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Implication For The Role Of Leptin-Induced Signaling As A Negative Regulator Of Dendritic Cell Function

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IMPLICATION FOR THE ROLE LEPTIN-INDUCED SIGNALING AS A NEGATIVE REGULATOR OF DENDRITIC CELL FUNCTION

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ABSTRACT

The incidence of obesity among the distinct world populations has become an ever growing concern. Central to the regulation of obesity is the adipocyte-derived hormone, and in more recent years cytokine, leptin. Leptin plays a central role in the maintenance of food consumption as well as the efficient use of peripheral energy stores. Leptin exerts its physiological role in energy homeostasis by regulating the expression of orexigenic peptides in the central nervous system. However, recent research has implicated leptin in other physiological roles including those of the immune system. As a pleiotropic cytokine, the absence of leptin has been shown to alter macrophage functions especially altering processes directly required for antigen processing. In addition, leptin has also been demonstrated to be critical in T cell proliferation. Having been implicated in these many roles, especially those of the immune system, we sought to determine whether the absence of a functional leptin protein would alter dendritic cell function in mice. Dendritic cells (DCs) are the most potent antigen presenting cells in the immune system and the only reported cells with the capability to activate naïve T cells, or those T cells that have never encountered antigen in the context of a major histocompatability complex. Our original focus was to determine whether dendritic cells that were procured from leptin-deficient mice were different when directly compared to age and sex matched wild type controls. Using flow cytometry, we measured the total detectable number of splenic DCs and their phenotype. Using a molecular approach, we investigated whether leptin-deficiency changed the capability of splenic DCs (sDCs) to acquire and process antigen. Using both approaches, we could detect no significant differences in any of the characteristics of interest. Our efforts shifted from DC phenotype and antigen processing to T cell

activation, a property for which DCs are very well known and one that sets them apart from the rest of the cells of the immune system. To investigate the effects of leptin-deficiency on sDC antigen presentation, we used a T cell hybridoma cell line, primary T cells, as well as the in vivo neutralization of *M. avium*. To our surprise, sDC that were isolated from leptin-deficient mice exhibited a higher capability to activate the ova peptide specific T cell hybridoma cell line. To further corroborate our initial data, sDCs procured from leptin deficient mice also demonstrated an increased ability to activate primary T cells in a mixed lymphocyte reaction. Our *in vitro* data was then substantiated by our *in vivo* findings that showed that sDCs from leptin-deficient mice could activate T cells to a higher extent relative to their heterozygous, age, and sex matched controls. It is critical to point out that the increased T cell activation was not due to a decrease in plasmacytoid DCs as they were detected in relatively equal numbers. Having demonstrated that leptin deficiency bestowed DCs with an increased capability to activate T cells, we then wanted to determine the potential mechanism responsible for our results. Leptin potentiates its effects by activating the JAK2/STAT3 pathway and others to a lesser degree. DCs require the activation of the NF-kB transcription factor for their survival as well as their activation and ultimately T cell activation and polarization. Given this, we designed a series of experiments that would allow determining whether the receptor expressed by our DCs was functional. In addition, we wanted to determine whether leptin induced signaling had a negative effect on NF-kB directly. Western blot analysis shows that STAT3 gets activated upon the addition of leptin to DC cultures, a result observed by both western blot analysis and flow cytometry on sDC and BM-DCs. Furthermore, western blot analysis demonstrated that's the

STAT3 was functional as SOCS3 expression was detected in a time dependent manner upon leptin treatment. Confocal microscopic analysis showed that the concurrent activation of the NF-kB and STAT3 pathways led to a significant decrease in the nuclear translocation of activated NF-kB. Immunoprecipitation (IP) assays were performed to help determine whether the observed inhibition of NF-kB nuclear translocation was the direct consequence of protein-protein interaction. Upon analysis of leptin-treated DCs, we were able to detect the presence of NF-kB in STAT3 specific IP. Our data therefore implies that in the absence of leptin there are no severe detectable consequences to DC total numbers, phenotype, antigen acquisition, or processing. However, in the absence of a functional leptin protein, DCs demonstrate a superior ability to activate T cells, both cells lines and primary. In addition, this phenomenon is also extended to an *in vivo* scenario as demonstrated by the effective neutralization of *M. avium* by sDC from leptin-deficient mice relative to the controls. Upon determining that plasmacytoid DCs (or regulatory DCs) were present in relatively equal numbers we set out to determine any implications that leptin induced signaling might have on DCs. Our experimental data suggests that these observations may be the direct outcome of leptin induced signaling. Our data showed that upon the addition of leptin to DC cultures in the presence of LPS, the detectable levels of NF-kB translocation into the nucleus was significantly decreased. Moreover, IP assays suggest that said decrease is very likely the product of complex formation between STAT3 and NF-kB. Whether the complex being formed consists of only these two transcription factors or involves other still needs to be elucidated. In conclusion, our data demonstrates that leptin-deficiency alone is not enough to disrupt DC numbers phenotype, antigen acquiring properties, or antigen

processing abilities. However, in the absence of leptin-induced signaling sDC become highly efficient T cell activators and it is possible that this observation is the direct consequence of STAT3/NF-kB interactions. These data would suggest that in an obese state, an individual would become highly susceptible to microbial infections or pathogenic insults as DCs would encounter an activation signal that would only get screened by the negative/regulatory leptin induced STAT3 signaling more prevalent during this state

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CHAPTER 1

OBESITY

Obesity has become a worldwide epidemic. The problem has scientists searching for therapies that will help prevent and/or alleviate the problem. Obesity is defined by the Centers for Disease Control as a "range of weight that is greater than what is generally considered healthy for a given height" (1). Obesity has been shown to increase the possibility of malfunction in several physiological processes leading to cardiovascular disease, osteoarthritis, respiratory problems, bladder disease, and diabetes, specifically non-insulin dependent Type II Diabetes Mellitus. Diabetes is defined as a resistance to insulin combined with increased circulating glucose levels (2). Although the manifestation of diabetes cannot be attributed to one factor alone, several have been suggested. Yki and colleagues reported that one possible contributing factor may be genetic. Diabetes demonstrates characteristics of genetic traits, although the mechanism for inheritance and the number of genes involved still needs to be determined (3). Environmental factors have also been suggested. For example, when Polynesians migrated to Caledonia and altered their living habits ("from traditional to industrial") the incidence of diabetes increased over a ten year period from 3% to 12% (4). Obesity however, among the different factors contributing to diabetes, is considered by far to be the major contributor to the occurrence of diabetes. Central to obesity and its predisposition to develop diabetes is resistance to insulin. Insulin resistance is a hallmark of and a good predictor of the development of diabetes. Insulin resistance is defined by the American Diabetes Association as the condition where the normal amount of insulin produced by an individual are no longer sufficient to produce a

normal response by fat cells, muscle cells, and liver cells. This in turn leads to an increase in circulating fatty acids in the serum (ADA). The lack of exercise is also a key contributor to the development of diabetes, individuals from the same population who do not exercise have a higher risk than those individuals that do (5).

While the exact cause for diabetes is not known, among the many factors involved, leptin-deficiency, as well as defective signaling through the leptin receptor, is a factor shared by many with diabetes; moreover, the lack of leptin signaling has also been shown to promote obesity (6). The focus of the project presented herein is on the effect of obesity, as a result of leptin-deficiency or altered leptin signaling, on immune function. More specifically, the current project assesses the impact of leptin-deficiency on the function of dendritic cells (DC), the only immune cell capable of initiative immune responses. The treatment of obesity and its accompanying syndromes, including Type II Diabetes, have become a financial and physical burden on the United States tax payers and the medical community. By better understanding the effects of leptin and the signaling pathways activated by it we may be able to identify a potential target for the treatment of one aspect of health problems associated with obesity.

LEPTIN

In 1950, Ingalls and colleagues identified a strain of mice that exhibited obesity. Puzzled by their observations, they reported their findings (7). In 1973, Coleman conducted a series of elegant parabiosis experiments in an attempt to identify the underlying cause of this phenotype. He surgically paired the obese mice with diabetic mice (also presenting with an obese phenotype) or with lean mice. When paired with

diabetic mice, the obese mice lost significant weight but succumbed to their condition and died. When paired with lean mice, the obese mice lost significant weight and survived for a longer time after surgery. It was then postulated that there might be a satiety factor produced by normal mice that was not present in obese mice. In addition, the results suggested that the diabetic mice produced the satiety factor present in normal mice but could not respond to it (8, 9). These studies ultimately led to the discovery of leptin and its receptor.

As it was later reported by Meinders and colleagues, the obese mice secrete a truncated version of the wild type leptin protein which renders it biologically inactive (10). Leptin is encoded by the obese gene. The truncation is the result of a non-sense mutation which changes the cysteine to thymidine at position 105, resulting in the coding of a stop codon. The premature stops leads to the expression of a protein that is no longer capable of binding to the leptin receptor. Moreover, it was later determined that the diabetic mice used in the parabiosis experiments indeed produce leptin but express a truncated version of the leptin receptor that is also non-functional (10).

Halaas and colleagues reported that the truncated receptor was non-functional because it was missing a significant portion of the carboxy terminus. In the leptin receptor, the carboxy terminus is the intracellular portion of the receptor and contains several key amino acid residue that are required as docking sites for several key signaling molecules (11). Chen and colleges identified a point mutation on nucleotide 108 from guanine to thymidine. This mutation directly results in the generation of a new site for alternative splicing thus giving rise to the aberrant gene product (12).

In 1994, Friedman and colleagues identified the satiety factor leptin, (from the Greek word “leptos” for thin), and cloned the gene, located on chromosome four in mice (the *ob* gene) (13). Leptin is a 16-kDa protein produced and secreted primarily by mature white adipose tissue with serum levels being proportionate to adiposity. Leptin has been implicated in the regulation of many physiological processes including reproductive function, glucose and insulin metabolism, among others. Leptin has also been reported to play a major role in regulating food intake and energy expenditure by signaling the satiety center in the hypothalamus of the energy status of the body (13); although this role of leptin is superseded by the lack of glucose, which functions as the primary signal for food intake.

In the hypothalamus, the presence of leptin acts as an anorexic peptide. Leptin signaling has been shown to lead to the release of several other anorexic factors that include proopiomelanocortin (POMC), corticotropin releasing factor (CRF), and bombesin. The release of these peptides has been linked to the negative regulation of neuropeptides that are orexigenic in nature, like neuropeptide Y, melanin concentrating hormone (MCH), Agouti-related peptide (AGRP), and others by regulating the mRNA levels of said peptides (14). In short, the presence of leptin in the central nervous system acts as a negative regulator of orexigenic peptides. This regulation is the direct result of leptin regulating the mRNA expression levels of a specific group of regulatory peptides.

Recently, research has shown that leptin is a pleiotropic cytokine/hormone involved in functions of the immune system as well (15, 16), which is further detailed below. In humans, mutations in the *ob* gene are rare but resistance to circulating leptin,

which promotes obesity, has been suggested by several groups (17). This phenomenon has been particularly described In the central nervous system where the regulatory system for food intake and energy expenditure reside. For example, in cases where leptin resistance has occurred, the levels of circulating leptin are no longer sufficient to incite a response to normal levels of leptin. Instead, the aberrant levels of leptin have little to no effect, therefore leading to disregulated appetite and thus obesity.

LEPTIN RECEPTOR and SIGNALING

Leptin exerts its physiological effects by binding to its receptor. Identified in 1995 by Tartaglia and colleagues, the leptin receptor is encoded by the diabetes *(db)* gene and is highly expressed in the arcuate nucleus of the hypothalamus, but has been shown to be expressed in other tissues and cell types, including cells of the immune system (18). The expression of the leptin receptor on the myriad of tissues provided the first evidence that leptin is a pleiotropic cytokine.

There are six isoforms of the leptin receptor, all of which are the result of alternative mRNA splicing. The long isoform of the leptin receptor expresses an intracellular domain crucial to signaling via the JAK2/STAT3 pathway. When leptin binds to its receptor it initiates a receptor homodimerization. This homodimerization leads to the recruitment and activation of the non-receptor associated Janus Kinase (JAK). Activation of the Janus Kinase leads to the phosphorylation of the transcription factor Signal Transducers and Activators of Transcription (STAT). Once phosphorylated, the STATs are then capable of dimerizing, this dimerization leads to nuclear translocation and gene expression as shown by Baumann and others (19-21) .

While leptin receptor heterodimers have been detected, their capability to activate signaling cascades has not reported. Leptin signaling is similar to signaling for multiple cytokine super family members (19).

Of the remaining five isoforms, soluble ObRe is the shortest isoform. Ge and colleagues demonstrated that the soluble receptor is the result both of gene expression induction as well as ectodomain shedding of the membrane spanning receptor isoforms (22). While the soluble isoform has not been reported as having signal transduction capabilities, it is thought to be responsible for transport of leptin through the blood-brain barrier as well as neutralization of circulating leptin in the serum (23). Neutralization of leptin is critical because it allows for reduced signal transduction after leptin has achieved its goal of reducing food intake. One can envision a situation where if left in the serum, leptin could potentially signal indefinitely leading to reduced food intake and maybe starvation or worse case scenario death due to abnormal signal by the JAK/STAT pathway.

The remaining four isoforms have short intracellular domains, none of which have been shown to activate the JAK/STAT pathway, a phenomenon that may be due to the absence of the STAT binding site present only on amino acid residue 1138. However, these four receptors have been reported to have the capability to activate the Mitogen-Activated Protein (MAP) kinase pathway (24-27). In addition, Bado and colleagues demonstrated that leptin binding to the long isoform receptor was capable of activating the NF-κB signaling cascade as well (28).

Leptin and its receptor share structural similarities with other cytokines and their receptors. Extracellular motifs of four cysteine residues, a tryptophan-serine-any amino

acid-tryptophan-serine (WSXWS) motif, as well as unique numbers of fibronectin III domains are several of the features shared between the leptin receptor and some class I cytokine receptors (25). These features are critical for activating cells of the immune system. For instance, interleukin (IL) -12 and IL-2 are critical elements mediating T cell activation, both of which share structural similarities with leptin (13, 15). On the other hand, the IL-6 receptor shares structural similarities with the leptin receptor, both are considered class I cytokine receptors. IL-6 is involved in many functions of the immune system including proliferation and differentiation of T cells (29). Given the importance of the above-mentioned interleukins in mediating functions of the immune system, and considering the structural similarities between leptin and its receptor with these immunomodulators, it is of particular interest to determine how leptin affects cells of the immune system.

Obesity and leptin resistance are tightly correlated. It has been suggested that leptin resistance leads to obesity. However, until recently, the mechanism responsible for said resistance had not been identified. Münzberg and colleagues reported that one possible mechanism for the resistance to leptin signaling, at least in the arcuate nucleus of the hypothalamus, may be due in part to the over-expression of the protein Suppressor of Cytokine Signaling 3 (SOCS3), a negative regulator of STAT proteins, including STAT-3 (30). In addition to deterring STAT-3 signaling, SOCS3 inhibits other signaling cascades, including Insulin Receptor Substrate (IRS), as well as PI3-Kinase. The inhibition of STAT-3 or other signaling molecules prevents signaling through a specific receptor thus preventing the expression of specific proteins in unwanted

circumstances. In the case of the leptin receptor, activation of SOCS3 functions as a negative feedback loop; excessive activation may lead to leptin resistance.

DENDRITIC CELLS

DCs are professional antigen presenting cells (APCs) responsible for the activation of naïve T cells (31, 32). Bone marrow hematopoietic stem cells (HSC) give rise to DCs, *in-vivo,* as well as *in-vitro,* through differentiation of precursor cells in the presence of specific growth or differentiation cytokines. Such cytokines include Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Interleukin-4 (IL-4), but others have also been implicated as possible contributors to this process. Initially, an HSCs encounter specific environmental cues in the form of cytokines that initiate differentiation into a myriad of cells that ultimately form part of the immune system. HSCs may differentiate into myeloid or lymphoid progenitor cells depending on the cues received. In the case of DC, HCSs must detect GM-CSF to differentiate into myeloid lineage precursor cells (33) and must receive secondary cues to give rise to DCs (34).

DCs are highly potent APCs and are the only APCs capable of presenting antigens to cells of the adaptive immune system due to highly specialized properties. DCs are found in two prevalent forms, immature or mature. As immature cells, DCs are well characterized by their enhanced capacity to acquire antigens. Once within the cell, said antigens are processed in the phagolysosome into peptides that later get transported to compartments containing MHC class II molecules where they are loaded onto antigen pocket of these molecules. The peptide-loaded MHC molecules are then transported to the cell surface and are presented to T cells. During this entire process,

DCs start undergoing the process of maturation, one that will allow them to increase the expression levels of certain cell surface markers required for optimal T cell interaction and thus T cell activation. This is believed to take place during the migration from the antigen acquisition site to the secondary lymphoid organs where T cells reside and the processed antigens get presented. It is this particular property to migrate from the site of antigen acquisition to the site of antigen presentation that sets DCs apart from other APCs. Macrophages (MΦ) and to some degree B cells, are recognized as APCs but neither one posses the capability to incite naïve immune responses (35). The ability of DCs to migrate from site of acquisition to the site antigen presentation is very likely due to the expression profile of multiple chemokine receptors. Chemokines are a particular set of cytokines secreted by a wide array of cells that function to traffic immune cells to specific cites (36). Immature DCs express chemokine receptors that target trafficking from the bone marrow to the peripheral tissues; chemokine receptors expressed by mature DCs are responsible for directing DCs to the T cell areas of draining lymph nodes from the peripheral tissue (37, 38).

DENDRITIC CELL SUBSETS

Dendritic cells are dispersed throughout an organism and are divided into subsets based on their differentiation lineage physical location. For example, DCs that are found in the skin are called Langerhans cells (LCs). Other DC populations include liver DCs, follicular DCs (in lymph node and spleen germinal centers), interstitial DCs (IDCs), and interdigitating DC (IDDCs) lymph node and spleen DCs. All of these DC populations are considered to be of the myeloid lineage. Myeloid DC originate from a

specific myeloid precursor and are distinguished from lymphoid derived DCs based on the lack of CD8 expression.

One of the original DC populations to be identified was the LC. Originally described by Paul Langerhans (39), it is now known that LCs reside within the epidermis, are a mobile cell population, and have a relatively slow turnover. LCs are derived from BM-SC and later migrate to the skin (40). Phenotypically, LCs are similar to other DC subsets. In contrast to other skin cells, they are the only cells within the epidermis to express CD1a, Fc receptors, and MHC class II. Like immature DCs in lymphoid tissues, immature LCs are not very efficient at activating naïve T cells but are highly efficient at antigen acquisition (41). LCs undergo a process called LC activation, which includes MHC II loading with peptide and increased expression of DC markers including CD80, CD86, and CD40 (42). Upon activation, LC increase the secretion of certain cytokines that include IL-1β, IL-6, and IL-12 amongst others (43) and also acquire the capability to efficiently activate naïve T cells, similar to their DC counterparts in secondary lymphoid tissues after migration.

Follicular DCs (FDCs) are yet another of the several DC subsets described to date. They are identified by the expression of CD21 and Ki-M4p (44). For the most part, FDCs are found in germinal centers of spleen and lymph nodes but their precursors remain an enigma. Like many cells, FDCs are affected by the presence or absence of particular cytokines. Lymphotoxin (LT) has been shown to be a potent FDC stimulator. In ectopic tissues, other than secondary lymphoid organs, FDCs networks have been established in the presence of high LT levels (45). In addition, TNF-α has also been demonstrated to have a regulatory role in FDC network formation. In the

absence of TNF-α, FDC networks are severely under-formed or simply fail to form (46, 47). In terms of function, FDCs are the only reported DCs that do not express MHC class II for antigen presentation. Instead, FDCs function as antigen (Ag) trapping cells that capture Ag and provide a sample of the acquired microenvironment for germinal center B cells (48). B cells obtain Ag through this process becoming efficient APCs themselves and thus gaining the capability to activate T cells (49).

Three additional DC subsets have been characterized, each having been accepted with different levels of enthusiasm. First, interstitial DCs are most commonly found in all non-lymphoid tissues and are considered to function primarily as surveyors. Interstitial DCs are characterized by whether they reside in superficial epithelium or non-epithelial tissue. Superficial epithelial DCs can be found in the skin or in other tissues like the gut or respiratory tract. Second, a distinct population of DCs has also been identified in the liver. Liver DCs reside solely in the liver and their function is thought to be typical of DCs found in other tissues (50) Some reports suggest that liver DCs, like other non-lymphoid tissue DC, are likely to be inferior immunostimulators (51). The third subset of DCs is a highly debated population. This subset of DCs was originally identified as plasmacytoid DCs (pDCs) and have slowly gained acceptance. pDCs are considered to be immuno-regulators rather than immuno stimulators. pDCs are capable of orchestrating the regulation of immune responses due mostly to their ability to secrete Type I interferons (52). They are thought to undergo complete differentiation in the bone marrow and leave to populate the periphery through the blood. While much more research is required to clearly establish this subset, it is becoming more apparent that DCs also function as regulators and not just stimulators.

Lymphoid DCs have also been characterized. Lymphoid precursor cells have been reported as having the capability to give rise to lymphoid-related DCs that are CD8+ and are thought to play an important role in T cell selection within the thymus. Vremec and colleagues demonstrated in mice that after irradiation, mice that were seeded with thymic precursors gave rise to CD8+ DCs but not to myeloid DCs, a phenomenon that was also observed *in vitro* by Saunders and colleagues (53, 54). It is interesting to point out that Saunders and colleagues reported that the absence of GM-CSF was necessary for the differentiation to occur in this DC lineage. To make matters more complicated, Traver and colleagues reported that lymphoid DCs could develop from myeloid precursors (55). These data may suggest that there exist plasticity in the ability of DCs to arise from several different progenitors or that differences in DCs phenotype, as well as function, are based on maturation status as well as micro-environment in which the DCs are in.

While different DC subsets have been identified, it is evident that for the most part their role is similar regardless of subset type (with the exception of pDCs). Most DCs function by acquiring then presenting antigens to T cells, or facilitate recognition of antigens as in the case of FDC and B cells. The majority of the subpopulations of DCs are of the myeloid lineage, which is considered to be the conventional DC population.

DENDRITIC CELL ENDOCYTOSIS

While DCs may respond to and produce a plethora of chemokines, their function as APCs is only possible upon acquisition of antigens, mainly, if not exclusively, through the process of endocytosis. While endocytosis is the general term employed to

describe the process by which DCs survey their surrounding environment, it may be subdivided into different subclasses depending on the mechanisms utilized to internalize the “cargo” and the type of “cargo”. The different subclasses of acquiring antigen include receptor-mediated phagocytosis, which requires the binding of particular molecule(s) to receptors on the surface of DCs; the receptors included are the mannose/β glucan, DEC205, Fc, scavenger, and complement receptors. Most of the receptor-mediated phagocytic pathways have the capability to initiate the signals for ligand internalization which require clathrin-coated mediated phagocytosis (56).

Pinocytosis is a specialized form of phagocytosis. It is the process by which a DC internalizes fluid-phase cargo. The fluid-phase includes micro and macropinocytosis and requires clathrin-coated vesicles but some exceptions have been described (57). Micropinocytosis is used to internalize particles less than 0.2 microns in size, while macropinocytosis allows particles from 0.2 - 5.0 microns in size to be internalized. This process allows DCs to sample the environment in a non-specific manner.

Potocytosis on the other hand, allows DCs to internalize GPI-anchored membrane proteins. Unfortunately, while this process allows for the uptake of specific antigens, the function of said uptake in generating a DC-driven immune response remains to be elucidated (58).

DENDRITIC CELL - T CELL INTERACTION

Along with neutrophils and MΦ, DCs are considered the most efficient phagocytes in the immune system. DCs can be considered either mature or immature depending on their activation status (59). Immature DCs are distinguished by their high

phagocytic capacity and low expression levels of cell surface markers. Once an immature DC has encountered and internalized extracellular material, it migrates to secondary lymphoid organs. During this migration, the DC undergoes a process of maturation; the extent to which this occurs is dependent on the environment where antigen was acquired. A non-inflammatory environment promotes little to no maturation while an inflammatory environment promotes strong maturation. The maturation can be measured as a function of the increased expression levels of specific cell surface markers like CD80, 86, 54, 40, and MHC class II as well as cytokine production. The cell surface markers are key components in the interaction between a DC and a T cell. CD 80 and 86 are co-stimulatory molecules on DCs and interact with CD28, their receptor on T cells. This specific interaction leads to the expression and secretion of IL-2, a potent regulator of T cell proliferation (60). CD54 is an intercellular adhesion molecule (ICAM) and its expression results in increased duration of the interaction between the DC and T cell. CD40 is a DC activation marker; interaction of CD40 with its ligand, CD40L (aka, CD154), signals to the DC that the T cell is receptive and that it has recognized the antigen being presented. This interaction in turn leads to increased expression of cytokines and the other cell surface markers on the DC (59, 61).

