ROLE OF LEPTIN IN THE INDUCTION OF OBESITY-RELATED INFLAMMATION AND INFECTION SUSCEPTIBILITY

by

LEA H. DIB

M.Sc., American University of Beirut, Beirut, Lebanon, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Obesity is a metabolic disease accompanied by a disruption in the immune system leading to systemic inflammation and susceptibility to infections. Leptin, the peptide secreted by adipocytes in proportion to fat mass, is primarily a metabolic hormone translating the body's energy status to the brain. Leptin is also a pro-inflammatory cytokine and leptin deficiency is associated with higher infection susceptibility and a protection against autoimmune diseases. Leptin's dual metabolic-immune function places this hormone as the link between metabolic disturbances of obesity and the immune system. In the following research projects, the contribution of leptin to both inflammation and infection susceptibility was investigated in a murine model of diet-induced obesity.

Chimeric mice with leptin receptor-deficient bone marrow were resistant to HFD-induced weight and fat mass gain. These mice exhibited less inflammation in the adipose tissue demonstrated by a blunted increase in *tnfa* and *il6* gene transcript levels, a higher prevalence of anti-inflammatory macrophages and a lower number of crown-like structures. Systemically, these mice showed a tendency towards higher insulin sensitivity. These outcomes were compared to those from mice with wild-type bone marrow. Obese and lean mice exhibited similar kinetics of bacterial clearance and systemic leptin changes following infection with *Ehrlichia chaffeensis in vivo*. Nevertheless, isolated "obese" peritoneal macrophages were significantly less phagocytic than macrophages from lean mice and supplementation with leptin significantly increased the obese macrophages' phagocytic activity with no effect on lean macrophages. A cell line, DB-1, derived from leptin receptor-deficient bone marrow was immortalized and characterized. This cell line has phenotypic and functional properties characteristic of macrophages, lacks the long isoform of the leptin receptor, and is unresponsive to leptin.

The data from the above mentioned studies suggest that leptin contributes to the inflammation of obesity. They also suggest that leptin affects macrophage function in obesity *in vitro* though more studies are required to assess leptin's contribution to infection outcomes *in vivo.* Finally, DB-1 cells provide a dependable tool to study the role of leptin in obesity-associated inflammation and immune system dysregulation further.

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Dedication

To my mother Maggy, my father Hanna and my brother Samer, your unconditional support, love and faith in me is my strength and driving force to keep moving forward.

To Jeanine, Ziad, Joyce and Laura for being my friends, my family, my support system…

Chapter 1 - Obesity: a condition of metabolic inflammation and compromised immune response

Prevalence and Implications of Obesity

Obesity, or the state of excess fat mass, is the 21st century's major epidemic disease (Caterson, Hubbard et al. 2004). Obesity rates have increased steadily for the past 30 years in both developed and underdeveloped countries. Currently, more than 1 billion adults are overweight (body mass index (BMI)>25) globally, and of those 300 million are obese (BMI>30). By 2015, 700 million people are estimated to be obese worldwide (http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/). Based on the latest National Health and Nutrition Examination Survey (NHANES), approximately 66% of the U.S. adult population is classified as overweight and more than 30% is obese (Ogden, Carroll et al. 2006). More alarming is the increased prevalence of childhood obesity; according to the NHANES data for 2007-2008, 32% of children and adolescents aged 2 to 19 years, are at or above the 85th percentile of BMI for age (Ogden, Carroll et al.). As for the economical burden associated with obesity, a 2003 study estimated medical expenditure attributed to obesity to equal $75 billion, equivalent to 9.1 percent of overall U.S. health expenditures (Finkelstein, Fiebelkorn et al. 2004).

A plethora of morbidities associated with excess weight further highlights the seriousness of this situation. Obesity is positively correlated with insulin resistance, diabetes mellitus (DM), atherosclerosis, cardiovascular disease (CVD), some forms of cancer and fatty liver disease (Pi-Sunyer 1993; Shoelson, Herrero et al. 2007) . In addition, abdominal obesity by itself, is a major risk factor for metabolic syndrome, a constellation of conditions that significantly increases the risk of coronary artery disease and other health problems, including diabetes and stroke (Grundy, Brewer et al. 2004). Although metabolic disorders such as DM, CVD and cancer are the major contributors to the increased morbidity and mortality rates in obesity, chronic and acute respiratory infections, including bronchitis, pneumonia, tuberculosis, septicemia, and other infections, also contribute to substantial adult mortality in obese individuals (Flegal, Graubard et

al. 2007). This highlights the fact that obesity-related morbidities are not limited to metabolic disturbances, but encompass immunological responses as well.

With the rapid growth in the number of overweight individuals, significant research currently focuses on illuminating the link between the plethora of morbidities, disease susceptibility and obesity.

Metabolic and Immune Systems: An Evolutionary Perspective

Throughout evolution, species survival depended on the ability to withstand starvation and the capacity to successfully clear pathogens (Dixit 2008). The scarcity of food and infectious epidemics and pandemics through evolution shaped a “thrifty” genetic profile. This genetic profile favors caloric storage of excess calories as fat and strong antibacterial immune responses (Levin, Lipsitch et al. 1999; Ames 2006). It is hypothesized that both metabolic and immune functions share common or overlapping pathways regulated through common key molecules and signaling systems (Hotamisligil 2006). In fact, primitive organisms such as Drosophila integrate the pathogen and nutrient sensing pathways such that nutrients can induce immune responses and pathogens can evoke and regulate metabolic actions (Levin, Lipsitch et al. 1999; Dionne, Pham et al. 2006). Toll-like receptors (TLRs) a family of pattern-recognition receptors, are an example of receptors that can be activated by both pathogens and nutrients (Senn 2006; Shi, Kokoeva et al. 2006). In fact, lipopolysaccharide (LPS), which is present on the cell wall of gram-negative bacteria, binds and activates TLR4. Fatty acids such as palmitate and oleate, which correlate with obesity (Gao, Nong et al. 2010), are also ligands for TLR4. Another example for the immune-metabolic interactions are the 5' adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) pathways. Both pathways are involved in energy state sensing of the body. In addition, current investigations focus on AMPK and mTOR as mediators of the effect of caloric restriction (CR) on increased longevity and improved immune functions (Jiang, Zhu et al. 2008).

A delicate balance exists between metabolic and immune functions. Depending on the situation, one of these functions is shut down in order to maximize efficiency of the other. To illustrate this concept, activation of inflammatory or stress responses to pathogens inhibit major anabolic signaling pathways such as the insulin/insulin-like growth factor (IGF), thus channeling

the body's energy towards fighting the infection rather than towards anabolic mechanisms (Garsin, Villanueva et al. 2003). This dual regulation has been efficient and essential to survival in the past eras where food supply was erratic and people led a less sedentary lifestyle. However, in our current times of food surplus combined with a sedentary lifestyle and hyperhygienic environments, our highly efficient metabolic and immune systems are facing alternative challenges. In fact obesity is associated with immune disturbances characterized by both chronic low grade inflammation (Hotamisligil 2006) and an increased susceptibility to infections (Falagas and Kompoti 2006). This introduction provides an overview of obesity, adipose tissue and immune mechanisms relevant to obesity. It will discuss aspects of obesity-associated immune disturbances and the role of leptin, a peptide involved both in metabolism and immunity, in relation to these disturbances (Figure 1.1). The latter two aspects will be discussed in greater details in the following chapters.

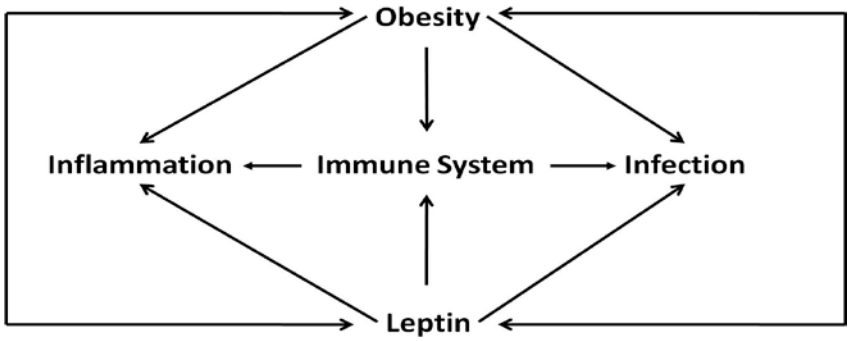

Figure 1.1 Relationships between obesity, leptin and the immune system.
Obesity and leptin affect the immune system independently. In addition, obesity and leptin are highly correlated suggesting that the coexistence of both can act synergistically on the immune system.

Leptin: A Pleiotropic Hormone

Leptin, the product of the *ob* gene in rodents and *LEP* gene in humans, is a 16 kDa peptide hormone predominantly secreted by white adipocytes (Zhang, Proenca et al. 1994; Ahima and Flier 2000). Leptin belongs to the family of long-chain helical cytokines characterized by a four α-helix bundle and shares structural similarities with type I cytokines and is referred to as an adipokine or adipocytokine (Otero, Lago et al. 2005). Leptin acts through its receptor (LepR) encoded by the *db* gene in rodents (Chen, Charlat et al. 1996). The leptin

receptor is a member of the class I cytokine receptor family (Friedman and Halaas 1998). LepR exists in six alternatively spliced isoforms that share the same extracellular domain but differ in the length of the intracellular domain (Lee, Proenca et al. 1996). The long isoform (LepRb) has a total length of 1162 amino acids and is responsible for most of leptin's biological activities (Tartaglia, Dembski et al. 1995). When leptin binds to LepRb, it recruits Janus tyrosine kinase 2 (JAK2) and activates the receptor, which then serves as a docking site for signal transducer and activator of transcription (STAT) factors, particularly STAT3 (Baumann, Morella et al. 1996). STAT3 then translocates to the nucleus and induces expression of other genes, including negative regulators, such as the suppressor of cytokine signaling 3 (Bjorbaek, El-Haschimi et al. 1999) and the protein tyrosine phosphatase 1B (Cheng, Uetani et al. 2002). Additional pathways are also involved in leptin signaling and include the mitogen-activated protein kinase (MAPK), the insulin receptor substrate 1 (IRS-1), and the phosphatidylinositol 3 kinase (PI3-K) (Martin-Romero and Sanchez-Margalet 2001).

Leptin is a pleiotropic hormone; its major role involves the regulation of neuroendocrine function and energy homeostasis (Chan, Heist et al. 2003). Leptin stimulates the production of anorectic neuropeptides and suppresses the action of orexigenic peptides in the arcuate nucleus of the hypothalamus (Cheung, Thornton et al. 1997; Schwartz, Seeley et al. 1997; Thornton, Cheung et al. 1997), where LepRb is highly expressed (Elmquist, Bjorbaek et al. 1998). Since leptin was identified as an anti-obesity hormone (Zhang, Proenca et al. 1994), it was expected that human obesity, similarly to the rodent model, will be characterized by leptin deficiency. However, contrary to these early assumptions, obese individuals almost always express high leptin levels (Considine, Sinha et al. 1996) and their obesity was due to leptin resistance in the hypothalamus rather than leptin deficiency (Banks and Farrell 2003; Munzberg and Myers 2005).

In addition to being the body's major energy sensor, leptin exerts other physiological effects that impact reproduction, hematopoiesis, angiogenesis and immunity (Wauters, Considine et al. 2000). Most of these effects are through a direct action of leptin on peripheral tissues and cells that express the functional long leptin receptor. Leptin plays an important role in the regulation of both innate and adaptive immunity (La Cava and Matarese 2004; Matarese, Moschos et al. 2005) with most immune cells expressing LepRb (Matarese, Moschos et al. 2005). T-cells (Batra, Okur et al. 2009), B-cells (Papathanassoglou, El-Haschimi et al. 2006),

neutrophils (Caldefie-Chezet, Poulin et al. 2003), monocytes (Raso, Pacilio et al. 2002), dendritic cells (Mattioli, Straface et al. 2005) and NK cells (Tian, Sun et al. 2002) all express LepRb, and leptin modulates the function both *in vivo* and *in vitro* (Papathanassoglou, El-Haschimi et al. 2006). In innate immunity, leptin modulates the activity and function of neutrophils by increasing chemotaxis and the secretion of oxygen radicals (Mancuso, Gottschalk et al. 2002; Caldefie-Chezet, Poulin et al. 2003). Leptin acts on monocytes and macrophages by stimulating phagocytic activity, the secretion of pro-inflammatory cytokines and expression of adhesion molecules (Lord, Matarese et al. 1998; Fantuzzi and Faggioni 2000). It also increases cytotoxic ability and the secretion of perforin and IL-2 by natural killer (NK) cells (Tian, Sun et al. 2002). In adaptive immunity, leptin affects the generation, maturation and survival of thymic T cells (Howard, Lord et al. 1999) and promotes the switch of naïve T-cells (TH0) towards T helper 1 (TH1)-cell immune responses by increasing interferon-γ (IFN-γ) and TNF secretion (Lord, Matarese et al. 1998).

Hence, leptin is actively involved in immune system activation. Indeed, in situations of caloric deprivation such as starvation, leptin levels are drastically decreased leading to an attenuation of energy demanding mechanisms such as reproductive and immune systems (Faggioni, Moser et al. 2000; Matarese, La Cava et al. 2002). On the other hand, in obesity, leptin levels are drastically increased to abnormal levels also negatively impacting these pathways.

Adipose Tissue: More than an Inert Energy Reservoir

White adipose tissue (WAT) is the only tissue in the body that can significantly change in size throughout lifespan and different levels of obesity (Hausman 2001). WAT is best known as the body's unlimited energy store. Nevertheless, adipose tissue is not just an inert fuel storage reservoir. Now recognized as an endocrine organ, adipose tissue secretes a multitude of substances involved in both energy homeostasis and inflammatory mechanisms through paracrine, autocrine and endocrine pathways (Harle and Straub 2006). Adipose tissue is a major source of adipocytokines and other soluble factors. Among the secreted adipocytokines are leptin, adiponectin, resistin and visfatin. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1),

angiotensinogen, retinol-binding protein-4, serum amyloid A (SAA) are also among the soluble factors secreted by the adipose tissue (Shimomura, Funahashi et al. 1996; Fried, Bunkin et al. 1998; Steppan, Bailey et al. 2001; Fukuhara, Matsuda et al. 2005). Thus, it is clear that adipose mass is a complex organ involved in many physiological processes.

The role of the adipose tissue in the induction of low-grade inflammation of obesity was first demonstrated by Hotamisligil and colleagues in 1993. This study showed that adipose tissue is an important source of the pro-inflammatory cytokine, TNF-α and its expression, in fat mass, increases with obesity. In the same study, neutralization of TNF-α using a TNF-α soluble antibody lead to an improvement in insulin sensitivity of obese animals. Although suggested in the early 1900s, this study demonstrated, for the first time, a direct mechanistic link between inflammatory mediators and metabolic disease (Hotamisligil, Shargill et al. 1993). The early studies used salicylates; the non-steroidal anti-inflammatory drug, for the treatment of diabetes and high blood sugar (Williamson 1901; Reid, Macdougall et al. 1957) suggesting since that time an interaction between immunity and metabolism.

WAT is divided into subcutaneous and abdominal or visceral stores that differ both at the physiological and pathological levels (Shoelson, Herrero et al. 2007). The two depots differ in their contribution to secreted proteins and cytokines; abdominal WAT produces higher levels of adiponectin (Motoshima, Wu et al. 2002), PAI-1, IL-8, IL-6 and IL-1β (Juge-Aubry, Somm et al. 2003; Bruun, Lihn et al. 2005) whereas subcutaneous WAT secretes higher leptin levels (Montague and O'Rahilly 2000; Fain 2006). The two fat depots also differ in cell size, metabolic activity, and potential role in insulin resistance (Johnson and Hirsch 1972; Krotkiewski, Bjorntorp et al. 1983; Gastaldelli, Miyazaki et al. 2002). Compared to other fat depots, visceral adiposity is highly correlated with increased risk of CVD and diabetes (Wang, Rimm et al. 2005; Kuk, Katzmarzyk et al. 2006). One possible explanation is the "portal hypothesis" which suggests that the direct access of visceral fat to the portal circulation allows direct delivery of free fatty acids to the liver. This hypothesis may explain the high correlation between this particular fat depot and insulin resistance (Kabir, Catalano et al. 2005). In addition to its anatomical location, visceral fat displays a higher lipolytic activity compared to subcutaneous fat (Montague and O'Rahilly 2000) releasing more free fatty acid into the circulation and directly to the portal system impacting the liver and leading to metabolic abnormalities (Bjorntorp 1990; Nielsen, Guo et al. 2004).

Obesity: Chronic Inflammatory State

Though beneficial in the context of energy balance, the close interplay between metabolic and inflammatory pathways, becomes deleterious if this balance is disrupted due to either nutrient scarcity or nutrient excess (Wellen and Hotamisligil 2005). Obesity, or excess fat mass, is currently defined as a state of chronic low-grade inflammation (Dandona, Aljada et al. 2004; Cancello and Clement 2006). Human and most rodent obesity models are characterized by an increased serum levels of C-reactive protein (CRP), IL-6, IL-18, P-selectin, TNF-α and leptin - most of those arising from the expanded adipose tissue mass (Greenberg and Obin 2006; Tilg and Moschen 2006). This sustained systemic elevation of inflammatory markers correlates with the development of insulin resistance and atherosclerosis, two hallmarks of the metabolic syndrome (Hotamisligil, Peraldi et al. 1996; Cottam, Mattar et al. 2004; Wellen and Hotamisligil 2005; Lazar 2006) (Figure 1.2). Indeed, the present consensus supports the fact that most of the obesity-associated diseases such as hypertension, diabetes, atherosclerosis, dyslipidemia and some forms of cancer are of inflammatory origin (Wellen and Hotamisligil 2005). Pro-inflammatory cytokines and inflammatory pathways such as TNF-α, IL-6, suppressor of cytokine signaling proteins (SOCS), ER stress, NF-κB and JNK signaling pathways are all up-regulated in obesity and provide different mechanisms for the metabolic-immune dysregulation observed in this state (Rui, Yuan et al. 2002; Croker, Krebs et al. 2003; Ozcan, Cao et al. 2004; Tilg and Moschen 2006). Among these mechanisms are, enhanced lipolysis leading to increased systemic levels of non-esterified free fatty acids (NEFA), suppressed gene expression of glucose transporter GLUT4, disrupted insulin signaling pathway through serine phosphorylation of IRS-1, and increased production of reactive oxygen species (ROS). All of these alterations lead to metabolic disease through insulin resistance and atherosclerosis (Feingold, Doerrler et al. 1992; Hauner, Petruschke et al. 1995; Hotamisligil, Peraldi et al. 1996; Stentz, Umpierrez et al. 2004).

