenishing the media every two day. On day eight, the cells were collected, enriched through positive selection using anti-CD11c antibodies and were left to rest with HBSS or PBS for 24 hours at 4°C. For splenic DCs, spleens of two C57BL/6 mice were obtained and treated with Collagenase II in order to separate the cells from the tissue. Red blood cells were lysed before enrichment of sDC via positive selection using anti-CD11c antibodies and were left to rest with HBSS or PBS for 24 hours at 4°C. Enrichment of both types of cells was done with the use of the AutoMacs system.

After the 24 hour resting period, the DCs were collected and plated in 96-well culture plates in the presence or absence of JSI-124 in the following concentrations for 24 hours at 37°C with 5% CO_2: 0.05uM, 0.5uM, 2.5uM, and

5uM. At the end of the 24 hour incubation period the cells were prepared for MTS, luminescence or computerized trypan blue exclusion viability assessment. For the assessment of viability via MTS, 20ul of the MTS solution was directly added to each treatment well and incubated for a maximum of 4 hours at 37°C with 5% CO_2, colorimetric chances were observed and results read at 490nm in fluoroskan. For the assessment of viability using luminescence, the treatment wells were transferred to white walled plates, 100ul of the Cell Titer Glo buffer was added to each well, incubated for10 minutes and samples read on luminoskan at 948 and/or 1200 voltqages. For the assessment of viability via computerized trypan blue exclusion, a 90ul sample of the cell culture was treated with 10ul of trypan blue, mixed, and 20ul of that sample was then placed in Cellometer SD100 cell counting chambers for viability assessment using the Cellometer Auto T4 microscope and Cellometer Auto Program software.

We were able to determine that viability of the dendritic cells was significantly inhibited by JSI at the 0.5uM concentration for both bone marrow-derived and splenic DCs (Figure 2b.2). Viability of the cells was drastically reduced to almost 50%. An insignificant reduction in cell viability was observed lower concentrations of the inhibitor and we therefor sought to determine what concentration would provide a significant decrease in STAT3 activation as a measure of phosphorylated STAT3. For this assessment, we employed the use of

JAWS II cell line, an immortalized murine dendritic cell line. JAWS II cells were collected and treated with JSI-124, with/without 160ng/ml of murine leptin, for 30 minutes at 37°C with 5% CO_2. Untreated/Media samples functioned as negative controls in order to compare background levels of the phosphorylated protein. The samples were then washed, fixed with 4% PFA, permeabilized with 90% methanol, and intracellular stained for flow cytometry against phosphorylated STAT3 (Y705).

We were able to observe that using JSI-124 inhibitor, significant loss of phosphorylated STAT3 was at the same concentration observed for significant loss of viability: 0.5uM concentration (Figure 2b.3).