If antigen being presented by DC is recognized by the interacting T cell, an immune response ensues. The nature of the response is modulated by DCs. DCs secrete cytokines, including IL-12 and IL-10, both of which are vital for T_H cell differentiation into T_H1 or T_H2 cells, respectively. IL-12 secretion by DCs drives the secretion of interferon gamma (IFN-γ) by T_H1 effector cells, which facilitates activation of

innate immune system cells like macrophages, promotes B cell isotype switching to IgG2a, and induces cytolytic activity of killer T cells. Alternatively, IL-10 secretion by DCs stimulates T_H cell differentiation to T_H2 profile. T_H2 effector cells secrete IL-4 which facilitates activation of innate immune cells such as mast cells, eosinophils, and B cells, and promotes B cell isotype switching to IgG1 and IgE. Both cytokines are crucial for T cell activation and driving the T cell response appropriate to the invading pathogen (32, 62, 63).

ACTIVATION of the NF-κB PATHWAY in DENDRITIC CELLS

Among the many signaling pathways activated during DC maturation/activation, Nuclear Factor- kappa B (NF-κB), a transcription factor ubiquitously expressed and known to regulate transcription of a plethora of genes, has been shown to be of particular importance. Artis and colleagues demonstrated that DC's derived from NF-κB knockout mice, failed to induce T cell production of IL-4, a hallmark of a T_H2 response (64). Additionally, Yoshimura and colleagues demonstrated that NF-κB coordinates the increased expression of the class II MHC, co-stimulatory molecules CD80, 86, and 40, as well as the secretion of IL-12 and TNF-α (65) both critical cytokines for the polarization of T_H1 cells. Once DCs receive a stimulatory signal, the NF-κB transcription factor undergoes canonical activation pathway. Inhibitor of Kappa B Kinase (IKK) phosphorylates the Inhibitor of kappa B (IκB) promoting its proteosomal degradation thus releasing NF-κB from its regulatory subunit, leaving it free to enter the nucleus to regulate gene expression. NF-κB regulates expression of its own regulator (IκB). Once

expressed, IκB can enter the nucleus where it binds NF-κB and returns it to the nucleus (66).

In DCs, a similar mode of activation is thought to occur. More specifically, NF-κB has been shown to be activated and required for multiple DC functions. For example, Yoshimura and colleagues showed that NF-κB was required for effective antigen presentation, the expression of MHC class II, cell surface markers (and thus DC activation) required for T cell activation, and cytokines. This phenomenon was observed *in vitro* as well as *in vivo* (65, 67). Moreover, Yanagawa and colleagues showed that NF-κB is required for the expression of IL-6, a key pro-inflammatory cytokine, and IL-12, a key cytokine in the polarization of T_H cells to the T_H1 profile.

LEPTIN and the IMMUNE SYSTEM

Leptin has been described as a multifunctional hormone/cytokine involved in many physiological processes. Central to this project is the involvement of leptin with cells of the immune system. Earlier work has shown that MΦ procured from leptin-deficient mice exhibit phenotypic abnormalities which include altered expression levels of several degradative enzymes, as well as increased expression of pro-inflammatory cytokines. Fung-Yee and colleagues reported that MΦ isolated from Lep^{ob} mice produced lower levels of uncoupling protein-2 mRNA, increased levels of Interleukin-6 as well as cyclooxygenase-2 (68). MΦ are similar to DCs in that they are the first line of defense against invading pathogens. MΦ phagocytose material encountered in the periphery and randomly sample the environment for pathogens, a mechanism shared with DCs. MΦ also function as APCs, however, they are incapable of stimulating naïve

T cells because they lack the ability to migrate. MΦ remain in peripheral tissue dealing with the invader while secreting chemokines to direct activated T cells to the target location. Thus, although similar, the differences between MΦ and DC may result in differential responsiveness or requirements for leptin.

Leptin has also been shown to increase functions of other cells of the innate branch of the immune system like natural killer cells and neutrophils where the addition of leptin increases cytokine production (68-72). Additionally, leptin has also been described as a positive regulator of adaptive immunity, causing an enhancement of T cell proliferation (69). Further support of leptin's role in immunity is provided by experimental models of autoimmune disease. Leptin-deficiency has been shown to protect mice from autoimmunity such as Experimental Autoimmune Encephalomyelitis and Experimental Arthritis (73, 74). Furthermore, it has been clearly established that mutations to the leptin protein or the leptin receptor lead to increased susceptibility to bacterial infections suggesting that leptin is critical for an effective immune response (75-77). Taken together, these data suggest that leptin plays a positive role in the function of the immune system at the level of both innate and adaptive immunity.

PROJECT OBJECTIVES

Obesity has become a worldwide health concern. The Center for Disease Control now estimates that 30 - 35% of adults in the United States currently meet the criteria to be considered obese, with an additional 50 – 60% of the population being classified as overweight (78). Estimates by the World Health Organization (WHO) report that since 1999, obesity has increased in almost every country in the world, even

in those countries affected by hunger (79). As more and more of the world's population become obese, the numbers of health complications that arise from obesity also increase, leading to global health care issues.

It has long been known that mounting an effective immune response requires a significant amount of ATP, the universal energy currency (80). Leptin is the energy "gage" of the body in humans, as well as in mice, and informs the brain of the energy stores available (17). Obesity has been directly associated with deficits in leptin signaling, particularly leptin resistance. In addition, the obese state has long been suspected and is now generally accepted as a state of increased inflammation, further linking obesity to leptin (81). Thus, obesity appears to be potentially caused and also perpetuated by high concentrations of leptin. Of interest, is that despite high levels of leptin, signaling for significant stores of available energy, obese individuals do not always mount effective immune responses.

Fifteen years after it was first cloned and characterized, leptin is now the focus of many research interests, due mainly to its pleiotropic nature. It is now well known that leptin is crucial in the maintenance of food intake and energy expenditure with resent research, however, turning its attention to other functions of this adipokine. As mentioned previously, leptin has also been shown to have a major effect on cells of the immune system. DCs are the only cells capable of activating naïve T cells (31, 32). On their own, leptin and DCs have very little cross talk, except that DCs express the functional form of the leptin receptor. This now becomes important, and begs the question as to what happens to DCs in obese individuals where leptin concentrations are extremely high. Determining the effects of leptin on dendritic cells is an issue that

must be addressed due to the important part that DCs play in bridging the innate and adaptive branches of the immune system.

The overall goal of this project was to use a murine obesity model to help determine the effects that leptin has on DCs. By using leptin deficient ($Lep^{ob-/ob-}$) and heterozygous ($Lep^{ob-/ob+}$) littermates as controls, effects of leptin on DC were indirectly determined by analyzing the effect of leptin-deficiency on DC phenotype and function. The first objective was to determine whether the absence of a functional leptin protein altered DC numbers, phenotype, antigen acquisition, and antigen processing. The second objective was to determine whether DCs that were procured from $Lep^{ob-/ob-}$ mice have altered capabilities to activate T cells. The ex vivo experiments were coupled with *in vivo* infection models to demonstrate that the *in vitro* culture assays were physiologically relevant. The third objective was to evaluate the leptin signaling pathway in DCs. We focused on the intersection of leptin signaling with DC signaling pathways that are critical for DC activation. This series of experiments were designed to investigate the effects of leptin on DC cell function and the ultimate impact on T cell activation. Given the increased incidence of obesity worldwide and the associated syndromes, it is critical to determine whether leptin and leptin-induced signaling, or altered leptin signaling affect immunity.

CHAPTER 2

Leptin-deficiency *in vivo* enhances ability of splenic dendritic cells to activate T cells

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ABSTRACT

Leptin is a pleiotropic cytokine/hormone that plays a critical role in food intake and energy expenditure. Leptin also participates in functions of the immune system, including those of antigen presenting cells. Here, we assess the effect of leptin-deficiency on splenic dendritic cell (sDC) function. sDCs isolated from leptin-deficient mice (Lep^{ob}) were evaluated *ex vivo* for phenotype and abilities to respond to inflammatory stimuli, to acquire and process antigens, and to present antigens to T cells. The data show that Lep^{ob} sDCs express activation markers similar to controls and respond similarly to lipopolysaccharide (LPS) activation or anti-CD40 crosslinking. In addition, antigen acquisition and processing by Lep^{ob} sDCs was similar to controls. However, compared to controls, Lep^{ob} sDCs elicited higher production of interferon-gamma (IFN-γ) in mixed lymphocyte reactions (MLR) and increased production of interleukin (IL)-2 by an antigen-specific T cell hybridoma. To assess Lep^{ob} sDC activation of T cells *in vivo*, Lep^{ob} and control mice were infected with a systemic administration of *Mycobacterium avium*. Lep^{ob} mice were significantly better at neutralizing the infection as measured by splenic bacterial load over time. This was mirrored with an increased percentage of activated T cells in the *M. avium* infected Lep^{ob} mice. Thus, although no changes were detected in sDC phenotype, activation, or acquisition or processing of antigen, sDCs from Lep^{ob} mice showed enhanced ability to activate T cells *ex vivo* and *in vivo*. These data demonstrate that leptin can modulate DC function and suggest that leptin may dampen T cell responsiveness in the physiological setting.

INTRODUCTION

Leptin, the product of the obese (*ob*) gene, is a 16-kDa protein produced primarily by mature white adipose tissue (13), with leptin serum levels being proportionate to adiposity (17). Research has shown that leptin plays an important role in food intake and energy expenditure (14), in addition to its roles in other physiological processes such as reproduction, glucose and insulin metabolism, and hematopoiesis. Leptin exerts its effects by interacting with its cognate receptor, which is encoded by the diabetes *(db)* gene (18, 26). Alternative mRNA splicing gives rise to six variants of the receptor, of which only the long isoform has been reported to be crucial for leptin signal transduction through the Janus kinase/Signal transducers and activators of transcription (JAK/STAT) pathway (11, 25-27). The long isoform of the leptin receptor is highly expressed in the arcuate nucleus of the hypothalamus, and to a much lesser degree in the lungs and kidneys, and has been detected in other organs and cells types including T cells, B cells, monocytes, and macrophages (24, 82).

Leptin has been shown to affect both the innate and adaptive branches of the immune system. For innate immunity, leptin modulates activity of natural killer cells (70) , macrophages (68, 69, 71), and neutrophils (72), potentiating function in all cell types and promoting the production of pro-inflammatory cytokines. For adaptive immunity, *in vitro* and *in vivo* experiments demonstrate that leptin positively influences T cell proliferation and increases Th1 cytokine production while suppressing Th2 (69, 83-85). These findings are further substantiated by bacterial infection and experimental autoimmune disease models. Leptin-deficient (Lep^{ob}) or leptin receptor-deficient (Lep^{db}) animals have impaired ability to clear or control infection by *Klebsiella pneumoniae* (76),

Listeria monocytogenes (75), and *Mycobacterium tuberculosis* (77) and are less susceptible to Experimental Autoimmune Encephalomyelitis (EAE) (74) and Experimental Arthritis (73). The leptin-deficient animals were characterized with low leukotriene synthesis (76) and a Th2 phenotype (73, 74). Similar experiments where leptin is administered exogenously have been shown to accelerate of EAE (86) and autoimmune diabetes in NOD mice (87); both models present with an increase in Th1 pattern of cytokine release. Taken together, the data support a model in which leptin exerts its effects on the immune system by promoting pro-inflammatory responses. Integration of the innate and adaptive immune responses is mediated by dendritic cells (DCs), which are the only reported cells capable of activating naïve T-cells (31, 32, 63). Leptin also modulates DCs: the addition of exogenous leptin to human monocyte-derived DC resulted in enhanced DC survival, induction of a Th1 response as measured by cytokine production by the treated DC and the responding T cells, and rearrangement of actin cytoskeleton, resulting in enhanced migratory capabilities (70, 88). DC derived from the bone marrow (BM) of Lep-^{db} or Lep-^{ob} mice showed the corresponding opposite results: poor survival, a Th2 or tumor growth factor beta (TGFβ) cytokine profile, and a poor capacity to stimulate allogeneic T cells (89, 90). Thus, leptin appears to also be a critical for optimal DC function.

Taken together, these data suggest that leptin is required for optimal cell-mediated immunity. Leptin potentiates innate immune cell activity (32, 69, 71, 91), including that of DCs (70, 88-90), and enhances T cell responsiveness (70, 83-85, 92). Specifically, leptin promotes survival and migration of DC and induces Th1-mediated inflammation while seemingly suppressing Th2-mediated responses. However, these

findings are based on DCs generated from stem cells of humans and mice. Data focused on DCs *in situ* is scant; one study found that leptin-deficiency increased the steady-state number of DCs in the epidermis (90). The functionality of this specific DC population was not ascertained.

Given the importance of DCs in the initiation and regulation of an immune response, the present study was designed to evaluate the effect of leptin-deficiency on mature splenic DCs (sDC). Our findings demonstrate that, while leptin is not crucial for the ability of sDCs to respond to inflammatory stimuli nor to acquire or process antigen, the absence of leptin enhances their ability to activate allogeneic and antigen-specific T cells *ex vivo*. Moreover, leptin-deficiency allowed for increased clearance of systemically administered bacteria that correlated with an increased percentage of activated T cells. The data demonstrate that for this specific population of DCs, leptin lessens function and underscores that stem cell-derived DCs are not necessarily representative of all *in situ* DC populations.

MATERIALS AND METHODS

Animals. Three week old female Lep^{ob} and their heterologous control littermates (C57Bl/6) were purchased from Jackson Laboratories and used for experiments at 8 weeks. Balb/c female mice, 6 – 8 weeks old, were also purchased from Jackson Laboratories. Animals were kept in a controlled environment with a constant temperature of 72^{o} Fahrenheit and a 12-12 light-dark cycle. Animals were fed standard pellet chow *ad lib*. All experiments were conducted in accordance with IACUC and University of Texas at El Paso guidelines.

Splenic dendritic cell isolation. To isolate sDCs, spleens were treated with collagenase D (Sigma-Aldrich; 1 mg/ml), teased apart and then treated a second time with collagenase D. The disintegrating spleens were filtered to remove cellular debris. Following a wash in Hanks Balanced Salt Solution (HBSS) (Invitrogen), sDCs were purified using Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotech) via positive selection, as described below.

Bone marrow-derived immature dendritic cells. To generate immature BM-DCs, bone marrow was flushed from long bones and red blood cells were lysed using an ammonium chloride buffer (0.15M NH_4Cl and 0.17 M Tris Base) (Sigma). The resulting cell population was induced to differentiate into DCs by culturing in RPMI (Invitrogen) media in the presence of 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 10 ng/ml Granulocyte-Macrophage-Colony

Stimulating Factor (Peprotech), and 10 ng/ml Interleukin-4 (Peprotech). Cells were incubated for eight days, refreshing the media every other day. DCs were enriched by MACS, as described below.

T cell hybridomas. Ovalbumin-specific CD4+ T cell hybridoma (80.10) cells were a generous gift from Dr. Phillipa Marrack (National Jewish Medical and Research Center). Hybridomas were kept in culture in hybridoma media (S-MEM media enhanced with tumor cocktail) and used every fourth day after passaging.

Magnetic Activated Cell Sorting (**MACS**). MACS was used to enrich splenic and bone marrow-derived DCs. Briefly, single cell suspensions of spleens were incubated in 30% mouse serum (Rockland) in MACS buffer (2% FBS in 1X phosphate buffered saline) (Hyclone). Antibody-conjugated magnetic beads (Miltenyi) against the dendritic cell marker CD11c, were added at a ratio of 90 µL for every 10^6 cells. Following incubation on ice, the cells were placed onto a positive selection magnetic column (Miltenyi) and washed 3 times with MACS buffer. Upon removal from the magnet, the enriched cell suspension was released from the column using pressure. Cell purity, as determined by flow cytometry, was consistently above 95%.

Western blot analysis. For detection of the leptin receptor, cell lysates from enriched control (C57Bl/6 littermates) or experimental (Lep^{ob}) BM-DCs were prepared using MEM-PER and general protease inhibitors (Pierce) following the manufacturers' instructions. Protein concentrations were determined using bicinchoninic acid assay

(Pierce). Proteins were separated on a 10% SDS-PAGE (Sigma Aldrich) and transferred to a nitrocellulose membrane (Bio-Rad). Following blocking with 3% non-fat dry milk (VWR), the membrane was incubated with mouse-specific monoclonal anti-leptin receptor antibody (Affinity BioReagents; 1:1000 dilution), then with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Chemicon; 1:5000 dilution). Immunoreactive bands were visualized using Super-Signal West Pico Chemiluminescent Substrate (Pierce) and exposed to x-ray film.

Reverse transcriptase polymerase chain reaction (RT-PCR). Messenger RNA (mRNA) was isolated from hypothalamus, Lep^{ob} BM-DCs, C57Bl/6 BM-DCs, and JAWS II cells using Invitrogen's Micro to Midi RNA Isolation Kit (Invitrogen) per the manufacturer's protocol. cDNA synthesis was performed using MMLV-RT and random hexamer primers (Promega). Alternatively, primers specific for the cytosolic domain of the leptin receptor long isoform were used to amplify the cDNA of interest. Forward primer, 5'-TGGATAAACCCTTGCTCTTCA-3'; reverse primer: 5'-GGTCTCAGAGCACCCAGGTA-3'. An amplicon product size of 121 bp was detected by agarose gel electrophoresis.

Flow cytometry. Specificity of staining was controlled with isotype-matched antibodies for all studies (BD Pharmingen). Flow cytometry analysis was performed on a Beckman-Coulter FC500 instrument. Samples were gated for live cells based on forward- and side-scatter parameters and 10,000 events per sample were collected and analyzed using CXP software (Beckman-Coulter).

Measurement of Ob-R and STAT-3 activation. sDC expression if the leptin receptor as well as receptor functionality, were determined by flow cytometry. Briefly, sDC single cell suspension were generated as mentioned above, subjected to anti leptin receptor for 1 hour, fixed in 1% paraformaldehyde (PFA)(Sigma) then analyzed on the flow cytometer. To determine the receptor's ability to activate the JAK2/STAT3 pathway, sDCs were analyzed for pY705. Briefly, sDCs were isolated, treated with 10nM leptin for 0,15, and 30 minutes, fixed with 1% PFA, permeabilized with Perm Buffer (BD Pharmingen) then stained with 20 uL antipYSTAT3 (BD Pharmingen) per sample, and later analyzed by flow cytometry.

Dendritic cell phenotype and cell numbers. To determine numbers of sDCs, single cell suspensions of experimental and control spleens were blocked with 30% normal mouse serum (NMS), incubated with fluorescein (FITC)-conjugated anti-class II MHC and biotinylated anti-CD11c (1:50 dilution), and finally incubated with streptavidin-conjugated PC5 (all reagents from BD Pharmingen). The number of sDCs was calculated by multiplying the percentage of class II MHC and CD11c positive cells by the total number of splenic cells obtained per spleen. To assess phenotype, enriched sDCs from experimental or control mice were blocked with 30% NMS, incubated with phycoerytherin (PE) conjugated antibodies (BD Pharmingen) against the cell surface markers CD 80, 86, 54, and 40 (each diluted 1:50). In each case, the cells were fixed with 1% paraformaldehyde (PFA Sigma Aldrich) and analyzed by flow cytometry.

Dendritic cell activation. To assess the response of enriched sDCs to inflammatory stimuli, DC cultures were treated with lipopolysaccharide (LPS) (Sigma Aldrich; 10 □g/mL) or were incubated with hamster anti-mouse-CD40 (HM40-3, 5 □g/ml) and goat anti-hamster immunoglobulin (BD Pharmingen). Following the activation period, the cells were blocked with 30% NMS, and labeled with PE-conjugated antibodies to CD80, 86, 54, and 40. The cells were then fixed with 1% PFA and were analyzed by flow cytometry.

Dendritic cell phagocytic activity. Enriched splenic DCs from Lep^{ob} and control mice were used to assess phagocytic activity. Phagocytosis was monitored using the Vybrant Phagocytosis Assay Kit (Molecular Probes) as per manufacturers' instructions. Briefly, cells were seeded at 1 x 10^5 cells per well in a 96-well flat bottom, black-walled plate and incubated with fluorescein-labeled *Escherichia coli* (*E. coli*) fragments. Following incubation, the cells were treated with trypan blue dye to quench fluorescence of any bacterial fragments that were not taken up by the DCs, i.e., remained extracellular. Phagocytosis was analyzed with a fluorescence plate reader. Alternatively, phagocytosis was measured by uptake of the green fluorescent protein-expressing *Mycobacterium avium* (*M. avium*) strain JC104 (a generous gift from Dr. Todd P. Primm, Sam Houston State University). The cells were plated in 12-well plates at 1 x 10^6 cells /well and co-cultured with the bacterial cells at a multiplicity of infection (MOI) of 5 bacteria per DC. After incubation, the DCs were washed with amikacin (Sigma-Aldrich; 200 □M) to remove extracellular *M. avium*. The infected cells were

stained with anti-CD11c to identify DC and assessed for bacterial internalization as a function of relative GFP fluorescence by flow cytometry.

Dendritic cell antigen processing. Enriched splenic DCs from Lep^{ob} and control mice were cultured in 96-well, flat bottom, black-walled plates in the presence of caged fluorescently-labeled ovalbumin (DQ ovalbumin) (Molecular Probes; 10 μg/mL). OVA-DQ fluorescence was detected with a fluorescence plate reader. Fluorescence was excited at λ505 and detected at λ515 at 10 minute intervals over a three hour and twenty minute time course.

Mixed Lymphocyte Reactions. Enriched splenic DCs from Lep^{ob} or control mice were used as stimulators of T cells enriched from lymph nodes of Balb/c mice. Splenic DCs were isolated as described above, irradiated in a XRad160 gamma-irradiator (Precision Instruments; 1200 rads), then co-cultured with responder T cells at 1:5 ratio. T cell activation was measured 96 hours later as function of IFN-γ production (measured by ELISA) or of proliferation. To assess proliferation, cells were labeled with [^{3}H]-thymidine (PerkinElmer, 0.5 □Ci) during the final 18 hrs of culture (overnight pulse). The cells were harvested and the cell-associated radioactivity was determined by β-counter. Un-enriched lymph node cells stimulated with Concanavalin A (Con A) were used as a positive control and T cells in culture medium were used as a negative control.

Antigen-specific presentation. Enriched splenic DCs from leptin-deficient and control mice were pulsed with increasing concentration of I-A^b-restricted ovalbumin peptide

323-339 (H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-OH)(Peptides International) and co-cultured with ovalbumin peptide specific T cell hybridoma cells (80.10) at a ratio of 1:5, DCs to T cells. T cell hybridoma activation was detected as a function of IL-2 production by ELISA after a 96-hour co-culture.

Enzyme-linked immunosorbant assay (ELISA). Cellular production of IFN and IL-2 was measured by ELISA. Microtiter ELISA plates were coated with capture antibody. Plates were blocked at room temperature with 3% bovine serum albumin in PBS. Supernatants from stimulated T cell and sDC co-cultures were added to the plates. Following binding of cytokines to the capture antibodies, the plates were incubated with biotin-conjugated anti-cytokine antibody, followed by HRP-labeled avidin (Biosource). The enzyme substrate O-phenylenediamine (Sigma-Aldrich) was utilized for color development. Cytokine concentrations were calculated against murine recombinant cytokines (BD Pharmingen).

***In vivo* infection of mice with *Mycobacterium avium*.** Following anesthetization, the mice were injected intraperitoneally with $1x10^8$ cells of *M. avium* strain MAC104 (obtained from The Institute for Genomic Research). At the indicated time post-infection, spleen and lungs were collected in HBSS and placed with 1 mm glass beads in a bead beater (Biospec Products Inc.); cells were disrupted for three continuous minutes. Serial dilutions (10^{-4}, 10^{-5} and 10^{-6}) of the tissue homogenates were plated onto Mycobacteria 7H11 agar (VWR) supplemented with Oleic Acid Dextrose Complex (OADC) (100 ml /liter) (VWR). Colonies were counted on day 14 and bacterial titers

were calculated by multiplying the number of colonies by the dilution factor to determine the number of colony forming units (CFU's), generating an average over the different dilutions plated.

Statistical analysis. Where applicable, the appropriate statistical analyses were used to determine any significant differences between control and experimental groups. P values of .05 or lower were considered to be statistically significant.