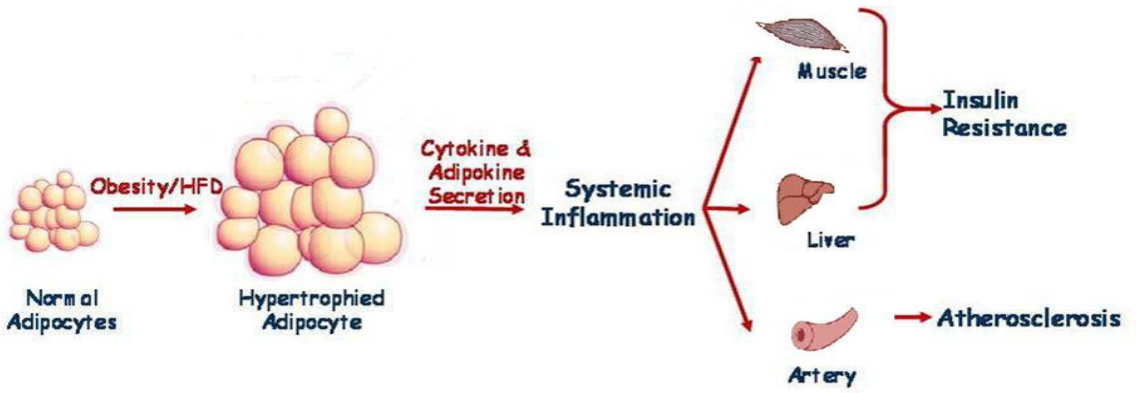

Figure 1.2 Model of inflammation-induced metabolic disease in obesity.
Obesity leads to adipose tissue expansion mainly through adipocyte hypertrophy. Hypertrophied adipocytes secrete cytokines and adipokines into the systemic circulation leading to the low grade inflammatory state reported in obesity. This inflammatory state is associated with cytokine-mediated insulin resistance and atherosclerosis.

Since inflammation seems to be at the center of obesity-associated metabolic disorders, current research focuses on identifying culprit molecules within the adipose tissue responsible for the chronic low grade inflammatory state of obesity.

Adipose Tissue Macrophages: Major Player in Obesity Associated Inflammation

Adipose tissue is a heterogeneous organ consisting of different groups of cells. The most abundant cells are the adipocytes (fat cells); other smaller cells constitute the stroma vascular fraction (SVF) and include preadipocytes, endothelial cells, fibroblasts, leukocytes and macrophages (Tilg and Moschen 2006). The fact that the adipose tissue consists of a multitude of cell types, resulted in a debate on the role of each in the secretion of the different adipose tissue-derived endocrine and paracrine regulators (Wisse 2004). Adipocytes secrete most of the endocrine hormones such as leptin and adiponectin, whereas other secreted proteins are derived primarily from the non-adipocyte fraction of adipose tissue (Fain 2006). In 2003, two large scale gene expression studies (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003) identified adipose tissue macrophages (ATMs) as the major source of inflammation in the adipose mass. These studies demonstrated that a significant increase in ATMs characterized both genetic and diet-induced obesity (DIO) in mice. In fact, ATMs make up to 50% of the total adipose tissue cells in extreme cases of obesity (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). In addition, adipose tissue gene expression of inflammatory cytokines such as TNF-α and macrophage inflammatory protein (MIP)-1α is derived almost entirely from macrophages (Xu,

Barnes et al. 2003; Fain 2006). Meanwhile, mature adipocytes secrete the majority of leptin, and adipocytes, macrophages, and non-macrophage SVF express IL-6 almost equally (Weisberg, McCann et al. 2003). Subsequent studies confirmed these data and further characterized this cell population of macrophages strengthening the role of ATMs in obesity-associated inflammatory status (Cinti, Mitchell et al. 2005; Lumeng, Bodzin et al. 2007; Lumeng, Deyoung et al. 2007; Lumeng, DelProposto et al. 2008). As a result, macrophages are currently considered as central players in obesity-induced inflammatory status and its associated metabolic diseases.

Resident and Obesity-Recruited Macrophages

Macrophages are present in the adipose tissue of both lean and obese subjects. In lean subjects, they form a constitutive part of the adipose tissue (AT) making up to 10% of the total AT cell percentage. Referred to as the resident or type II macrophages, these macrophages are $F4/80^{+}$, $CD11c^{-}$ and $MGL1^{+}$ and display the anti-inflammatory or M2 gene transcripts such as *Il10, Mgl1* and *Arg1* (Lumeng, Bodzin et al. 2007; Lumeng, DelProposto et al. 2008). These macrophages are dispersed in the interstitial space between adipocytes (Cinti, Mitchell et al. 2005) and are believed to have a role in preserving AT integrity and maintenance. With the onset of obesity and the expansion of fat mass, another population of bone-marrow derived macrophages is recruited to the adipose tissue. The newly recruited macrophages may comprise up to 50% of the total adipose tissue cells in cases of extreme obesity (Weisberg, McCann et al. 2003) (Figure 1.3).

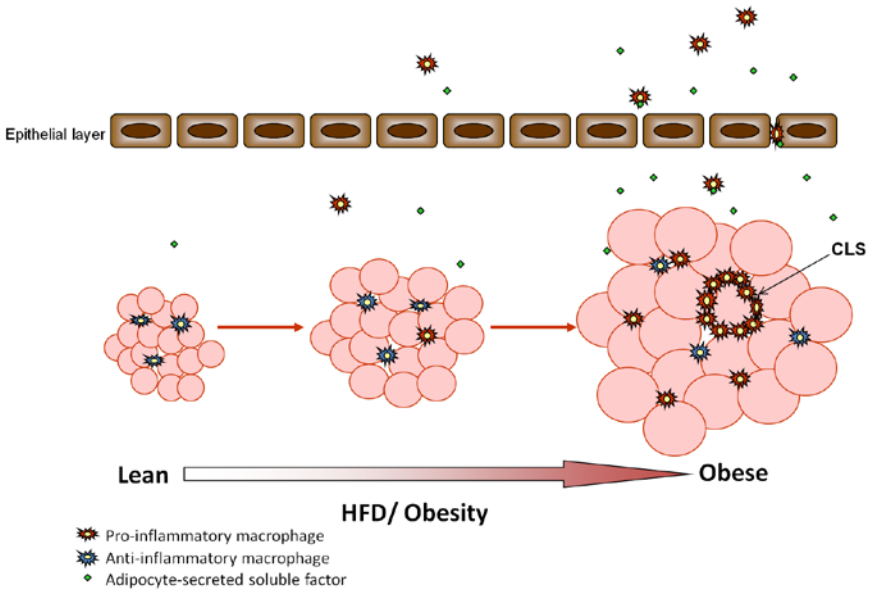

Figure 1.3 Model of ATM recruitment into the adipose tissue in obesity.
In the lean state, resident (anti-inflammatory) macrophages are dispersed between adipocytes. With obesity, adipocytes increase in size and secrete soluble factors that recruit pro-inflammatory macrophages that form crown-like structures (CLS) surrounding dying adipocytes

These infiltrated macrophages differ from the resident macrophages in number, distribution, phenotype and function. The recruited macrophages display the classical or pro-inflammatory phenotype (Lumeng, Bodzin et al. 2007) and are referred to as type I or pro-inflammatory macrophages. With high transcript expression of the M1 or pro-inflammatory gene array such as *Tnfa*, *Il6* and *Nos2* (Lumeng, Deyoung et al. 2007), the recruited macrophages are F4/80^{+}, CD11c^{+} and MGL1^{-} (Lumeng, Bodzin et al. 2007; Westcott, Delproposto et al. 2009). These type I macrophages aggregate around dying adipocytes forming crown-like structures or CLS and have a role in the clearance of dying adipocytes (Cinti, Mitchell et al. 2005; Murano, Barbatelli et al. 2008) and adipose tissue angiogenesis (Pang, Gao et al. 2008); two conditions resulting from weight gain and the expansion of fat mass (Strissel, Stancheva et al. 2007; Murano, Barbatelli et al. 2008).

Although many of the steps relating obesity to the development of inflammation-induced metabolic diseases (more recently described as the "metaflammation" syndrome (Hotamisligil 2006) have been identified, the exact physiological or secretory factor in the expanded adipose tissue triggering the onset of pro-inflammatory ATMs recruitment is yet to be identified.

Adipose Tissue Macrophage Recruitment

Obesity is characterized by an enhanced recruitment of inflammatory macrophages into the adipose tissue (Hotamisligil, Shargill et al. 1993; Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). The factors behind this increased recruitment are yet, not well defined, though many soluble physiological factors have been investigated. T-cell infiltration, adipose tissue hypoxia, increased angiogenic demand, increased levels of free fatty acids and soluble factors such as MCP-1, osteopontin and sLIGHT all contribute to ATMs recruitment, though none of these factors can independently, account for this process (Weisberg, Hunter et al. 2006; Kintscher, Hartge et al. 2008; Pang, Gao et al. 2008; Rausch, Weisberg et al. 2008; Bertola, Deveaux et al. 2009; Kosteli, Sugaru et al. 2010; Kim, Jeong et al. 2011). Chemokine (C-C motif) ligand 2 (CCL2) and its receptor chemokine (C-C motif) receptor 2 (CCR2) are to date, the best studied chemokine ligand and receptor pair for inducing macrophage accumulation in white adipose tissue. CCL2 and CCR2 recruit monocytes and macrophages to sites of inflammation (Maus, Wellmann et al. 2005). In addition, CCL2 is highly expressed in the adipose tissue of obese human subjects and rodents (Sartipy and Loskutoff 2003; Charo and Taubman 2004; Bruun, Lihn et al. 2005). Adipose tissue from transgenic diet-induced obese (DIO) mice over-expressing CCL2 contains increased number of ATMs. These mice also have a higher prevalence of insulin resistance and glucose intolerance compared to DIO WT controls (Kamei, Tobe et al. 2006; Kanda, Tateya et al. 2006). These studies suggested CCL2 as the major chemoattractant agent for ATMs. However, experiments with CCL2 and CCR2 deficient mice produced inconsistent results. Some studies showed that CCR2 KO and CCL2 KO mice on a high fat diet (HFD) have a lower inflammatory response and decreased ATMs compared to WT mice on the same dietary regimen (Kanda, Tateya et al. 2006; Weisberg, Hunter et al. 2006). In addition, the ATMs displayed an anti-inflammatory or alternatively activated profile as compared to the DIO WT controls (Lumeng, Deyoung et al. 2007). On the other hand, additional studies showed that CCL2 is not critical for adipose tissue macrophage recruitment (Chen, Mumick et al. 2005; Inouye, Shi et al. 2007; Kirk, Sagawa et al. 2008). Inouye et al. and Kirk et al. both demonstrated a slight increase in ATMs in DIO CCL2 KO mice as compared to WT DIO mice (Inouye, Shi et al. 2007; Kirk, Sagawa et al. 2008). Chen et al. reported no differences in the number of ATMs or in plasma insulin or glucose levels in DIO CCR2 KO mice compared to DIO WT controls (Chen, Mumick et al. 2005). *In vitro*, CCL2 was reportedly

a potent chemoattractant for monocytes and macrophages (Gruen, Hao et al. 2007). However, these experiments used much higher CCL2 concentrations than the physiological levels seen in obesity thus undermining the role of CCL2 in physiological obesity.

In conclusion, these observations strengthened the belief that other soluble factors, probably endogenous to fat mass and positively correlated with fat mass, play a major role in ATM recruitment in obesity.

Leptin and ATM Recruitment

Leptin is a good candidate for the soluble factor responsible for the recruitment and pro-inflammatory differentiation of macrophages into the adipose tissue. As mentioned previously, leptin is secreted by adipocytes and its secretion is proportional to fat mass and adipocyte size. *In vitro* studies showed leptin to be a potent chemoattractant for monocytes and macrophages at levels comparable to those measured in obesity (Gruen, Hao et al. 2007). By increasing the expression of adhesion molecules in epithelial cells, leptin enhances diapedesis of monocytes through an epithelial layer (Curat, Miranville et al. 2004). In addition, leptin induces pro-inflammatory or Th1 cytokine secretion by macrophages and monocytes (Lord, Matarese et al. 1998) while inhibiting a Th2 response. *In vivo* studies showed that although leptin deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice have increased recruitment of pro-inflammatory ATMs, the degree of macrophage infiltration was significantly less than expected for their bodyweight when compared to WT DIO mice (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). These data suggested that the absence of leptin or leptin signaling leads to a decrease in ATMs recruitment in these genetic models of obesity. Finally, human obesity is characterized by high leptin levels and a prevalence of M1 macrophages in the adipose tissue (Clement, Viguerie et al. 2004). Upon minor weight loss (around 5% of total body weight), leptin levels are drastically decreased below that expected for the weight loss (Infanger, Baldinger et al. 2003). This is accompanied with a shift in ATMs from an M1 to an M2 phenotype (Clement, Viguerie et al. 2004). It can be argued that the shift in ATM phenotype correlates better with the decrease in leptin rather than with the amount of weight or fat mass loss. The evidence presented above and the fact that human obesity is usually a hyperleptinemic state, suggest leptin is a strong candidate to enhance recruitment of pro-inflammatory ATMs in the obese state.

Obesity, Inflammation and Infection

Since obesity is a state of chronic low grade inflammation with upregulated levels of pro-inflammatory cytokines, one might conjecture that this pre-existing inflammatory status would impart an advantage to mounting a specific, pro-inflammatory response to clear foreign pathogens. However, evidence demonstrates the opposite; obese individuals are more susceptible to infections and have a delayed pathogen clearance compared to normal weight controls. Indeed, epidemiological and observational studies established a strong link between obesity and the development of several types of infections (reviewed in (Falagas and Kompoti 2006). Obese people are at higher risk of developing nosocomial, surgical site, odontogenic, respiratory, urogenital, skin, bone and liver infections when compared to their leaner counterparts (Falagas and Kompoti 2006).

Both genetically obese (leptin and leptin receptor deficient) and DIO mice exhibit a blunted immune response as well as a delayed pathogen clearance when infected with either bacterial or viral agents (Mancuso, Gottschalk et al. 2002; Amar, Zhou et al. 2007; Smith, Sheridan et al. 2007). In addition, compared to lean controls, infected DIO mice have a higher mortality in response to influenza virus infection (Smith, Sheridan et al. 2007). *In vitro* studies demonstrated impaired phagocytic and bactericidal activity by macrophages isolated from either obese humans or mice (Krishnan, Trost et al. 1982; Mancuso, Gottschalk et al. 2002). Furthermore, these "obese" macrophages exhibit a blunted cytokine (Amar, Zhou et al. 2007) and a defective oxidative response (Zhou, Leeman et al. 2009) upon exposure to an *in vitro* bacterial challenge.

Leptin and Infection Susceptibility in Obesity

Leptin is an adipokine that perfectly illustrates the metabolic-immune dichotomy of adipose tissue secreted factors. Genetically obese mice lacking either leptin (*ob/ob*) or its receptor (*db/db*) are more prone to opportunistic infections and are resistant to autoimmune diseases (Sanna, Di Giacomo et al. 2003; Ikejima, Sasaki et al. 2005), highlighting the role of functional leptin and leptin signaling in generating normal immune responses. In fact, restoring leptin levels to normal in either *ob/ob* or starved mice normalized the immune responses in bacteria challenged mice (Mancuso, Gottschalk et al. 2002; Sanna, Di Giacomo et al. 2003;

Mancuso, Huffnagle et al. 2006). Similarly, leptin supplementation of macrophages isolated from *ob/ob* mice normalized their phagocytic activity (Mancuso, Gottschalk et al. 2002). Leptin acts on macrophages through activation of JAK/STAT signaling pathway, leading to stimulation of phagocytosis, production of oxygen and nitrogen reactive species, and secretion of pro-inflammatory cytokines (Shirshev and Orlova 2005).

Leptin signaling and regulation is impaired in obesity, leading to leptin resistance at the central nervous system and possibly peripheral levels (Klein, Horowitz et al. 2000; Schwartz, Woods et al. 2000). Influenza infection decreased serum leptin in DIO mice whereas the lean group maintained normal leptin concentration. The decrease in leptin in the obese group correlated with a higher infection rate (Smith, Sheridan et al. 2007). In a separate study, leptin levels significantly increased after influenza infection in lean mice while this response failed to occur in obese mice. Additionally, leptin increase correlated with an improved pathogen clearance (Karlsson, Sheridan et al. 2010). Although these studies reported leptin levels in their studies, they did not further investigate if serum leptin change is a causative factor in the infection outcome in DIO mice.

Dissertation Research Summary

In the following chapters, the role of leptin is investigated in the context of diet-induced obesity on both adipose tissue inflammation and infection susceptibility. A DIO murine model lacking leptin signaling in bone marrow cells was used to investigate the role of leptin in the recruitment of pro-inflammatory macrophages to the adipose tissue after a 16 weeks period of HFD feeding. The outcomes assessed in this study included parameters of adipose tissue inflammation and anthropometric and systemic metabolic measures. This study is thoroughly described and discussed in Chapter 2. Briefly, the DIO mice with leptin receptor deficient bone marrow showed resistance to HFD in terms of body and fat mass weights. These mice exhibited higher insulin sensitivity at the systemic level and displayed less inflammation in their adipose tissue in terms of inflammatory genes transcript levels, the prevalence of anti-inflammatory macrophages and the decreased number of crown-like structures. Leptin's contribution to infection outcomes in obesity was investigated using also a DIO murine model. Obese mice and their lean counterparts were injected with *Ehrlichia chaffeensis* and systemic and bacterial

clearance was monitored for 9 days. This study is described in details in Chapter 4. Briefly, no significance difference was detected in the kinetics of bacterial clearance between lean and obese mice *in vivo*. Systemic leptin was decreased in both groups after the infection, while inflammatory cytokines increased. Nevertheless, macrophage phagocytic activity was decreased *in vitro* in the obese mice and supplementation with leptin restored that function back to normal levels. Finally, in Chapter 3, a leptin receptor-deficient cell line, DB-1, is immortalized and characterized. This cell line displays phenotypic and functional characteristics of macrophages and is unresponsive to leptin's chemotactic effects.

Chapter 2 - Leptin Receptor Contributes to the Metabolic and Inflammatory Profile of High Fat Fed Mice

Abstract

Obesity is characterized by an increased recruitment of pro-inflammatory macrophages to the adipose tissue leading to systemic inflammation and metabolic disease. We investigated the role of leptin receptor in the induction of obesity-associated inflammation. We generated radiation chimeric C57BL/6J mice reconstituted with either leptin receptor-deficient ($LepR^{-/-}$) or wild type (WT) bone marrow and kept them on a high fat diet (HFD) for sixteen weeks. Anthropometric, serological, and gene expression data, adipose tissue morphology and macrophage content were assessed. Mice reconstituted with $LepR^{-/-}$ bone marrow, were resistant to HFD-induced weight and fat mass gain. Epididymal adipocytes in these mice showed a 50% reduction in the expression of *Tnfa* and *Il6* and their epididymal fat contained significantly fewer crown-like structures compared to mice with WT bone marrow. Most of these macrophages expressed Mgl1 and were CCR2 negative, indicative of an anti-inflammatory phenotype. Systemically, no difference in serum levels of TNF-α and IL-6 was reported amongst any of the groups, but mice reconstituted with $LepR^{-/-}$ bone marrow showed a tendency towards better insulin sensitivity. These data suggest that leptin, through its receptor contributes to the recruitment of pro-inflammatory macrophages in the adipose tissue of obese mice and the expression of the obese phenotype.

Introduction

Obesity is the epidemic disease of this century (Caterson, Hubbard et al. 2004). WHO estimate that in 2015, 700 million adults will be obese worldwide (http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/). Obesity is positively correlated with a plethora of diseases (Pi-Sunyer 1993; Shoelson, Herrero et al. 2007) and a higher susceptibility to infections (Falagas and Kompoti 2006). Therefore, investigating the link

between the increase in fat mass and the development or exacerbation of disease is of utmost importance.