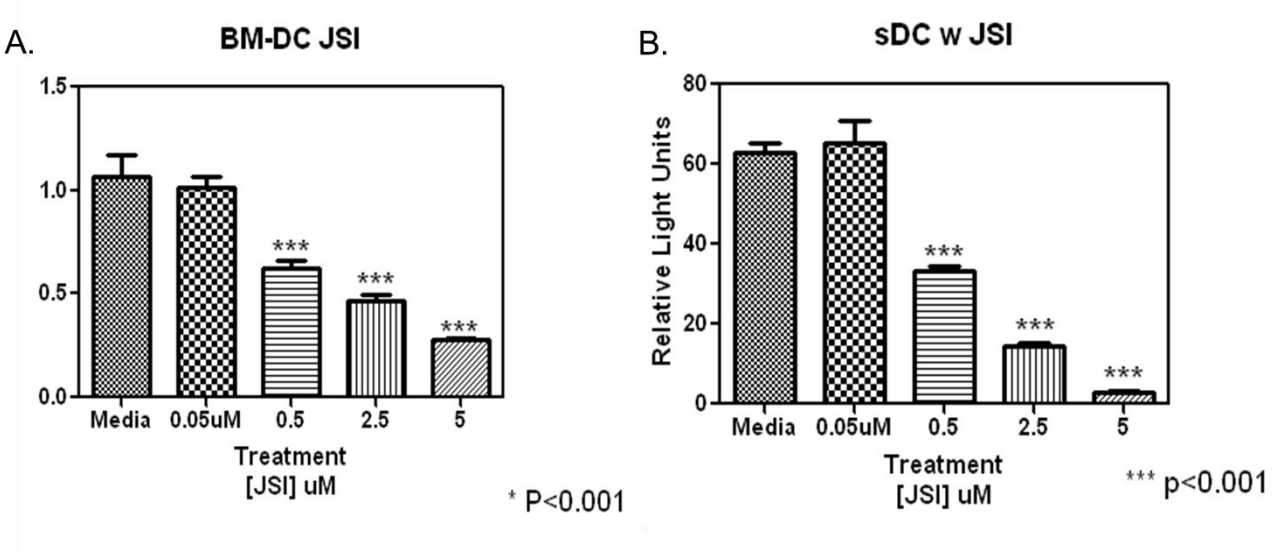


Figure 2b.2. JSI Inhibitor Significantly Reduces Viability of Dendritic Cells at 0.5uM. Purified CD11c+ bone marrow-derived and splenic DC were treated with indicated concentrations of JSI-124 for 24 hours. The cells were evaluated by MTS, luminescence, or computerized trypan blue exclusion. Significant reduction in viability was observed at the 0.5uM concentration in both bone marrow-derived and splenic dendritic cells. Results are the mean ± SEM and representative of 3 individual experiments. Statistical analysis was using One-way ANOVA with a Tukey Multiple Comparison test.

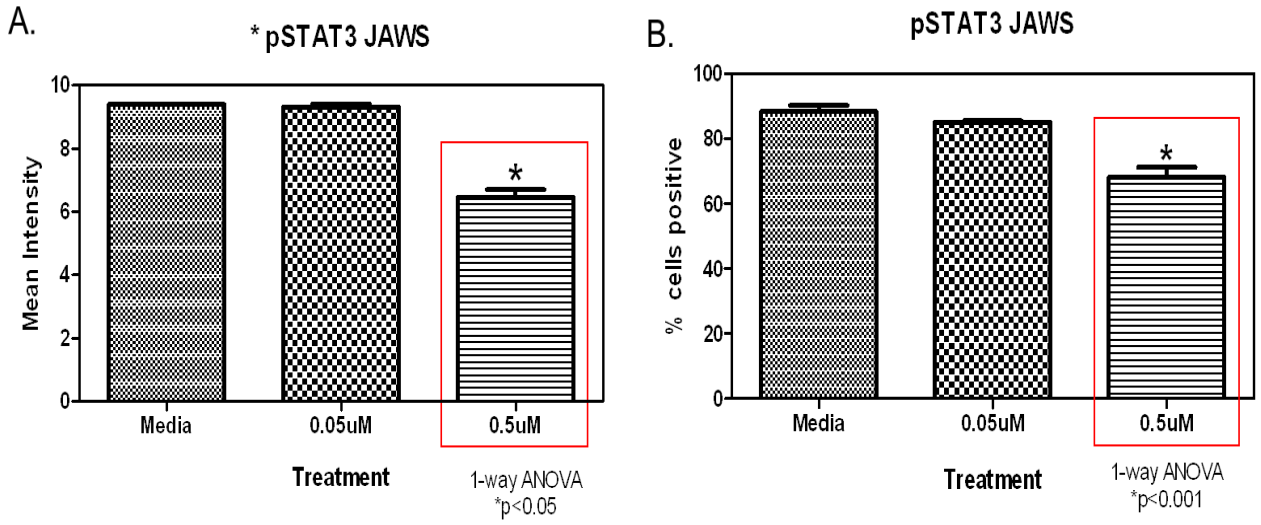


Figure 2b.3. Significant Inhibition of pSTAT3 by JSI-124 at 0.5uM on JAWS. JAWS II cells were treated with indicated concentrations of JSI-124 for 30 minutes. After indicated time frame, the cells were harvested, fixed and permeabilized, and finally stained with anti-murine phospho-STAT3. The cells were evaluated by flow cytometry gating for CD11c+ cells. The data is presented as the mean ± SEM of duplicate samples and representative of two individual experiments. Statistical analysis was performed using 1-way ANOVA with a Tukey Multiple comparison posttest.

Taking the Nefedova and colleagues studies into consideration we proposed to determine if, in obese individuals, the inhibition of STAT3 activation via the leptin receptor but still allowing MAPK & PI3K signaling pathways to continue their role, may lead to enhanced immune functions. The Jak2/STAT3 signaling pathway is essential in almost all cellular functions and development and after determining cell viability and STAT3 activity in dendric cells. Because dendritic cells are the link to between both branches of the immune system with the ability to activate naïve T cells, ***we sought to determine the effects of acute inhibitor exposure on these cells and their ability to activate T cells with the use of T cell hybridomas (TCH)***.