RESULTS

Murine DCs express the long isoform of the leptin receptor

To assess the effects of leptin on DC function and consequently T cell activation, we first determined whether DCs express the primary signaling isoform of the leptin receptor (Ob-Rb). Leptin interacts with six types of receptors (Ob-Ra, -Rb, -Rc, -Rd, -Re, and -Rf) which in turn are encoded by a single gene (*db*) (4). Ob-R isoforms share a similar extracellular ligand-binding domain at the amino terminus, but differ at the intracellular carboxy-terminus. All of the Ob isoforms, excluding Ob-e which is a soluble receptor, have a transmemberane domain; however, Ob-Rb, the long isoform, is the only receptor isoform that can signal intracellularly (6, 8). We confirm here previous reports from Mattioli and colleagues (11) as well as Lam and colleagues (18), that murine DCs express the long isoform of the leptin receptor. Messenger RNA (mRNA) for Ob-Rb was detected by RT-PCR analysis using primers specific for intracellular signaling motifs unique to the long isoform of the leptin receptor. As templates for the RT-PCR, mRNA was isolated from purified BM-DC samples, from JAWS II cells, and from the hypothalamus, which is known to express high levels of Ob-Rb. BM-DCs were enriched by magnetic bead sorting to a purity of >98% (as determined by flow cytometry) for the presence of the DC marker CD11c (data not shown). The data showed that DCs express the mRNA encoding the leptin receptor long isoform, as evidenced by the presence of a specific 121 bp amplicon (Figure 1A). The identity of the 121 bp product was verified by DNA sequencing (data not shown). To determine protein expression, Western blot analysis was conducted. Upon resolution of whole DC

lysates from C57Bl/6, Lep^{ob}, and Jaws II DCs, a 125-kDa band was detected, which corresponds to the long isoform of the leptin receptor (Figure 1B).

To determine whether the absence of leptin alters DC function, the current studies were focused on splenic DCs (sDCs), working from the assumption that this population had developed, differentiated, and seeded peripheral tissue entirely within the leptin-deficient environment. This is in contrast to bone marrow (BM)-DCs, which are exposed to bovine leptin in the fetal calf serum used to supplement the media and, by nature of the culture, do not seed peripheral tissue. Splenic DCs were therefore also assessed for the presence of the leptin receptor. To address concerns about sufficient numbers of sDCs for Western blot analysis and purity of sDCs for RT-PCR, the presence of the leptin receptor on sDCs was analyzed by flow cytometry. As shown in Figure 1C, >97% of the CD11c-positive splenic C57Bl/6 and Lep^{ob} cells express the leptin receptor. The antibody used in these studies however, identifies all isoforms of the leptin receptor since it is specific for the extracellular domain. The leptin signal is transmitted primarily through the JAK-STAT pathway (5, 6, 7, 8). JAK2 associates constitutively with conserved box 1 and 2 motifs in the intracellular domain of Ob-Rb. Ligation of Ob-Rb by leptin results in autphosphorylation of JAK2, tyrosine phosphorylation of the cytoplasmic domain of Ob-Rb, and tyrosine phosphorylation of the downstream transcription factor STAT3. STAT3 then undergoes homodimerization and nuclear translocation for the transactivation of target genes.

The expression of functional Ob-Rb by sDCs could therefore be confirmed with the detection of activated molecules known to play a role in the leptin signaling pathway. The leptin-treated sDCs were therefore analyzed by flow cytometry for the presence of phosphorylated STAT-3. Splenic DCs from leptin receptor-deficient mice (Lep^{db}) were used as controls. As presented in Figure 1D, the percentage of sDCs with phosphorylated STAT-3 is higher in C57Bl/6 mice than in Lep^{db} mice. Although the extent of STAT-3 activation was relatively low upon leptin treatment, likely due to the low sensitivity of the assay, it is clear that treatment of sDCs with leptin induces signaling through the leptin signaling pathway. Therefore, sDCs must also express the active signaling (long) isoform of the leptin receptor.

Splenic DC numbers and phenotypes are similar between C57Bl/6 and Lep^{ob} mice

Evaluation of the impact of leptin-deficiency on DCs began with an overall assessment of the sDC population. The spleens of C57Bl/6 and Lep^{ob} mice were analyzed for total DC numbers and the sDC were analyzed for phenotype. The percentage of CD11c and class II MHC positive cells was determined by flow cytometry. No detectable differences were observed in sDC percentages or actual cell number between the two genotypes (Figure 2A). The CD11c and class II MHC positive cells were further analyzed for the relative expression levels of antigen presenting cell (APC) surface markers CD80, CD86, CD54, and CD40. Again, no detectable differences were observed between control and experimental sDC populations (Figure 2B). These results suggest that the ability for DCs to populate the spleen and that sDC differentiation remains unaltered in the absence of leptin.

C57Bl/6 and Lep^{ob} splenic DCs respond similarly to external stimuli

Given the role of DCs as antigen presenting cells and activators of naïve T cells, it was critical to assess whether the absence of leptin would alter sDCs function, beginning with the ability to respond to inflammatory stimuli, which is required for optimal interaction and activation of T cells. To determine this, enriched sDCs were exposed to LPS, to mimic bacterial stimulation, or to CD40 crosslinking, to simulate activation upon interaction with T cells. Following LPS or anti-CD40 treatment, the sDCs were stained for the presence of APC activation markers and were analyzed by flow cytometry (Figure 3). The results demonstrate that there were no detectable differences, since the extent of stimulation (up-regulation of APC activation markers) was similar between the two genotypes regardless of the stimulus. The results imply that splenic DCs, which differentiate in a leptin-deficient environment, retain their ability to respond to external stimuli.

Lep^{ob} splenic DCs possess normal antigen acquisition and processing capabilities.

To determine whether leptin-deficiency compromises the ability of sDC to acquire antigen, enriched populations of Lep^{ob} and C57Bl/6 sDCs were fed bacteria that were either fluorescently-labeled or that expressed GFP. Enriched sDCs were first exposed to fluorescently-labeled *E. coli* fragments. Immediately after incubation, the bacterial media was removed and external fluorescence was quenched by treating the cells with trypan blue. Phagocytosis was then measured as a function of fluorescence intensity.

The results showed no significant differences in phagocytosis of the *E.* coli cells between Lep^{ob} and control sDC (Figure 4A). To determine whether this observation was pathogen-specific, ingestion of GFP-expressing *M. avium* was also assayed. To remove extracellular bacteria, the cells were washed with amikacin following the incubation period. The sDCs were fixed and analyzed by flow cytometry to identify $CD11c^{+}$ cells that were also GFP-positive. The results showed that there were no significant differences in phagocytosis of the GFP-expressing *M. avium* by the Lep^{ob} sDCs when compared to the controls (Figure 4B). However, acquisition of protein antigens is meaningless if the APC is unable to convert the proteins to peptides for antigen presentation. Thus, antigen processing was also assessed. Enriched sDCs from Lep^{ob} and C57Bl/6 mice were incubated in the presence of DQ-Ovalbumin, which is caged fluorescein-labeled ovalbumin. Antigen processing was determined as function of fluorescence intensity released upon proteolytic removal of the cage. As shown in Figure 4C, Lep^{ob} sDCs reveal similar antigen processing capabilities relative to controls. Thus, neither antigen acquisition nor antigen processing is compromised in sDCs that have differentiated in a leptin-deficient environment.

Lep^{ob} splenic DCs induce stronger T cell responses in MLRs and antigen specific assays

The final and ultimate act of DCs is to present antigen to T cells for the initiation of adaptive immunity. To evaluate whether leptin-deficiency alters the ability of sDCs to activate T cells, mixed lymphocyte reactions and antigen-specific T cell activation assays using a T cell hybridoma cell line were conducted. Enriched and irradiated

sDCs from Lep^{ob} or C57Bl/6 mice were used as stimulators of primary T cells enriched from lymph nodes of Balb/c mice. T cell stimulation was measured after 72 hours of co-culture as a function of INF-γ production and the comparative rate of tritiated-thymidine incorporation after 96 hours of co-culture. As shown in Figure 5A and 5B, Lep^{ob} sDC elicited a higher response than the control C57Bl/6 sDCs. To further substantiate these findings, similar studies were conducted with the ovalbumin-specific T cell hybridoma, 80.10. T cell activation was measured as a function of IL-2 production. Again, the data demonstrates that Lep^{ob} sDCs were more efficient stimulators of T cells than C57Bl/6 sDCs (Figure 5C). While surprising, the results show that the absence of leptin enhances the ability of splenic DCs to activate T cells in an MLR as well as in a peptide-specific T cell assay. These data suggest that leptin may be exerting inhibitory effects on sDCs as pertains to their ability to activate T cells.

Lep^{ob} mice are more efficient at neutralizing M. avium infections when compared to wild type controls.

To determine whether the findings in MLRs and antigen-specific assays were not the result of an *in vitro* artifact, mice were infected with *M. avium*. *M. avium* is an environmental organism encountered in soil and water (Q). This organism is known to primarily infect and replicate in macrophages (M□s) (T) while control of the bacteria requires the presence of activated $CD4^+$ T cells (R, S). Although effective phagocytes, M□ are unable to initiate naïve T cell responses, suggesting that they are more likely to be critical for the maintenance of an *M. avium* T cell response *in situ*. In contrast, DCs are potent APCs, showing clear superiority in inducing primary immune responses (U),

and are likely responsible for induction of *M. avium*-specific T cell-mediated immunity. Therefore, C57Bl/6 and Lep^{ob} mice were intraperitoneally infected with $1x10^8$ bacteria to mimic a systemic infection. Every 15 days post-infection up to 60 days, the bacterial load in the spleen and lungs was determined to assess extent of infection. In a similar trend to previous results, leptin-deficient mice were significantly better at controlling the infection *M. avium* than the control mice as evidenced by better containment of overall bacterial loads in the spleen (Figure 6A). Lung infection was undetected in both genotypes. Moreover, splenic bacterial loads directly correlated with the percentage of activated splenic T cells (Figure 6B), suggesting that in Lep^{ob} mice the bacteria was managed by a slightly larger and perhaps more effective T cell population. These results further substantiate the *in vitro* findings and demonstrate that leptin-deficiency enhances sDC-mediated T cell activation.

DISCUSSION

Circulating concentrations of leptin are proportional to adiposity, making leptin a biomarker for body fat and a reflection of individual energy balance (14). The primary function of leptin is in the regulation of appetite and energy expenditure, but studies over the years have provided evidence to suggest that it is a pleiotropic adipokine that influences reproduction, glucose metabolism, angiogenesis, production of surfactant, and immunity (18, 26). Recent data have demonstrated that human and murine DCs express the signaling isoform of the leptin receptor and that addition of exogenous leptin promotes DC survival, alters DC morphology leading to increased migration, and pushes DCs to induce a Th1 cytokine profile (70, 88-90). Given the important role of DCs in immunity and the enhancement of DC function induced by leptin, the current investigation was designed to determine the impact of leptin-deficiency on a population of DCs that had differentiated and matured entirely within a leptin-deficient environment.

To assess the influence of leptin-deficiency on splenic DC (sDC) function we first ascertained whether sDCs express the long isoform of the leptin receptor, a requirement for leptin to exert its effects (Figure 1). Our findings show that sDCs express the long isoform of the leptin receptor and that the receptor is functional, suggesting that sDCs are capable of responding to circulating leptin. We continued with an assessment of sDC numbers and phenotype, both of which were equivalent between cellular suspensions from leptin-deficient and C57Bl/6 control spleens (Figure 2). These data suggest that the numbers of DCs required to mount an immune response are not altered by the absence of leptin nor is the ability of the sDCs to interact with

naïve T cells since cell surface markers remained present on the DCs despite the lack of leptin. Our data however, is in contrast to findings made by Macia and colleagues, which demonstrate that leptin-deficient mice present with more DCs in the spleen than their normal counterparts. Similar findings were made for the epidermis of these mice where higher numbers of epidermal DCs were found in leptin-deficient mice compared to control animals (90). Spontaneous migration of epidermal DCs from skin explants of leptin-deficient mice appeared normal, as did inducible migration *in vivo* (90). In our studies, the sDCs from leptin-deficient mice responded normally to inflammatory stimuli (Figure 3) and were able to acquire and process antigen similar to control sDCs (Figure 4). Thus, the despite differing in cell numbers, the two studies demonstrate that peripheral DCs are functionally normal. It is possible that seeding of peripheral tissues by DCs varied between the two studies as a result of genetic differences, since the leptin-deficient mice used by Macia and colleagues possess only 70% of the C57Bl/6J background, whereas ours possess an entirely C57Bl/6 genetic background.

Antigen presentation to naïve T cells in the draining lymph nodes of peripheral tissue is one of the characteristics that set DCs apart from other antigen presenting cells. DCs are critical for the initiation and regulation of an immune response and must therefore be evaluated for their ability to induce T cell immunity. Bone marrow-derived DCs from leptin-deficient mice have been shown to be less immunogenic than controls (90); we therefore hypothesized that sDCs from Lep^{ob} mice would likely be suboptimal APCs. Despite appearing functionally normal in regards to activation, antigen acquisition, and antigen processing, sDCs from leptin-deficient mice were surprisingly different than

controls in their ability to activate T cells. Our results demonstrate that Lep^{ob} sDCs were more effective activators of T cells in an MLR and in an antigen-specific T cell assay (Figure 5). Differentiating and maturing completely within the leptin-deficient environment seems to have produced a distinctly unique population of DCs, allowing for enhanced immunogenicity. Our *in vivo* data substantiated the *ex vivo* data in that clearance of a systemic bacterial infection was superior in the leptin-deficient environment and was accompanied with an increase in the percentage of activated T cells (Figure 6).

Earlier findings have demonstrated that Lep^{ob} or Lep^{db} mice are more susceptible to bacterial infections and less susceptible to the induction of experimental autoimmune disease (73-77), due in large part to the need for leptin by the responding T cells. In fact, the administration of leptin reverses T cell responses to normal. Our data, however, demonstrate enhanced T cell responsiveness in Lep^{ob} mice. This may be due to differences in the route of administration, therefore targeting different populations of DCs. Thus, it might be the specific population of DCs and their microenvironment that affects whether or not they are influenced by leptin and to what extent. Indeed, intestinal DCs have been recently reported to be functionally modulated by products of adjacent intestinal epithelial cells (93) and BM-DCs have been shown to become tolerogenic when exposed to products of thymic stromal cells (94). Both reports provide evidence that the local microenvironment can impact DC function, which may explain the unique functional ability of sDCs isolated from Lep^{ob} mice.

In summary, we have evaluated a specific population of DCs to determine the effect of leptin-deficiency on DC function. We report that, for splenic DCs, the absence of leptin enhances DC induction of T cell responsiveness *ex vivo* and *in vivo*. Evaluating the *in vivo* effect of leptin in this model of leptin-deficiency (Lep^{ob} mice) is particularly complicated by obesity and its associated metabolic syndrome. The outcome, however, is nonetheless likely directly associated with leptin-deficiency, since the background levels of phosphorylated STAT-3 in Lep^{ob} sDCs is intrinsically lower than that observed for sDCs of C57Bl/6 mice (data not shown), demonstrating that direct leptin signaling affects sDCs. The data therefore imply that in the physiological setting, sustained exposure to normal levels of leptin may function to temper T cell responses induced by splenic DCs. This may be of particular importance when considering potential anti-leptin therapies proposed for treatment of excess inflammation and increased susceptibility to infections (95). Excess leptin, commonly caused by obesity, has been shown to reduce Th1 responses, to alter inflammatory responses, and to result in an increased susceptibility to pulmonary and urinary tract infections (95). Excessive reduction of leptin by anti-leptin therapies to counteract these adverse symptoms could potentially bring on a new series of complications, such as increased immunogenicity, as highlighted by the data presented in this report. It is therefore critical to evaluate the effects leptin, and any pleiotropic molecule, on all cells of the immune system and on subpopulations within individual cellular compartments to strike an appropriate balance of immune responsiveness.

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DISCLOSURES

The authors have no financial conflict of interest.

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FIGURE LEGENDS

FIGURE 1. Murine DCs express the long isoform of the leptin receptor.

Single cell suspensions of Lep^{ob} and C57Bl/6 bone marrow cultures were enriched for dendritic cells by magnetic bead separation, selecting for cells that expressed CD11c. The enriched cells were divided in half for two different assays to assess the presence of the long isoform of the leptin receptor: (A) Western Blot analysis and (B) RT-PCR. JAWSII cells (an immortalized DC cell line) were also assessed. (A) Western blot analysis was performed using a leptin receptor specific antibody. The 125 kDa band corresponds to the long isoform of the leptin receptor (Ob-Rb); the 100 kDa band corresponds to the short isoform of the receptor. Actin (18kDa band) serves as a loading control. (B) RT-PCR of isolated mRNA was performed using primers specific to the cytoplasmic domain of the leptin receptor and cDNA products were separated on an agarose gel. The cytoplasmic domain yields a 121 bp cDNA species. From left to right: Lane 1 = DNA ladder; Lane 2 = positive control (hypothalamus); Lane 3 = bone marrow-derived DCs from Lep^{ob} mice; Lane 4 = bone marrow-derived DCs from C57Bl/6 mice; and Lane 5 = Jaws II cells. (C) Single cell suspensions of C57Bl/6 and Lep^{ob} spleens were analyzed by flow cytometry to determine the presence of the leptin receptor on splenic DC. The splenocytes were gated for CD11c and analyzed for the cell surface expression of Ob-R. (D) Single cell suspensions of C57Bl/6 and Lep^{db} spleens were stained with anti-CD11c, fixed, permeabilized, stained with anti-py705 STAT-3 and then analyzed by flow cytometry to determine the presence of activated STAT-3. The

splenocytes were gated for CD11c and analyzed for the expression of phosphorylated STAT-3. All data is one of three representative experiments.

FIGURE 2. Lep^{ob} splenic DC numbers and phenotype are similar to control splenic DC.

(A) Single cell suspensions of C57Bl/6 and Lep^{ob} spleens were analyzed by flow cytometry to determine the percentage of DC by gating for cells staining positive for MHC class II and CD11c; the percentages were converted to actual cell numbers based on the total splenic population. (B) Splenocytes were further analyzed for the expression of antigen presenting cell-specific cell surface markers, CD86, CD80, CD54, and CD40 specifically for the MHC class II and CD11c positive cells. The data is presented as the mean ± SEM of duplicate samples and is one of five representative experiments.

FIGURE 3. Splenic DC from Lep^{ob} mice respond normally to □-CD40 and LPS stimulation.

Enriched splenic DC from C57Bl/6 and Lep^{ob} mice were incubated with hamster □-mouse-CD40 followed by incubation with goat □-hamster Ig or were treated for 48 hr with 10 ug/ml of LPS. The cells were stained with antibodies against MHC class II and CD11c to identify the DC population and with the indicated DC surface markers. The cells were assessed by flow cytometry. Data is presented as the mean ± SEM for 2 mice per genotype and is one of two representative experiments.

FIGURE 4. Lep^{ob} splenic DCs possess normal antigen acquisition and processing capabilities.

(A) Enriched Lep^{ob} and control splenic DCs were co-cultured with FITC-labeled *E. coli* fragments. The cells were treated with trypan blue to quench extracellular fluorescence and were analyzed by flow cytometry to assess phagocytosis as a function of fluorescence intensity. Control = background (cells alone). (B) Enriched Lep^{ob} and control splenic DC were co-cultured with GFP-expressing *M. avium* (MOI = 5). The cells were washed with 200 uM amikacin to remove extracellular bacteria. The cells were stained with anti-MHC class II and anti-CD11c to identify DC and were assessed for bacterial internalization as a function of relative GFP fluorescence. (C) Lep^{ob} and control DC were enriched and pulsed with caged fluorescently-labeled ovalbumin (OVA-DQ) and were monitored for fluorescence emission every 10 minutes for 320 minutes. All data is presented as the mean ± SEM for duplicate samples for each animal and is one of two representative experiments

FIGURE 5. Splenic DC from Lep^{ob} mice are strong activators of primary or antigen-specific T cells.

Enriched and irradiated splenic DCs from C57Bl/6 and Lep^{ob} mice were used as stimulators of T cells enriched from lymph nodes of Balb/c mice. T cell stimulation was measured at 96 hrs as a function of IFN-γ production by ELISA (A) and tritiated-thymidine incorporation (B). Enriched splenic DC from C57Bl/6 and Lep^{ob} mice were pulsed with increasing concentrations of ovalbumin peptide and used as antigen presenters to an ovalbumin-specific T cell hybridoma (80.10). T cell responses were

measured as a function of IL-2 production (C). Data is presented as the mean ± SEM of triplicate samples and is one of three representative experiments. ND = none detected. P value as indicated in figure determined by One way ANOVA.

FIGURE 6. Leptin-deficient mice neutralize *M. avium* infections more efficiently than control mice.

Lep^{ob} and control mice were infected with $1x10^8$ *M. avium* cells via an intraperitoneal administration. On the indicated days post-infection, splenic bacterial loads quantified (A) and the percentage of activated T cells (CD69-positive) in the spleen (B) were determined for CD4+ cells by flow cytometry. For (A), data is presented as the mean ± SEM of three different animals; for (B), data is presented as the mean ± SEM of triplicate samples. The data is one of three representative experiments. P value as indicated in figure determined by Student T test.

Figure 1

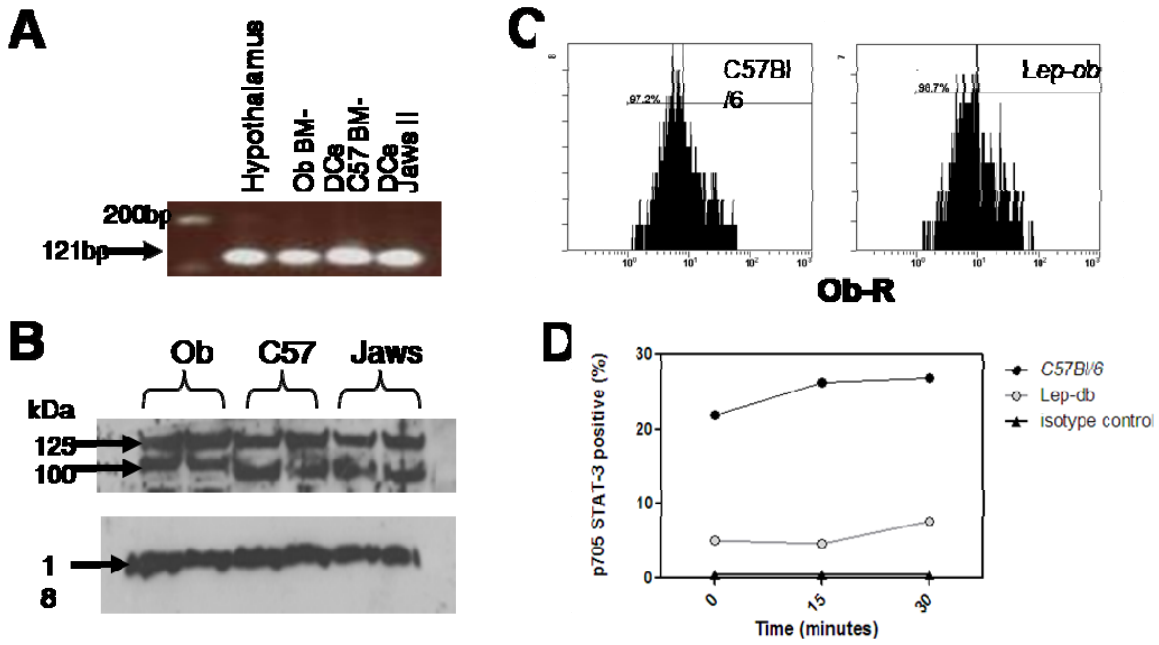

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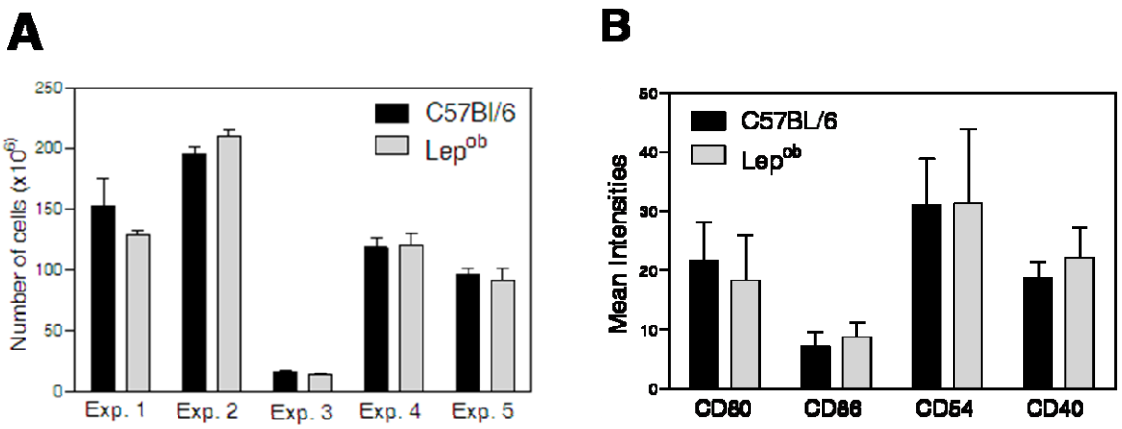
Figure 2