The close interplay between metabolic and inflammatory pathways is beneficial in the context of energy balance but becomes deleterious if this balance is disrupted; whether in the case of nutrient scarcity or nutrient excess (Wellen and Hotamisligil 2005). Indeed, obesity is currently defined as a state of low grade inflammation (Dandona, Aljada et al. 2004; Cancello and Clement 2006) characterized by increased levels of inflammatory markers such as C-reactive protein (CRP), interleukin-6, and -18 (IL-6, IL-18), P-selectin, tumor necrosis factor alpha (TNF-α) and leptin; most of these arising from the expanded adipose tissue mass (Greenberg and Obin 2006; Tilg and Moschen 2006). This chronic low-grade inflammation is believed to be the origin of most of the obesity-associated diseases (Wellen and Hotamisligil 2005; Hotamisligil 2006).

Macrophages have been identified as the major secretory cells in adipose tissue contributing to systemic and local inflammation in obesity (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). Adipose tissue macrophage (ATM) recruitment is increased dramatically (Weisberg, McCann et al. 2003) with the expansion of fat mass comprising up to 50% of adipose tissue cells in the obese state. Whereas macrophages are present in the adipose tissue of both lean and obese subjects, they differ in number, distribution, phenotype and function. In the lean state, resident (Type II) macrophages display an anti-inflammatory phenotype. They are $F4/80^{+}$, $CD11c^{-}$, $MGL1^{+}$(Lumeng, DelProposto et al. 2008), express higher numbers of M2 gene transcripts such as *Il10*, *Mgl1* and *Arg1* (Lumeng, Bodzin et al. 2007), are dispersed in the interstitial space between adipocytes (Cinti, Mitchell et al. 2005) and are believed to have a role in preserving adipose tissue (AT) integrity and maintenance. On the other hand, obesity recruited (Type I) macrophages, display a pro-inflammatory phenotype (Nguyen, Favelyukis et al. 2007; Lumeng, DelProposto et al. 2008) have a high transcript level expression of *Tnfa*, *Il6* and *Nos2* (Lumeng, Deyoung et al. 2007) and are $F4/80^{+}$, $CD11c^{+}$, $MGL1^{-}$ (Lumeng, Bodzin et al. 2007; Westcott, Delproposto et al. 2009). They form crown-like structures surrounding necrotic-like adipocytes and are believed to have a role in the clearance of dying adipocytes (Cinti, Mitchell et al. 2005; Murano, Barbatelli et al. 2008).

Many factors are capable of inducing macrophage infiltration and activation in adipose tissue. Adipocyte death, hypoxia, angiogenesis, T-cell infiltration and certain chemokines have all been studied and demonstrated as candidates to promote ATM recruitment

(Cinti, Mitchell et al. 2005; Wu, Ghosh et al. 2007; Ye, Gao et al. 2007; Kintscher, Hartge et al. 2008; Pang, Gao et al. 2008; Rausch, Weisberg et al. 2008; Wang, Wood et al. 2008). CCL2, also known as monocyte chemotactic protein-1(MCP-1), and its receptor, CCR2, are the best studied chemokine-ligand receptor pair that promotes macrophage accumulation in white AT. Some reports showed a high correlation between CCL2 and CCR2 expression in ATM recruitment in obese individuals (Kamei, Tobe et al. 2006; Kanda, Tateya et al. 2006; Weisberg, Hunter et al. 2006), however more recent studies failed to show this correlation (Inouye, Shi et al. 2007; Kirk, Sagawa et al. 2008). These data suggest that other factors, probably endogenous to fat mass and related to obesity, play a major role in ATM recruitment.

Leptin plays a major role in both innate and adaptive immunity (La Cava and Matarese 2004). The long leptin receptor isoform (LepRb), responsible for leptin's physiological activities (Tartaglia, Dembski et al. 1995; Baumann, Morella et al. 1996), has been identified on most immune cells including monocytes and macrophages (Raso, Pacilio et al. 2002; Tian, Sun et al. 2002; Caldefie-Chezet, Poulin et al. 2003; Matarese, Moschos et al. 2005; Mattioli, Straface et al. 2005; Papathanassoglou, El-Haschimi et al. 2006; Batra, Okur et al. 2009). Some have suggested that leptin initiates the recruitment and differentiation of macrophages in the AT (Xu, Barnes et al. 2003). Leptin is a potent chemoattractant for monocytes and macrophages *in vitro* (Gruen, Hao et al. 2007). It increases the expression of adhesion molecules in epithelial cells and it enhances diapedesis of monocytes through an epithelial layer (Curat, Miranville et al. 2004). In addition, leptin induces a pro-inflammatory or Th1 cytokine secretion by macrophages and monocytes (Lord, Matarese et al. 1998) while inhibiting a Th2 response. Indeed, leptin deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice exhibit less macrophage infiltration in AT than would be expected for their body weight (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003).

Since human obesity is often characterized by a hyperleptinemic state, we tested the role of the LepRb on white blood cells in the recruitment of ATMs. We demonstrated that the absence of the long leptin receptor isoform ameliorates the expression of the obese phenotype as indicated by attenuated weight gain, a higher insulin sensitivity index and decreased inflammation in the adipose tissue of diet-induced obese (DIO) mice.

Materials and Methods

Animals- Five-week old wild type (WT) male C57BL/6J mice, and 10-week old male WT and *db/db* mice on the C57BL/6J background, were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed individually in sterile micro-isolators under specific pathogen-free conditions at 30-40% controlled humidity and a 12-hour light/dark cycle. Animals were allowed a one week adaptation period prior to any intervention. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals with the approval and monitoring of the Institutional Animal Care and Use at Kansas State University and the University of Kansas-Medical Center.

Materials and Reagents- Primary labeled monoclonal anti-mouse CD3, B220 F4/80 and CD11b and their respective isotype controls were purchased from eBioscience (San Diego, CA). For immunohistochemistry, primary labeled monoclonal anti-mouse antibodies against MGL1 and F4/80 and their respective isotype controls were purchased from AbD Serotec (Raleigh, NC). Anti-mouse CCR2, its secondary (goat anti-rabbit IgG) antibody and isotype control antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Lethal Total Body Irradiation and Bone Marrow Transplantation (BMT) - Thirty-six, 6-week old WT mice were irradiated with a single 10 Gy dose from a ^{137}Cs γ source. Bone marrow cells were collected from eleven-week old WT and *db/db* mice by flushing long bones (femora, tibiae and humeri) with sterile PBS containing 50 µg/ml gentamycin (Sigma-Aldrich, St. Louis, MO). Recipient mice were each reconstituted with $2x10^7$ bone marrow cells by intravenous injection through the retro-orbital sinus. There were 16 mice in each WT bone marrow and *db/db* bone marrow treatment groups. Four irradiated mice were not reconstituted and used to confirm irradiation effectiveness. Reconstituted mice were housed in laminar flow isolators and received acidified water supplemented with 100 mg/l neomycin and 10 mg/l polymyxin B sulfate 1 week prior and 2 weeks post-irradiation according to an established protocol (Lim, Timmins et al. 2008). Thereafter, acidified water was provided for all mice. Mice were monitored for symptoms of radiation sickness and mortality. Reconstituted mice received an irradiated normal fat diet (NFD, D12450B, 10% kcal from fat, Research Diets Inc., New Brunswick, NJ) for six weeks at the end of which reconstitution was assessed. At that time, mice were moved to standard housing where half of each bone marrow reconstitution group was randomly assigned to

the NFD or switched to a high fat diet (HFD, D12492, 45 kcal% from fat, Research Diets Inc.) for an additional sixteen weeks. Sixteen additional age- and gender-matched WT mice received the same treatments and randomization without the irradiation and transplantation and served as the irradiation effect controls for the study. The study design is outlined in Figure 2.1.

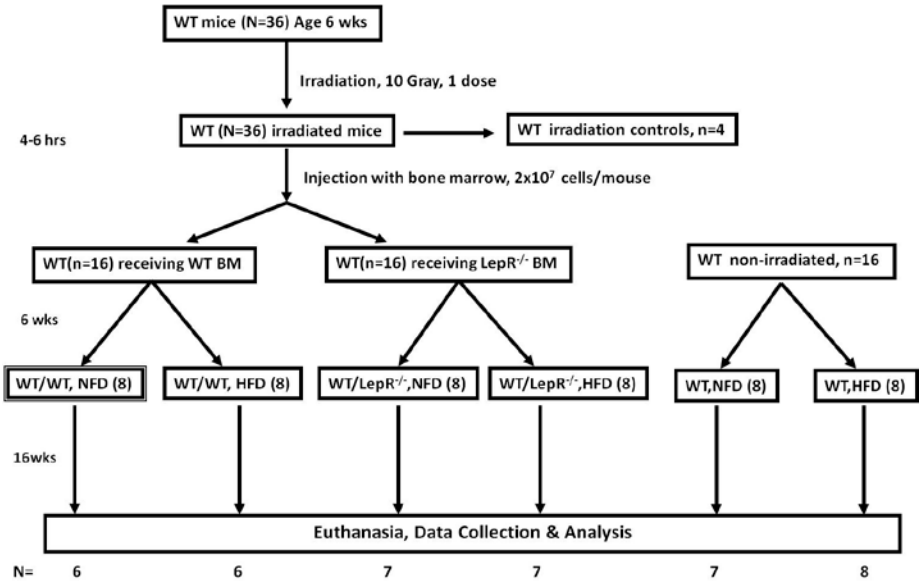

Figure 2.1 Diagram of the study
BM= bone marrow, $LepR^{-/-}$= leptin-receptor deficient

Assessment of Irradiation and Transplantation Effectiveness- Blood was collected through the retro-orbital sinus from non-transplanted mice three days post-irradiation to confirm that mouse hematopoietic systems were destroyed by radiation treatment. Blood was collected also from age, strain and gender-matched WT non-irradiated mice for comparison. Red blood cells were lysed in hypotonic buffer, centrifuged and the resulting pellet was assessed by flow cytometry (FACSCalibur, Becton Dickson, San Jose, CA) for the presence of CD3, B220, F4/80 and Ly6G to determine the percentages of T-cells, B-cells, macrophages and neutrophils. Blood was collected from all transplanted mice six weeks post-irradiation and T-cell and B-cell percentages were assessed using similar techniques.

Anthropometric and Serological Measurements- Body weight and food intake were measured weekly. Blood was collected at 0, 8 and 16 weeks after the dietary intervention. Prior to each collection, mice were fasted for 4 hrs, anesthetized with isoflurane and eyes were treated with one drop of proparacaine hydrochloride (0.5%). Approximately 0.4 ml of blood was

collected from the retro-orbital sinus of each mouse. Serum was separated from clotted blood and stored at -80°C. Fasting blood glucose was measured at each blood withdrawal using a digital glucometer (Contour Blood Glucose Monitoring System, Bayer, Mishawaka, IN). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: fasting blood glucose (mg/dl) × fasting insulin (μU/ml)/405.

Serum Cytokine/Adipokine Analysis- Insulin, leptin, MCP-1, IL-6 and TNF-α were measured in serum from fasted mice by fluorescent immunoassay using a Milliplex MAP kit for mouse serum adipokine (Millipore, St. Charles, MO) according to the manufacturer's instructions. The samples were processed on a Luminex 100, and results were analyzed with MasterPlexQT software (MiraiBio, South San Francisco, CA, USA).

Adipose Tissue Digestion, Adipocyte Collection and ATM Sorting- Epididymal, inguinal, mesenteric and retroperitoneal fat pads were dissected from all mice, weighed, and were collectively constituted the total dissectible fat. Epididymal and inguinal fat pads were finely minced and added to Krebs Ringer buffer (KRB) solution supplemented with 1 mg/ml type II collagenase (Sigma-Aldrich). Homogenates were incubated at 37°C for 10 minutes and then moved to a shaking incubator for an additional 40 minutes incubation at 37°C with agitation at a speed of 0.1 x *g*. The cell suspension was filtered through nylon cell strainers (100 μm; Fisher Scientific, Pittsburg, PA) and centrifuged at 300 x *g* for 1 minute. Floating adipocytes were collected and washed with KRB and RNA was extracted using TRI reagent (MRC, Cincinnati, OH) similar to the method described by Chomczynski et al. (Chomczynski and Sacchi 1987). The stroma-vascular fraction (SVF) pellet was washed twice with KRB. Cells were blocked with 50% normal goat serum in PBS for 30 minutes at 4°C and incubated with F4/80 and CD11b antibodies for 1 hour at 4°C followed by 2 washes in PBS. ATMs were then sorted by co-expression of F4/80 and CD11b using a MoFLO XDP cell sorter (Becton Dickinson, Franklin Lakes, NJ). Double positive cells were sorted directly into TRI reagent for RNA extraction.

RNA Isolation and Gene Expression Quantification- RNA was extracted from adipocytes and sorted macrophages as described above. RNA was purified and DNAse treated using E.Z.N.A Total RNA kit and RNase Free DNase Set (Omega Bio-Tek, Norcross, GA). RNA quality was assessed by microfluidics (Agilent Bioanalyser, Agilent Technologies, Bracknell, UK) and RNA was considered of acceptable quality if the RNA integrity number (RIN) was equal or higher than 8. RNAs were reverse transcribed into cDNA with the RT² First Strand

cDNA Kit (SABiosciences, Frederick, MD). cDNA of transcripts of interest were assessed by real-time PCR using a customized SYBR Green real-time PCR detection superarray from SABiosciences (Frederick, MD). The superarray format was a 96-well, 12 genes/8 samples template. Sequences coding for *tnfa, il6, Ccl2, Ccr2, mgl1, lep, lepr*, *adipoq* and *Pparg* were targeted by the assay. *Actb* (β-actin) was used as the internal standard for normalization and RT and genomic DNA were assessed for each sample as quality controls. Fluorescence was assessed on a real-time thermal cycler (iCycler, Bio-Rad, Hercules, CA). Results were calculated using the $2^{-\Delta\Delta CT}$ method as directed by the superarray manufacturer protocol and expressed in fold increase/decrease of the gene of interest in all groups vs. the non-irradiated NFD group.

Immunohistochemistry and Adipose Tissue Morphology- Samples from epididymal fat were fixed in 10% formaldehyde for 24 hours prior to embedding in paraffin. A total of 4 sections, 100 µm apart, were cut per mouse tissue. Each section was 10 µm thick. Sections were deparaffinized with xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by immersing the slides in citrate buffer (10 mM citric acid, 0.05% Tween20, pH 6.0) for 30 minutes at 90-95°C. Non specific binding was blocked by incubating the slides for 30 minutes in homologous serum at 37°C. Three sections per mouse were incubated overnight with anti-mouse F4/80, MGL-1 and CCR2 antibodies and 1 section with the respective isotype controls at 4°C. The CCR2 secondary antibody was added and slides were incubated for 1 hour at room temperature. Non-labeled sections were subjected to the same treatment with no antibody added to determine the background autofluorescence level of the adipose tissue. Microscope light settings were set to minimize autofluorescence and fluorescence from non-specific antibody binding. Samples were examined using a laser scanning confocal microscope (Zeiss LSM 5 PASCAL, Zeiss, Thornwood, NY). Aggregates of macrophages surrounding a single adipocyte known as crown-like structures (CLS, (Cinti, Mitchell et al. 2005) were identified by the presence of F4/80 and CCR2 on cells forming the "crowns" surrounding adipocytes. Adipocyte size was obtained from perimeter tracings using Image J software. Fat cell number was calculated per cm^2 of adipose tissue in each field.

Liver pathology- Entire livers were dissected from each animal and weighed. Approximately, a 200 mg liver sample was fixed in 10% formaldehyde for 24 hours and embedded in paraffin. Deparrafinized 10 µm sections were H&E stained. Samples were coded and scored by two blinded observers for microvesicles (lipid droplets with visible nuclei),

macrovesicles (lipid droplets with no visible nuclei) and structure (irradiating structure from visible blood vessels) for 2 sections per mouse. The rating scale ranged from 1 to 3, with 1 being the least amount of micro- and macrovesicules and the most conserved structure and 3 the highest amount of micro-and macrovesicules with a disrupted structure of hepatocytes.

Statistical Analysis- Anthropometric and serologic data were analyzed as an analysis of covariance model (ANCOVA) with a 2 (diet) x 3 (irradiation) factorial treatment structure in a completely randomized design. Initial weight was the covariate and was used to calculate adjusted treatment means. When the diet x Irradiation interaction was significant contrasts on the adjusted diet x irradiation means were performed to test whether the change in responses between HFD and NFD is the same magnitude between the three irradiation treatments. The ANCOVA analysis was performed using the GLM procedure in SAS (SAS Institute Inc. Cary, SC). For all other response variables (for which adjustment for initial weight was not necessary), regular analysis of variance (ANOVA) was performed and differences between the six treatments were calculated using the LSD method. The ANOVA analysis was performed using SPSS software (SPSS Inc. Chicago, IL). For all analyses, statistical significance was considered at $p<0.05$.

Results

Total Body Irradiation and BMT

Mice were irradiated and reconstituted with bone marrow from WT and *db/db* donors to test the hypothesis that leptin, through its long receptor, plays a role in macrophage recruitment and adipose tissue inflammation in obese animals. Unreconstituted mice had no (<1% of normal) circulating leukocytes, T-cells, B-cells, neutrophils or macrophages by day 3 post-irradiation (data not shown). Therefore, the residual recipient leukocytes contamination was minimal in these radiation chimeras. At 6 weeks post-transplantation, irradiated mice were completely reconstituted. There were no statistical differences in either B-cell percentages (56.1 ± 10.3% vs. 51.8 ± 8.9%) or T-cells percentages (16.3 ± 3.4% vs. 17.6 ± 2%) between transplanted and non-irradiated control mice, respectively. Therefore, mice were considered immunosufficient, moved

to standard facilities, and divided into NFD and HFD treatment groups as described in the Materials and Methods (Figure 2.1).