Bone marrow-derived and splenic dendritic cells were obtained as mentioned above and left to rest for 24 hours. Upon the resting period, both cell types were pretreated with the inhibitor for 30 minutes at 37ºC with 5% CO_2 with the indicated inhibitor concentrations: 0.05uM, 0.5uM, 2.5uM and 5uM. Treated dendritic cells were then cocultured with T cell hybridoma cells in a 96-well round bottom tissue culture plate at 100,000 cells/well with a ratio of 1:4 (DC: T cell) and Con A at 5μg/ml at 37ºC with 5% CO_2 for 72 hours. After the 72 hour period, supernatants were collected for IL-2 and IL-12 production measured by ELISA. A significant reduction in dendritic cell ability to induce a T cell response as a measure of TCH ability to produce IL-2 was observed at 2.5uM concentration for

bone-marrow-derived dendritic cells and at 0.5uM concentration in splenic dendritic cells (Figure 2b.4). At the same time, the dendritic cells had a significant reduction in their ability to produce IL-12. Bone-marrow-derived dendritic cells had a significant loss in Il-12 production observed at 0.05uM concentration and splenic dendritic cells at 0.5uM concentration (Figure 2b.5). This data demonstrated that with a 30 minute pre-treatment of the cells with JSI-124 not only inhibits the Jak2/STAT3 signaling pathway in these cells but also reduces their immunogenic abilities.

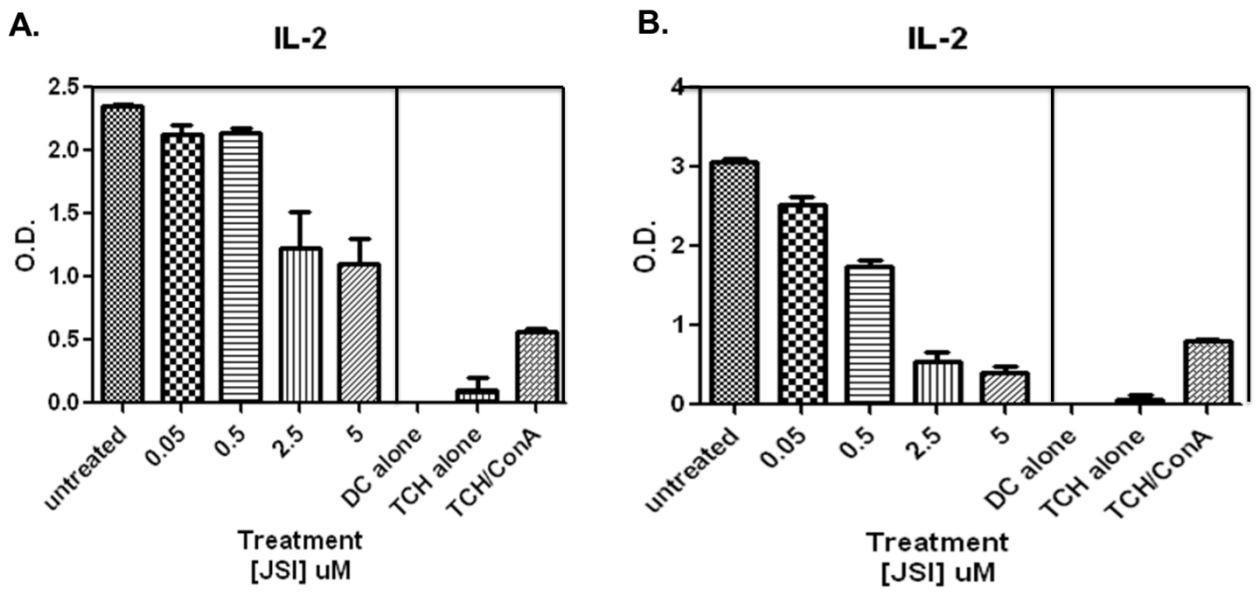


Figure 2b.4. JSI -124 treated DC reduced TCH activation. Purified CD11c+ bone marrow-derived (A) and splenic DC (B) were treated with indicated concentrations of JSI-124 for 30 minutes at 37°C. The cells were then collected, washed in PBS to remove residual inhibitor and cocultured with T cell Hybridomas (TCH**)** and Con A for 72 hours at 37°C with 5% CO2. Supernatants were collected and IL-2 production was determined by sandwich ELISA. Results are the mean ± SEM and representative of 1 individual experiment.