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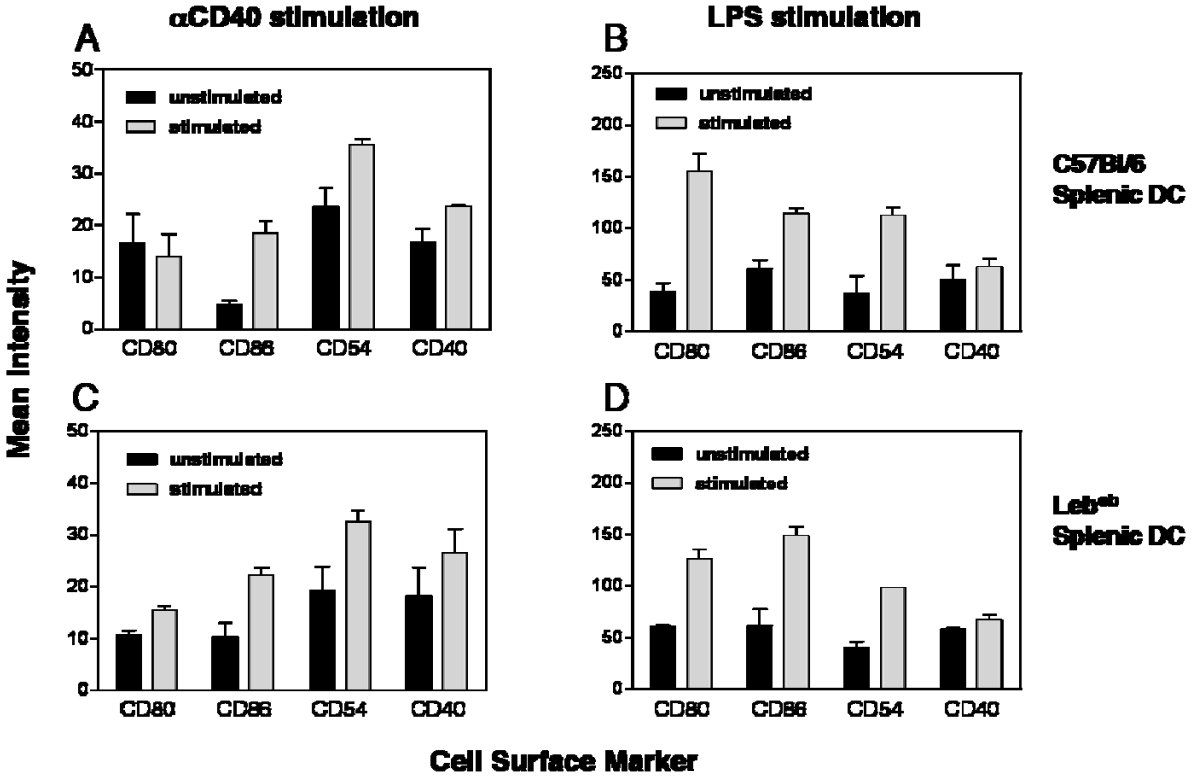

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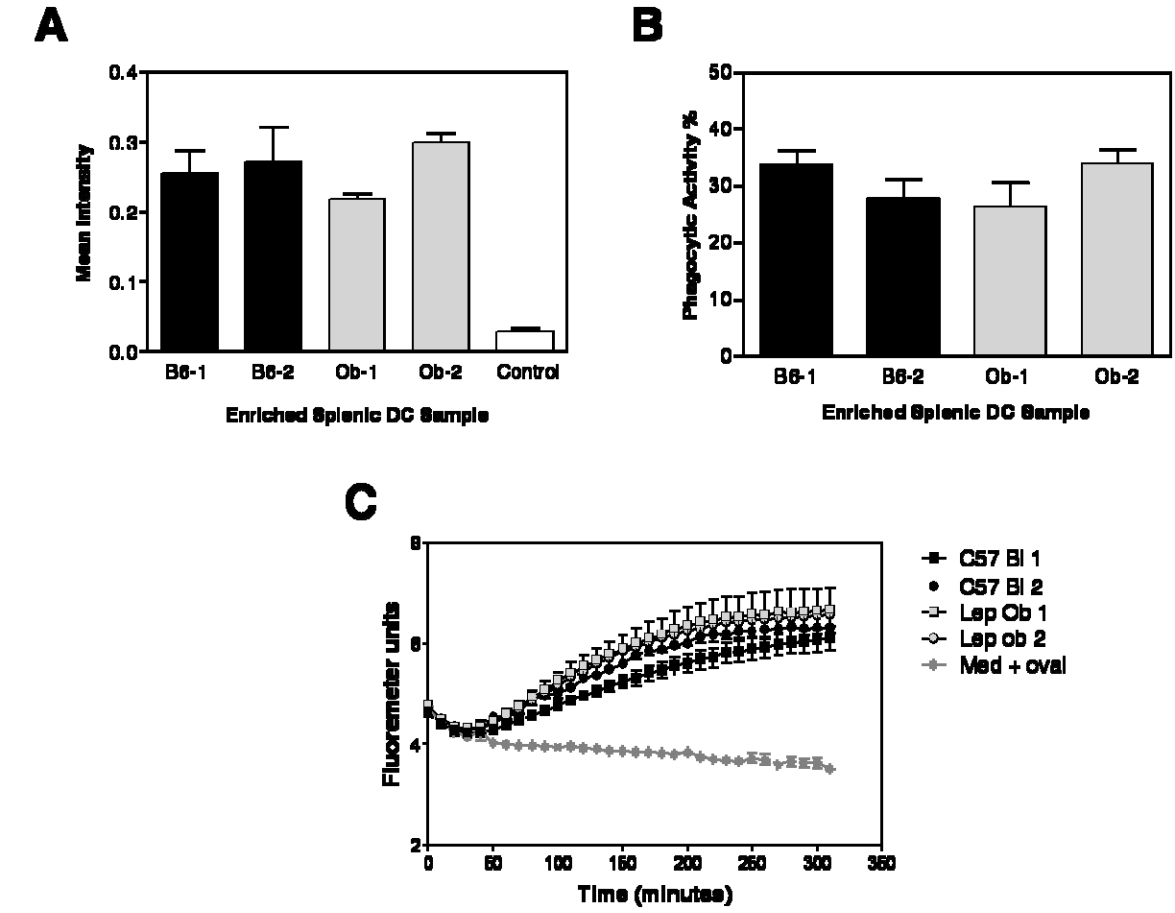

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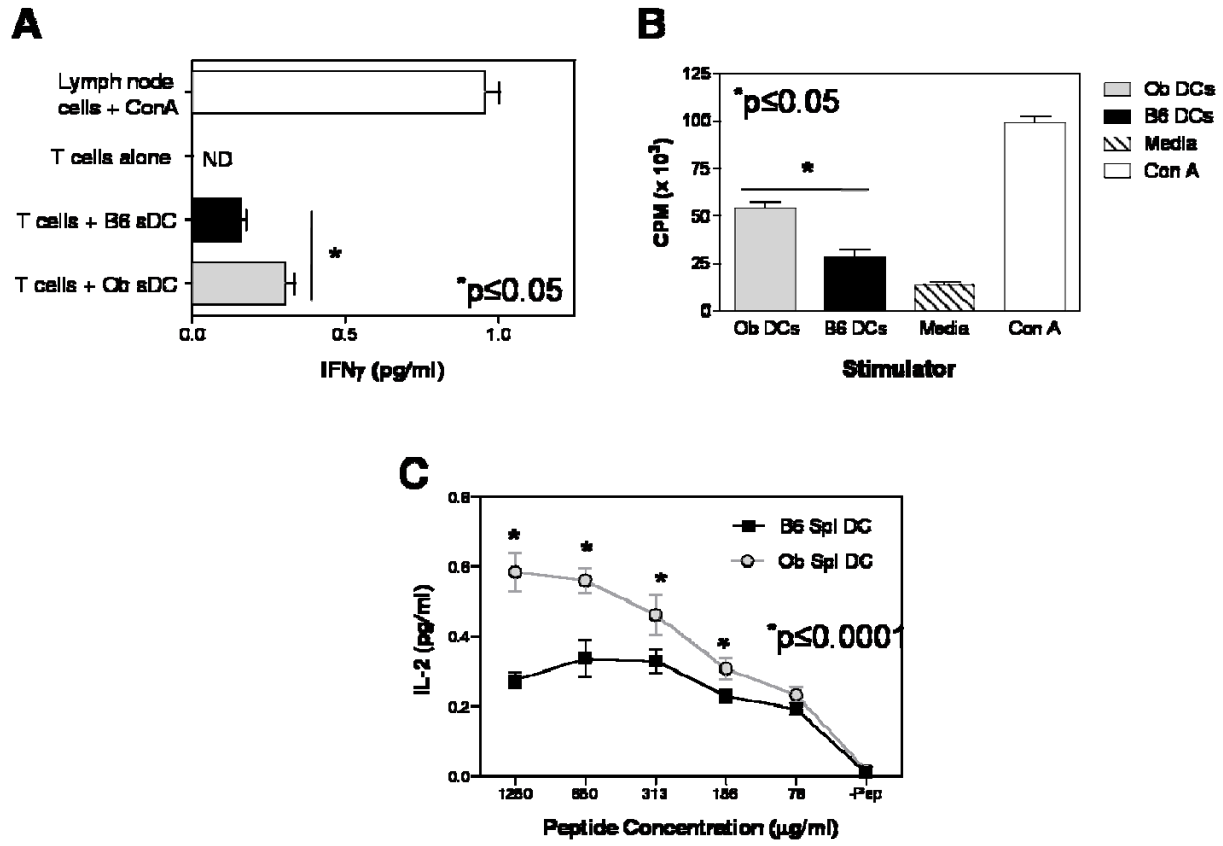

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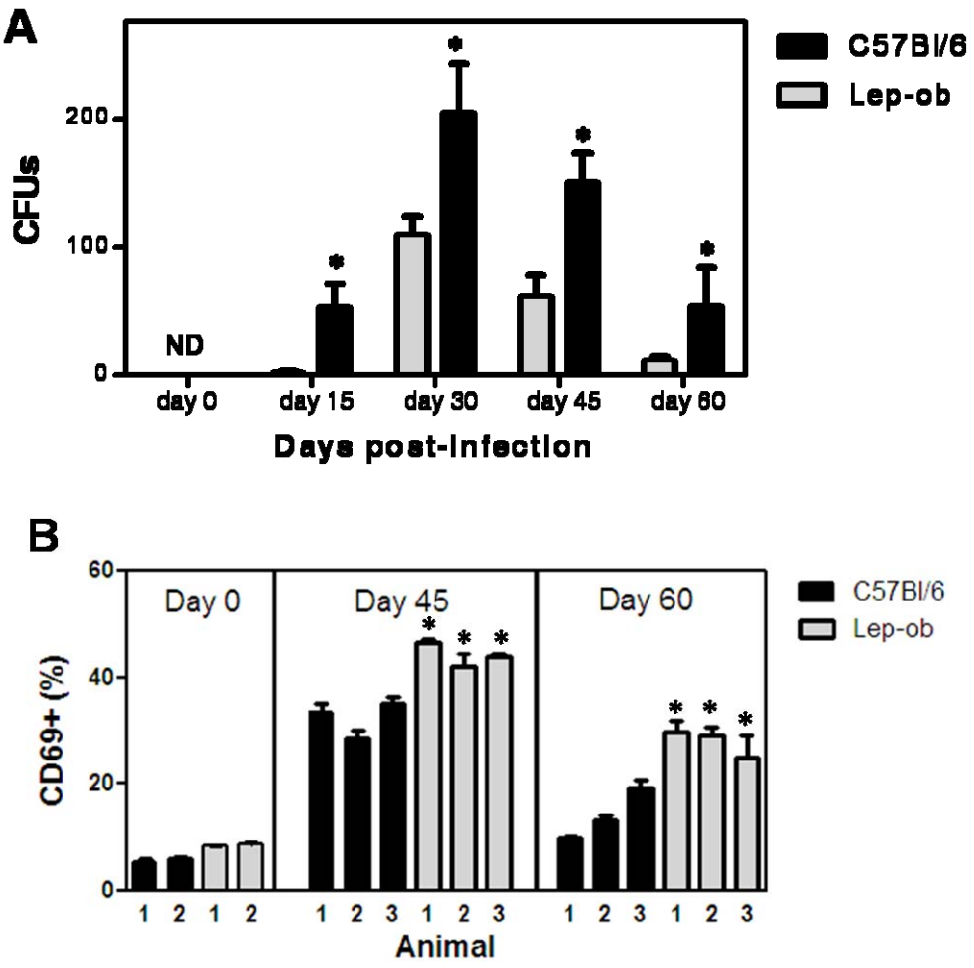

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CHAPTER 3

Leptin-induced STAT3 activation in Bone Marrow derived Dendritic cells leads to NF-kB/STAT3 interactions and represents a possible mechanism for decreased DC mediated T cell activation.

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ABSTRACT

The immune system is a complex network of cells that function cooperatively to help maintain the body clear of pathogenic invasions. Dendritic cells form part of the innate branch of the immune system and are the most potent antigen presenting cells as well as the only cells capable of activating naïve T cells. Communication between cells of the immune system is made possible by cytokines. Leptin is a cytokine/hormone that is produced adipocytes and has been shown to have different effects on macrophages and T cells. We reported previously that sDCs from leptin mutant mice exhibit a higher T cell activation capability in vitro as well as in vivo relative to age and sex matched heterozygous littermates. In an attempt to elucidate a potential mechanism for our observations we conducted a series of experiments. Initially, we sought to determine whether the leptin receptor expressed by DCs was functional and capable of activating STAT3. Next, NF-kB localization after TNF-α treatment was measured, as well as the interaction between STAT3 and NF-kB. Our data show that sDCs express a functional long isoform of the leptin receptor as measured by western blot and flow cytometric analysis. Upon differentiation, bone marrow derived dendritic cells (BM-DCs) treated with TNF-α demonstrated a significant decrease in NF-kB nuclear translocation when co incubated with murine recombinant leptin (mrleptin) relative to controls, as determined by confocal microscopy. After treatment with leptin BM-DC lysates were immunoprecipitated with anti STAT3 antibodies and blotted for NF-kB (p65). Western blot analysis detected an immunoreactive band approximately 65 kDa using NF-kB p65 specific antibodies; while our findings show that STAT3 and NF-kB co-immunoprecipitate, they do not demonstrate that the interaction is direct. Our data

suggests that leptin-induced STAT3 activation is sufficient to significantly reduce NF-kB nuclear translocation and that it does so by direct interaction with NF-kB. In conclusion, the interaction between STAT3 and NF-kB may explain the significant reduction in NF-kB nuclear translocation and may explain why in the absence of leptin, DCs are more immunogenic.

INTRODUCTION

Dendritic cells are cells of the innate immune system that survey the environment which they populate. In instances where they encounter antigens, said antigens get processed and then presented to T cells. DCs are the most potent APCs and the only reported cell type with the capability to stimulate naïve T cells; those T cells that have never encountered antigen in the context of a major histocompatability complex (MHC) (96). It is this particular property of DCs that lead us to investigate whether the absence of leptin in a mouse model would lead to an alteration in DC function when compared to heterozygous littermate controls. Our findings have demonstrated that sDCs procured from leptin mutant mice have an increased ability to stimulate T cells, a phenomenon that was observed in vitro using a T cell hybridoma cell line, primary T cells, as well as in vivo (97). None-the-less, our findings failed to elucidate the potential mechanism (s) responsible for our observations. We, therefore, set out to determine whether the signaling pathways involved in DC function and activation, particularly the NF-kB pathway, as well as one critical in DC differentiation and the primary signaling pathway in leptin, JAK2/STAT3, were involved.

Dendritic cells arise from bone marrow hematopoietic stem cells (HSC) as a direct consequence of environmental cues in the form of cytokines. In vitro differentiation of mouse HSC to DCs in the presence of GM-CSF and IL-4 has been demonstrated and repeated many times(98). Throughout the differentiation process from HSC to immature DCs (iDCs), a myriad of signaling pathways, including the NF-kB

and JAK/STAT pathways, get activated which allow for differentiation to occur. NF-kB's role in functions of the immune system, both innate and adaptive, has been extensively studied (99). For example, Gugasyan and colleagues demonstrated that in B cells, NF-kB plays a crucial role in B cell activation (100). Ouaaz and colleagues demonstrated the requirement for multiple subunits of NF-kB and normal DCs development as well as survival. The data did however show that when a single subunit was missing there were no adverse effects (101). These results suggest that NF-kB is required for DC development and that in its absence; DCs undergo aberrant development and have decreased survival. In addition to being critical for DC development and survival, NF-kB has been demonstrated to be necessary during DC/T cell interactions and is required for optimal T cell activation via CD40 ligation. O'Sullivan and colleagues showed that the p50, cRel, and RelA NF-kB subunits are critical in the regulations of genes directly involved with dendritic cell function. For example, p50 and cRel regulate genes that are directly involved in T cell responses; i.e. CD80, 86, 54, 40, and MHCII after LPS activation. In addition, they also demonstrated the requirement for the different subunits in the expression of cytokines, like IL-12 which plays a crucial role in the polarization of T cells to the T_H1 phenotype. These data demonstrates the key role that NF-kB plays in DC function (102). Taken together these data would suggest that any alteration s to the NF-kB signaling pathway, or others, during obesity may lead to aberrant DC function.

In addition to determining the relation between the NF-kB pathway and optimal DC function, determining whether changes to STAT3 activation would alter DC function is critical. Leptin exerts its physiological effects by binding to the leptin receptor and by

activating the JAK/STAT3 signaling pathway (11, 18, 25). Binding of leptin to its receptor initiates a signaling cascade that starts off with receptor dimerization; this dimerization may result in homo- or heterodimers. Upon dimerization, the receptor-associated Janus kinase (JAK) transphosphorylate and get activated. The JAKs can then phosphorylate tyrosine residues on the receptor intracellular domains that will become docking sites for other signaling molecules. Of importance to us is the STAT3 transcription factor, but other molecules like the SHP2 have been reported to have the capability to dock and initiate a variety of signaling cascades like Mitogen Activated Kinase (MAPK) and Extracellular Regulated Kinase (ERK) (11, 25, 103-105). Upon phosphorylation of the intracellular residue Y1138, a docking site necessary for STAT3 is generated and thus allows for recruitment, phosphorylation, and activation of STAT3 by the JAK (103, 106). In leptin receptor signaling, JAK activation and function is tightly regulated based on the isoform of the receptor which is present (105). Alternative mRNA splicing gives rise to six different isoforms of the leptin receptor and ectodomain shedding gives rise to the only soluble form of the receptor (22, 24). Thus, in the presence of the short isoforms of the receptor, the alternative pathways reported above are activated (25, 104, 107).

We reported previously that the absence of leptin did not alter the number of sDCs nor did it alter their phenotype, ability to acquire or process antigen. The absence of leptin did, however, enhance T cell activation as mediated by DCs. Our data, however, did not address a potential mechanism that might explain our observations. The present study was designed to help determine whether leptin-induced STAT3 activation is

involved in negative regulation of DC mediated T cell activation. Our data shows that addition of murine recombinant leptin (mrleptin) to mouse stem cell cultures decreases the number of CD11c positive DCs. In addition, leptin-induced signaling significantly reduced the nuclear translocation of NF-kB and this reduction seemed to be due to complex formation, including STAT3 and NF-kB, as determined by IP assay. Our data suggests that in the presence of leptin, STAT3 activation may serve as a negative regulatory signal. This is important given the dramatic increase in obesity around the world, a health condition clearly marked by increased levels of circulating leptin. Individuals who are obese may then become more susceptible to infections and may experience decreased immune functions.

MATERIAL AND METHODS

Animals. Three week old female Lep^{ob} and their heterologous control littermates (C57Bl/6) were purchased from Jackson Laboratories and used for experiments at 8 weeks. Animals were kept in a controlled environment with a constant temperature of 72^{o} Fahrenheit and a 12-12 light-dark cycle. Animals were fed standard pellet chow *ad lib*. All experiments were conducted in accordance with IACUC and University of Texas at El Paso guidelines.

Splenic dendritic cell isolation. To isolate sDCs, spleens were treated with collagenase D (Sigma-Aldrich; 1 mg/ml), teased apart and then treated a second time with collagenase D. The disintegrating spleens were filtered to remove cellular debris. Following a wash in Hanks Balanced Salt Solution (HBSS) (Invitrogen), sDCs were stained for flow cytometry as described below.

Bone marrow-derived immature dendritic cells. To generate immature BM-DCs, bone marrow was flushed from long bones and red blood cells were lysed using an ammonium chloride buffer (0.15M NH_4Cl and 0.17 M Tris Base) (Sigma). The resulting cell population was induced to differentiate into DCs by culturing in RPMI (Invitrogen) media in the presence of 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), Granulocyte-Macrophage-Colony Stimulating Factor (10 ng/ml, Peprotech), and Interleukin-4 (10 ng/ml, Peprotech). Cells were

incubated for eight days, refreshing the media every other day. DCs were enriched by MACS, as described below.

Magnetic Activated Cell Sorting (**MACS**). MACS was used to enrich bone marrow-derived DCs (BM-DCs). Briefly, single cell suspensions of BM-DCs were incubated in 30% mouse serum (Rockland) in MACS buffer (2% FBS in 1X phosphate buffered saline) (Hyclone). Antibody-conjugated magnetic beads (Miltenyi) against the dendritic cell marker CD11c, were added at a ratio of 90 µL for every 10^6 cells. Following incubation on ice, the cells were placed onto a positive selection magnetic column (Miltenyi) and washed 3 times with MACS buffer. Upon removal from the magnet, the enriched cell suspension was released from the column using pressure. Cell purity, as determined by flow cytometry, was consistently above 95% as determined by Flow cytometry.

Flow cytometry. Specificity of staining was controlled with isotype-matched antibodies for all studies (BD Pharmingen). Flow cytometry analysis was performed on a Beckman-Coulter FC500 instrument. Samples were gated for live cells based on forward- and side-scatter parameters and 10,000 events per sample were collected and analyzed using CXP software (Beckman-Coulter).

Measurement of STAT-3 activation. sDC expression if the leptin receptor as well as receptor functionality, were determined by flow cytometry. Briefly, sDC single cell suspension

Immunoprecipitation Assays. Immunoprecipitation assays (IPs) were conducted in the following manner. Cells were treated for the indicated time. Upon completion of treatments cells were washed and whole cell lysates were generated and IPd using anti STAT3 antibodies. Immunoprecipitates were then probed with anti NF-kB (p65) in western blot analysis as described before. Immuno reactive nbands were visualized using chemiluminescence on x-ray film.

RESULTS

We reported previously that splenic DCs were more efficient activators of T cells in vitro as well as in vivo (97). However, our data failed to elucidate a possible mechanism responsible for our observations. We set forth to determine whether leptin-induced STAT3 activation could explain some of our findings. Initially we had to determine whether sDCs expressed the long isoform of the receptor as well as the functionality of the receptor. Using flow cytometric analysis, we were able to detect the presence of a leptin receptor in sDCs isolated from C57Bl/6 heterozygous control mice or leptin deficient (Lep^{ob}) experimental mice (Figure 1A). Our data shows the presence of a functional receptor as well in sDCs. To determine whether BM-DCs expressed a functional receptor, western blot analysis was performed. Our results indicate that the DCs generated from C57Bl/6 bone marrow express the leptin receptor, are capable of activating the STAT3 pathway, as a function of phosphorylation of tyrosine 705 of the STAT3 protein (pY705STAT3). Furthermore, the activated STAT3 is a functional protein and can regulate gene expression as the detectable levels of the Suppressors Of Cytokine Signaling 3 (SOCS3) protein increase as the concentration of the pY705 STAT3 increases (B). In addition to western blot analysis, flow cytometry was used as a second method to substantiate our findings. BM-DCs were treated for the indicated time and then analyzed to detect the presence of activated STAT3. Our data shows a significant difference in STAT3 activation in control C57Bl/6 DCs relative to DCs obtained from leptin receptor deficient (Lep^{db}) mice (C and D). Lep^{db} mice lack the expression of a functional long isoform of the leptin receptor and thus serve as a control

that allows for the direct comparison of STAT3 activation between the two groups (C57Bl/6) versus a known negative control (Lep^{db}). Upon confirmation of our findings and those of others (70, 90, 97) that dendritic cells, both splenic and bone marrow derived, express the leptin receptor and that further it is a functional isoform, it is very likely that either population of dendritic cells is capable of responding to leptin in vivo or in vitro. These data would suggest that if leptin were the source of a potential negative feedback loop that DCs would be capable of being affected by it. In addition, depending on the signaling mechanism affected by the possible inhibition, dendritic cell function may be compromised.