Mice Reconstituted with Leptin Receptor-Deficient Bone Marrow were Resistant to Weight and Fat Mass Gain after 16 Weeks HFD

Non-irradiated and reconstituted irradiated mice were weighed and food intake was assessed weekly to test whether transplantation with leptin receptor-deficient bone marrow or whole body irradiation affects weight gain. Fat pads were dissected and weighed at study termination. Six weeks after bone marrow reconstitution, irradiated mice weighed significantly less that the non-irradiated controls (23.9 ± 1.2 vs. 28.3 ± 0.9 g respectively, $p<0.0001$). There were no signs of malabsorption such as diarrhea or bloody stools in any of the mice. Due to the difference in starting weights, all comparisons were made after adjusting for baseline weight using ANCOVA (Raubenheimer 1992). We assessed the effect of both diet (HFD vs. NFD) and irradiation regimen (Irr-WT, Irr-*db* and Non-Irr) and the interaction of both (Diet x Irradiation). Both the model and the Diet x Irradiation interaction were significant for weight and fat mass parameters ($p<0.05$, Figure 2.2). This shows that there is combined effect of both diet and irradiation on weight and fat mass beyond the added effect of diet and irradiation alone. Contrasts analysis (Abdi & Williams 2010) was performed, in case of a significant model, to assess whether the measured difference between HFD and NFD is statistically different between the 3 irradiation treatment groups (Non-Irr, Irr-WT and Irr-*db*). The difference in mean adjusted final weights between HFD and NFD was smaller in the Irr-*db* group (32 ± 1g vs. 31 ± 1g) compared to either the Irr-WT (35 ± 1 vs. 31 ± 1, $p<0.05$) or the Non-Irr groups (35.5 ± 1 vs. 28 ± 1, $p<0.002$) (Figure 2.2-A). Significant differences were not observed in weight and weight change between Non-Irr and Irr-WT groups ($p>0.1$). The same was seen in adjusted total dissectible fat, epididymal and inguinal fat depots where HFD feeding had the least effect in the Irr-*db* group compared to Irr-WT ($p>0.05$) and Non-Irr groups ($p<0.02$) (Figure 2.2-D, E, F). Differences in weight and fat mass in the different irradiation groups were not a result of caloric intake given that no statistical difference in overall caloric intake was calculated between the different treatment groups ($p>0.05$, Figure 2.2- C). These data suggest that replacing the bone marrow with leptin receptor-deficient cells provided some resistance to high fat diet-induced weight and fat mass gain.

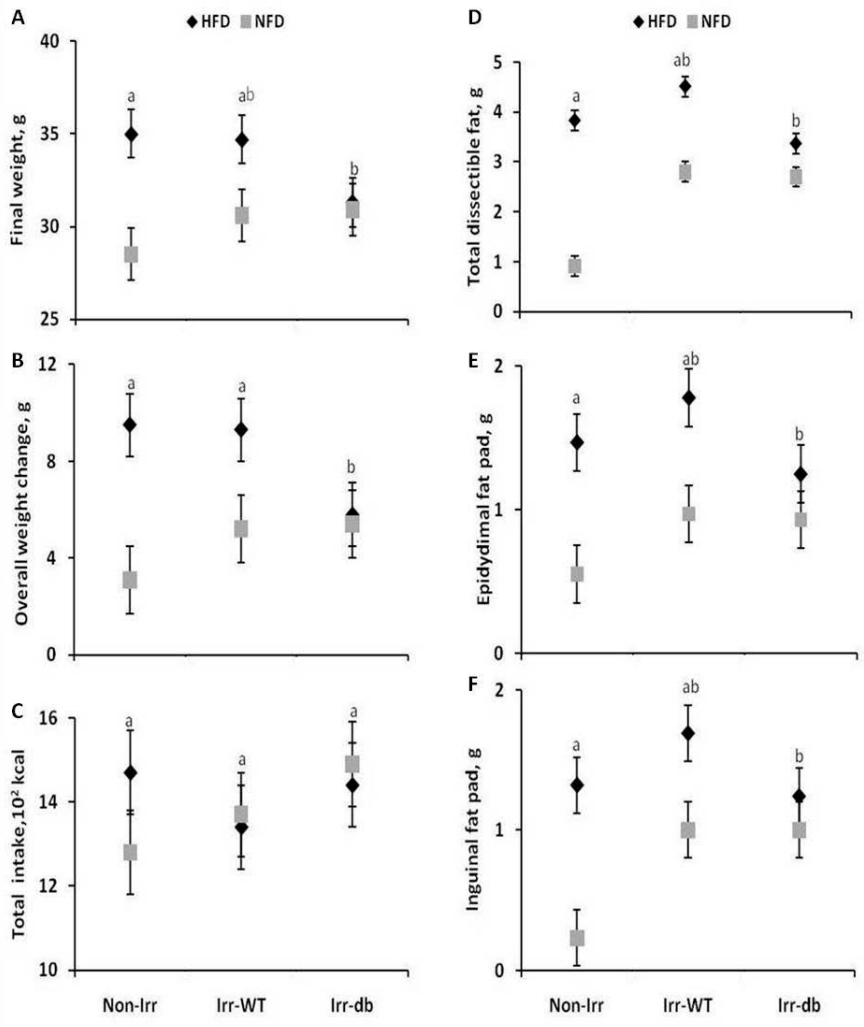

Figure 2.2 Mice reconstituted with leptin receptor-deficient bone marrow (Irr-*db*) have minimal difference between HFD and NFD in weight (A), total weight change (B), total dissectible fat (D), epididymal (E) and inguinal fat pads (F) compared to Non-Irr and Irr-WT mice.
Analyses are done for means adjusted for initial weight using 2 way interaction (Diet and Irradiation) and comparing mean differences within irradiation groups using contrast analysis (see Materials and Methods). Groups with different letters have means differences between HFD and NFD significantly different ($p<0.05$).

Adipocyte Size Increased in Irr-db Mice on HFD without Change in Fat Pad Weight

Adipose tissue morphology in epididymal fat pads of all mice was examined to determine if the smaller weight and fat mass gain in Irr-*db* mice was secondary to adipocyte dysregulation. There was a decrease in the number of adipocytes/cm^2 of tissue (31.3 ± 5.2 vs. 19.2 ± 3.3 and 45.6 ± 5.5 vs. 24.9 ± 5.1, $p<0.05$) and a corresponding increase in adipocyte mean size (5.2 ± 1.6 vs. 2.6 ± 0.4 and 3.7 ± 1.2 vs. 1.8 ± 0.2, $p<0.05$) in Non-Irr and Irr-*db* HFD groups, respectively, compared to their NFD controls (Table 2.1 and Figure 2.3).

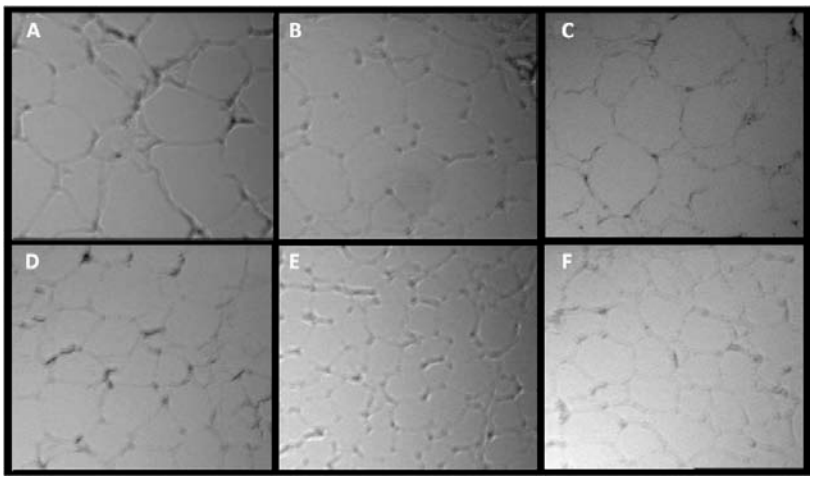

Figure 2.3 A HFD leads to an increase in adipocyte size in all mice.
Deparaffinized epididymal fat sections (10µm thick) visualized with a confocal microscope at 200 magnification. Representative picture from each experimental group. A,B,C HFD non-irradiated, irradiated with WT bone marrow and irradiated with db/db bone marrow. D,E,F NFD non-irradiated, irradiated with WT bone marrow and irradiated with db/db bone marrow

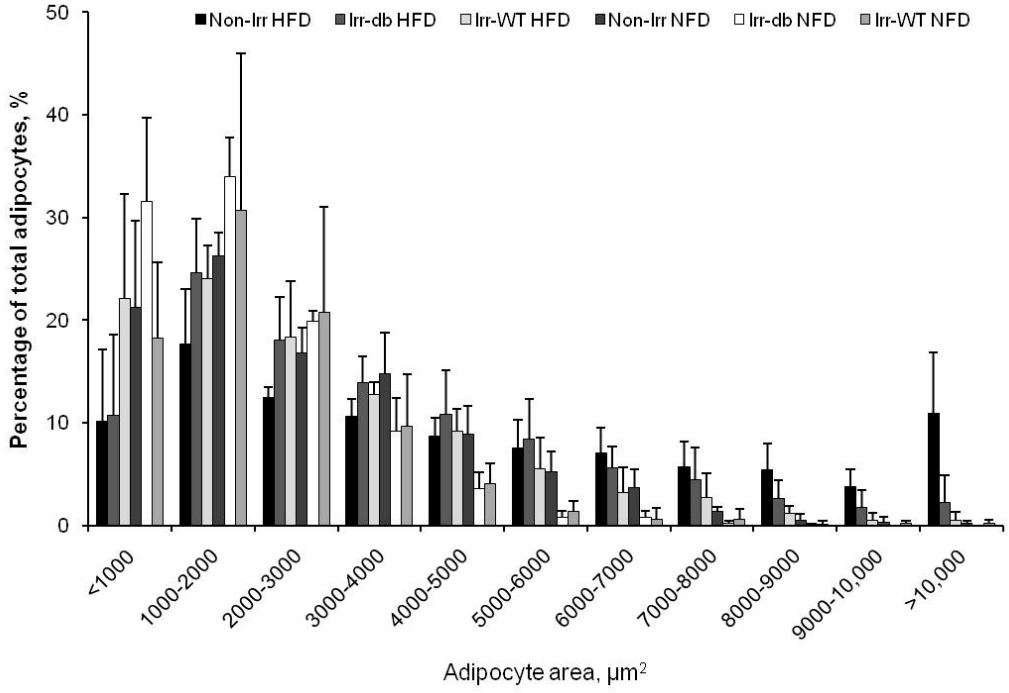

Figure 2.4 A HFD in Non-Irr and Irr-db mice led to an increase in the percentage of larger adipocytes.
Adipocyte size distribution by area size (μm^2) measured using Image J. Thirteen to fifteen photomicrograps were examined per mouse.

Table 2.1 Epididymal adipose tissue morphology, adipocyte mean size and number per group.

Parameter	Non-Irradiation		Irradiated-WT		Irradiated-db		p-value*
	HFD	NFD	HFD	NFD	HFD	NFD	
Epididymal fat pad (g)	$2.3 \pm 0.3^{a,\#}$	1.1 ± 0.2^{d}	$1.4 \pm 0.4^{b,\#}$	$0.5 \pm 0.1^{d,e}$	0.9 ± 0.4^{b}	0.5 ± 0.0^{e}	0.001
Number of adipocytes/cm^2 ($x10^3$)	$19 \pm 3^{a,\#}$	31 ± 5^{d}	32 ± 7^{b}	$40 \pm 3^{d,e}$	$25 \pm 5^{a,b,\#}$	46 ± 5^{e}	0.0001
Mean adipocyte area (μm^2) ($x10^3$)	$5.2 \pm 1.6^{a,\#}$	2.6 ± 0.4	2.7 ± 0.6^{b}	2.0 ± 0.2	$3.7 \pm 1.2^{a,b,\#}$	1.8 ± 0.2	0.001

*p-value between groups as per ANOVA

a,b &d,e groups with same superscript are not statistically different as per LSD at $p<0.05$ (a,b for HFD groups and d,e for NFD groups). #statistically different from its NFD group as per LSD.

Both Non-Irr and Irr-*db* groups on HFD had a larger fat cell size distribution compared to their NFD controls (Figure 2.4). This trend was less pronounced in the Irr-WT group which had the fewest number of adipocytes larger than $8000\mu^2$ (Figure 2.4). Hence, irradiation did not compromise the ability to store, synthesize or take up triacylglycerol (TAG). In particular, the Irr-*db* group responded to the HFD feeding in a similar fashion as the Non-Irr group as judged by their increased adipocyte size. Although Irr-*db* mice had increased numbers of large adipocytes with a significant increase in mean adipocyte area when on a HFD compared to Irr-*db* on NFD, the total epididymal fat pad weight did not differ significantly between Irr-*db* HFD and NFD groups (0.9 ± 0.4 vs. 0.5 ± 0.04 g, $p>0.05$) (Table 2.1). On the other hand, no significant difference in adipocytes size and number per area was measured between HFD and NFD in the Irr-WT treatment group. However, epididymal fat pads weights were significantly higher in the HFD group compared to its NFD control (1.4 ± 0.4 vs. 0.5 ± 0.1 g, $p<0.01$, Table 2.1). These data suggest that adipose tissue in the Irr-*db* mice responded differently to HFD than the Irr-WT group.

Liver Histology Shows no Increase in Ectopic Fat Deposition in Irradiated Mice

Fat is stored in the liver when the adipose tissue fails to take up fatty acids efficiently secondary to adipocytes dysfunction. Liver steatosis was measured in all mice to determine whether adipocyte fatty acid uptake was altered by irradiation. The lower fat mass gain in the Irr-*db* mice on a HFD would be expected to lead to ectopic fat deposition in the liver if it were functioning normally. All groups accumulated some fat in the liver, and all mice on a HFD had a higher fat content than their NFD counterparts as shown by the presence of large enucleated fat droplets or macrovesicules (Figure 2.5).

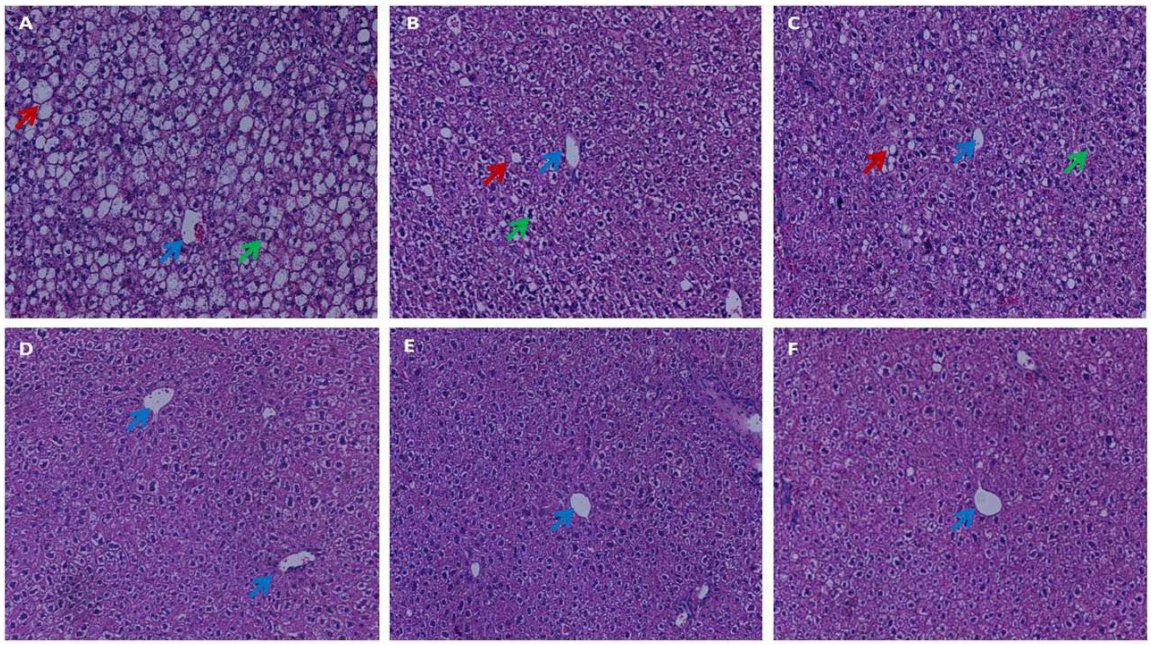

Figure 2.5 Irradiated mice on HFD show less fat deposition in the liver compared to the non-irradiated group. Deparaffinized liver sections (10µm sections) observed under inverted microscopy in brightfield at 200 magnification. A, B, C: HFD non-irradiated, irradiated db, irradiated WT respectively. D, E, F: NFD non-irradiated, irradiated db, irradiated WT respectively. Red arrows point at examples of macrovesicules, green arrows at examples of microvesicules and blue arrows at blood vessels.

Liver weights, and liver weights as percentage of the total body weight were highest in the Non-Irr group on HFD compared to the 2 irradiated groups on HFD (2.1 ± 0.6 vs. 1.3 ± 0.3 and 1.2 ± 0.2 g; 4.9 ± 0.6 vs. 4.1 ± 0.6 and 4.3 ± 0.4 % respectively, $p<0.05$, Table 2.2). Structure and microvesicules were not different in any of the groups ($p>0.05$). However, significantly more macrovesicules, a measure of more severe steatosis, were observed in the Non-Irr HFD group compared to its NFD counterparts (2.44 ± 0.6 vs. 1.0 ± 0.0, $p<0.05$, Table 2) and compared to the both Irr-WT and Irr-*db* on HFD (2.44 ± 0.6 vs. 1.75 ± 0.6 and 1.81 ± 0.7 respectively, $p<0.05$, Table 2). Irr-*db* on HFD also had a higher score for macrovesicules compared to its NFD control (1.81 ± 0.7 vs. 1.06 ± 0.2, $p<0.05$, Table 2.2). These data show that that there was no compensatory uptake of fatty acids in the livers of irradiated mice.

Table 2.2 Liver steatosis parameters

Parameter	Non-Irradiation		Irradiated-WT		Irradiated-db		
	HFD	NFD	HFD	NFD	HFD	NFD	p-value*
Liver weight(g)	$2.1 \pm 0.6^{a,\#}$	1.6 ± 0.2^{d}	1.3 ± 0.3^{b}	$1.4 \pm 0.2^{d,e}$	1.2 ± 0.2^{b}	1.3 ± 0.2^{e}	0.001
Percentage liver weight/body weight (%)	4.9 ± 0.6^{a}	4.8 ± 0.4	$4.1 \pm 0.6^{b,\#}$	5.1 ± 0.4	$4.3 \pm 0.4^{b,\#}$	5.1 ± 0.5	0.001
Microvacuoles score&	2.7 ± 0.5	2.3 ± 0.6	2.4 ± 0.8	2.4 ± 0.7	2.7 ± 0.4	2.6 ± 0.6	NS
Macrovacuoles score	$2.4 \pm 0.6^{a,\#}$	1.0 ± 0.0	1.7 ± 0.6^{b}	1.5 ± 0.5	$1.8 \pm 0.7^{b,\#}$	1.1 ± 0.2	0.001
Structure score	1.6 ± 0.7	1.3 ± 0.5	1.6 ± 0.6	1.7 ± 0.6	1.7 ± 0.6	1.2 ± 0.4	NS

*p-value for ANOVA between all groups

$^{a,b\ \&d,e}$ groups with same superscript are not statistically different as per LSD at $p<0.05$, (^{a,b} for HFD groups and ^{d,e} for NFD groups). $^{\#}$statistically different from its NFD group as per LSD. $^{\&}$The overall score was calculated as the mean value from 2 observers rating 5-6 mouse samples per group (2 slides per mouse). The scoring system was from 1 to 3, number 1 for the least amount of microvacuoles, macrovacuoles and the best conserved structure and 3 for the highest presence of microvacuoles, macrovacuoles and the disrupted structure.