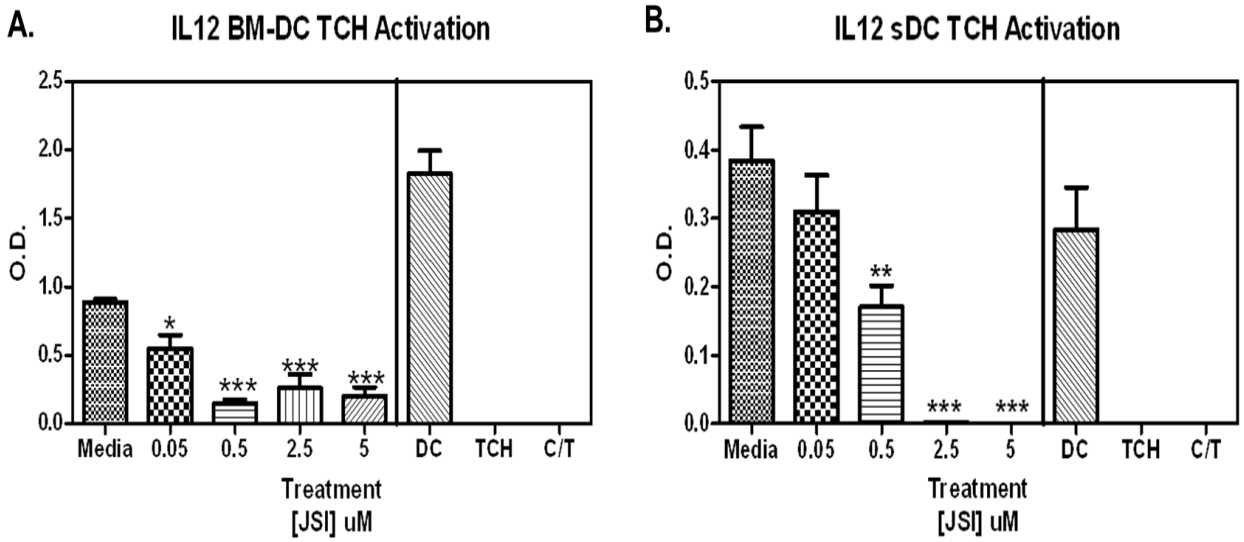


Figure 2b.5. JSI treatment reduced DC cytokine production. Purified CD11c+ bone marrow-derived (A) and splenic DC (B) were treated with indicated concentrations of JSI-124 for 30 minutes at 37°C. The cells were then collected, washed in PBS to remove residual inhibitor and cocultured with T cell Hybridomas (TCH) and Con A for 72 hours at 37°C with 5% CO2. Supernatants were collected and IL-12 production was determined by sandwich ELISA. Results are the mean ± SEM and representative of 1 individual experiment.

Chapter 2b Conclusion

Our data demonstrated that JSI-124 not only serves its purpose to inhibit/reduce phosphorylation of STAT3 but also decreased dendritic cell viability by almost 50% at the same concentration needed to see a reduction on STAT3 activation and a decreased ability of both cell types to induce a T cell response as a measure of cytokine production. It is necessary to state that these results do not contradict any of the Nefedova and colleagues studies. Nefedova and colleagues demonstrated increased dendritic cell in the presence of TDF (Nefedova et al 2005) which they had also previously demonstrated that these TDF induced hyperactivation of STAT3 not only in cancerous cells but also in dendritic cells. Reducing levels of active STAT3 to below normal levels is also not beneficial for optimal DC function as shown by my studies. Optimizing DC activity by lowering hyperactivated levels of phosphorylated STAT3 to normal levels is our projected mechanism of immunotherapy.

Chapter 3

Overall Discussion

Prevalence of obesity in the United States and in the world overall has reached epidemic proportions (Lorincz 2006). Obesity is associated with a low level state of chronic inflammation identified by abnormal cytokine production and intracellular signaling. Obesity has also been identified to play a role in cancer development (Housa et al 2006, Lorincz and Sukumar 2006). Many epidemiological studies support the increased risk of cancer in obese individuals but the exact mechanism still remains unidentified and the search for therapeutic targets is still in process (Garofalo and Surmacz 2006, Cowey and Hardy 2006, Marmot et al 2007). Some of the cancers showing a 1.5 to a 3 fold increased risk in obese as compared to lean individuals are, but not limited to: breast cancer, endometrial cancer, colon, and kidney (Housa et al 2006).

Leptin levels have been shown to be increased in obese individuals and as a result these individuals have also become resistant to its beneficial immunological effects. Nonetheless, functional leptin receptors have been found in a variety of cancer cell and tissues such as breast, colon, and prostate (Frankenberry et al 2004, Frankenberry et al 2006, Cirillo 2008, Ratke et al 2009). Much data also supports

a direct and functional role of leptin in cancer processes such as its initiation and progression that can later result in metastatic development (Lang and Ratke 2009). Leptin has now been shown to directly affect all functions within the body, both in the CNS and PNS, and to add to leptin effects on the body, data strongly supports leptin aiding effects on cancerous cell development and growth.