Armed with the knowledge that DCs cells express a functional leptin receptor isoform, we then wanted to determine whether leptin deficiency was capable of altering the generation of plasmacytoid DCs (pDCs) in vivo. Plasmacytoid dendritic cells are a relatively new sub-population of DCs that has been reported to have a major role in immunogenic regulation (52, 108). Given their important role in regulating immunity, we performed flow cytometric analyses on splenic single cell suspensions generated from heterozygous controls or Lep^{ob} mice to determine the percent age of pDCs. Our results indicate the absence of a functional leptin protein does not have any significant effects on the percent of detectable pDCs (Figure 2). These findings suggest that the observed increase in T cell activation by sDCs reported previously(97) were a direct consequence of leptin deficiency and not the result of the absence of the “regulatory” plasmacytoid DC population, thus further implying that leptin may indeed be functioning as a negative regulator of DC function.

In order to determine whether leptin was having a negative effect on dendritic cells, we next investigated its effects on DC differentiation from HSC. HSC cultures were generated in the usual manner (98). While in culture, cells were supplemented with physiological concentrations of leptin from day zero until day eight. On the final day of culture DCs were analyzed to determine whether the presence of the recombinant leptin had any adverse effects on the differentiation process. Flow cytometric analysis was performed on the BM-DC cultures and gates were set for live cells expressing the CD11cand CD45Rα markers, bath putative pDC markers. Unexpectedly, the percent of DCs (CD11c+ and CD45Rα) that were detected in our media control were significantly higher relative to the leptin treated group (Figure3). While we reported previously that there were no significant differences in the number of sDCs in Lep^{ob} mice relative to heterozygous controls, the leptin concentration in those mice may not have been comparable to those used here. Non-the-less, the concentrations used here were well within the range of concentrations in humans (109-111)and thus the data provides an insight as to what might occur in the case that HSC are exposed to these concentrations of leptin and how it might affects DC differentiation. It is worth pointing out that in our previous findings the animal model used lacked a functional leptin protein and thus our observations here are expected if leptin is having a regulatory effect.

Leptin, through the interaction with its receptor, has been well documented as being capable of activating multiple signaling pathways, particularly the JAK2/STAT3 pathway when signaling through the long isoform (11, 25, 104, 112). Upon determining that leptin was capable of inhibiting DC differentiation we wanted to determine whether

addition of exogenous leptin would alter the Nuclear Factor-kappa B (NF-kB) transcription factor and more specifically its nuclear translocation as NF-kB has been shown to be critical for DC function by several groups (65, 101, 102, 113). After enrichment, BM-DCs were treated with LPS, leptin, LPS and leptin, or left untreated then stained with Cellomics HitKit and analyzed by confocal microscopy. Our data showed that when left untreated, BM-DCs had minimal levels of NF-kB in the nucleus (Figure 4a). Treatment with LPS or leptin alone demonstrated an increase in nuclear translocation relative to media (Figure 4 B&C). However, in the presence of LPS and leptin simultaneously, BM-DCs exhibited a significant decrease in the amount of nuclear translocation of NF-kB (Figure 4d). Leptin has been reported as having the capability to activate the NF-kB pathway. However, the JAK2/STAT3 pathway remains the ideal signaling cascade activated when the long isoform of the leptin receptor is activated. Our results imply that in the presence of a known NF-kB activator, in this case LPS, leptin induced STAT3 activation is functioning as a negative regulator. It has been documented that transcription factors have the capability to inhibit other transcription factors as they compete for limited co-factors required for their function. For example, Luo and colleagues demonstrated that STAT5 inhibits NF-kB signaling via protein – protein interactions (114). Furthermore, Hoentjen and colleagues showed that in DCs, STAT3 was capable of inhibiting NF-kB recruitment to the IL-12 promoter, a known cytokine in DCs function and a major contributor to T helper cell differentiation into the TH1, proimflammatory, profile (115). Additionally, several other groups have reported similar interactions and of particular interest in dendritic cells. Nefedova and colleagues demonstrated that the hyper activation of the STAT3 resulted in abnormal differentiation

of dendritic cells (116). Additionally, this phenotype could be reversed by using a STAT3 specific inhibitor (JSI-124) (117, 118). Our results, therefore, suggest that leptin induced STAT3 activation may be directly inhibiting NF-kB and thus is a plausible mechanism responsible for the enhanced T cell activation by DCs previously reported by us (97).

In order to determine whether the leptin induced STAT3 activation culminated in a direct interaction between the two transcription factors, NF-kB and STAT3, we performed immuno precipitation (IP) assays. Upon treatment of BM-DCs with physiological concentrations of leptin for the indicated times, cell lysates were generated and then subjected to IP using STAT3 monoclonal antibodies. Once completed, the IP pellet was boiled and proteins were resolved on an SDS-PAGE gel and detected for the presence of NF-kB. Once immuno reactive bands were visualized we were able to detect a band of approximately 65kDa, corresponding to NF-kB (Figure5). Our data clearly shows that when the STAT3 pathway is activated by leptin these proteins are interacting, as evidenced by our western bot. It is therefore possible that STAT3 is playing a negative regulatory role in DCs and that this regulation is affecting dendritic cells and their ability to efficiently activate T cells, in vitro as well as in vivo.

Our preliminary data shows that in the presence of exogenous leptin, BM-DCs are present at significantly lower percentages relative to controls. When in the presence of a known NF-kB activator, leptin induced STAT3 activation has the capability of significantly decreasing nuclear translocation of NF-kB, an observation that may be the

direct consequence of protein-protein interactions. To help elucidate the potential mechanism for our observations, we propose the following model (Figure 6)

DISCUSSION

Dendritic cells now represent one of the most widely research cell types in science. Known for their uncanny and unmatched function as antigen presenting cells, DCs are crucial and a major link between the innate and adaptive branches of the immune system. Our interests in determining whether leptin-deficiency had any effects on DC function lead us to investigate and later report that leptin-deficiency, while not a requirement for normal DC percentages, expression of cell surface markers, antigen acquisition or presentation, the absence of a functional leptin protein did have a positive effect on T cell activation both in vitro and in vivo (97). Originally described as a satiety factor, leptin has been shown to directly affect cells of the immune system like T cells, macrophages, and DCs (68, 76, 83). Given our observations reported previously, we sought to determine the mechanism(s) responsible.

In our present study we set out to determine whether the preferential signaling pathway activated by leptin, STAT3, was involved in negatively regulating the NF-kB transcription factor. NF-kB has been described by others as being a critical transcription factor in DC survival as well as in DC function (64, 65, 101, 102, 113, 119). Initially, we sought to determine the whether leptin induced signaling altered DC differentiation in bone marrow cultures. Our data shows that dendritic cells express a functional leptin receptor as STAT3 phosphorylation of tyrosine residue 705 was detected. In addition, the SOCS3 gene product was also detected, a gene that is regulated by STAT3 (Figure 1) thus confirming the functionality of the receptor. These

data show that in the event that leptin were secreted by adipose tissue or other cells like regulatory T cells (92), DCs would be capable of responding to it.

To determine whether addition of recombinant murine leptin would alter HSC differentiation to DCs, leptin was added to BM-DCs culture and later assessed to determine the percentage of cells that were DCs as a function of staining positive for CD11c, a well established cell surface marker for DCs. Upon flow cytometric analysis, our data shows that leptin treated cell cultures exhibited a significant decrease in the percent of DC generated from bone marrow HSC when directly compared to untreated group (Figure 3). We therefore suggest that the signaling cascades activated in the presence of physiological concentrations of leptin are detrimental to the DC differentiation process and serve as a negative regulator of DC differentiation.

Our findings demonstrate that it is possible that DC differentiation may be affected in the presence of leptin in cell cultures, however, the effects that leptin or the lack of leptin might have on regulatory DC (plasmacytoid DCs (pDCs)) needed to be addressed. In a previous publication, we reported that sDCs were superior T cells activators than control DCs. We wanted to determine whether leptin affected the presence pDCs, which play a critical role in modulating immune responses. Single cells suspensions from leptin deficient or heterozygous controls were analyzed by flow cytometry to assess the percentage of pDCs. Our data shows that the detectable percentages of pDCs are not aberrant in leptin deficient mice relative to controls (Figure

2). Given the role that pDCs contribute to the regulation of immune responses (52). We report here that our previous findings are more than likely the direct effect of leptin on CD11c+ conventional DCs and not the result of any effects on CD11c and CD45Rα positive plasmacytoid dendritic cells. These data therefore implies that while addition of exogenous leptin, at concentrations that are physiologically relevant, has an effect on the number of BM-DCs that can be generated in vitro, while the effects on the generation of the subpopulations of DCs in vivo, in this case pDCs, remains unaffected, at least not detectable by flow cytometry.

Our data propose that leptin serves as a negative regulator of DC differentiation without altering the percentages of pDCs in vivo. We wanted to determine the possible function of leptin induced STAT3 signaling. It is now well accepted that transcription factors act as negative regulators of other transcription factors. Many times this inhibition is the result of direct protein-protein interactions. In the case of dendritic cells it has been reported previously that STAT3 is capable of inhibiting NF-kB gene regulation and that said inhibition is the result of STAT3 directly inhibiting NF-kB function (115, 118). We therefore designed and performed a series of experiments that would allow us to determine the effects of leptin induced signaling on NF-kB nuclear translocation, a hallmark of NF-kB activation. BM-DCs were generated and then enriched and then treated with leptin, LPS, leptin and LPS, or left untreated. The cells were then processed with Cellomics HitKit and then analyzed by confocal microscopy and the presence of NF-kB was quantified as a function of fluorescence intensity in the nucleus. Our results demonstrate that both leptin and LPS alone are sufficient to activate NF-kB, while media alone showed baseline levels of activation. However,

when compared to LPS positive control, LPS/leptin treatment showed a significant decreased in NF-kB translocation (Figure 4). Our data suggest that leptin induced signaling in the presence of a putative NF-kB activator inhibits NF-kB activation, thus our results are similar to those published previously. These results are of particular interest given the role that NF-kB plays in DC function and in their survival. By altering NF-kB's cellular localization, DC function may be jeopardized and lead decreased T cell mediated immune responses

To help determine whether leptin induced STAT3 activation led to the inhibition of NF-kB via protein-protein interactions we carried an IP assay. Cells were subjected to a time course with leptin, cell lysates were generated then immuno precipitated using STAT3 monoclonal antibodies and then IP pellet was analyzed by western blot analysis using NF-kB specific monoclonal antibodies. A 65 kilo Dalton immunoreactive band was detected corresponding to NF-kB (Figure 5). Our data shows that in the presence of leptin induced signaling activated STAT3 and NF-kB interact. This interaction then probably leads to the inhibition of NF-kB activity only, as the same treatment resulted in a time response for the expression of a STAT3 regulated gene, SOCS (Figure1). Our results are line with others that reported that multiple STATS are capable of inhibiting NF-kB by protein-protein interactions (114). Nefedova and colleagues reported that in the case of DCs, specific inhibition of the JAK2/STAT3 pathway with the chemical inhibitor JSI-124 resulted in an increased up-regulation of MHCII as well as an increase in T cell activation in allogeneic or antigen specific reactions both of which require an activated NF-kB(118).

We reported previously that sDCs isolated from leptin deficient mice were superior T cell activators relative to heterozygous controls. In order to help determine a potential mechanism, we utilized BM-DCs and determined that DCs express a functional leptin receptor capable of activating the JAK2/STAT3 pathway. Our data showed that dendritic cells express a functional receptor and that once activated, STAT3 is capable of complex formation with NF-kB. Obesity has become a worldwide pandemic. It is now estimated that more than 50% of Americans are overweight. Alarmingly, even populations in famine stricken countries have seen an increase in obesity. During an obese state, circulating serum levels of leptin are increased due directly to an increase in white adipose tissue. In the hypothalamus, leptin signals the brain of the energy stores by activating the JAK/STAT pathway. This activation leads to the expression of anorexic peptides that then signal the periphery and offer a satiety signal. In dendritic cells, leptin is capable of activating the same signaling pathways. It is then of grave concern that during an obese state the increased opportunities for activating the JAK/STAT pathway soar. In situations where leptin signaling coincides with an NF-kB activator, like activation through a toll like receptor, or direct T cell/DC interaction, DC function may be compromised, thus leading to reduced immunity. This would in turn put human health at risk given that DCs are considered to be the bridge between innate and adaptive immunity. Given our findings, some precautionary measures must be taken to help reduce the risk of hyper activating the JAK/STAT pathway so that the required NF-kb signaling necessary for DC function may not be disrupted.

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FIGURE LEGENDS

Figure 1. Splenic and Bone marrow derived dendritic cells express a functional leptin receptor.

Bone marrow derived and splenic DCs were treated with mouse recombinant leptin for the indicated time. Whole cell lysates were then generated and analyzed by western blotting or cells were analyzed by flow cytometry to determine the functionality of the leptin receptor as a function of STAT3 phosphorylation on tyrosine residue 705. (A) Western blot analysis of BM-DCs using antibodies specific to STAT3, phosphorylated Tyrosine 705 (pY705), and SOC3. (B) Flow cytometric analysis was used to detect STAT3 activation in BM-DCs and isotype control antibodies were used to confer specificity of pY705 antibodies. BM-DCs were gated for CD11c and analyzed for the expression of phosphorylated STAT-3. (C). Graphed flow cytometry data for (B). Flow cytometric analysis of splenic DCs for phosphorylated STAT3 on tyrosine residue 705 (D). All data is one of three representative experiments.

Figure 2. In vivo leptin deficiency does not alter the plasmacytoid dendritic cell population in the C57Bl/ 6 leptin receptor deficient mouse model.

Single cell suspensions from C57Bl/6 and leptin receptor deficient mice were obtained and analyzed for the presence of CD45Rα+ plasmacytoid dendritic cells (pDCs). pDCs were identified as those cells that were gated for CD11c and stained positive for CD45Rα; data is presented as percentages and is one of two independent experiment with two mice per group.

Figure 3. Differentiation of Hematopoietic Stem Cells to Bone Marrow derived Dendritic cells is altered by the presence of murine recombinant leptin at physiological concentrations in vitro.

Bone marrow stem cells were isolated and treated with 10 ηM murine recombinant leptin from day zero to day eight. On day eight, DCs were stained with anti CD11c and analyzed by flow cytometry for total DCs in the cell cultures.

Figure 4. Nuclear Factor-kappa B (NF-kB) nuclear translocation is reduced by mouse recombinant leptin in bone marrow derived dendritic cells in vitro.

Enriched bone marrow derived dendritic cells were treated with TNF-α (10ρM) in the presence or absence of leptin (10nM) then analyzed by confocal microscopy to determine the cellular localization of NF-kB using the Cellomics HitKit® as specified by the manufacturer. Graphical representation of mean intensity for all sub figures (E).

Figure 5. In vitro treatment of Bone marrow derived dendritic cells with leptin causes NF-kB and STAT3 to co-immunoprecipitate. BM-DCs were generated and then treated for the indicated time, whole cell lysates were obtained then lysates were immunoprecipitated with anti STAT3 specific antibodies. Western blot analysis were performed using NF-kB (p65) specific antibodies. Data is representative of two independent experiments.

Figure 6. Proposed Model for the interaction between STAT3 and NF-kB

We propose the following model as a potential mechanism of action for leptin-induced signaling in dendritic cell function. Initially, when leptin is detected by DCs, it is through the interaction with the leptin receptor. In instances where leptin interacts with the long isoform of the leptin receptor, the JAK2/STAT3 signaling cascade is initiated. Binding of leptin to the receptor initiates conformational changes that lead to receptor dimerization. It is this dimerization event that leads to the recruitment of the JAK proteins which then phosphorylate specific tyrosine residues on the receptor which serve as docking sites for multiple proteins, among these the STAT3 protein. Upon recruitment to the docking sites, the STAT3 proteins undergo JAK2 mediated phosphorylation on tyrosine residue 705, a critical and necessary step required for STAT3 activation and dimerization which then allows nuclear translocation and ultimately gene transcription/ regulation. As a final gene product, STAT3 regulates the expression of SOCS3, a negative regulator of STAT3 signaling. We propose, however, that upon activation of the JAK2/STAT3 pathway by leptin, in the presence of a secondary signal capable of activating the NF-kB pathway, like LPS, the activated STAT3s inhibit or compete for limited co-factors required for transcriptional activity.

FIGURES

Figure 1

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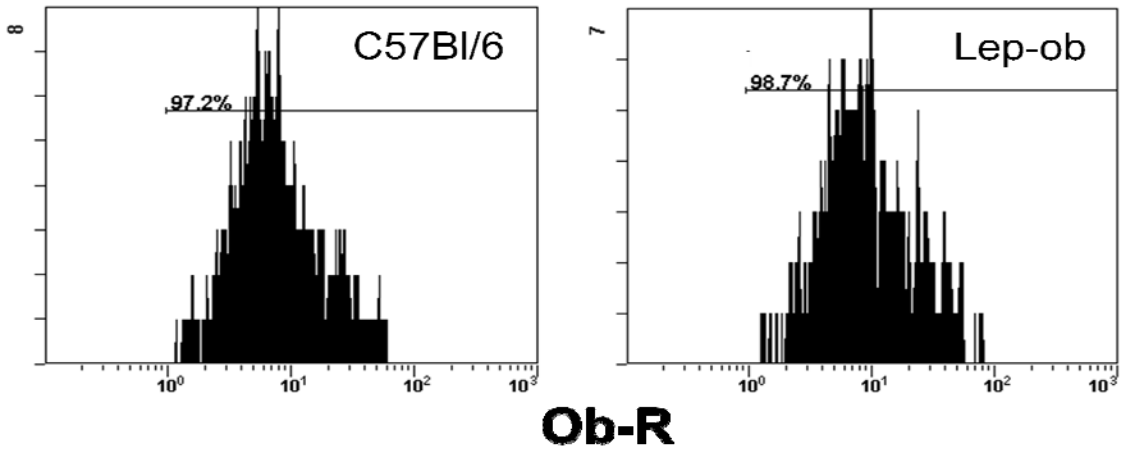

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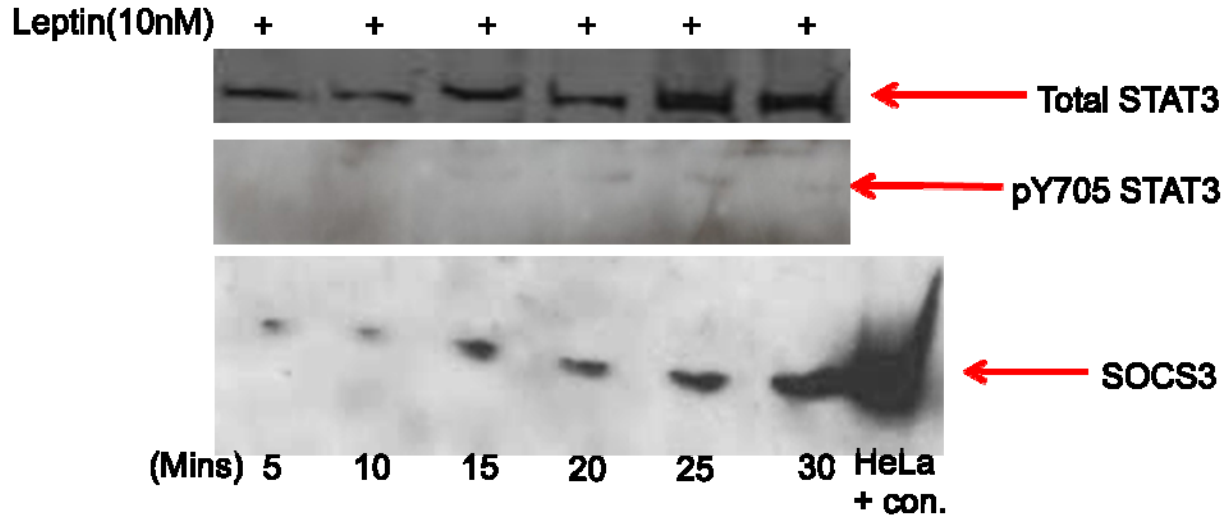

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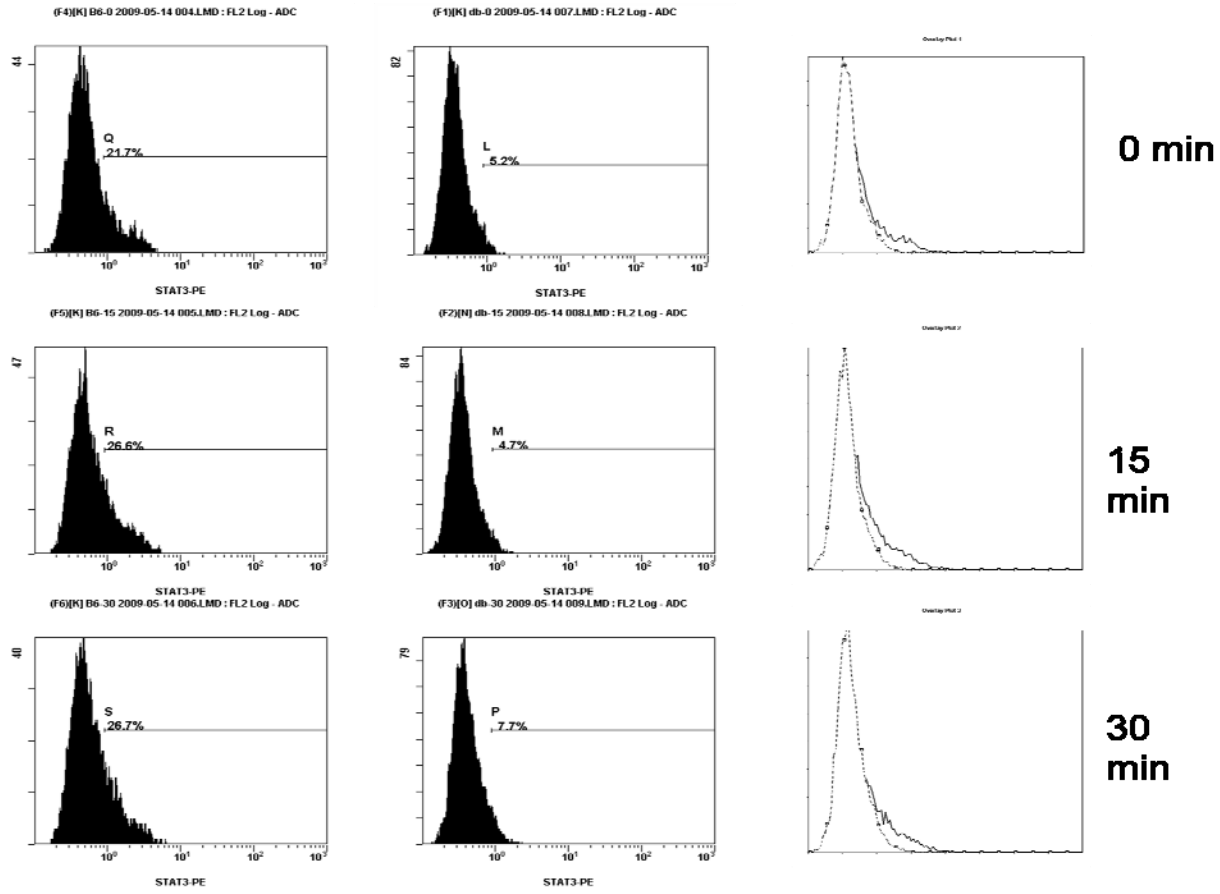
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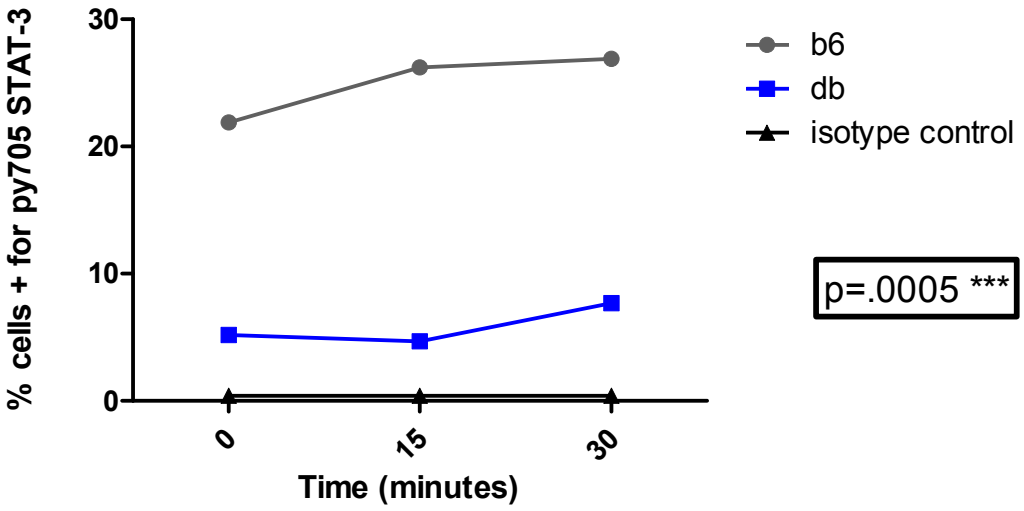
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Figure 2