Gene Expression in Irr-db Adipocytes Shows a Blunted Increase in Inflammatory Genes Compared to Irr-WT Adipocytes

Adipose tissue during obesity is characterized by an increase in inflammatory gene expression. Adipocyte transcript expression was calculated for relevant genes in obesity-associated inflammation to see if leptin receptor expression had an impact on the level of inflammation in adipocytes. Epididymal adipocytes were analyzed and gene transcript levels were assessed using a customized superarray as described in the Materials and Methods. Non-Irr mice on the NFD were used as controls for calculations of fold changes for the specific genes of interest since they constitute age, gender and strain-matched counterparts for all groups. TNF-α and IL-6 were increased in the Non-Irr on HFD compared to the NFD control (41 fold, $p<0.05$, and 6 fold respectively, Table 2.3). In the Irr-WT on HFD, both TNF-α and IL-6 were increased (4 and 3 fold respectively, Table 2.3) whereas in the Irr-*db* group on HFD group this increase was only by 2 fold for both (Table 2.3). Leptin gene expression was increased 2 fold in the HFD groups except for the Irr-*db* group consistent with the adipose tissue data showing no increase in fat mass in the group on HFD. Adiponectin, an anti-inflammatory adipokine (Mandal, Pratt et al.), was significantly decreased in the Non-Irr group on the HFD (2 fold, $p<0.05$) with no change in the other groups. CCL2 and its receptor CCR2 were both upregulated in Non-Irr and Irr-WT groups on HFD (5 to 8 fold increase and 3 to 5 fold increase respectively, Table 2.3). No change was measured in the Irr-*db* groups (Table 2.3). Peroxisome proliferator-activated receptor gamma (PPARγ) involved in adipogenesis and lipid uptake in adipocytes (Barak, Nelson et al. 1999; Rosen, Sarraf et al. 1999) was upregulated in all irradiation groups regardless of the dietary allocation (2 to 3 fold increase, Table 2.3). These data suggest that replacing the bone marrow with leptin receptor-deficient cells led to an attenuation of inflammatory and insulin resistance parameters at the adipocytes level.

Table 2.3 qRT-PCR-based fold differences in mRNA abundance in epididymal fat pad adipocytes in control (Non-Irr on NFD) vs.all other groups

Gene	Non-Irr HFD	Irr-WT-HFD	Irr-WT NFD	Irr-*db* HFD	Irr-*db* NFD
Tnf	↑41*	↑4	↑2	↑2	No change#
Il6	↑7	↑3	↑3	↑2	No change
Lep	↑2	↑2	No change	No change	No change
lepr	↑3	↑2	↑5	No change	↑2
Ccl2	↑5**	↑3	↑2	No change	No change
Ccr2	↑8	↑5*	↑3	No change	No change
Mgl1	↑4***	↑2	↑2	No change	No change
Pparg	No change	↑2	↑3	↑2*	↑2*
Adipoq	↓2	↑2	No change	No change	No change
Actb	No change	No change	No change	No change	No change

*p<0.05 , **p<0.01, ***p<0.001 vs Non-Irr NFD group
#No change was assumed if fold change was equal or less than 1.5

Irradiated Mice with db/db Bone Marrow have Less Inflammatory ATMs and Exhibit Fewer CLS in their Adipose Tissue

Obesity is associated with increased pro-inflammatory ATMs. These ATMs form CLS surrounding dying adipocytes (Cinti, Mitchell et al. 2005). We tested whether the deficiency of LepRb in monocytes/macrophages affected the inflammatory phenotype of adipose tissue under a HFD. Cells in the SVF from collagenase-digested fat pads were labeled with F4/80 and CD11b antibodies to identify double positive macrophages by FACS analysis. No difference in percentage $F4/80^+/CD11b^+$ ATMs was measured between HFD and NFD in either Irr-WT or Irr-*db* groups (ANOVA, $p>0.05$, Table 2.4). As expected, the percentage ATMs in epididymal fat was more than 3-fold higher compared to that in inguinal fat pads (average 31 ± 3 vs. 8 ± 2, $p< 0.0001$, Table 2.4) with no statistical difference between any of the groups.

These data suggest that replacing the bone marrow with leptin receptor-deficient cells shifted ATMs towards an anti-inflammatory phenotype in DIO mice.

Table 2.4 Percentage sorted $CD11b^{+}/F4/80^{+}$ macrophages from epididymal and inguinal fat depots of all mice.

Parameter	Non-Irradiation		Irradiated-WT		Irradiated-db		p-value*
	HFD	NFD	HFD	NFD	HFD	NFD	
Epididymal fat	34 ± 4&	26 ± 2	33 ± 7	32 ± 10	33 ± 7	28 ± 8	NS
Inguinal fat	10 ± 3.5	6 ± 4	10 ± 7	6 ± 4	10 ± 10	6 ± 4	NS

*ANOVA done for each fat pad between all groups
& p,0.05 between HFD and NFD group within same irradiation group as per student t-test

Inguinal fat contained fewer macrophages than epididymal fat and displayed no increase in inflammatory or metabolic genes where animals received a HFD. Therefore, we determined macrophage localization and phenotype only in the epididymal adipose tissue using immunohistochemistry (IHC). Deparaffinized epididymal fat sections were probed for F4/80 as the general marker for macrophages, CCR2 as the marker for Type I or pro-inflammatory macrophages and Mgl1 as the marker for Type II or anti-inflammatory macrophages using a laser scanning confocal microscope. The Non-Irr group showed the highest amount of labeling for both F4/80 and CCR2 antibodies. Mice receiving the HFD had increased labeling of F4/80 and CCR2 compared to NFD groups in both the Non-Irr and Irr-WT groups. Mice in the Irr-*db* group showed no labeling for CCR2 in either the HFD or NFD groups. All $F4/80^{+}$ macrophages were $Mgl1^{+}$ and $CCR2^{-}$ (Figure 2.6). These data demonstrate that ATMs in irradiated mice on a HFD display a pro-inflammatory $CCR2^{+}$ phenotype after reconstitution with WT BM but an anti-inflammatory $Mgl1^{+}$ phenotype after reconstitution with *db/db* BM. To confirm that ATMs in Irr-db mice on HFD were different from WT reconstituted chimeras we examined CLS in epididymal fat pads. The number of CLS/section in Irr-*db* mice on HFD was significantly less than the number in both Irr-WT mice on HFD (0.2 ± 0.5 vs. 0.6 ± 0.8, $p<0.05$, Figure 2.7) and the Non-Irr group on HFD (0.2 ± 0.5 vs. 1.7 ± 1.3, $p<0.05$, Figure 2.7).

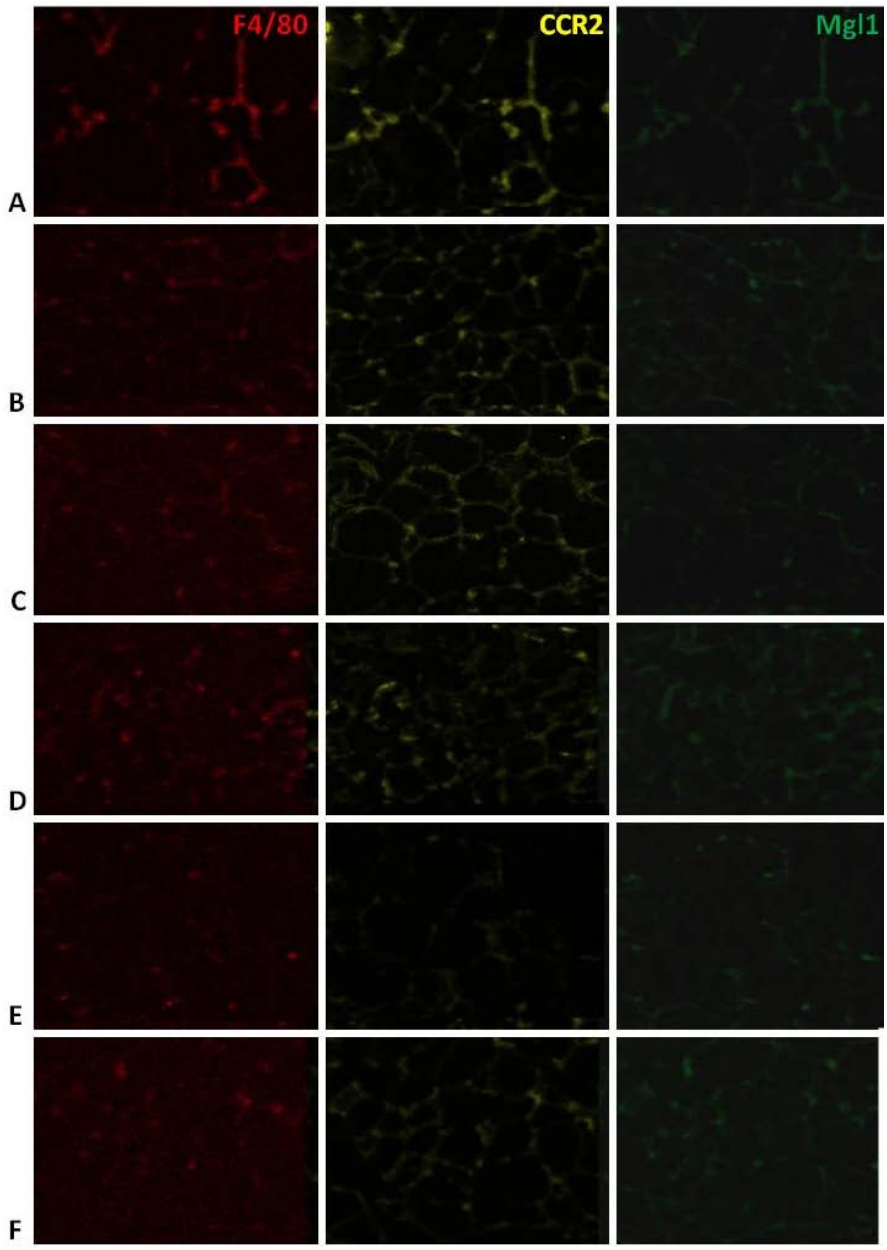

Figure 2.6 Irr-*db* mice on a HFD (panels E) have a prevalence of $CCR2^{-}$/ $Mgl1^{+}$ anti-inflammatory $F/4/80^{+}$ macrophages.
Paraffin-embedded epididymal fat sections (10µm sections) observed under laser scanning confocal microscope at 200 magnifications. A= Non-Irr HFD, B=Non-Irr NFD, C=Irr-WT HFD, D=Irr-WT NFD, E=Irr-*db* HFD, F=Irr-*db*

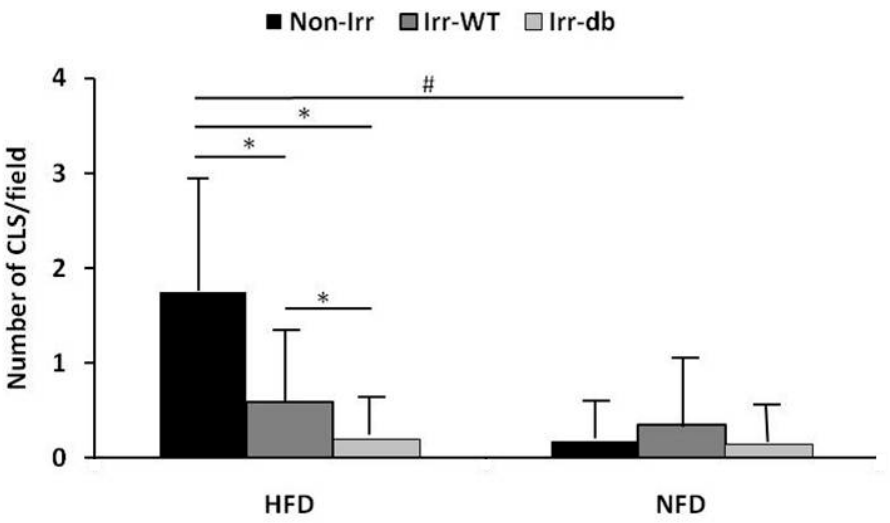

Figure 2.7 Irr-*db* mice on HFD have fewer crown-like structures (CLS) in epididymal fat pads.
Quantification of CLS/field in epididymal fat of all experimental mice. *Significance as per LSD within same diet group, p>0.05. # Significance as per LSD within same irradiation group, p>0.05

Irr-db Mice have an Improved Systemic Metabolic Profile

Diet-induced obesity leads to systemic metabolic and inflammatory disturbances. We measured fasting serum levels of TNF-α, IL6, MCP-1, leptin, insulin and glucose in all mice at 0, 8 and 16 weeks of the study. No differences were observed in IL-6, TNF-α or MCP-1 amongst any of the groups at any measured time point (data not shown). Paralleling fat mass data, leptin levels were highest in the Non-Irr HFD group compared to Irr-WT HFD and Irr-*db* HFD (40 ± 7, 22 ± 8 and 12 ± 9 ng/ml respectively, $p<0.01$, Figure 2.8). Fasting insulin and the HOMA-IR index ere measured to assess glucose metabolism in all experimental mice. Both measures were highest in the Non-Irr HFD group compared to both Irr-WT HFD and Irr-*db* HFD groups (4 ± 2 ng/ml vs. 2 ± 0.5 ng/ml and 1.3 ± 0.2 ng/ml for insulin respectively and 48 ± 32 vs. 22 ± 6.5 and 16 ± 5 for HOMA-IR respectively, $p<0.01$, Figure 2.8). Contrast analysis as described in Materials and Method was performed for these 2 parameters at week 16 to test if the magnitude change between HFD and NFD was similar across the 3 groups. The Irr-*db* treatment group had the smallest increase in these 2 parameters and a blunted response after a HFD with the difference between HFD and NFD almost reaching significance compared to that in the Non-Irr group (2 vs. 0.2 ng/ml difference for insulin, $p=0.07$ and 26 vs. 4 unit difference for HOMA-IR, $p=0.06$, Figure 2.8). No significant difference was measured between Non-Irr and Irr-WT (Figure 2.8).

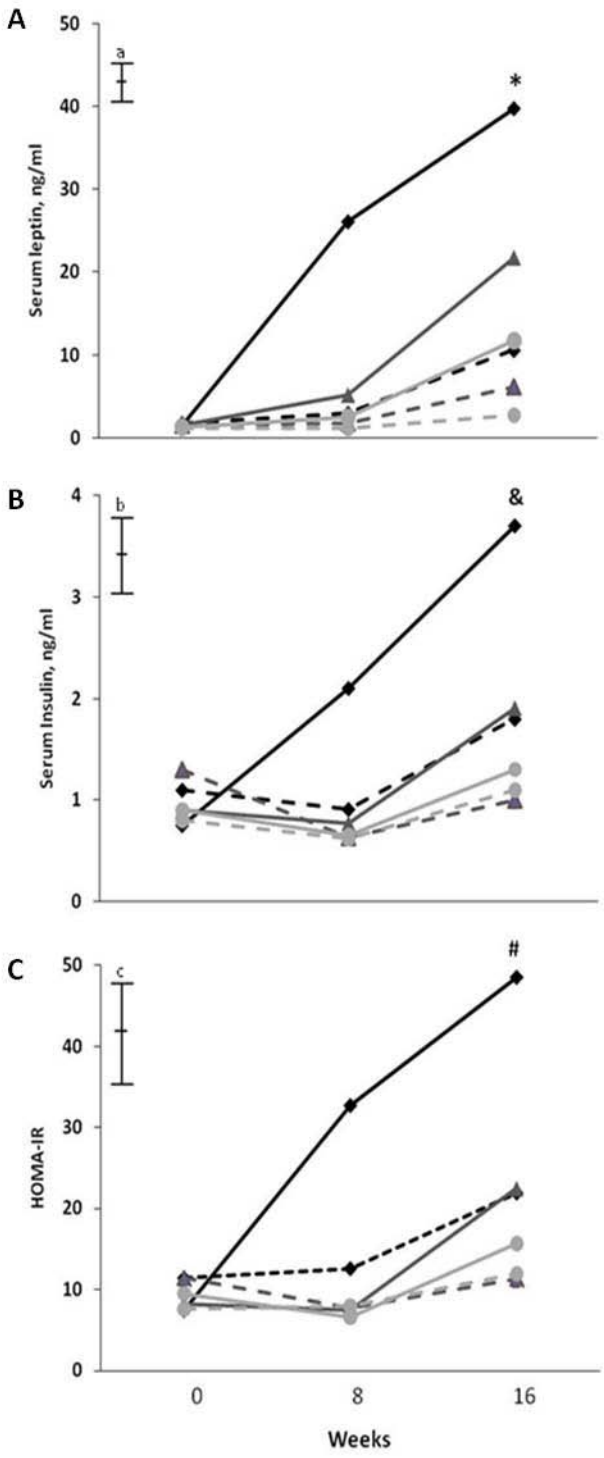

Figure 2.8 Mice reconstituted with leptin receptor-deficient bone marrow (Irr-*db*) have minimal difference between HFD and NFD in serum leptin (A), serum insulin (B), and HOMA-IR (C) compared to Non-Irr and Irr-WT mice. Fasting serum levels of leptin and insulin and measures of HOMA-IR at 0, 8 and 16 weeks of dietary treatment. Full lines represent HFD groups, dotted lines are for NFD groups. Black (♦) for Non-Irr, dark grey (▲) and light grey (●) for Irr-db. Difference between HFD and NFD significantly different in Irr-WT versus Irr-db at $^{*}p<0.05$. $^{\&}p=0.07$, $^{\#}p=0.06$. $^{a, b, c}$ SEM(±) for serum leptin, serum insulin and HOMA-IR respectively.

Discussion

Obesity induces a state of low grade inflammation arising from an inflamed adipose tissue characterized by the presence of inflammatory macrophages (Hotamisligil, Shargill et al. 1993; Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). The factors behind this increased recruitment are not completely defined, though many factors have been investigated. However, none of these factors are exclusively responsible for this process (Weisberg, Hunter et al. 2006; Kintscher, Hartge et al. 2008; Pang, Gao et al. 2008; Rausch, Weisberg et al. 2008; Bertola, Deveaux et al. 2009; Kosteli, Sugaru et al. 2010; Kim, Jeong et al. 2011). We hypothesized that leptin plays an important role in the recruitment of inflammatory macrophages and the exacerbation of the resulting inflammatory and metabolic disturbances in obesity. We successfully constructed chimeric mice that lacked the LepRb on circulating leukocytes in order to test our hypothesis. This allowed us to examine the impact leptin has on the recruitment of ATMs in a DIO model. We concluded that the lack of LepRb expression on bone marrow cells attenuates the inflammatory profile of the adipose tissue and alleviates weight and fat mass gain in DIO mice.