Cancer is rapidly becoming the leading cause of death in the United States and the biomedical research community continues its search for treatments and possibly a cure (Aldrich et al 2010). Several drastic and invasive methods for treating of cancer patients include surgery, radiation, chemotherapy and/or a combination of two or more of these methods and sometimes it is still not enough. Cancer immunotherapies range from vaccines to cytokine-based and monoclonal antibodies and even the genetic engineering of dendritic cells (Aldrich et al 2010, Boudreau et al 2011). Vaccine modalities currently underway include vectors expressing tumor antigens (Pise-Masison et al 1998), DNA vaccines (Prud'homme 2005), and APC-based vaccines (Palucka et al 2007). Cytokine-based immunotherapy is the direct attempt to stimulate the immune system with the systemic administration of immunostimulatory molecules such as IL-21 and IFN-α, which can mediate an anti-tumor immune response without adverse side effects (Eriksen et al 2009). The use of monoclonal antibodies as a cancer immunotherapeutic approach has several advantages. To say the least, immune

effectors can easily be increased by increasing antibody dosage, is an alternative to vaccines when autoimmunity is of concern, and they can directly targeted against tumor cells and be used to block immunosuppressive molecules (Onizuka et al 1999, Hernandez et all 2001, Aldrich 2010).

Several cancer immunotherapeutic targets have been shown to be promising but dendritic cells provide the ideal cancer immunotherapeutic platform due to their direct link into the adaptive immune response. Dendritic cells have been genetically modified to enhance tumor antigen presentation, enhance stimulatory molecules and decrease inhibitory molecules, and even modified to constitutively produce Th1 polarizing cytokines and chemokines (Boudreau et al 2011). The approach in dendritic cell modification is known as adoptive cellular therapy and it utilizes the patient's own immune cells to promote the rejection of already established tumors. This method allows direct manipulation of immune cells *ex vivo* with antigens, cytokines, or even intracellular or surface cellular modifications.

Leptin signaling in dendritic cells may also serve as a direct target for increased immunogenicity and specific anti-tumor response. We have proposed the following mechanism for the enhanced function of these cells in obese individuals (Figure 3.1) (see page 52). By specifically mutating the STAT3 binding site on the leptin receptor, the receptor's production of its own negative

feedback loop will be inhibited allowing for the continued signaling of the other signaling pathways activated by leptin. Leptin has positive immunogenic effects in both the innate and adaptive immune systems. The reason why the immune system fails upon chronic leptin stimulation is due to the activation of its own negative feedback loop shutting down the intracellular signaling pathways of the receptor as a phenomenon known as leptin resistance. The mutation, in this case, will not alter any phenotypic characteristics of the dendritic cells and, in the presence of leptin, the cells will have enhanced performance of antigen acquisition, processing, and presentation to T cells giving rise to an antigen-specific anti-tumor T cell response and ultimately tumor regression.