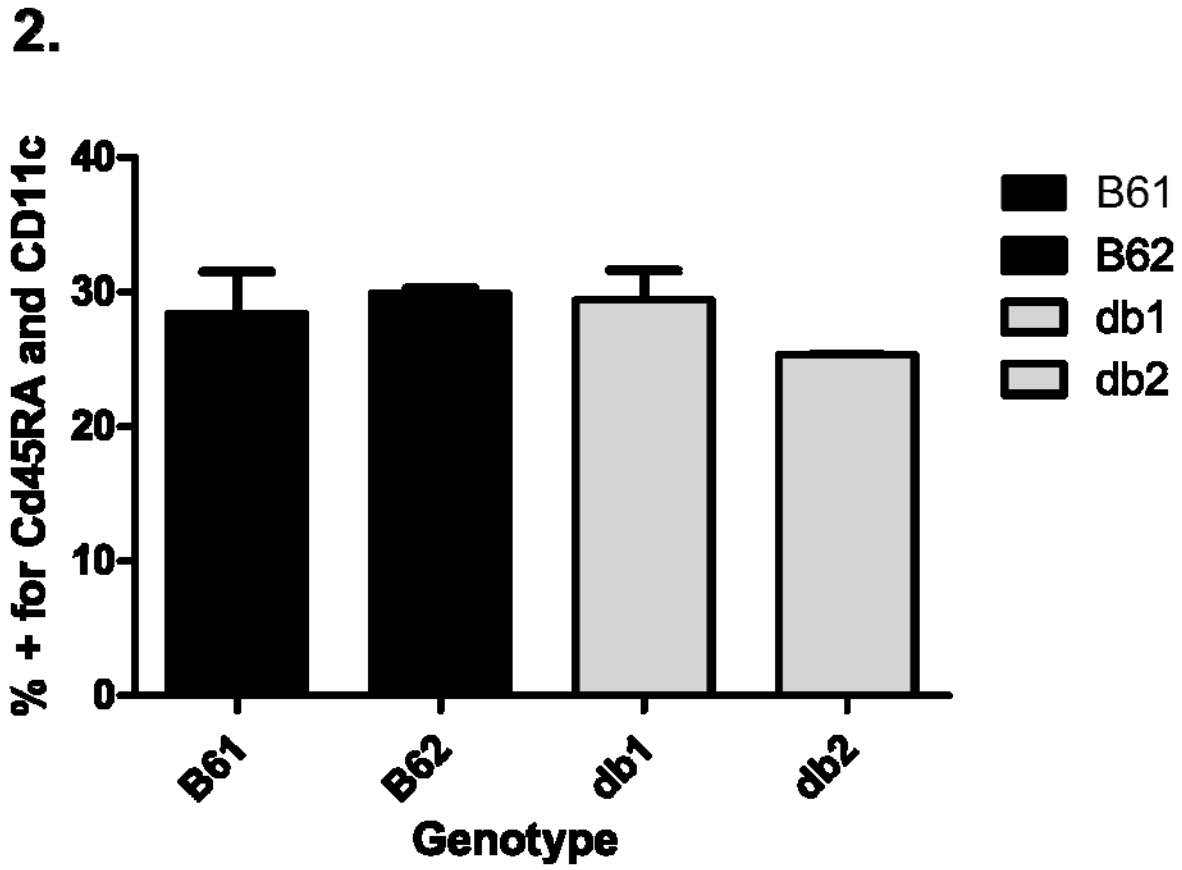

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Figure 3

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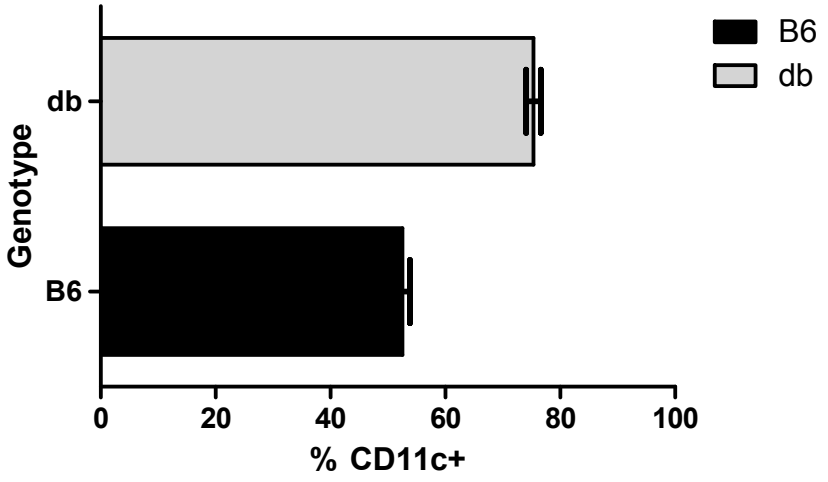

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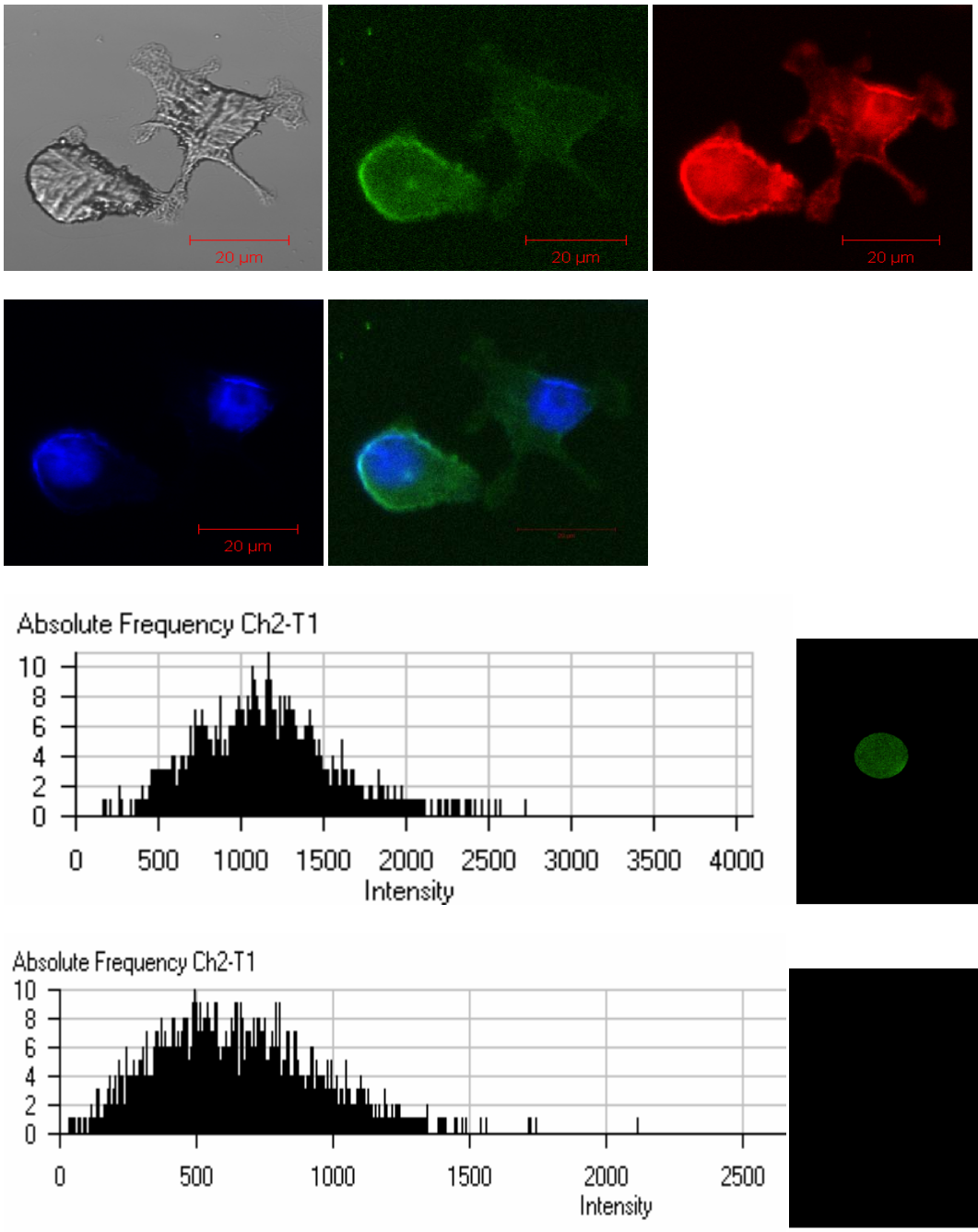
Figure 4

A.



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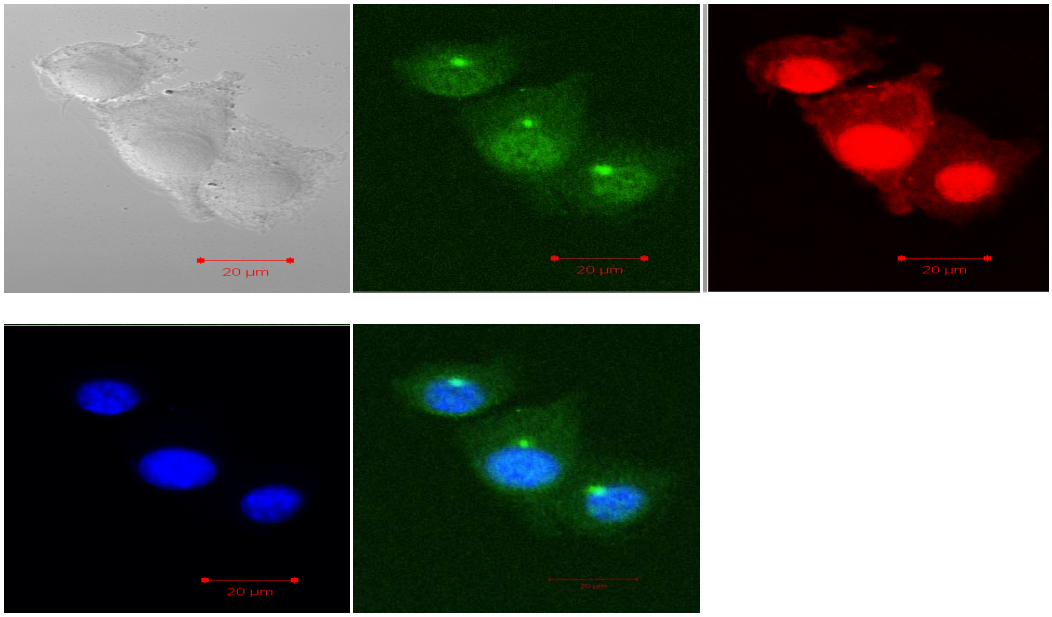
B.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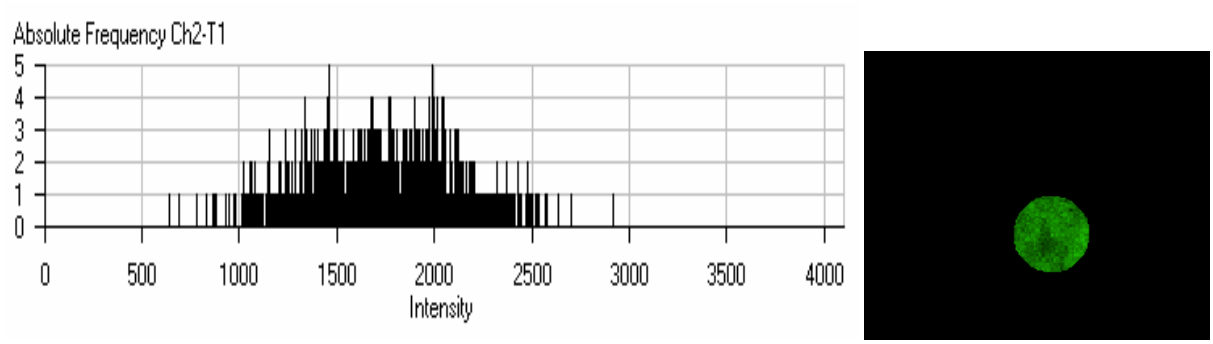
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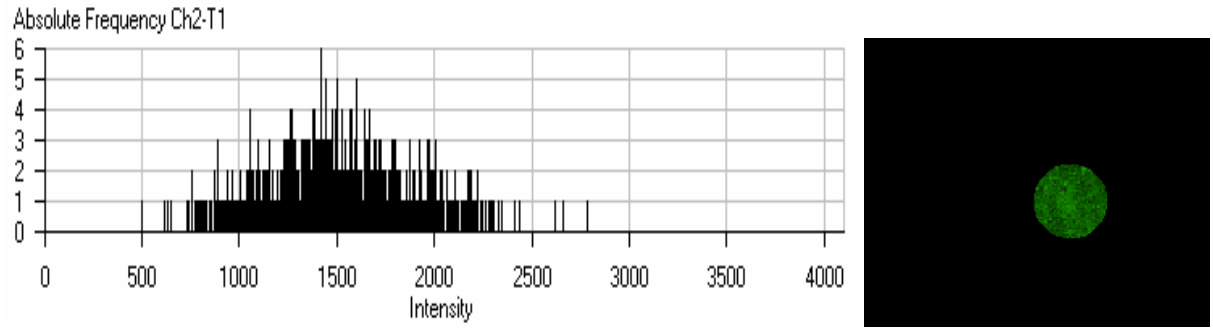
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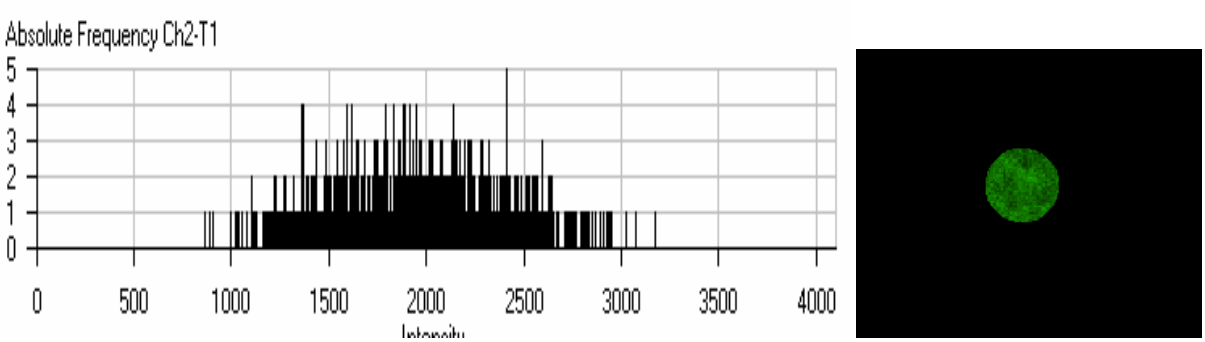
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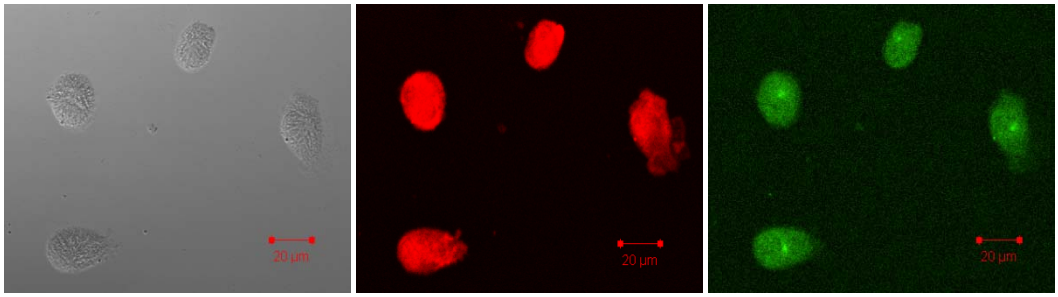
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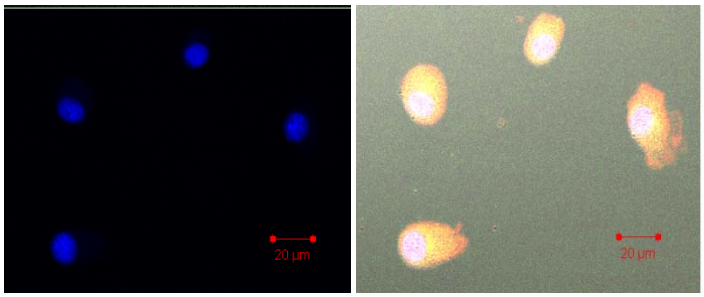
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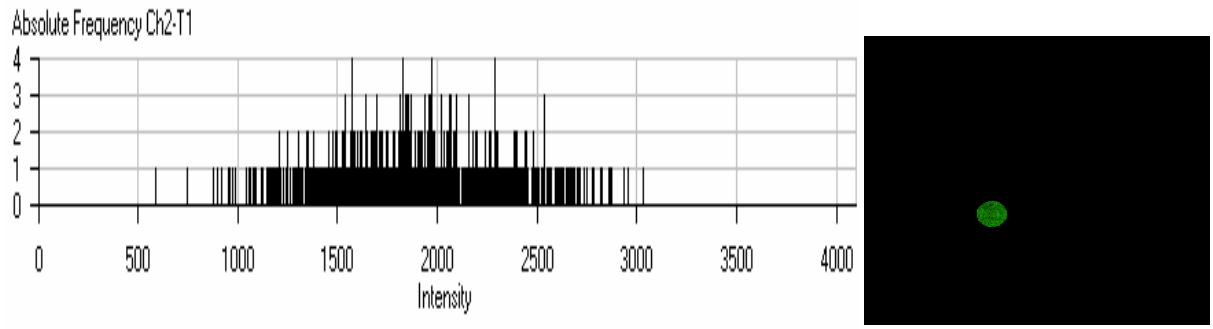
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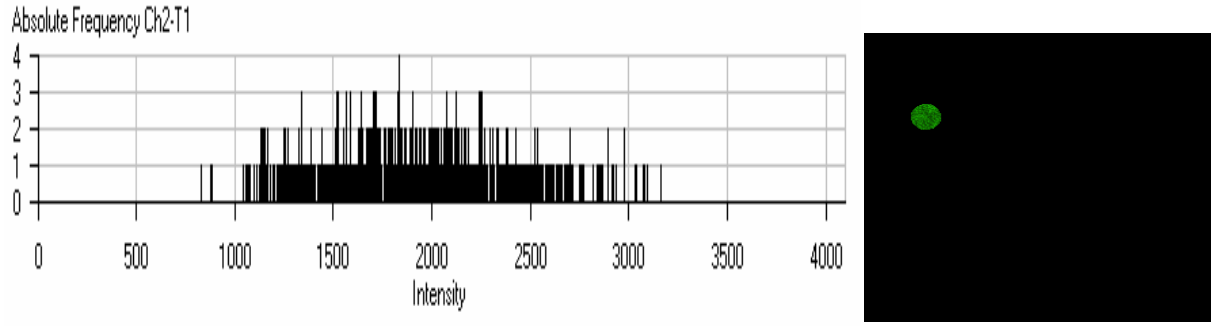
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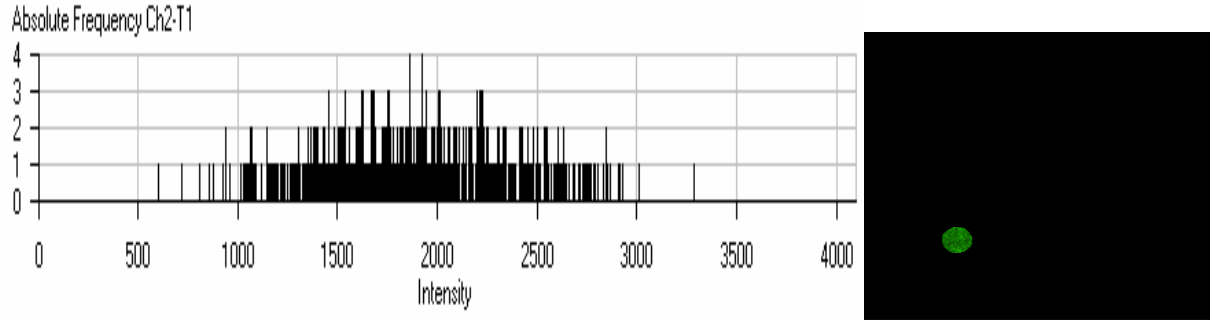
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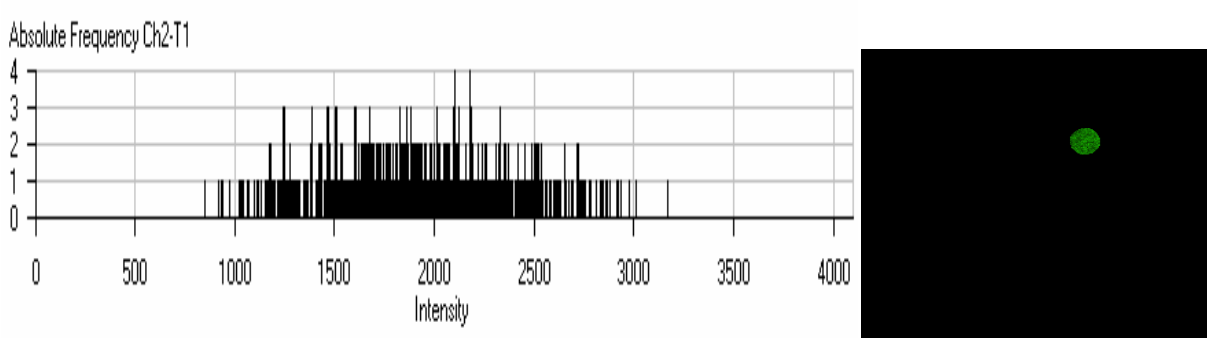
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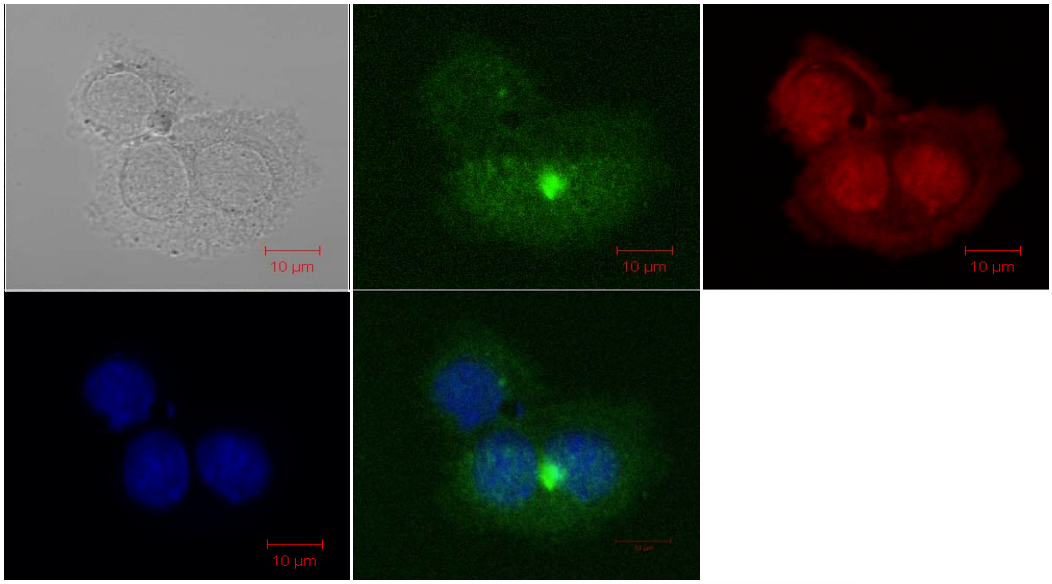
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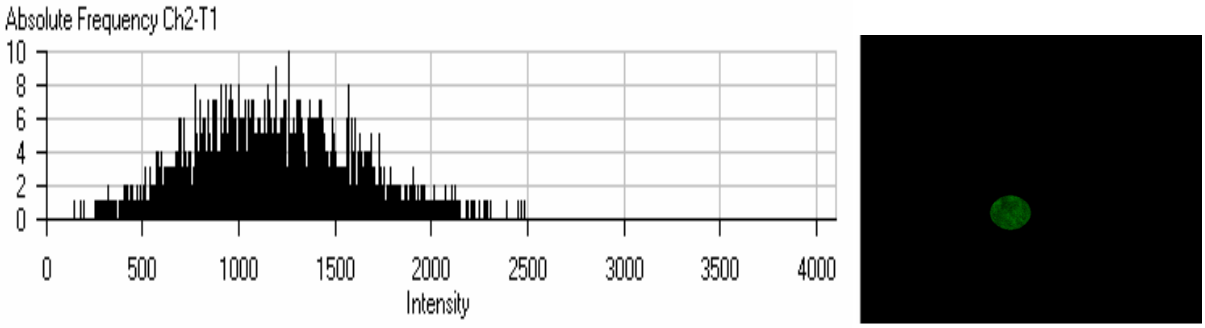
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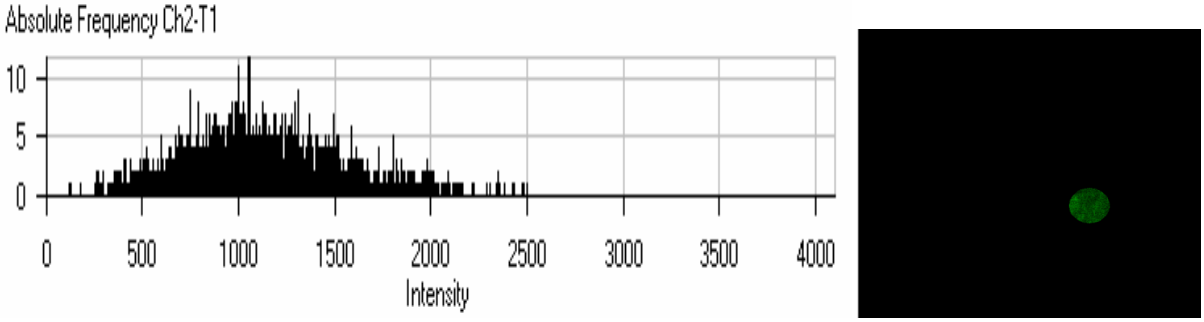
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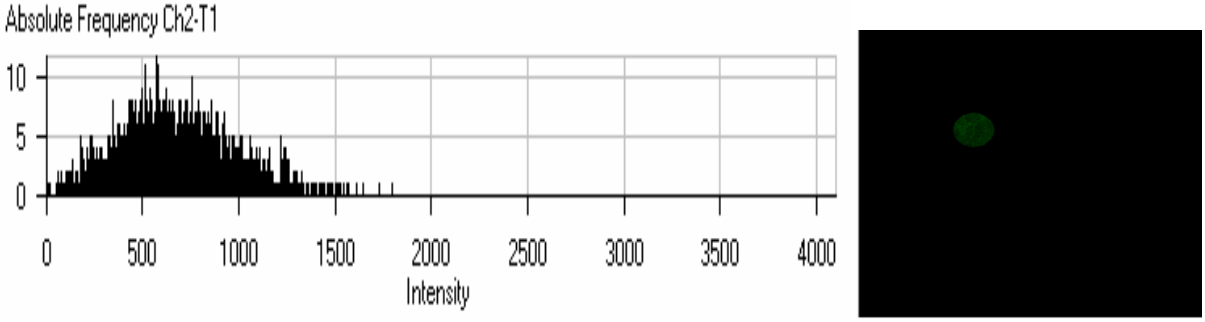
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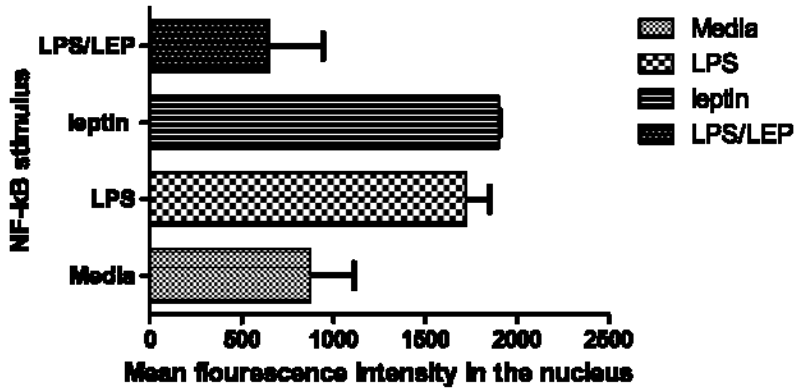