The lack of leptin signaling on monocytes/macrophages was accompanied by significantly less CLS in epididymal fat of DIO mice. These data suggest that the absence of LepRb was diminishing the inflammation in the adipose tissue because CLS numbers correlate with adipose tissue inflammation (Strissel, Stancheva et al. 2007; Murano, Barbatelli et al. 2008). This conclusion is supported by a prevalence of $Mgl1^{+}$ macrophages (assessed by qRT-PCR and indirect immunofluorescence) with few $CCR2^{+}$ macrophages in epididymal fat depot. Mgl1 is associated with macrophages in the pre-obese fat and CCR2 is associated with pro-inflammatory macrophages (Lumeng, Bodzin et al. 2007). Normal mice and mice reconstituted with WT bone marrow had more $CCR2^{+}$ macrophages when fed a HFD. These molecular expression patterns are consistent with the description of macrophages in adipose tissue of obese and non-obese animals (Lumeng, Bodzin et al. 2007; Lumeng, Deyoung et al. 2007; Lumeng, DelProposto et al. 2008). These data are also consistent with observations that leptin induces a pro-inflammatory phenotype in T-cells (Lord, Matarese et al. 1998) and primes macrophages to secrete inflammatory cytokines in septic conditions (Shapiro, Khankin et al. 2010). In addition, we and others have demonstrated that leptin is a potent chemoattractant for monocytes/macrophages

(Curat, Miranville et al. 2004; Gruen, Hao et al. 2007) and it is a major chemokine in adipocyte conditioned medium (Chapter 4). Although the chimeric mice had a decreased inflammatory phenotype in ATMs after a HFD as measured by CLS numbers and $Mgl1^{+}/CCR2^{-}$ ATMs, we were not able to find a difference in the percentage $F4/80^{+}/CD11b^{+}$ sorted macrophages in the epididymal or inguinal SVF between treatment groups. Only normal non-irradiated mice on HFD had more $F4/80^{+}/CD11b^{+}$ macrophages in their epididymal fat compared to their NFD controls. It is possible that the isolation procedure with collagenase affected our ability to measure accurately surface expression of these molecules (Ruan, Zarnowski et al. 2003). Our data indicate that leptin contributes to the M1 to M2 switch of ATMs in DIO mice though more studies are warranted to assess the role of leptin in the actual recruitment process.

Mice with leptin receptor deficient bone marrow had minimal increase in adipocytes inflammatory genes transcript levels. TNF-α and Il-6 adipocyte gene transcript levels were increased in all mice on HFD but mice with leptin receptor deficient bone marrow had only a 2 fold increase in these transcript levels compared to a 4 fold increase in WT transplanted mice. These data also suggest that LepRb has an impact on macrophage activation. There is cross talk between macrophages and adipocytes, with each cell type affecting metabolism and secretory function of the other (Suganami, Tanimoto-Koyama et al. 2007; Yamashita, Soga et al. 2007; Xie, Ortega et al. 2010). The prevalence of anti-inflammatory macrophages in the adipose tissue of leptin receptor-deficient bone marrow mice might explain the attenuated inflammatory profile of adipocytes in this model. The local increase in TNF-α directly contributes to insulin resistance at the adipose tissue level through suppression of glucose transporter GLUT4 gene expression (Hauner, Petruschke et al. 1995; Xie, Ortega et al. 2010), induction of serine phosphorylation of insulin receptor substrate 1 (Hotamisligil, Peraldi et al. 1996) and stimulation of lipolysis in adipocytes (Feingold, Doerrler et al. 1992).

In addition to its role in promoting macrophage recruitment in obesity, CCL2 induces insulin resistance in an autocrine fashion on adipocytes by decreasing insulin-stimulated glucose uptake (Sartipy and Loskutoff 2003; Weisberg, Hunter et al. 2006). Though, we did not measure any of the downstream insulin resistance markers in fat tissue, both CCL2 and CCR2 gene transcript levels were increased in adipocytes following a HFD in all mice with WT bone marrow. No change in either of these gene transcript levels was detected in mice with leptin receptor-deficient bone marrow. The absence of change in this chemokine or its receptor on

adipocytes from leptin receptor-deficient bone marrow mice along with the minimal increase in TNF-α suggests that adipose tissue in the leptin receptor chimeric mice has greater insulin sensitivity compared to WT counterparts.

Along with an attenuated inflammatory phenotype in the adipose tissue, chimeric mice with leptin receptor deficient bone marrow were resistant to weight and fat mass gain when on a HFD. We believe that this effect is not secondary to the lethal total body irradiation intervention. A difference in body weight and fat pads weights was measured between HFD and NFD mice when they were transplanted with WT bone marrow. This difference that was blunted in the mice reconstituted with leptin receptor deficient bone marrow. This would be consistent with previous studies that concluded that limiting or preventing inflammation in fat may lead to a decrease in weight gain in rodent models of DIO (Davis, Gabler et al. 2008; Chiang, Bazuine et al. 2009).

Radiation had an impact on the mice used in this study. Total body irradiation by itself can affect adipose tissue and prevent weight gain with no effect on food intake or malabsorption complications (Ablamunits, Weisberg et al. 2007; Okada, Kobayashi et al. 2007; Poglio, Galvani et al. 2009). All the irradiated mice exhibited a stunted growth following the six weeks of reconstitution. Nevertheless, mice reconstituted with WT bone were responsive to the HFD and gained weight until the end of the sixteen weeks of dietary intervention though the magnitude was lower than that observed in the non-irradiated mice. At the end of the study, both subcutaneous (inguinal) and intra-abdominal (epididymal) adipose tissue depots also seemed to be affected by radiation treatment. Nevertheless, this effect cannot account for the total absence of weight gain in the leptin receptor-deficient bone marrow mice, suggesting leptin signaling in bone marrow cells to be an independent factor in affecting weight gain as a response to a HFD. Studies looking at the effect of radiation on anthropometric parameters in mice showed that in the short term (7 days), the lack of weight gain was due to a decrease in both fat cell size and number (Poglio, Galvani et al. 2009) while long term studies showed either no difference in adipocytes morphology between irradiated and non-irradiated groups (Okada, Kobayashi et al. 2007) or a decrease in adipocyte hyperplasia but not hypertrophy (Ablamunits, Weisberg et al. 2007). None of the previous studies examined the combined irradiation and diet-induced obesity in mice. The long-term studies used *ob/ob* mice (Ablamunits, Weisberg et al. 2007; Okada, Kobayashi et al. 2007) while short-term studies looked at WT mice (Poglio, Galvani et al. 2009). None of the studies included dietary intervention. In our study, irradiated mice on NFD

transplanted with either WT or *db/db* bone marrow gained less fat mass accompanied by decreases in adipocyte number and size in the epididymal depot. When switched to a HFD, Irr-WT and Irr-*db* mice responded differently. While no significant difference in fat pad weight between HFD and NFD was detected in Irr-*db* mice, HFD led to an increase in adipocyte size with a slight decrease in adipocyte number. In the Irr-WT mice the opposite was observed, as HFD increased fat pad weight due to hyperplasia with no effect on adipocyte size. The lack of weight and fat mass gain in Irr-*db* mice cannot be explained by adipocyte dysfunction because TAG uptake and adipogenesis were not compromised. This hypothesis is also supported by PPAR-γ gene transcript level in adipocytes, the transcription factor involved in adipogenesis and adipocyte differentiation (Barak, Nelson et al. 1999; Rosen, Sarraf et al. 1999). PPAR-γ gene transcript level was increased in all irradiated mice irrespective of dietary or bone marrow treatment. In addition, the absence of enhanced ectopic fat deposition in the livers of the irradiated mice also supports the conclusion of a functional adipose tissue in these mice.

Our chimeric mice were more insulin sensitive compared to the WT mice based on HOMA-IR and insulin measures. This supports the role for the LepRb in the metabolic disorders caused by obesity and a HFD which generally lead to tissue specific and general insulin resistance. Other studies looking at DIO and insulin resistance used a higher fat concentration in their diets (60%) (Weisberg, Hunter et al. 2006; Westcott, Delproposto et al. 2009; Yang, Youm et al. 2010) compared to our study (45% calories from fat). Therefore, use of a more aggressive dietary approach could potentially lead to a higher difference between the different HFD treatment groups.

In summary, the LepRb contributed to the pathogenesis of obesity and the associated inflammatory phenotype in this study. Although the impact was masked by the radiation treatment, we have been able to dissect out another component in this complex physiological response. Future work may benefit by manipulation of leptin signaling in an adipose tissue specific manner as an experimental model and as a possible therapeutic target in efforts for the treatment of obesity and its complications.

Chapter 3 - Establishment and Characterization of DB-1: A Leptin Receptor Deficient Murine Macrophage Cell Line

Abstract

Metabolic and immune mediators activate many of the same signal transduction pathways. Therefore, molecules that regulate metabolism often affect immune responses. Leptin is an adipokine that exemplifies this interplay. Leptin is the body's major nutritional status sensor, but it also plays a key role in immune system regulation. We immortalized and characterized a leptin receptor-deficient macrophage cell line, DB-1, to provide an *in vitro* tool to investigate the link between leptin and innate immunity. The cell line was created using cells from the bone marrow of leptin-receptor deficient (*db/db)* mice. Bone marrow cells were differentiated into macrophages by culturing them with recombinant mouse monocyte colony stimulating factor, and passaged when confluent. This treatment was maintained for six months. The cells spontaneously immortalized at approximately passage 20. Cells were cloned twice by limiting dilution cloning prior to characterization. The macrophage cell line is diploid and has a three-day doubling time. The cells are MAC-2 and F4/80 positive and have phagocytic activity similar to primary macrophages from WT and *db/db* mice. DB-1 cells are responsive to stimulation with IFN-γ as measured by increase in *Nos2* transcript levels. In addition, DB-1 macrophages are not responsive to the chemotactic signaling of adipocyte conditioned media nor leptin as compared to primary WT macrophages. DB-1 cells provide a dependable tool to study the role of leptin or the leptin receptor in obesity-associated inflammation and immune system dysregulation.

Introduction

Leptin, the product of the *ob* gene, is a 16 kDa peptide hormone predominantly secreted by white adipocytes (Zhang, Proenca et al. 1994; Ahima and Flier 2000) and is structurally similar to type I cytokines (Otero, Lago et al. 2005). Leptin signals through its receptor (LepR)

encoded by the *db* gene (Chen, Charlat et al. 1996). The LepR exists in six alternatively spliced isoforms (Lee, Proenca et al. 1996). The long isoform, LepRb, is a member of the interleukin-6 receptor family of class 1 cytokine receptors, and is responsible for most of leptin's physiological activities (Tartaglia, Dembski et al. 1995; Baumann, Morella et al. 1996).

Leptin is involved in a plethora of physiological actions, and plays a major role in the regulation of neuroendocrine function and energy homeostasis (Chan, Heist et al. 2003). Leptin stimulates the production of anorectic neuropeptides and suppresses the action of orexigenic peptides in the arcuate nucleus of the hypothalamus (Cheung, Thornton et al. 1997; Schwartz, Seeley et al. 1997; Thornton, Cheung et al. 1997), where LepRb is highly expressed (Elmquist, Bjorbaek et al. 1998). This hormone also exerts other physiological effects that impact reproduction, hematopoiesis, angiogenesis and immunity (Wauters, Considine et al. 2000). Leptin's effects are exerted through the expression of LepRb on different types of tissues and cells throughout the body. Furthermore, leptin plays an important role in the regulation of both innate and adaptive immunity (La Cava and Matarese 2004; Matarese, Moschos et al. 2005), and different immune cells express LepRb - which supports the notion that leptin plays a direct role in immune function (Matarese, Moschos et al. 2005). T-cells (Batra, Okur et al. 2009), B-cells (Papathanassoglou, El-Haschimi et al. 2006), neutrophils (Caldefie-Chezet, Poulin et al. 2003), monocytes (Raso, Pacilio et al. 2002), dendritic cells (Mattioli, Straface et al. 2005) and NK cells (Tian, Sun et al. 2002) all express LepRb, and leptin modulates their functions *in vivo* and *in vitro* (Papathanassoglou, El-Haschimi et al. 2006) .

Leptin induces diapedesis of monocytes and macrophages through an epithelial layer *in vitro* and the production of pro-inflammatory cytokines from both cell types (Curat, Miranville et al. 2004; Gruen, Hao et al. 2007). Leptin augments lipopolysaccharide (LPS)-induced cytokine secretion (Gainsford, Willson et al. 1996; Loffreda, Yang et al. 1998) via interleukin-1 receptor-associated kinase 1 (IRAK-1) up-regulation, (Vaughan and Li 2010) and potentiates IFN-γ-induced expression of nitric oxide synthase (Raso, Pacilio et al. 2002) in macrophages.

Non-genetic obesity is characterized by a hyperleptinemic state (Considine, Sinha et al. 1996) and a compromised immune system (Falagas and Kompoti 2006). Additionally, there is increased pro-inflammatory macrophage recruitment in the adipose tissue of obese individuals (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003), and "obese" macrophages have impaired phagocytic activity (Krishnan, Trost et al. 1982; Mancuso, Gottschalk et al. 2002).

Mechanisms that promote a proinflammatory state in obese animals and people and their higher susceptibility to infections remain unclear. This study central hypothesis is that leptin contributes directly to this inflammation by regulating macrophage function. The objective here was to create a continuous macrophage cell line to allow for investigation of LepRb's impact on macrophage function. The properties of this new leptin receptor-deficient macrophage cell line, DB-1 are detailed in this paper.

Materials and Methods

Animals - Male leptin receptor-deficient (*db/db*) mice as well as gender and aged-matched wild-type (WT) counterparts were purchased from Jackson Laboratories (Bar Harbor, ME). All animal procedures were performed following the Guide for the Care and Use of Laboratory Animals with prior approval and regular monitoring of the Institutional Animal Care and Use Committee (IACUC) at Kansas State University.

Reagents - Biomedium was prepared using Dulbecco's modified Eagle's medium (DMEM, Atlanta Biologicals, Lawerenceville, GA) supplemented with 5% Nu Serum (Collaborative Biomedical Products, Bedford, MA), 5% fetal bovine serum (FBS, Atlanta Biologicals), 10% Opti-miminum essential media (MEM) reduced serum medium (Invitrogen, Carlsbad, CA), 50 μg per ml gentamycin (Sigma-Aldrich, St Louis, MO) and 1.5 ng per ml rmM-CSF (R&D Systems, Minneapolis, MN). Cell culture medium was prepared using DMEM supplemented with 10% Opti-MEM, 15 mmol/L HEPES and 100 μg per ml gentamycin. Recombinant mouse leptin (rm-Leptin) and IFN-γ were purchased from R&D Systems Inc. Fluorescent spheres were purchased from Invitrogen and phorbol 12-myristate 13-acetate (PMA) from Sigma.

Collection of Peritoneal Macrophages - Peritoneal exudate macrophages from WT and *db/db* mice were induced by i.p. injection of 1.5 ml of sterile, 2.9% thioglycollate (DIFCO, Detroit, MI). Four days after injection, mice were anesthetized via Isoflurane (IsoFlo, Abbott, Abbott Park, IL) inhalation and euthanized via cervical dislocation. Peritoneal exudate macrophages were collected by washing the peritoneal cavity twice with 12 ml of ice-cold PBS.

Differentiation of Bone Marrow Derived Macrophages - Bone marrow cells from *db/db* mice were collected by flushing the humeri, tibiae and femora as described previously

(Armstrong, Nelson et al. 1993). The undifferentiated cells were resuspended in Biomedium, counted and plated at a density of 3-5×10^6 cells per 100-mm dish. Cells were incubated at 37°C in 8% CO_2 and non-adherent cells were removed three days post-seeding by washing the plates with PBS and replacing with fresh Biomedium. Adherent cells were dispersed with trypsin/EDTA (10% trypsin) to passage the cells. Cells were passed twenty times until spontaneous immortalization occurred. For activation and phagocytosis assays, bone marrow cells from *db/db* and WT mice were allowed to differentiate for 7-10 days in Biomedium before primary macrophages were used.

Limiting Dilution Cloning of Cell Lines - Cells were dispersed with trypsin/EDTA and resuspended in DMEM supplemented with 10% FBS (DMEM10). Cells were counted and each dilution was plated in 48 wells of a Costar 96-well, flat-bottom tissue culture plate. A 1:10 serial dilution scheme was used starting at 100 cells/well in 100µl DMEM10 and ending at 0.01 cell/well. Cells were then incubated at 37°C in 8% CO_2. Only clones that grew from dilutions having less than a 30% positive growth were selected and expanded. The limiting dilution was repeated twice to ensure pure clones. Two final clones were selected and characterization experiments were performed using these clones.

Genotyping of Cell Line - Transcript expression of LepRb gene (NCBI 16847) was assessed using qRT-PCR and the following primers: *leprb* forward 5'- GCA ACC CAC CAT GAT TTC ACC ACA-3'; reverse 3'-AGG ATT CCT GCC TCA CCA GTC AAA-5'. The internal standard used was β-actin (NCBI number 11461) with the following primers: *Actb* forward, 5'- TGT GAT GGT GGG AAT GGG TCA GAA-3'; *Actb* reverse, 3'- TGT GGT GCC AGA TCT TCT CCA TGT-5'. Fold change in transcript level of each cell type was quantified using the method described by Pfaffl (Pfaffl 2001), and the formula employed WT macrophages as the reference group:

$$\text{Fold change} = E^{\Delta ct \text{ target gene (reference-exp)}} / E^{\Delta ct \text{ housekeeping gene (reference-exp)}};$$

where E is the efficiency of the primers, and the housekeeping gene is β-actin.

Cell Cycle Determination - Cell cycle determination and ploidy of the cell line was determined using flow cytometry. Briefly, cells were dispersed, collected and counted. Two x 10^6 cells were transferred to 12×75-mm polystyrene tubes (Falcon, Brookings, SD). Vindelov's propidium iodide (PI) solution was added and cells were incubated for 5-10 minutes at 4°C

(Vindelov, Christensen et al. 1983). Ploidy was analyzed with singlet discrimination to detect PI-stained mononuclear cells.

Cell Line Proliferation Rate - Cell growth doubling time of the leptin receptor-deficient macrophage cell line, DB-1, was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 1 $x10^5$ cells were seeded in 6-well plates. Three wells were treated with MTT reagent for 2 hours at each time point. Subsequently, the supernatant was removed and IsoPBS (Isopropyl alcohol with 50% PBS and 0.2% 1N HCL) was added to dissolve formazan crystals. Color intensity was quantified at 570 nm spectrophotometerically (Packard SpectraCount, Packard Instrument Company, Meriden, CT).

Antibody Phenotyping of Macrophage Cell Lines and Flow Cytometry - DB-1 cells, peritoneal macrophages and bone marrow-derived macrophages were probed with antibodies raised against the following surface markers: CD11b, F4/80, MAC-2, Ly6G (e-Bioscience, San Diego, CA) and Ly6C (BD Pharmigen, San Diego, CA). Appropriate isotype controls were used for each antibody as the background labeling for FACS analysis. Briefly, macrophages were recovered and plated at 1×10^5 cells per well of 96-well tissue culture plates. Fc receptors were blocked by incubating the cells with 50% goat serum in PBS for 30 minutes at 4°C. Antibodies and respective isotype controls were added at the appropriate concentrations and incubated for 1 hr at 4°C. Cells were washed twice with Hank's buffered salt solution (HBSS). Cells were transferred to 12×75-mm polystyrene tubes, washed once with HBSS and resuspended in 200 µl of 1% formalin/HBSS. The cells were placed on ice and the amount of labeling of each marker was assessed by flow cytometry using a FACS Caliber analytical flow cytometer (Becton Dickson, San Jose, CA) measuring 5,000 events per sample. Data analysis was performed with WinList software (Verity Software House, Topsham, ME). The amount of labeling of each marker was assessed after subtracting the percent positively labeled cells in the isotype control groups.