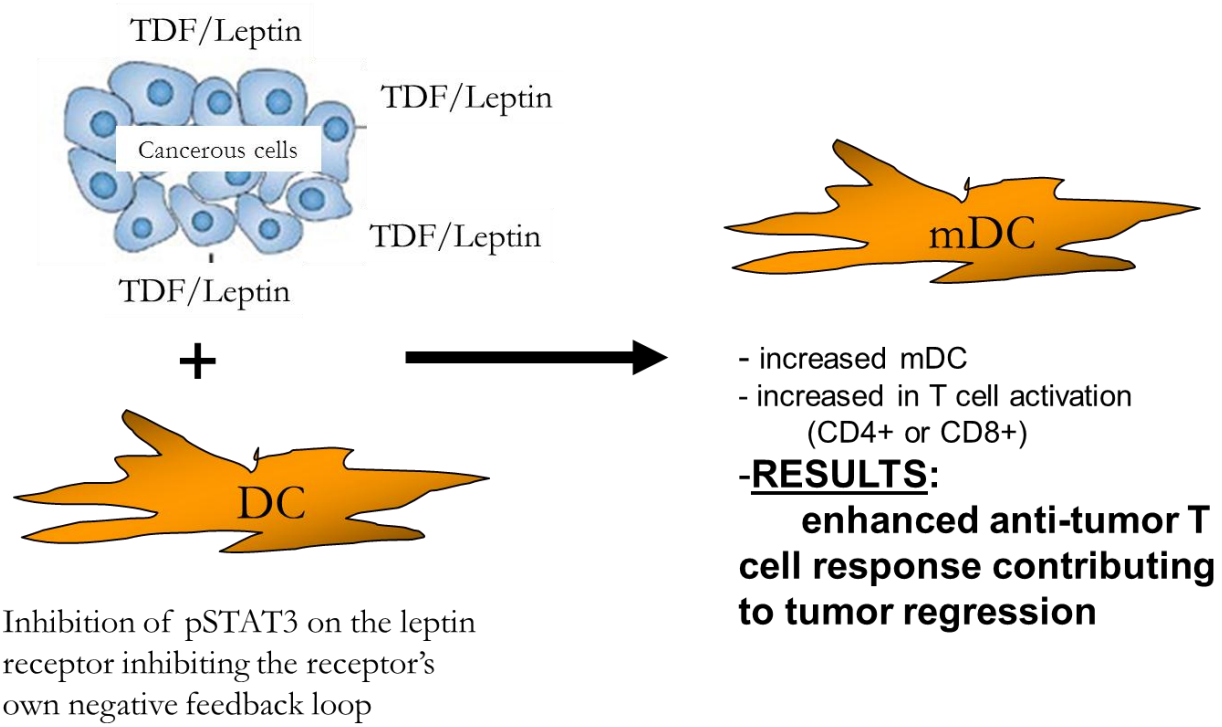


Figure 3.1. Proposed mechanism for enhanced DC function and anti-tumor T cell response. The direct inhibition of the leptin receptor's negative feedback loop can increase the differentiation of mature dendritic cells increase T cell activation leading to an enhanced anti-tumor T cells response ultimately resulting to tumor regression.

It cannot be certain that any one specific method will be the determining factor for the treatment of cancer or its prevention. The application of immunotherapies and immune cell modifications are vital and a captivating quest of today's medicine. Although several immunotherapeutic techniques have been approved by the Food and Drug Administration (FDA), adoptive cellular therapy has provided with the most impressive results and expectations. A combination of therapeutic techniques may be the answer to maximizing immune responses against the different cancers and the expansion of different treatment options to cancer patients by replacing harsh treatments currently in use may be the hope these patients need for ultimately finding a cure.

Chapter 4

Materials and Methods

Medias and Buffers

- RPMI 1640 (Invitrogen)
- Iscove's Complete Media (Invitrogen)
- Hanks Buffered Salt Solution (Thermo Scientific)
- Phosphate Buffer Solution (Invitrogen)

Cytokines, Peptides, Proteins and Chemicals

- Interleukin-4 (IL-4) (Peprotech) reconstituted in HBSS to a final concentration of 10ug/ml, aliquoted and stored at -80°C.
- Granulocyte colony Stimulating Factor (GM-CSF) (Peprotech) reconstituted in HBSS to a final concentration of 10ug/ml, aliquoted and stored at -80°C.
- Fetal Bovine Serum (Invitrogen) aliquoted and stored at -20°C.
- Leptin (murine recombinant) (Alpha Diagnostic) dissolved in 15mM HCL and 7.5mM NaOH, diluted in PBS, aliquoted, and stored at.
- Curcubitacin-I (Sigma Aldrich), also known as JSI-124, reconstituted in DMSO to a final concentration of 1mM, aliquoted, and stored a t-20°C.
- Concanavalin A (Con A) (Sigma Aldrich) dissolved in PBS to a final concentration of 1mg/ml, aliquoted, and stored at -20°C.

Antibodies

- Anti-CD11c (BD Pharmingen) 1:50 dilution
- Anti-pSTAT3 (Tyr705) (Cell Signaling) 1:200 dilution
- Anti-pMAPK (Thr180/Tyr182) (Cell Signaling) 1:800 dilution
- Anti-Akt (Thr308) (Cell Signaling) 1:100 dilution
- Streptavidin (BD Pharmingen) 1:50 dilution
- Alexa Fluor conjugated IgG (H+L), $F(ab')_2$ fragment (Cell Signaling) 1:1000 dilution

Animals

C57BL/6 mice purchased from Jackson Laboratories or bread in-house. Animals were maintained at 72° F in a 12 hour day/night cycle. Animals were fed a standard diet *ad lib*, and housed with a maximum of 5 mice per cage. Animals were then used between six to ten weeks of age.

Dendritic Cell Isolation

Bone Marrow-Derived Dendritic Cell Isolation. To generate immature BM-DCs, bone marrow from the femurs and tibias of two C57BL/6 mice were extracted and red blood cells were lysed. The remaining population of cells was cultured for 6 days in the presence or absence of leptin (160 ng/ml), replenishing

RPMI (Invitrogen) culture media containing 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 10 ng/ml Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) (Peprotech), and 10 ng/ml Interleukin-4 (IL-4) (Peprotech) every two days. BM-DCs were enriched using autoMacs as described below with a small portion of enriched cells stained for flow cytometry against DC-specific surface marker CD11c to assess purity of cells.