Media vs. Leptin *
LPS vs. L/L *
Leptin vs. L/L **

***= 0.05**
****=0.0023**

Figure 5

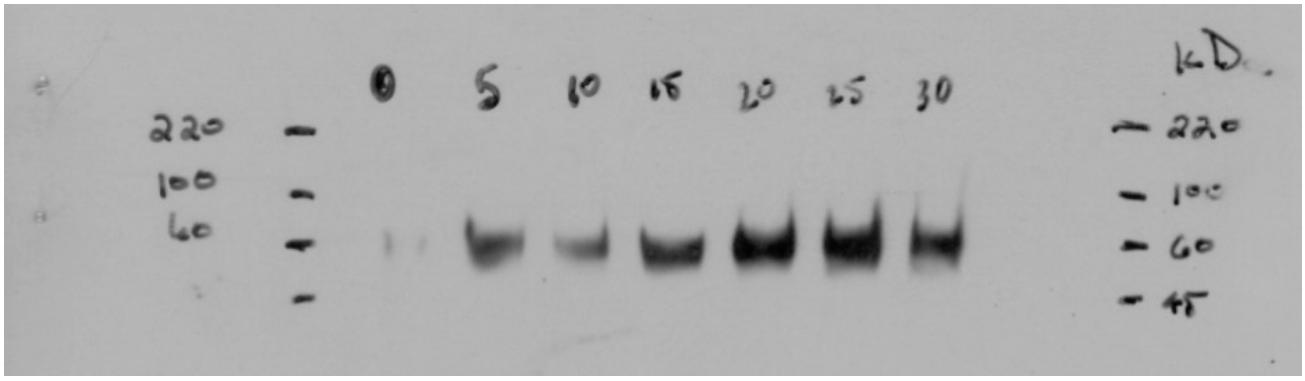

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Figure 6

CHAPTER 4

EXTENDED DISCUSSION

There is an ever growing health concern. The incidence of obesity has increased at an extraordinary rate. It is now estimated that more than 35 % of US residents are either overweight or obese. Alarmingly, children's obesity has also increased from 15% in the late 1970's to 33% in 2003. (CDC). It is now well documented that obesity is accompanied by a plethora of health disparities like hypertension, high cholesterol levels, increased risk for some cancers, as well as Type II Diabetes (CDC) . Given the complications associated with obesity, it is critical to determine whether the constant state of inflammation that accompanies obesity has any adverse effects on the immune system.

Overweight and Obesity are defined by the CDC as-" Overweight and obesity are both labels for ranges of weight that are greater than what is generally considered healthy for a given height. The terms also identify ranges of weight that have been shown to increase the likelihood of certain diseases and other health problems". Upon its identification and cloning in 1994 by Zhang and colleagues (13), leptin quickly became the focal point of a multitude of research laboratories. It is now well accepted that leptin plays a critical role in the regulation of multiple physiological processes, including that of caloric intake as well as the efficient use of peripheral energy stores (11, 13, 18, 109). With-in the past ten years, evidence has emerged that implicates leptin in other physiological functions, as would be expected given the expression of the leptin receptor in a myriad of tissues (24). However, of critical importance to us is the data

that shows that leptin has an effect on cells of the immune system like macrophages and T cells (15, 68, 76, 83). The purpose of this study then was to determine whether the absence of a functional leptin protein would alter dendritic cell function.

This project was designed to determine whether leptin deficiency caused a change in the total number of sDCs, their phenotype, their ability to respond to external stimuli, one that would mimic a pathogen (LPS) and one that would mimic the direct interaction with a T cell (anti CD40), and lastly, their ability to activate T cells, both in vitro as well as in vivo. Our hypothesis stated that we expected to observe differences in DC function and once the differences where know we were going to investigate a possible mechanism.

The first objective was to determine whether leptin deficiency altered the total number of dendritic cells that were present in the spleen of experimental leptin-deficient mice relative to the WT controls. We determined that in the absence of leptin, sDC were present in relatively similar numbers when directly compared to WT controls. Additionally, sDCs from experimental mice also exhibited similar phenotypes as well as equal responses to external stimuli. Furthermore, leptin-deficiency did not affect their ability to acquire and process antigen as we detected no significant differences. Initially our data suggests that in the absence of leptin there seems to be no adverse effects on the phenotype of dendritic cells nor is there is a difference in their ability to acquire and process antigens. We therefore suggest that from an immunogenic/ immunomodulatory

stand point, these sDC would have no problem responding to a pathogenic insult, acquiring and processing antigens and as a result activate T cells.

To further investigate whether our proposal that these sDCs would function normally, we designed and conducted a series of experiments where our goal was to determine whether leptin-deficiency altered DC function. Splenic DCs were isolated, enriched and then irradiated and later placed in a 1:5 ratio with T cells, either T cell hybridomas or primary T cells. T cells activation was then determined as a function of IL-2 production for the hybridomas and T cell proliferation and IFN-γ by primary T cells. Unexpectedly, our data shows that in the absence of leptin sDCs have an increased T cell activation. It is likely that in the presence of leptin sDC are receiving an inhibitory signal and that this inhibitory signal may be interfering with the function of DCs. In order to further substantiate our in vitro observations, we infected sex and age matched heterozygous controls with M. avium. The infections were done via intra-peritoneal injections and on the indicated dates the mice were euthanized and three different tissues were collected, liver, lungs, and spleen. The tissues were minced and the supernatants were plated on *M. avium* selecting agar plates. After 15 days of incubation, *M. avium* CFUs were counted for all three tissues. Our data shows that we could not detect any CFUs in the liver nor could we detect CFUs in the lung. The later results were surprising as *M. avium* is well known for thriving in the microenvironment provided by the lung. However, in line with our initial findings, spleen samples generated from control mice had a significantly higher number of CFUs when directly compared to the experimental group. To determine whether this was a function of T cell activation, flow cytometry was

performed on T cells isolated from the spleen homogenates. The data clearly demonstrates that T cells had a higher detectable level of CD69, a T cell on or off marker, in the experimental group relative to the controls. Our results suggest that in the presence of leptin sDC are inferior T cell activators when compared to sDC from leptin-deficient mice. We therefore propose that leptin is indeed activating a signaling cascade that in turn is providing an inhibitory effect to DCs and their ability to stimulate T cells. Current research has provided data that suggest that activation of one transcription factor has the potential to serve as a negative regulator of other transcription factors (114, 115, 120). These data suggest that activating multiple transcription factors in DCs may lead to an aberrant execution of their function.

To help determine whether leptin induced signaling was indeed the culprit and providing an inhibitory signal, confocal microscopy, as well as immunoprecipitation assays, was utilized. Dendritic cell activation as well as the expression of multiple cell surface markers is regulated by the NF-kB transcription factor (65, 67). We designed and carried out a set of experiments that would allow us to measure NF-kB activation as a function of nuclear translocation using confocal microscopy. In addition we also performed an immunoprecipitation assay to help determine whether leptin induced signaling would modulate the protein-protein interaction between STAT3 and NF-kB. Our data showed that when DCs were treated with leptin or LPS alone, there was a significant increase in the amount of detectable NF-kB in the nucleus relative to the media alone treatment. Our data is in line with previous reports that have demonstrated that leptin is capable of activating a handful of signaling pathways, including NF-kB

((25). In support of our proposal, however, we detected a significant decrease in the amount of NF-kB in the nucleus in DCs treated with LPS and leptin simultaneously. As was the case in our in vitro and in vivo experiments investigating T cell activation, the presence of leptin seems to be inhibiting the entrance of the NF-kB transcription factor into the nucleus when STAT3 activation is occurring concomitantly. These result suggest that in the event that a DC should encounter an activating signal that requires the presence of an active NF-kB protein, the response to said activation would more than likely encounter a road block as the regulation of gene transcription would be inhibited by the presence of leptin induced signaling, a situation that may not be welcome. Next, we wanted to determine whether the decrease in NF-kB nuclear translocation was the result of the activation of STAT3. Upon processing of our samples, we were able to ascertain that STAT3 and NF-kB co-immunoprecipitate. These results confirm that STAT3 is interacting with NF-kB and that said interaction is causing a decrease in NF-kB and leading to decreased DC function. It is interesting to note that leptin treatment alone was capable of inducing NF-kB activation whereas the simultaneous treatment with LPS and leptin resulted in a significant decrease. Whether STAT3 and NF-kB are directly interacting or whether there is a complex forming needs to be further investigated. We have shown thus far that leptin has the potential for providing a negative regulatory signal when present during DC activation; Knowing this will help educate individuals on the need to maintain optimal body weights and body mass indexes. In addition, it may help point out a potential target for STAT3 mediated inhibition in specific cases like undesired inhibition of dendritic cell activation. Furthermore, using leptin like in case of autoimmunity may help dampen the

immunogenicity of DCs. However, the ever present problem of targetting said treatments to DCs only may present a challenge.

CONCLUSIONS

In conclusion, our data demonstrated that when hematopoietic stem cells differentiate into dendritic cells in mice, the absence of leptin is not a requirement as we detected relatively similar total number of sDCs within the spleen. Our data also demonstrates that leptin is not a requirement for DCs to express the canonical DC markers like CD80, 86, 54, 40, and MHCII. Additionally, leptin does not seem to be required for DCs to respond to external stimuli like LPS or anti CD40 nor is it required for antigen acquisition and processing. Unexpectedly, leptin inhibits dendritic cell function, both in vitro and in vivo as was determined in mixed lymphocyte reactions, antigen specific reactions, as well as in vivo. We therefore suspected that leptin was exerting a negative effect on dendritic cells. Upon further investigation, we conclude that the presence of leptin in DC cultures renders DC inferior T cell activators. This phenomenon was determined by confocal microscopy and our data showed a decrease in NF-kB nuclear localization relative to controls. In addition we determined that the observable fact was due to interactions between activated STAT3 and NF-kB. Our findings are in line with previous reports that indicate that transcription factors negatively regulate each other and that such regulation is the direct consequence of competition for reduced cofactors required for transcriptional activity. We therefore conclude that in an obese state, in the presence of a pathogenic microbe or immunogenic insult, DC mediated T cell activation will be aberrant because serum leptin levels during these conditions are reported to be as high as 300ng/ml, a concentration that is considerably higher than those used in these studies. So, it is possible that in the presence of DC activating signals, like LPS, CD40L interaction, or other cytokine signaling which require NF-kB activation, leptin

induced STAT3 activation will provide a negative feedback signal to dendritic cells and thus block or retard the immune response as a direct result of NF-kB inhibition. Therefore, careful consideration must be taken when attempting to utilize leptin as an adjuvant in conjunction with immuno therapies as there is a potential for inhibitory affects on cells of the immune system.

FUTURE DIRECTIONS

Our initial results have demonstrated that sDC isolated from leptin deficient mice show no significant differences in their phenotype or ability to acquire and process antigen (Figure 2, 3, and 4 Chapter 2). However, when presenting antigen to T cells, whether a cell line or primary cultures, sDCs from leptin deficient mice were superior T cell activators, a phenotype that was consistent even in an in vivo setting (Figure 5 and 6 chapter 2). Furthermore, in an attempt to elucidate a possible mechanism responsible for our observations, we determined that leptin induced signaling resulted in decreased NF-kB nuclear localization and that this phenomenon was very like the results of protein-protein interaction between the STAT3 and NF-kB, as determined by immunoprecipitation assays (Figure 5 chapter 4 and 5).

Our data clearly demonstrate the role of leptin on dendritic cell function; we therefore propose the possible future directions. We suggest using a diet induced model. This will allow for a representative model of human obesity as well as one where high caloric intake is the culprit. In addition to being an obesity model, mice being fed this high caloric diet serve as a Type II diabetes model as well. It is expected that by using this model we will be able to confirm our original findings and observe that in the presence of excess leptin, DC function will be diminished. Additionally, by utilizing this model, leptin's role can be clearly determined as a high concentration of leptin is expected to be in circulation. Furthermore, data may be generated as to the possible mechanism responsible for leptin resistance as one has not been clearly established.

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APPENDIX

A Small Library of Naphthoquinones Induce Apoptosis of Murine Fibroblasts Using Multiple Mechanisms

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ABSTRACT

The synthesis of compound libraries that generate reactive oxygen species (ROS) and their concurrent assessment as selective reagents for probing and modulating biological function remains an undeveloped area in Chemical Biology. We report the results of our efforts towards the synthesis of substituted 1,4-napthoquinone libraries and their utilization as reagents for the induction of apoptosis. Using a murine fibroblast cell line (L929), twelve synthetic naphthoquinone compounds were screened for cytotoxicity. The compounds were first assessed for their ability to inhibit cell survival. The compounds presented with three categories of cytotoxicity: no toxicity (25%), intermediate toxicity (50%), and high toxicity (25%). Cytotoxic compounds were further studied for the ability to induce cellular death via an apoptotic mechanism. Flow cytometric analysis for Annexin-V-positive staining and assessment of active caspase 3 demonstrated that the toxic compounds induced apoptosis. However, both cytotoxic and non-cytotoxic compounds promoted mitochondrial depolarization and one cytotoxic compound did not promote mitochondrial depolarization, highlighting the varied cytotoxicity mechanisms. Due to the potential of the naphthoquinones to promote redox reactions, representative compounds in each category were assessed for the induction of reactive oxygen species (ROS). Colorimetric assays for ROS production definitively demonstrated an increase in intracellular ROS over time upon exposure to the cytotoxic naphthoquinones. Moreover, the cells were only partially protected from apoptosis upon treatment with the cytotoxic naphthoquinones when catalase, superoxide dismutase, or ascorbic acid was present. Thus, of the molecules assayed, nine were cytotoxic to murine fibroblasts and induced an apoptotic death. However, the mechanism of action may or may not involve compromising mitochondrial membrane potential and is partially due to the production of ROS. Therefore, naphthoquinone activity is not

uniform even upon a single eukaryotic cell line suggesting that any library of biologically active naphthoquinone compounds requires thorough study.

INTRODUCTION

Apoptosis is a complex series of changes that occur to a cell as it systematically destroys itself. It was first described over 30 years ago as a mechanism of cell death responsible for the removal of cells within living tissue [9443]. We now know that this process is responsible for a number of physiological processes such as cell death in development, normal tissue turnover, and immune homeostatis [9444]. It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia, or viral infection [9444]. The converse is that apoptosis is also responsible for the detrimental process by which cells die prematurely causing many chronic degenerative processes such as neurodegenerative diseases and heart failure []. The role of apoptosis in both normal physiological processes as well as in patho-physiological processes has been supported by the demonstration of 'apoptotic' and 'anti-apoptotic' signaling pathways []. Together, this suggests that cellular survival and/or death may be promoted or inhibited by exogenously manipulating apoptotic pathways.

Evidence has demonstrated that quinones are particularly effective at inducing apoptosis [] and provide a rich source of unique reagents that can be exploited for the purpose of chemotherapy. Quinones are aromatic compounds naturally present in bacteria and eukaryotes and are often involved in the biochemistry of energy production and serve as vital links in electron transport in the form of ubiquinones []. The biological activity is related to the acceptance of one and/or two electrons to form the corresponding radical anion or dianion species [9527]. Quinones are also natural defensive products made by plants and have been employed as anti-fungal agents, broad-spectrum anti-bacterials, and as anti-malarial drugs [9529,9530,9532]. Of note, are the anti-malarial naphthoquiones []. Moreover, quinones have been shown to be effective anti-cancer agents [9531,9528] and form one of the largest classes of

cytotoxins used as anti-cancer drugs []. The clinically used quinones are extensively substituted anthroquinones or *p*-benzoquinones or naphthoquinones with reactive or heterocyclic groups []. Here, twelve different 1,4-naphthoquinones were screened against the murine fibroblast cell line, L929, for the induction of cytotoxicity. The twelve compounds were synthesized through novel organic chemistry schemes described elsewhere [] and served as a platform library for future compound design. The naphthoquinones presented with varied abilities to induce cell death, which was accomplished through the activation of apoptosis.

RESULTS

Compound effects on cellular viability

A total of twelve compounds were screened (Figure 1 and Table 1) for effects on viability of a murine fibroblast cell line (L929). To determine if the compounds were cytotoxic, the cells were cultured in the presence of ten two-fold dilutions of each compound and were measured for viability. Cytotoxicity was measured as an inverse function of cell survival using a colorimetric viability assay. As indicated in Figure 2, the effects of the different compounds were placed into three groups with respect to cytotoxicity: (1) no cytotoxicity (no observed difference from DMSO vehicle control) (Fig. 2A); (2) low to intermediate cytotoxicity (the compound inhibited cell survival in a dose-responsive manner) (Fig. 2B and 2C); and (3) high cytotoxicity (> 50% inhibition of cell survival at the lowest concentration examined) (Fig. 2D). The concentration of each compound that induced 50% inhibition of survival (IC_{50}) is listed in Table 1. Of note are the compounds used as controls: compound **5** (plumbagin) and **7** (juglone), both which are naturally occurring. Plumbagin is found in the roots of the Plumbago plant; juglone is found in the leaves, roots and bark of plants in the Juglandaceae family, particularly the black walnut [].

Both compounds have been found to be quite cytotoxic in a number of model systems [], including the L929 system, verifying that the new synthetic scheme being used to produce the naphthoquinones generates accurately cytotoxic compounds.

Compound effect on cellular morphology

To assess the effect of our small library of compounds on the morphology of the cells, the fibroblasts were cultured in 4-chamber well slides in the presence of each compound at the IC_{50}. Untreated and DMSO-treated cells were used as controls as previously described (DMSO was diluted similar to the DMSO-suspended compound with the highest IC_{50}). Following a 24 h incubation, the slides were processed (as described in Materials and Methods) to assess cellular morphology by light microscopy as shown in Figure 3. Untreated cells presented with two general morphologies: adherent-fibrous and semi-adherent rounded, which is typical of this cell line in different phases of the cell cycle (Fig. 3A). Similar morphologies were seen for cells treated with the vehicle control (Fig. 3B). In contrast, hydrogen peroxide (H_2O_2) treated cells were rounded, granular, and vesiculated, which is typical of dying cells (Fig. 3F). At a single concentration (60 μg/ml), the compounds either had no effect on the cells, as depicted by compound **9** (Fig. 3E), or were detrimental to cell survival, as depicted by compounds **2** and **5** (plumbagin) (Fig. 3C and 3D), further demonstrating that members of our small compound library were cytotoxic.

Cytotoxic compounds induce apoptosis

We assessed the mechanism by which the cytotoxic compounds (intermediate and high) inhibited cell survival. Cells die by two different mechanisms: necrosis or apoptosis []. Necrosis

is an uncontrolled cell death, characterized by cell membrane lysis and release of the intracellular contents, leading to an inflammatory response, with edema and damage to the surrounding cells. Apoptosis is a controlled cell death that keeps the intracellular content of the dying cell sequestered. Upon induction of apoptosis, the cell shrinks, loses contact with neighboring cells or surrounding matrix and the plasma membrane shows a bubbled appearance; small membrane bound bodies break off containing intracellular material, which are quickly removed by phagocytes or by neighboring cells.

To distinguish between these two mechanisms of cell death upon treatment with our naphthoquinone compounds, we employed Annexin V staining. The L929 cells were treated with the IC_{50} for the intermediate cytotoxic compounds or with the lowest concentration tested for the highly cytotoxic compounds and were then stained with Annexin V and assessed by flow cytometry (Figure 4A). Very little necrosis was induced by any of the compounds (<10% above background, data not shown). In contrast, all the cytotoxic compounds (**2**, **3**, **4**, **5**, **6**, 7, **9**, **10**, and **11**) induced a high level of apoptosis above that induced by medium alone.

In parallel experiments, the cells were also assessed for the induction of active caspase 3. Apoptosis is a tightly regulated process that is mediated through caspases, which are a family of cysteine proteases. They destroy cells by targeting a number of proteins for degradation, resulting in a breakdown of cellular structure and function that culminates in cell death. The final caspase activated in the apoptotic pathway is caspase 3 []. Using compounds **2**, **5**, and **9** as representative compounds for the three groups of cytotoxic compounds (intermediate, high, and no cytotoxicity, respectively), assessment of caspase 3 also demonstrated that the cytotoxic compounds promoted apoptosis (Fig. 3B). Compound **2** and **5** induced active caspase 3

equivalent to that of the H_2O_2 positive control. Thus, the cytotoxic naphthoquinone compounds primarily inhibit cell survival by inducing apoptosis.