Phagocytosis Assays - Bone marrow-derived, peritoneal and DB-1 macrophages were dispersed with trypsin/EDTA, recovered, and 1×10^6 cells were seeded per well in 24-well tissue culture plates. Cells were incubated at 37°C in 8% CO_2 for 2 hours. Cytochalasin D from *Zygosporium mansonii* (Sigma Aldrich) was added at a final concentration of 10 µM to control plates to inhibit phagocytosis. These plates were incubated at 4°C for 2 hrs to further inhibit phagocytosis. Experimental plates were kept at 37°C. Red fluorescent beads (diameter, 0.2 µm;

Invitrogen) were added to each well to a final concentration of 0.1%. Phagocytosis was stopped after 60 min by centrifuging the 24-well plates at 350×g for 5 min, removing the supernatant, and washing twice with PBS. The cells were transferred to 12×75-mm polystyrene tubes, washed twice with PBS and resuspended in 200 µl of 1% formalin/PBS. The cells were placed on ice and phagocytosis was assessed by flow cytometry and measuring 10,000 events per sample. The percentage of phagocytic cells in each experimental group was assessed after subtracting the percentage phagocytic cells of the control plates.

Activation of DB-1 with rm-IFNγ - DB-1 cells were stimulated with IFN-γ, to determine whether DB-1 macrophages respond to this stimuli in the same fashion as primary macrophages and expression of *nos2* was assessed. Briefly, primary WT, *db/db* and DB-1 macrophages were plated separately in 24-well plates at a density of 1 x 10^6 cells per well and incubated with or without IFN-γ (100 U/ml, R&D Systems, Minneapolis, MN) for 16 hours. RNA was collected using TRI reagent (MRC, Cincinnati, OH) and further purified with DNAse using the E.Z.N.A Total RNA kit (Omega Bio-Tek, Norcross, GA). Gene expression of *nos2* (NCBI number 18126) was quantified using qRT-PCR with the following primers: *nos2* forward, 5'- CTG CTG GTG GTG ACA AGC ACA TTT-3'; *nos2* reverse, 3'- ATG TCA TGA GCA AAG GCG CAG AAC-5'. β-actin was quantified using the previously mentioned primers. Fold change in transcript level of each cell type was quantified using the Pfaffl method as described above.

Adipocyte Collection and Conditioned-Medium Preparation - Adipocytes from epididymal and inguinal fat pads of obese and lean male C57BL/6 mice were collected in cell culture medium, pooled and minced into small pieces. Tissue was digested in Krebs Ringer Buffer (KRB) containing 1 mg/ml type II collagenase (Sigma-Aldrich) in a shaking incubator for 40 min at 37°C. The digested tissue was filtered through nylon cell strainers (100 µm; Fisher Scientific, Pittsburg, PA), washed with KRB, and separated by centrifugation for 1 min at 350 x *g*. The floating adipocytes were collected and cultured in DMEM at 37°C, 8% CO_2 for 24 hrs. The adipocyte-conditioned medium (ACM) containing all factors released by the adipocytes was collected and used for migration experiments with thioglycollate elicited peritoneal and DB-1 macrophages. Control medium (CM) was obtained using the same protocol but without adding adipocytes. Media were stored at -80°C until use.

Migration Assay - Wild-type peritoneal macrophages were collected as described above and suspended in serum-free culture medium. Cells were placed in the upper chamber of a

polystyrene filter (5 μm pore size, 6-transwell format; Corning, Lowell, MA). Cells were allowed to adhere for 3 hours, at which point medium in the lower chamber was replaced with CM (negative control), ACM, CM supplemented with 10ng/ml rm-leptin, or CM supplemented with 2 μM PMA. After 3 hrs, cells that had not migrated and remained in the upper chamber were removed by gently swiping the filters with cotton tips. Filters were fixed in 2% ethanol for 5 minutes. Cells located on the lower side of the filter were stained with Diff-Quick (Dade Diagnostics, Aguada, PR), and counted as migrated cells. These cells were quantified from 5-10 fields/condition and cell type.

Statistical Analysis - Results are reported as mean ± standard deviation (std) unless otherwise specified. ANOVA was performed and least significant differences (LSD) were calculated when appropriate. Analysis was conducted using SPSS software (version 17.0, SPSS Inc. Chicago, IL). Statistical significance was determined at p-value <0.05.

Results

Establishment of DB-1 cell line

An immortal leptin receptor-deficient cell line from mouse bone marrow-derived macrophages was established. Bone marrow cells from *db/db* mice were collected and cultured in Biomedium as described in the Materials and Methods section. Differentiated macrophages were passaged at confluency. Cells were kept in Biomedium for 6 months when the cells appeared to grow at a stable rate after twenty passages. At that time, we did two consecutive limiting dilution clonings. The DB-1 cells were expanded from one clone selected from the second cloning procedure. DB-1 cells lost their dependency to M-CSF after the cloning process. They were re-expanded and shifted to a regular medium, DMEM supplemented with 2% Nu serum and 2% FBS. DB-1 cells exhibit morphological properties characteristic to macrophages, and are adherent with multiple processes (Figure 3.1).

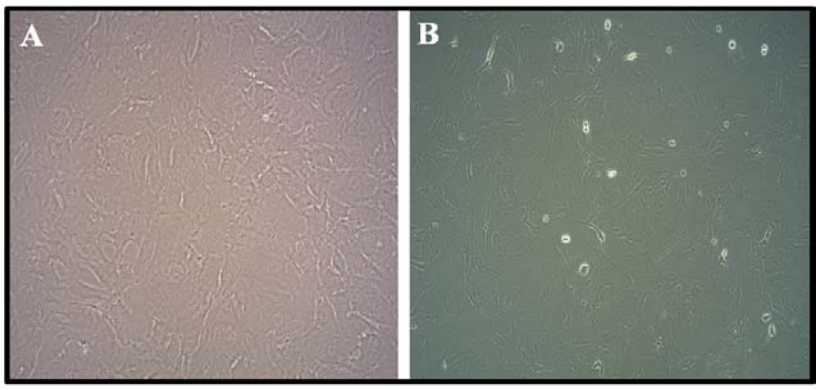

Figure 3.1 Morphological aspects of DB-1 cells using light photo micrographs. A, B in culture flask at magnification 10x and 20x, respectively.

Quantification of gene transcript levels of LepRb was performed to confirm the leptin-receptor deficient genotype of DB-1 cells using qRT-PCR. Similarly to primary peritoneal *db/db* macrophages, DB-1 had low levels of LepRb transcripts, and expression ranged from 0.1 to 12 % of WT macrophages ($p<0.05$, Table 3.1).

Table 3.1 Relative gene expression of LepRb in DB-1 and peritoneal *db/db* macrophages compared to WT peritoneal macrophages.

Cell type	Percentage fold expression compared to WT macrophages[§]
db/db macrophages[#]	1.4 (0.1-3.5)[*]
DB-1 macrophages	1.05 (0.1-12)[*]

[#]Results from 4-6 samples per group, are reported as median (range).
[§]Fold expression was calculated according to Pfaffl method (Pfaffl 2001) using WT values as control group and β-actin as the reference gene.
[*]$p<0.05$ compared to 100% expression in WT macrophages

Cell cycle analysis was conducted using flow cytometry (Vindelov, Christensen et al. 1983) to assess DB-1 ploidy and cell cycle distribution. The DB-1 cell line was homogenous consisting of 100% diploid cells, of which 90-95% cells were in G1 phase, 3 to 9% in G2 phase and 0 to 2% in S phase.

DB-1 cell growth was analyzed via a MTT assay, and cell doubling time was estimated to be approximately 3 days (Figure 3.2).

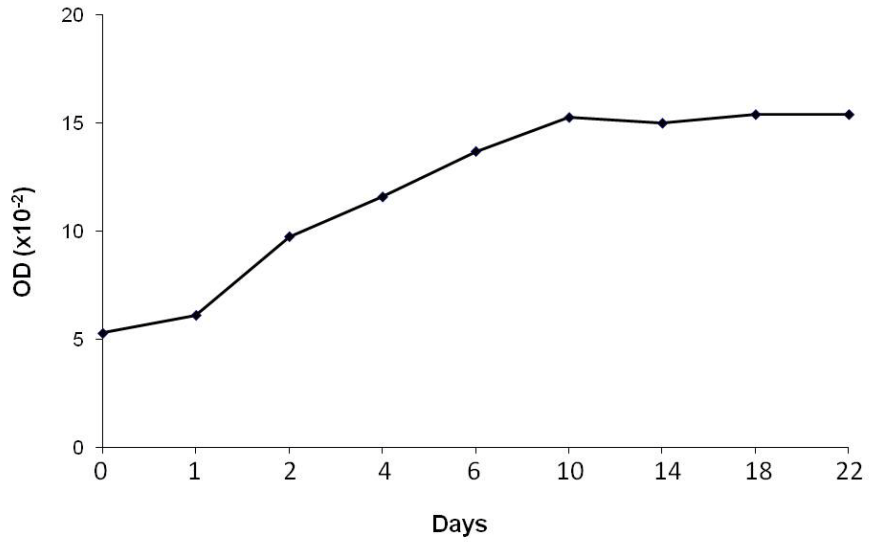

Figure 3.2 DB-1 cells exhibit a 3 day doubling time.
Cell growth was assayed at different days after seeding of $1x10^5$ DB-1 cells in 6-well plates using MTT. Each time point value is the mean of triplicate readings. OD= Optical density.

Cell growth plateaued at day 10 and the cells remained stable until day 22 when the experiment was terminated. The slow growth rate along with the growth arrest at confluency is consistent with a cell line that is non-transformed and contact inhibited (Schuler, Jefferson et al. 1977).

Cell Surface Phenotype

Cell surface antigens of DB-1 cells were compared to those of primary peritoneal and bone marrow-derived macrophages to verify that DB-1 cells belong to the monocyte/macrophage lineage. Primary macrophages expressed similar levels of F4/80 and MAC-2, regardless of their expression of LepRb (Figure 3.3). Bone marrow-derived macrophages exhibited less expression of CD11b and higher expression of Ly6C compared to the peritoneal macrophages. All cell types, including the DB-1 cells displayed minimal expression of Ly6G, the granulocyte marker, on their surface. DB-1 cells displayed MAC-2 in similar amounts as both bone marrow-derived

and peritoneal WT and *db/db* macrophages. In contrast, F4/80 was minimally expressed and CD11b was not expressed at all in DB-1 macrophages. Ly6C was expressed by DB-1 cells at an intermediate level between that of the bone marrow-derived and peritoneal macrophages (Figure 3.3). These data suggest that the DB-1 cells are intermediately matured in the macrophage lineage (Leenen, Melis et al. 1990) with high MAC2 expression, lower expression of F4/80 and low levels of Ly6C.

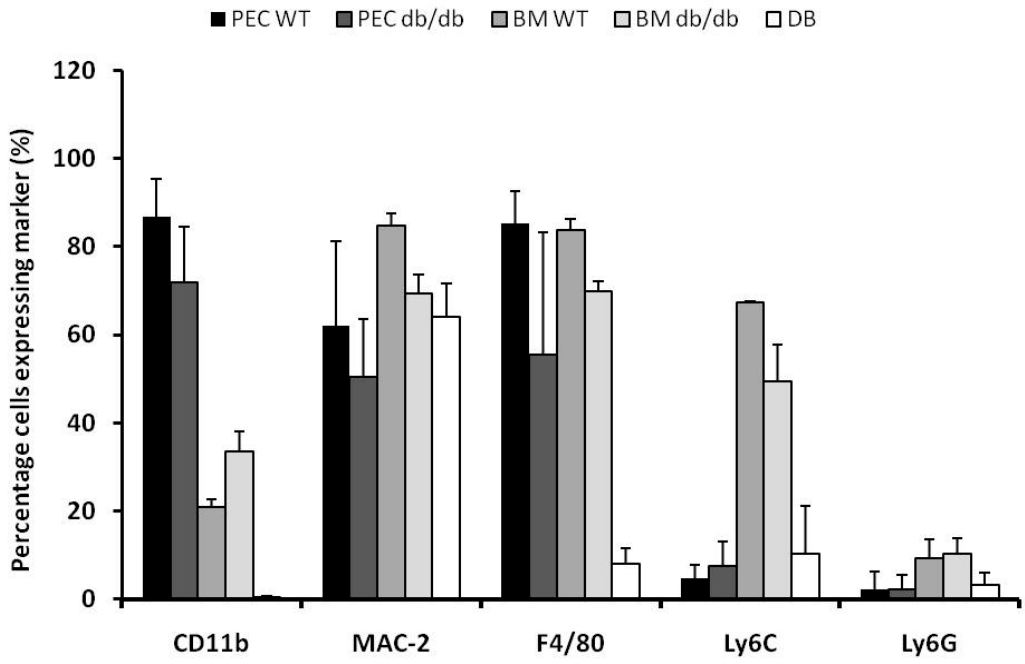

Figure 3.3 DB-1 cells exhibit macrophage lineage cell surface markers.
CD11b, MAC-2, F4/80, Ly-6C and Ly6G cell surface expression in peritoneal (PEC WT) macrophages, peritoneal (PEC db/db) macrophages, primary WT bone marrow (BM WT), primary *db/db* bone marrow (BM db/db) and DB-1 macrophages (DB) using flow cytometry. Data represent means ± std, from two independent experiments, n=2-5 replicates per experiment, each n from one or a pool of 2 mice.

Phagocytic Activity

Phagocytic activity of DB-1 cells was measured and compared to that of primary macrophages in order to assess whether DB-1 cells display functional macrophage characteristic. We concluded that all five cell-types - including the DB-1 cells – exhibited no statistical difference in their phagocytic indices based on a one-hour phagocytosis assay (Figure 3.4, $p>0.05$).

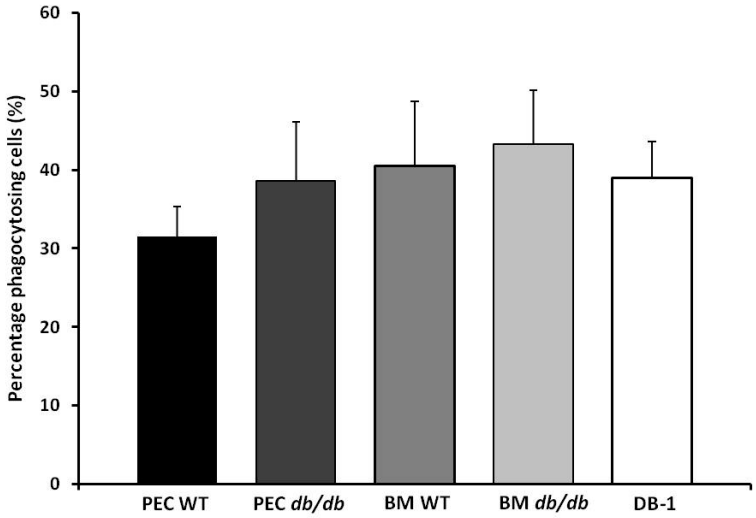

Figure 3.4 DB-1 cells exhibit the same phagocytic activity as peritoneal (PEC) and bone-marrow (BM) derived WT and *db/db* macrophages.
Cells were incubated with fluorescent beads and phagocitic activity assessed via the percentage of phagocytosed beads using flow cytometry. Data represent means ± std, from one or two independent experiments, n=3-6 replicates per experiment, each n from one or a pool of 2 mice.

DB-1 Respond to IFN-γ Stimulation

DB-1 macrophages were exposed to IFN-γ and transcript levels of *Nos2* were quantified to determine if DB-1 cells respond to a stimulus in a similar fashion as primary macrophages. *Nos2* transcript levels were higher after IFN-γ treatment with levels similar to peritoneal *db/db* macrophages ($p<0.05$, Table 3.2).

Table 3.2 Relative gene expression of *nos2* in DB-1 and peritoneal *db/db* macrophages after stimulation with IFN-γ compared to non-stimulated cells.

Cell type	Percentage fold up-regulation compared to no treatment group[$]
db/db macrophages[#]	1.4 (0.1-3.5)[*]
DB-1 macrophages	1.1 (0.1-12)[*]

[*]Results from 5-8 samples per group, are reported as median (range). [$]Fold expression was calculated according to Pfaffl method (Pfaffl 2001) using no treatment values as control group and β-actin as the reference gene for each macrophage type. [*]$p<0.05$ compared to 100% expression in control non-stimulated macrophages

Migration Assay

Migration assays were performed using both peritoneal WT and DB-1 cells to quantify the response of these cells to the chemotactic effect of adipocyte conditioned medium or leptin. DB-1 macrophages migrated when activated with PMA ($p<0.05$), but that they failed to respond to the chemotactic signals from the ACM as well as the medium supplemented with leptin (Figure 3.5). On the other hand, primary WT macrophages exhibited a significant increase in migration when cultured with either the ACM or leptin ($p<0.01$; comparison to medium alone) with no statistically significant difference between the two media ($p>0.05$; Figure 3.5); suggesting that most of the ACM chemotactic potential is mediated by leptin, and dependent on the presence of a functional LepRb.

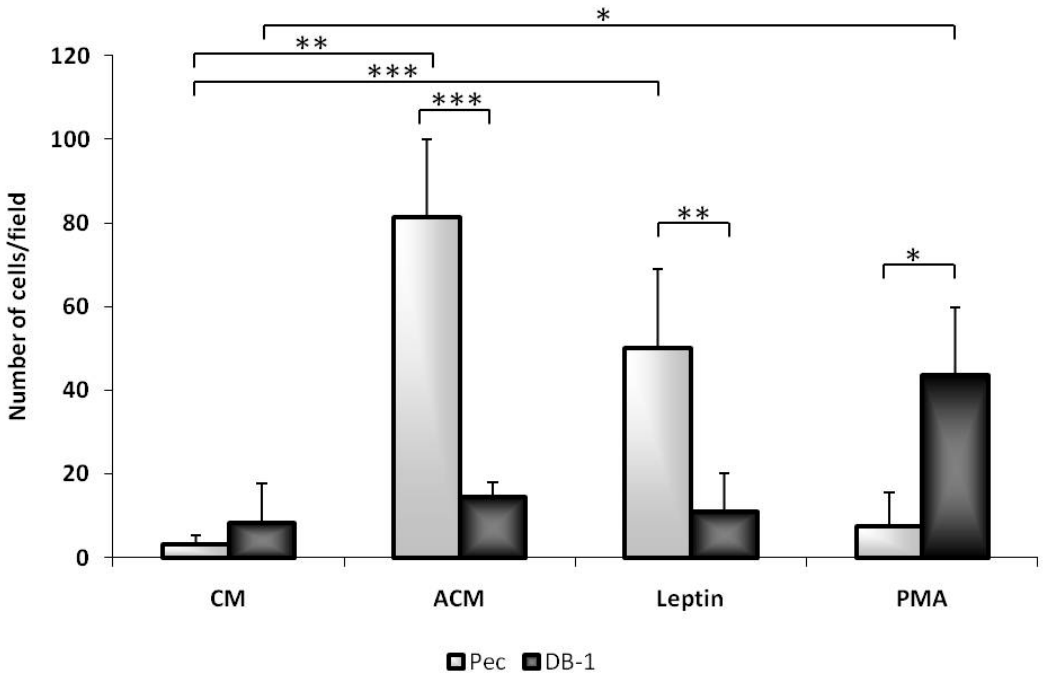

Figure 3.5 DB-1 cells fail to migrate following stimulation with adipocyte-conditionned media or leptin.
Migration assay was carried using a transwell system. The lower chamber was supplemented with control medium (CM), adipocyte conditionned medium (ACM), CM supplemented with 10ng/ml rm-leptin or CM supplemented with 2 µM phorbol 12-myristate 13-acetate (PMA). Values are means ± std. Data of 1 experiment with n=3 replicates per treatment group and cell type. * $p< 0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Pec= WT peritoneal macrophages.