Splenic Dendritic Cell Isolation. To generate sDCs, spleens were isolated from C57BL/6 mice. Spleens were then treated with Collagenase D (Sigma-Aldrich, 1mg/ml) for 30 minutes at 37°C then teased apart and retreated with collagenase D for another 30 minutes again at 37°C. Desingrated spleens were then filtered through a nylon mesh to isolate overall cell population. Red blood cells were lysed then washed in Hanks Balanced Salt Solution (HBSS) (Invitrogen). sDCs were enriched using autoMacs as described below with a small portion of enriched cells stained for flow cytometry against DC-specific surface marker CD11c to assess purity of cells.

T cell Hybridomas

T cell hybridomas specific for ovalbumin are maintained in Dulbecco's Modified Eagle Medium (D-MEM) enhanced with 10% FBS and tumor cocktail at 37°C with 5% CO2.

Cell Enrichment

Cell enrichment was possible using CD11c anti-mouse microbeads with autoMACS cell sorter purchased from Miltenyi Biotec and performed as per manufacturer's recommendations. autoMACS cell sorter was prepared and primed prior to cell population preparation. A single-cell suspension of 10^8 total cells was established prior to procedure and cell populations had to be kept cold and used with pre-cooled reagents for optimal results. Cell suspension was centrifuged at 200xg for 10 minutes. Pellet was resuspended in 400ul of 50% normal mouse serum and buffer solution and incubated on ice for 15 minutes. To the sample, 100ul of CD11c MicroBeads was added, mixed well, and incubated for 15 minutes at 4°C. Upon incubation, the cells were washed in 10mls of buffer and centrifuged at 200xg for 10 minutes with supernatant completely aspirated then resuspended in 500ul of buffer for magnetic separation. To the autoMACS sorter, add the tube containing the cell sample in the uptake port and two other tubes for collecting labeled cell populations in pos2 port and unlabeled cell populations in neg1 port. The "Posseld" program was chosen for positive selection of the sample and positive fractioned cells were collected from pos2 outlet port.

Treatments

Cells were collected and treated with the following

- Untreated/Media
- 160ng/ml Leptin
- 0.05uM JSI
- 0.5uM JSI
- 2.5um JSI
- 5uM JSI

Viability Assays

Cell Titer 96 Aqueous One Solution (MTS). MTS purchased from Promega and stored at -20°C. On the day of assessment, 20ul of the MTS stock is added to each sample well of a flat bottom, 96-well plate and incubated at 37°C for 2 hours. After the incubation period absorbance is read at 490nm.

Cell Titer-Glo Luminescent Cell Viabillity Assay. Cell Titer-Glo purchased from Promega and stored at -20°C. On day of assessment, the Cell Titer-Glo reconstituted lyophilized enzyme/substrate mixture is thawed and let to reach room temperature before using. In white-wall plates, 100ul of the Cell Titer-Glo reagent is added to the cultured samples. The contents is mixed for 2 minutes on an orbital shaker to induce cell lysis. The culture plate is incubated at room

temperature for 10 minutes and luminescence recorded at 0.25 – 1 second time intervals.

Computerized Trypan Blue Exclusion. The Cellometer Auto T4 micrsocope from Nexcelom Bioscience was used to perform computerized cell viability. Using a 96-well plate for mixture, 90ul of the cell culture sample and 10ul of Trypan Blue is mixed and pipette up and down to homogenize sample. 20ul of the homogenized sample is added onto an SD100 cell counting chambered slide. Viability was determined with the use of the Cellometer Auto Program which provided percentage of viable cells per sample.

Cellular Staining

Surface Marker. To determine cell phenotype and percentage of cells acquired from culture or organ, a sample of the cell harvest was obtained and blocked with 30-50% normal mouse serum (NMS) for 15 minutes at room temperature. The sample was then incubated with FITC-conjugated or PE-conjugated anti-CD11c antibody (BD Pharmingen) at a 1:50 dilution for 45 minutes. Finally, the sample was washed in either phosphate buffer solution (PBS) or HBSS then 400ul of 1% paraformaldehyde (Sigma Aldrich) was added to fix the cells and assess by flow cytometry.