Not all cytotoxic compounds activated the intrinsic apoptotic pathway

Although a number of stimuli can trigger the process of apoptosis, there are two major signaling pathways of apoptosis; the death receptor pathway (extrinsic) and the death receptor-independent or mitochondrial pathway (intrinsic). To determine if the cytotoxic compounds were promoting apoptosis through the mitochondrial pathway, the DePsipher™ Kit from Trevigen was used. DePsipher is a unique cationic dye that indicates the loss of the mitochondrial potential. The dye readily enters cells and fluoresces bright red in its multimeric form within healthy mitochondria. If the mitochondrial membrane potential were to collapse, the DePsipher reagent cannot accumulate within the mitochondria. In these cells, the reagent remains in the cytoplasm as a green fluorescent monomeric form. As shown in Figure 5A, all the cytotoxic compounds, with the exclusion of compound **5**, promoted mitochondrial depolarization. The data suggests that the majority of the members of our small library of naphthoquinone compounds induce apoptosis in L929 cells by activating the intrinsic apoptotic pathway. Compound **5** (plumbagin) is of particular interest because it is one of the most cytotoxic compounds (Fig. 2) yet its mode of action is different than any of the other cytotoxic compounds tested as is demonstrated by the depolarization profile in Figure 5B.

Cytotoxic compounds promote production of reactive oxygen species

The mechanism of cytotoxicity of naphthoquinones is still under investigation but two theories dominate the literature []. Quinone compounds are proposed to exhibit one or both of

these two mechanisms. The first mechanism is the promotion of reduction-oxidation (redox) reactions. The compounds are proposed to catalytically cycle and generate oxidative radicals such as hydrogen peroxide and superoxide, which then damage the cell. The second mechanism, alkylation, is when the quinones are activated inside the cells and become covalently attached to proteins, DNA, or other targets. L929 cells were treated with the representative compounds (**2**, **5**, and **9**) and were assessed by a fluorometric assay for the formation of reactive oxygen species (ROS) (Figure 6A). The vehicle control (DMSO-treated) and compound **9**-treated cells induced ROS equal to that generated in non-treated cells (medium). The intermediate cytotoxic compound (**2**) and the highly cytotoxic compound (**5**) both induced levels of ROS equal to that induced by H_2O_2 (although not at the same rate).

To further assess the formation of oxidative radicals upon treatment with the cytotoxic naphthoquinone compounds, the compound-treated cells were concomitantly treated with the antioxidant proteins superoxide dismutase (SOD), catalase, or a combination of the two. SOD converts superoxide to hydrogen peroxide and catalase converts hydrogen peroxide to water and free oxygen, thus protecting the cells from potential ROS damage. The cells were treated with the three representative compounds (**2**, **5**, and **9**) in the absence or presence of the oxidative radical inhibitors. Following a 48 h incubation, the cells were assessed for viability.

As shown in Figure 6B, the addition of catalase partially protected compound **2**-treated cells, enhancing cell survival in the presence of the compound. The addition of SOD did not protect cells against compound **2** and the combination of catalase and SOD did not protect the cells against compound **2** beyond that provided by catalase alone. The addition of catalase, SOD, or a combination of the two also did not protect cells treated with the highly cytotoxic compound **5**. In parallel experiments, the cells to be treated with the compounds were first pre-

loaded for 4 h with ascorbic acid. Ascorbic acid is a small molecule which sacrificially reacts with and eliminates oxidative radicals, and was used as a non-protein control for protection against ROS. As shown in Figure 6B, the cells treated with either compound **2** or **5** were partially protected from apoptosis when pre-treated with ascorbic acid. Thus, the data demonstrates that the cytotoxic naphthoquinone compounds induce oxidative radicals (Figure 6A) however, as shown in Figure 6B, this is unlikely the only mechanism by which the compounds induce cell death.

DISCUSSION AND CONCLUSION

Twelve naphthoquinone compounds produced by novel organic synthetic schemes were screened for cytotoxicity against a murine fibroblast cell line; two of the compounds (plumbagin and juglone) occur naturally and were used as internal controls for synthesis. The compounds presented with three categories of cytotoxicity: no cytotoxicity, low to intermediate cytotoxicity, and high cytotoxicity. The cytotoxic compounds were analyzed further and were determined to predominantly induce apoptosis via an intrinsic (mitochondrial) apoptotic pathway that was partially promoted by the production of oxidative free radicals. However, additional mechanisms appear to play a role in the mode by which these compounds kill cells. All of the cytotoxic compounds induced reactive oxygen species but the treated cells were only partially protected by the anti-apoptotic agents SOD and/or catalase or ascorbic acid.

The additional cytotoxic mechanisms induced by our compound library is particularly underscored by compound **5** (plumbagin), which was shown to promote the generation of ROS but does not activate mitochondrial depolarization (nor are its actions protected by SOD and/or catalase or ascorbic acid). This is in contrast to what has been seen in other mammalian cell

model systems [9562], where changes in mitochondrial membrane potential were observed. Thus, plumbagin is unique in our model system in being highly cytotoxic and induces apoptosis (evidenced by the activation of caspase 3) yet does not promote mitochondrial depolarization, which suggests a different mechanism of action from the other cytotoxic quinones.

Thus, our small library of compounds has provided a group of synthetically generated naphthoquinones that mediate cytotoxicity in a eukaryotic system. The mechanism of action of this group of compounds is mediated primarily through ROS production but is accompanied by additional modes of action. Of significance is that a number of the screened compounds (9 out of 12) possess pro-apoptotic activity as determined by a number of assays in a well-defined eukaryotic cell system, offering a number of reagents for future study and highlighting the potential interest of examining a larger synthetic naphthoquinone compound library created by a novel synthetic scheme.

EXPERIMENTAL APPROACHES

Compounds

2,3-disubstituted naphthoquinones were synthesized on solid support utilizing the Dotz reaction with solid supported Fischer carbine complexes as recently described []. Compounds were dissolved in DMSO at 20 mg/ml and stored frozen at -20^{o}C.

Cell culture

The murine fibroblast cell line, L929, was a generous gift from Kenneth S. K. Tung (Univ. of Virginia, Charlottesville, VA). The cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 Units/ml Penicillin, 100

μg/ml Streptomycin, and 1X Glutamax (all cell culture reagents were purchased from Invitrogen Corp., Grand Island, NY). The cells were grown at 37° C, 5% CO_2 and were used for experiments when in expansion phase of culture (at approximately 70% confluency).

MTT viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assays were carried out as per manufacturers' instructions (Sigma). The L929 cells were plated at 50,000 cells per well in a 96-well flat-bottom plate (Becton-Dickinson Labware, Franklin Lakes, NJ). The cells were cultured with decreasing concentrations of the compounds (in duplicate), beginning at 0.50 mg/ml and decreasing by half over eleven additional dilutions. As controls, cells remained untreated (media alone) or were treated with equivalent dilutions of dimethylsulfoxide (DMSO, vehicle control) (Sigma). Following a 48 h incubation, the MTT reagent was added at 10% of the total volume per well. Following a 4 h incubation, the formed crystals were solubilized by removing approximately ¾ of the supernatant and adding 50 μl of solvent (10% Triton X and 1% 12 N HCl in isopropanol) (Sigma). All wells were vigorously pipetted (without forming bubbles) to help dissolve the crystals. The plates were then read in a 96-well plate spectrophotometer at 570 nm.

Microscopy of compound-treated cells

Cells were grown at a density of 1 x 10^5 cells per well in a four-well chambered slide (Nalgene Nunc,) in the presence of medium alone, the indicated compound (IC_{50}), DMSO, or H_2O_2. The cells were cultured for 24 h at 37^{o}C, 5 % CO_2. After the 24 h incubation, the cells were observed at 40X using a Zeiss Axiovert 200 microscope. Digital images were captured

using the AxioCam HR digital camera and were processed with Axiovision Software, Version 4.1.

Flow cytometry assessment of apoptosis

L929 cells (500,000 cells/well) were cultured in 12-well plates for 48 h in the presence of medium, DMSO, or compound (IC_{50}). The cells were then washed in FACS buffer (phosphate buffered saline + 2% fetal bovine serum), resuspended in 50 μl of buffer and then incubated with 30% normal mouse serum (Sigma) to prevent non-specific staining, for 15 min. at room temperature. The cells were then treated with 5 μl of FITC-conjugated annexin V (1 mg/ml stock) and 2 μl of propidium iodide (5 mg/ml stock). The cells were then mixed gently and incubated at room temperature for 15 minutes in the dark followed by an analysis by flow cytometry within the hour. 10,000 events per sample were collected and analyzed by EXPO32 software.

Detection of active caspase-3

The assay was performed as per the manufacturers' instructions (BioSource International, Camarillo, CA). The L929 cells were plated at 3 x 10^6 cells per well in 6-well plates. The cells were then treated with the indicated compounds for 24 h. After treatment with the compounds, the cells were centrifuged, washed twice with 1X PBS, were re-suspended in 50 μL of chilled Cell Lysis Buffer and were incubated for 10 min. on ice. The samples were then centrifuged and the cytosolic extracts were then transferred to 96-well flat-bottom plates. Reaction buffer (5 μL of 1.0 M DTT to 500 μL of 2X Reaction buffer), made just prior to use, and was added to each well. DEVD-pNA substrate (5 μL) was then added to each well and the samples were incubated

in the dark for an additional 2 h. The plates were then read on a spectrophotometer at a wavelength of 405 nm.

Detection of mitochondrial membrane depolarization

L929 cells (5 x 10^4 cells/well) were cultured in 12-well plates for 48 h in the presence of medium, DMSO, camptothecin (4 μM, positive control) or compound (IC_{50}). The cells were then washed in FACS buffer resuspended in 100 μl of buffer. DePsipher (5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (5 μl per sample) (Trevigen, Inc., Gaithersburg, MD) was then added. The cells were incubated at room temp., in the dark for a minimum of 15 min. The cells were assayed within the hour by flow cytometry. 10,000 events per sample were collected and analyzed by EXPO32 software.

Detection of Reactive Oxygen Species (ROS) by DHCF-DA

Cells were plated at 50,000 cells per well in 96-well plates, protected and unprotected by 1 mM ascorbic acid. Following a 4 h incubation period, the cells were then treated with the indicated compounds at the IC_{50} or with H_2O_2 as a positive control. Twenty-four hours later, the plates were centrifuged and the supernatants were collected. The adherent cells were treated with 20 μL of 0.25 % trypsin (Invitrogen) for 2 min. The cells were then removed and transferred to black-walled 96-well plates. Dichlorofluorescein-Diacetate (10 μM) (Invitrogen - Molecular Probes), was then added to each well. The plates were then immediately read on a fluorescent plate reader using excitation wavelength of 485 nm and emission wavelength of 538 nm. Fluorescence was determined every 10 min over a 320 min. period.

Ascorbic acid protection assay

Prior to the viability assay, the cells were treated for 24 hrs with 1 mM ascorbic acid (Sigma). The cells were then thoroughly washed with Hank's Balanced Salt Solution (HBSS (Invitrogen) and the MTT viability assay was conducted as described above.

Catalase and super oxide dismutase (SOD) protection assay

L929 cells were plated at 50,000 cells/well in a 96-well flat-bottom plate. Catalase (1 mg/ml), SOD (0.5 mg/ml), or combinations of the two were added to the cells. The cells were also treated with the indicated compounds at a concentration that inhibited 50% of cellular viability or with media or DMSO as controls. Following a 48 h incubation period, cellular viability was assessed as described above (MTT assay).

FIGURE LEGENDS

Figure 1. **Structures of quinone library**.

All compound structures are based on the core structure (upper left), except for **1**, **8**, and **10**. Compound **9** is vitamin K1.

Figure 2. **Inhibition of cellular viability by naphthoquinone compounds.**

L929 cells were grown in 96-well plates for 48 hrs in the absence or presence of decreasing concentrations of vehicle control (DMSO) or decreasing concentrations of the indicated compound. The cells were then assessed for viability by an MTT assay. The compounds presented with no toxicity (Group I), low to intermediate toxicity (Group II), and high toxicity (Group III) (as described in the text). Compounds **2**, **5**, **8**, and **11** are representative examples of each group. The data is presented as the mean ± SEM of triplicate wells and is one of three representative experiments.

Figure 3. **Induction of abnormal cellular morphology by naphthoquinone compounds**.

The cellular morphology of L929 cells, grown in chamber well slides for 24 hrs in the presence of media alone (A), vehicle control (DMSO) (B), compound **2** (C), **5** (D), **9** (E), or H_2O_2 (F), was assessed by light microscopy (40 X). The cells were treated with 0.06 mg/ml of each compound, an equivalent volume of DMSO (vehicle control), or 600 □M H_2O_2 (positive control).

Figure 4. **Induction of apoptosis by napthoquinone compounds**.

L929 cells were grown in 12-well plates for 48 hrs in the presence of media, vehicle control (DMSO), or the indicated compound (concentration = IC_{50}). The cells were harvested, washed, stained with Annexin V-FITC and were assessed by flow cytometry within 30 minutes of staining (A). Percentage of apoptotic non-treated cells (background) was approximately 10 – 20%. In parallel experiments, cells grown in the presence of media, vehicle control (DMSO) or the indicated compound were assessed for the presence of active caspase 3 using a colorimetric assay kit (BioSource) (B). Compounds **2**, **5**, and **9** were used as representative compounds with intermediate, high, or no toxicity, respectively. Data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

Figure 5. **Compound-induced apoptosis is primarily mediated through an intrinsic pathway**

L929 cells were grown in 12-well plates for 48 hrs in the presence of media (-), vehicle control (V), or the indicated compound (concentration = IC_{50}). Treatment with campothecin (4 □M) was used as a positive control (+). The cells were harvested, washed, stained with DePsipher (assesses mitochondrial depolarization) and were assessed by flow cytometry within 30 minutes of staining. Data is presented as the mean ± SEM of duplicate wells (A) or as representative dot plots (B). The data is one of two representative experiments (experiment one is represented in (A) and experiment two is represented in (B)).

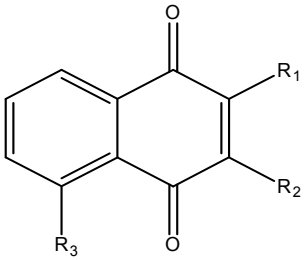
Figure 6. **Compound-induced apoptosis is mediated by ROS formation.**

L929 cells were grown in 96-well plates in the presence of media, vehicle control (DMSO), or the indicated compound (concentration = IC_{50}). The cells incubated with the compounds were also treated with catalase (1 mg/ml), superoxide dismutase (SOD, 0.5 mg/ml), or a combination of the two. A separate group of cells were pre-treated for four hours with 10 □M ascorbic acid, were washed and were then grown in the presence of media, vehicle control (DMSO), or the indicated compound. At 48 hrs, all groups of cells were assessed for viability (A). The data is presented as the mean ± SEM of five replicate wells and is one of three representative experiments. Cells grown in 96-well plates were also assayed at 24 h for the formation of intracellular reactive oxygen species (ROS) utilizing a fluorimetric assay (Molecular Probes) (B). The data is presented as the mean ± SEM of triplicate wells and is one of three representative experiments.

FIGURES

Figure 1

core quinone structure



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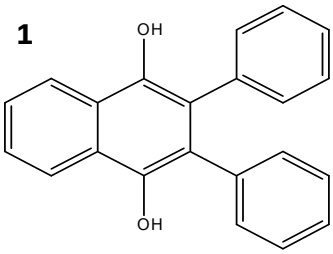

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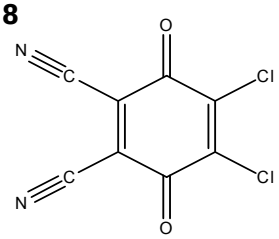

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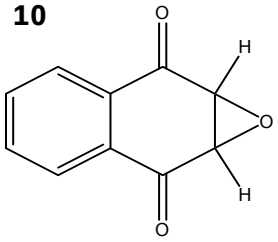


Compound	Position R_1	Position R_2	Position R_3
2	Ph	Ph	H
3	CH_3	CH_3	H
4	OH	$CH_2CH=C(CH_3)_2$	H
5	CH_3	H	OH
6	H	H	H

7	H	H	OH
11	$CH_2CH_2CH_3$	H	H
12	CH_2CH_3	CH_3	H

Figure 3

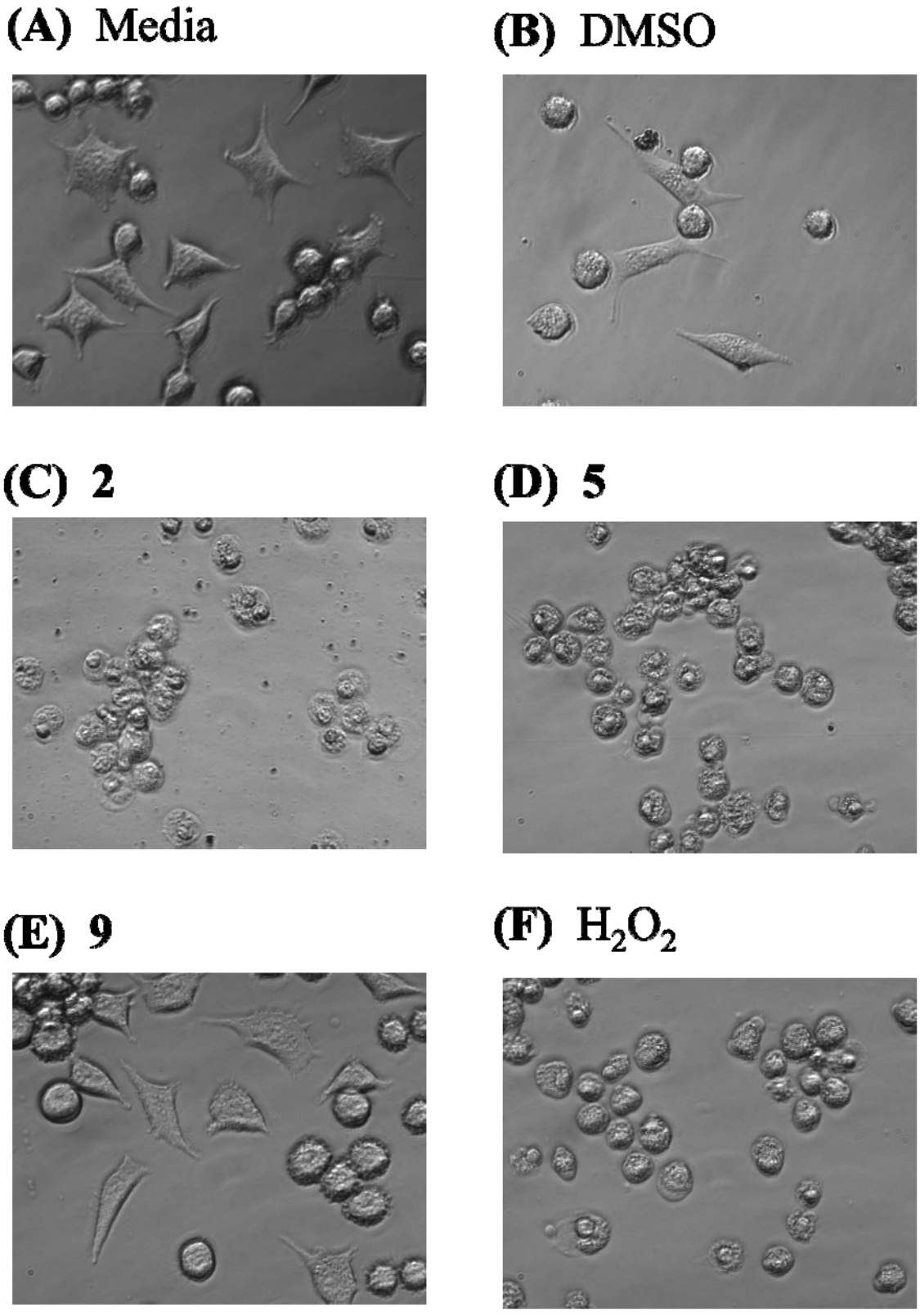
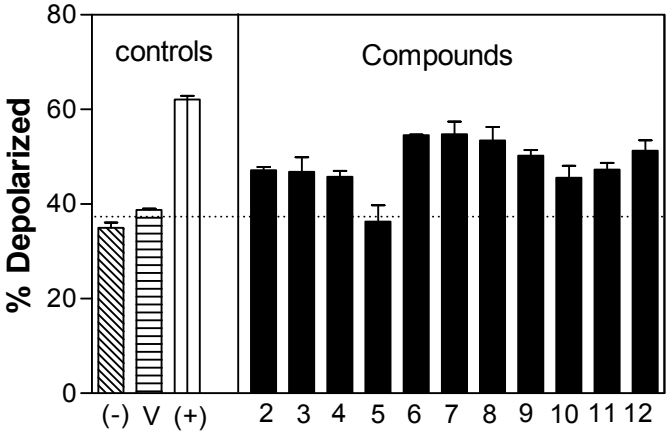


Figure 4

Figure 5

A


80
60
40
20
0
% Depolarized
controls
Compounds
(-) V (+)
2 3 4 5 6 7 8 9 10 11 12

B

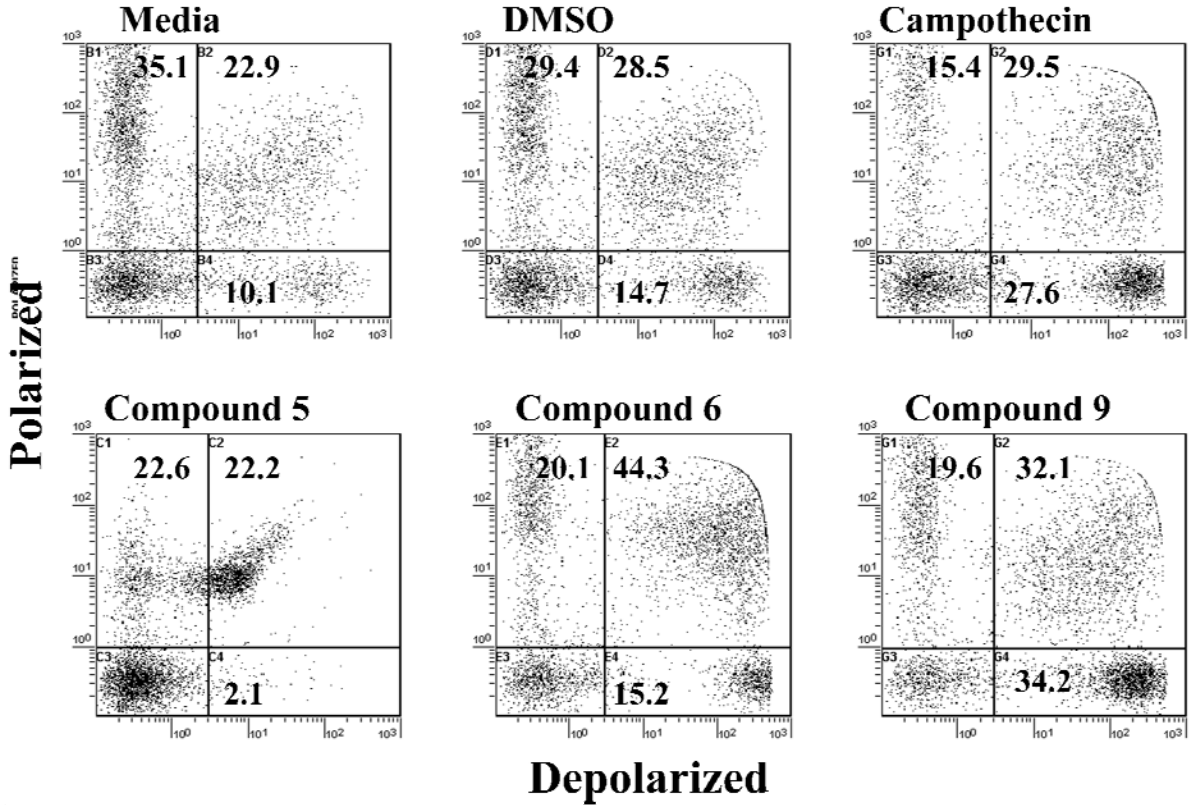

Media
35.1
22.9
10.1
DMSO
29.4
28.5
14.7
Campothecin
15.4
29.5
27.6
Compound 5
22.6
22.2
2.1
Compound 6
20.1
44.3
15.2
Compound 9
19.6
32.1
34.2
Polarized
Depolarized

Figure 6

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CURRICULUM VITA

Oscar Ramirez was born in Ciudad Juarez, Chihuahua. The first son of Oscar Ramirez, Sr. and Rosa Lilia Ramirez, Oscar graduated for Gadsden High School in May of 1989 and received a Bachelor's degree from New Mexico State University in May of 2003. In the Fall of 2004 he was admitted into the doctorates program at the University of Texas at El Paso. Soon after he was offered and accepted a position as laboratory research assistant to Dr. Kristine Garza, a position held until his graduation. Oscar has been awarded travel grants to the SACNAS and AAI national conferences and has also given several oral presentations at regional and national scientific meetings. Upon completion of his degree in July 2009, Oscar has agreed to join the laboratory of Virginia Borges, M.D., at the University of Colorado Health Science Center/Anschutz Cancer Research Center on August 15, 2009.

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