Intracellular Staining. Intracellular statining was performed as per Cell Signaling recommendations. Cells were collected and washed in either PBS or HBSS and fixed with a final concentration of 4% paraformaldehyde for 10 minutes at 37°C. Cells were immediately chilled on ice for one minute then permeabilized with ice-cold methanol to a final concentration of 90% methanol, added slowly while gently vortexing. The cells were incubated on ice for 30 minutes then washed two times to remove both paraformaldehyde and methanol. The cells were then blocked with 30-50% NMS for 15 minutes at room temperature. Samples were then incubated with purified, biotinylated, streptavidin-conjugated, or fluorochrome-conjugated antibodies (Cell Signaling, BD Pharmingen) with appropriate dilution for 45 minutes at room temperature protected from light. Cells were then washed in PBS or HBSS and 400ul of 1% paraformaldehyde was added for either immediate assessment or storing for a later time. If a secondary antibody was required, after the 45 minute incubation and wash, the secondary antibody was then added with the ppropriate dilution and incubated for 30 minutes protected from light. Upon completion of incubation, the cells were washed and 400ul of 1% paraformaldehyde was added for either immediate assessment or storing for a later time.

Flow Cytometry

Flow cytometry analysis for the assessment of cell surface and intracellular staining performed using the FC500 flow cytometer with the CXP software where ten thousand events, gated for live cells based on forward- and side-scatter parameters, was collected and analyzed for percentage and mean intensity expression.

Phagocytosis

Phagocytic ability of dendritic cells was assessed using the Vybrant Phagocytosis Assay Kit as per manufacturers' recommendations. Treated cells in the presence or absence of the inhibitor will be seeded at 1 x 10^5 cells per well in a 96-well, flat bottom, black-walled plate and incubated with fluorescein-labeled *E. coli* fragments. After a 2 hour incubation at 37°C, the cells were treated with Trypan Blue for quenching of the fluorescence of any extracellular bacterial fragments. Negative controls were as follows: media with DCs alone, media with fragments alone, and media with fragments and Trypan Blue (to determine complete quenching of extracellular fragments). Positive control were activated DCs from a non-treated sample. Phagocytosis assessment was observed via fluorescence plate reader.

TCH activation

Treated enriched primary bone marrow-derived or splenic dendritic cells and T cell hybridoma cells were cocultured in a 96 well round bottom tissue culture plate at 100,000 cells/well with a ratio of 1:4 (DC: T cell) and Con A at 5μg/ml at 37ºC with 5% CO_2 for 72 hours. Negative control wells contained the following: DC alone and TCH alone. Positive control wells contained DC, TCH, and Con A. Upon incubation period, supernatants were collected for IL-2 and IL-12 production.

Enzyme Linked ImmunoSorbent Assay (IL-2 and IL-12)

Production of IL-2 by T cells and IL-12 by dendritic cells was measured by ELISA as per manufacturer's recommendations (Biosource Invitrogen). Microtiter ELISA plates were coated with appropriate capture antibody overnight at 4°C. Plates were then rinsed with washing buffer (Triton X in 1X PBS) and blocked at room temperature with 3% bovine serum albumin in 1X PBS or Assay Diluent for at least 1hr then washed with washing buffer. ELISA plates are washed and supernatants from the different samples along with the appropriate standards were added to the plates. After a 2 hour incubation that allows for the cytokines to bind to the capture antibody, the plates were washed then incubated with biotin-conjugated anti-cytokine antibody, followed by HRP-labeled avidin (0.05ug/ml).

The enzyme substrate 3,3’5,5 ‘-tetramethyllbenzidine (TMB) is added for 30 minutes, protected from light, and reaction is stopped using a stop solution (2.5N H_2SO_4). Plate analyzed on a spectrophotometer with an absorbance set at 450nm and cytokine concentration determined.

Statistical Analysis

Statistical analyses were obtained using GraphPad Prizm 5 software.

Chapter 5

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Curriculum Vita

Lorena De los Santos was born in Oaxaca de Juarez, Oaxaca, Mexico. She is the second daughter of Miguel Angel and Patricia De los Santos. Lorena graduated from Mountain View High School in May of 1999 and proudly joined the US Navy that same year. After her military service, she initiated her collegiate studies in El Paso, Texas in the summer of 2003. She obtained her Associate of Arts degree in Psychology in December of 2004 from El Paso Community College and later two Bachelor of Science degrees in Psychology and Microbiology from the University of Texas at El Paso (UTEP) in May of 2007. Lorena was admitted into the graduate program at UTEP that summer. Lorena conducted all her graduate research in the laboratory of Dr. Kristine Garza where she worked as a research assistant funded by the RISE. Lorena has been employed by the United States Navy, Surface Warfare Center as a microbiologist reporting for duty upon completion of her Masters.

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