Discussion

A leptin-receptor deficient cell line, DB-1, was derived from murine bone marrow. This cell line has phenotypic and functional properties characteristic of macrophages. It is diploid, lacks the long isoform of the leptin receptor, and is unresponsive to leptin.

Characteristics of DB-1 cells are comparable to *ex vivo* bone marrow-derived and thioglycollate-elicited peritoneal WT and primary leptin receptor-deficient macrophages. We show that DB-1 cells exhibit higher levels of MAC-2 expression and lower levels of Ly6C, indicating that these cells belong to the macrophage lineage and have immortalized at a intermediate-to-late stage of macrophage differentiation (Leenen, de Bruijn et al. 1994). The DB-1 Ly6C expression is intermediate between that of peritoneal macrophages and seven-day, M-CSF-differentiated bone marrow-derived macrophages. The difference in primary Ly6C expression in bone marrow compared to peritoneal macrophages suggests that 7-day *in vitro* differentiation is not enough to yield fully mature macrophages (Chan, Leenen et al. 1998). The DB-1 macrophages are responsive to IFN-γ, which supports the conclusion that these cells are mature macrophages; given that NOS2 production is a property of mature macrophages (MacMicking, Xie et al. 1997).

DB-1 cells are phagocytic *in vitro*. The strong phagocytic activity of the DB-1 cells supports the notion that these cells are in the macrophage lineage (Leenen, Jansen et al. 1986). In this study, the LepRb did not contribute to macrophage phagocytosis. The lack of the leptin gene (*ob/ob*) as well as the leptin receptor gene (*db/db*) have been associated with an enhanced susceptibility to infections and a higher mortality rate in rodents following bacterial infection (Mancuso, Gottschalk et al. 2002; Ikejima, Sasaki et al. 2005; Ordway, Henao-Tamayo et al. 2008; Park, Rich et al. 2009). This increased risk of infection has been attributed to a decrease in phagocytic and bactericidal activity of *ob/ob* and *db/db* macrophages *in vitro* (Loffreda, Yang et al. 1998; Mancuso, Gottschalk et al. 2002; Park, Rich et al. 2009). The difference in phagocytic activity between DB-1 cells and that of *ob/ob* and *db/db* primary macrophages evaluated in other studies might be explained by the fact that inert fluorescent beads were used in this study compared to actual infectious agents (Loffreda, Yang et al. 1998; Mancuso, Gottschalk et al. 2002; Park, Rich et al. 2009). However, DB-1 macrophages's ability to phagocytose fluorescent *Ehrlichia chaffeensis* bacteria was comparable to that of primary macrophages (data not shown).

Therefore, the difference between our study and data presented by others may be due to other physiological factors such as a compromised oxidative response (Hsu, Aronoff et al. 2007) in immune cells or inefficient leukotriene production (Mancuso, Gottschalk et al. 2002); products that were compromised in primary *db/db* and *ob/ob* macrophages. It is also possible that physiological factors associated with diabetes such as hyperglycemia may have an impact. Parks and collegues showed similar infection rates in mice between 8-11 week-old *db/db* mice and WT controls but older (20 weeks and more) *db/db* mice were significantly more susceptible to *S. aureus* infection (Park, Rich et al. 2009). The cell line would not be subject to the physiological regulation of the *in vivo* macrophages.

Obesity is associated with an increase in inflammatory macrophage recruitment to the expanding fat mass (Weisberg, McCann et al. 2003; Xu, Barnes et al. 2003). Studies have recognized MCP-1 as the major adipocyte-derived chemokine responsible for this effect (Kanda, Tateya et al. 2006; Weisberg, Hunter et al. 2006). Nevertheless, recent studies have questioned this chemokine's role in the enhanced recruitment of macrophages into the adipose tissue of obese mice (Inouye, Shi et al. 2007). Leptin is another candidate adipokine/chemokine that might be responsible for this recruitment effect. Indeed, our data show that adipocyte-conditioned media as well as leptin-supplemented media have the same positive chemotactic effect on WT macrophages. DB-1 cell line, which lacks the functional LepRb, failed to respond to both leptin-supplemented and adipocyte-conditioned media, although this cell line responded better than the WT macrophages to PMA. Therefore, LepRb has a physiological function and DB-1 cells are refractory to leptin and supports the studies of Gruen *et al*, who have shown leptin's chemotactic effects at levels as low as 1 pg/ml (Gruen, Hao et al. 2007).

The body's nutritional status, ranging from starvation or obesity, affects immune function (Dixit 2008). In an era characterized by increasing obesity rates (Ogden, Carroll et al. 2006), it is of central importance that the immunological consequences of excessive body weight be further elucidated. The newly established DB-1 cell line can serve as an important and reliable tool to study the link between leptin and innate immune function.

Chapter 4 - Role of Leptin in Obesity-Associated Infection Susceptibility

Abstract

Obesity is associated with an increased risk of infection and compromised immune responses. Leptin plays a role in both innate and adaptive immunity and its plasma concentration changes in response to infection, therefore we hypothesized that obesity would affect the host response to an experimental infection by *Ehrlichia chaffeensis*. We experimentally challenged three groups of male C57Bl/6J mice, obese, lean and young lean with *E. chaffeensis*. Serum leptin, IL-6 and TNF-α were measured at 0, 3, 6 & 9 days post-infection and bacterial infection was assessed 6 and 9 days post-infection using qRT-PCR. Obese mice experienced the most weight loss after infection. All groups had a similar increase in inflammatory cytokines and leptin was decreased similarly in all groups. Although, no differences were measured between lean and obese mice in the kinetics of bacterial clearance, we did see some differences in some individual immune functions. "Obese" peritoneal macrophages were significantly less phagocytic than macrophages from lean mice. Supplementation with 500 ng/ml rm-leptin significantly increased the obese macrophages' phagocytic activity but had no effect on lean macrophages. Leptin treatment did not affect phagocytosis by macrophages from uninfected mice.

Introduction

Epidemiological and observational studies have established a strong link between obesity and the development of several types of infections. Obese people have a higher risk of developing nosocomial, surgical site, odontogenic, respiratory, urogenital, skin, bone and liver infections compared to leaner counterparts (Falagas and Kompoti 2006; Serrano, Khuder et al. 2010). In the March 2010 report, the Centre for Disease Control (CDC) declared obesity to be an independent risk factor for hospitalization and death due to the 2009 H1N1 epidemics (http://www.cdc.gov/h1n1flu/in_the_news/obesity_qa.htm) in Canada, New Zealand, Australia

and the U.S.A. (Miller, Markewitz et al. ; Kumar, Zarychanski et al. 2009; Louie, Acosta et al. 2009; Webb and Seppelt 2009).

Infection is also affected by obesity in rodents. Genetic and diet-induced obese (DIO) mice exhibit a blunted immune response and a delayed pathogen clearance when infected with either bacterial or viral agents (Mancuso, Gottschalk et al. 2002; Amar, Zhou et al. 2007; Smith, Sheridan et al. 2007). Additionally, infected DIO mice have a higher mortality rate compared to lean controls secondary to influenza viral infections (Smith, Sheridan et al. 2007; Karlsson, Sheridan et al. 2010

). Macrophages isolated from either obese humans or mice have an impaired phagocytic and bactericidal activity *ex-vivo* (Krishnan, Trost et al. 1982; Mancuso, Gottschalk et al. 2002). These "obese" macrophages exhibit a blunted cytokine response (Amar, Zhou et al. 2007), a defective oxidative response and increased levels of carboxyl-terminal modulator protein (CMTP), an inhibitor of Akt phosphorylation in macrophages (Zhou, Leeman et al. 2009; Zhou, Leeman et al. 2011). Therefore, there are many factors that contribute to the defective activation of "obese" macrophages after bacterial challenge.

Leptin is a small peptide mainly secreted by adipocytes in proportion to adipose tissue mass and the degree of obesity (Considine, Sinha et al. 1996). This hormone is a pleiotropic adipocytokine involved in energy homeostasis, reproduction, hematopoeisis and innate and adaptive immunity (Matarese, Moschos et al. 2005). Leptin induces a TH1 pro-inflammatory response, activates NK cells, stimulates macrophage cytokine secretion and is a potent chemokine for neutrophils and monocytes (La Cava and Matarese 2004). Genetically obese mice lacking either leptin (*ob/ob*) or its receptor (*db/db*) are more prone to opportunistic infections and are resistant to autoimmune diseases (Sanna, Di Giacomo et al. 2003; Ikejima, Sasaki et al. 2005), thus highlighting the role of leptin in generating normal immune responses. Restoring leptin concentrations to normal in either *ob/ob* or starved mice (low leptin levels) lead to a normalization of immune responses and a restoration of normal bacterial clearance (Mancuso, Gottschalk et al. 2002; Sanna, Di Giacomo et al. 2003; Mancuso, Huffnagle et al. 2006). The same has been observed *in vitro* where supplementation of *ob/ob* macrophages with leptin restored macrophage phagocytic activity (Mancuso, Gottschalk et al. 2002).

In obesity, leptin signaling and regulation is impaired, leading to leptin resistance in central and possibly peripheral tissues (Klein, Horowitz et al. 2000; Schwartz, Woods et al.

2000). Karlsson et al. (2010) and Smith et al. (2007) reported that, contrary to the acute and sharp increase in leptin levels seen in lean mice post-infection; DIO mice have an acute systemic decrease in leptin levels after influenza infection. Since leptin is involved in the activation of innate and adaptive immunity, the decrease in DIO mice might explain their ineffective viral clearance, though this link was not established conclusively (Smith, Sheridan et al. 2007; Karlsson, Sheridan et al. 2010). Therefore, there is a need to show a direct cause and effect between obesity and infection and establish the mechanisms involved.

Ehrlichia chaffeensis is an obligate, macrophage/monocyte-tropic intracellular bacterium. In humans, it causes monocytic ehrlichiosis, which can be lethal in the young, elderly, and/or immunocompromised subjects (McNabb, Jajosky et al. 2008). *E. chaffeensis* is classified as a Gram-negative bacterium, but it lacks the genes necessary for the synthesis of peptidoglycan or lipopolysaccharide (LPS) (Lin and Rikihisa 2003). Though *E. chaffeensis* is taken up by macrophages, these cells fail to clear the infection by themselves allowing the bacteria infection to persist through yet undefined evasion mechanisms (Ganta, Cheng et al. 2004). Nevertheless, immunocompetent mice clear *E. chaffeensis* infection by 10-14 days through a process requiring both innate and adaptive immunity players and involving macrophage activation, major histocompatibility complex class II (MHCII) molecules, and $CD4^+$ helper T-cell responses (Ganta, Cheng et al. 2007).

We hypothesized that the leptin response to *E. chaffeensis* infection would be impaired in obese mice and would compromise the immune response. *E. chaffeensis* was a good choice of challenge agent because it was opportunistic and prolonged infections would reveal changes to the host defense system.

Materials and Methods

Animals- Male leptin receptor-deficient (*db/db*) mice and gender and aged-matched wild-type (WT) counterparts were purchased from Jackson Laboratories (Bar Harbor, ME). After 1 week adaptation, mice were randomized to receive either the high fat diet (HFD, D12492, 45 kcal% from fat, Research Diets Inc., New Brunswick, NJ) (the obese group OB) or the normal fat diet (NFD, D12450B, 10% kcal from fat, Research Diets Inc., New Brunswick, NJ) (the lean group LE) for sixteen weeks. At the end of the sixteen weeks, another mouse group, 6-weeks old,

was added (young group YO) and all mice were infected as described below. All animal procedures were performed following the Guide for the Care and Use of Laboratory Animals with the prior approval and regular monitoring of the Institutional Animal Care and Use Committee (IACUC) at Kansas State University.

E. chaffeensis Quantification and Mouse Infections- E. chaffeensis was grown in canine DH82 cells as described previously (Luce-Fedrow, Von Ohlen et al. 2009). Cultured bacteria from 100 mm culture plates were harvested when 80 to 100% of the confluent DH82 cells were infected. The cell suspension was diluted 1:1 in phosphate-buffered saline (PBS), and 0.5 ml of the suspension ($\sim 5 \times 10^6$ cells) was injected intraperitoneally (i.p.) per mouse as described previously (Ganta, Wilkerson et al. 2002). Aliquot of the injection suspension were sampled to assess the number of *E. chaffeensis* bacteria per inocula using qRT-PCR with established primers (Ganta, Wilkerson et al. 2002). Cycle threshold (CT) values were plotted on standard curves generated by plotting the log number of bacteria versus the corresponding threshold cycle value and number of bacteria per inocula was calculated as described by our group (Luce-Fedrow, Von Ohlen et al. 2009). Infected mice were sacrificed and evaluated 6 and 9 days post-infection.

RNA Isolation and E. chaffeensis rRNA Gene-Specific qRT-PCR- RNA from peritoneal exudate cells, spleen, lungs and epididymal fat tissue samples was extracted 6 and 9days post-infection, using the TRI reagent/chloroform method according to manufacturer's procedure. RNA was purified and DNAse treated using E.Z.N.A Total RNA kit and RNase Free DNase Set (Omega Bio-Tek, Norcross, GA). Quality of the extracted RNA samples was analyzed using the Agilent Bioanalyser (Agilent Technologies, Bracknell, UK). qRT-PCR was performed using a TaqMan-based real-time RT-PCR as previously described (Sirigireddy and Ganta 2005). The internal control gene used was β-actin (NCBI number 11461) with the following primers: *Actb* forward, 5'- TGT GAT GGT GGG AAT GGG TCA GAA-3'; *Actb* reverse, 3'- TGT GGT GCC AGA TCT TCT CCA TGT-5'. Fold change in transcript level for each tissue and mouse group was quantified using the method described by Pfaffl below formula (Pfaffl 2001), and using young (YO) group as the reference group:

$$\text{Fold change} = E^{\Delta ct \text{ target gene (reference-exp)}} / E^{\Delta ct \text{ housekeeping gene (reference-exp)}};$$

where E is the efficiency of the primers, and the housekeeping gene is β-actin.

Serum Cytokine/Adipokine Analysis- Mice were anesthetized systemically using isoflurane and locally with proparacaine hydrochloride and blood was collected from the retro-

orbital sinus at 0, 3, 6 and 9 days post-infection. Serum was separated and frozen at -80°C until analysis. Serum leptin and IL-6 were measured by fluorescent immunoassay using a Milliplex MAP kit for mouse serum adipokine (Millipore, St. Charles, MO) according to the manufacturer's instructions. The samples were processed on a Luminex 100, and results were analyzed with MasterPlexQT software (MiraiBio, South San Francisco, CA, USA).

Fluorescent Labeling of Bacteria for Injection- E. chaffeensis was purified as described above and was labeled with fluorescein as previously described (Luce-Fedrow, Von Ohlen et al. 2009). Briefly, fluorescein isothiocyanate (FITC) was dissolved into 0.2 M Na_2CO_3 (pH 9.5) buffer to a final concentration of 5 mg per ml. One milliliter of the FITC solution was added to the cell-free *E. chaffeensis* pellet, mixed gently, and incubated with rocking for 15 min at room temperature (protected from light). The FITC-bacterium mixture was then added to 4 ml of 135 mM NaCl-10 mM phosphate buffer (pH 7.4) and incubated for 5 min at room temperature. The mixture was then centrifuged at 10,000 x *g* for 5 min and the pellet was resuspended in 2.83% Na_2HPO_4 (pH 8.5), and the mixture was centrifuged again. The resulting pellet was resuspended in sterile PBS and subsequently washed three times with sterile PBS. The bacterial pellet was resuspended in sterile PBS for the phagocytic assay and an aliquot was taken to measure bacterial number as described above.

Collection of Peritoneal Macrophages - Peritoneal macrophages were collected from infected OB, LE and YO mice at day 9 post-infection by washing of the peritoneal cavity twice with 12 ml of ice-cold PBS. Peritoneal exudate macrophages from non-infected OB, LE and YO mice were induced by i.p. injection of 1.5 ml of sterile, 2.9% thioglycollate (DIFCO, Detroit, MI). Four days after injection, mice were anesthetized with Isoflurane (IsoFlo, Abbott, Abbott Park, IL) inhalation and euthanized via cervical dislocation.

Phagocytosis Assays - Peritoneal macrophages were collected from infected and non-infected OB, LE and YO groups as described above. One × 10^6 cells were seeded per well in 24-well tissue culture plates. Cells were supplemented with or without recombinant mouse leptin (rm-leptin, Sigma-Aldrich, St. Louis, MO) at concentrations of 500 or 5000 ng/ml and were incubated at 37°C in 8% CO_2 overnight. Cytochalasin D from *Zygosporium mansonii* (Sigma Aldrich) was added at a concentration of 10 µM to control plates to inhibit phagocytosis (Mordica, Woods et al. 2009). These plates were incubated at 4°C for 2 hrs to further inhibit phagocytosis. Experimental plates were kept at 37°C. One hundred µl of fluorescein-labeled

bacteria were added to each well. Phagocytosis was stopped after 60 min by centrifuging the 24-well plates at 350×g for 5 min, removing the supernatant, and washing twice with PBS. The cells were transferred to 12×75-mm polystyrene tubes (Falcon, Brookings, SD), washed twice with PBS and resuspended in 200 µl of 1% formalin/PBS. The cells were placed on ice and phagocytosis was assessed by flow cytometry (FACS Caliber, Becton Dickson, San Jose, CA) measuring 10,000 events per sample. The percentage of phagocytosis in the experimental groups was assessed after subtracting the percentage phagocytosis of the negative control treatment group.

Statistical Analysis - Results are reported as mean ± standard deviation (std) unless otherwise specified. ANOVA was performed and least significant differences (LSD) were done when appropriate. Analysis was conducted using SPSS software for Windows version 17.0 (SPSS Inc. Chicago, IL). Statistical significance was determined at p-value <0.05.

Results

Animal Model of Infection

Mice in the obese group (OB) and age-matched lean group (LE) were 24 weeks old and had an average weight of 40.9 ± 7.1 g and 30.2 ± 2.6g respectively ($p<0.05$, Table 4.1). Young mice (YO) were 6 week old with an average weight of 20.3 ± 0.7g, statistically different from the other two groups ($p<0.05$, Table 4.1). Young mice were used as a comparison for the age effects that might be induced by the 16-week diet protocol.

Obese Mice had the Highest Percentage Weight Loss Secondary to Infection

Weight loss is often used as a measure of pathogen stress (Iandolo and Chapes). Therefore, percentage weight loss was calculated at different time points post-infection as a measure of morbidity due *E. chaffeensis* infection. Both the obese (OB) and the age-matched lean (LE) groups lost weight at day 3 compared to baseline (4 and 3% respectively) with only the OB having a significant weight loss ($p<0.05$). Unlike LE mice, OB mice failed to recover lost weight by study termination. In fact, at day 6, average weight loss in the OB group was still depressed 4% compared to their baseline weight while LE mice had completely regained the lost

weight. Lean young mice (YO) did not lose any weight, in fact at study termination they gained an average of 8% compared to their baseline weight ($p<0.05$, Figure 4.1).

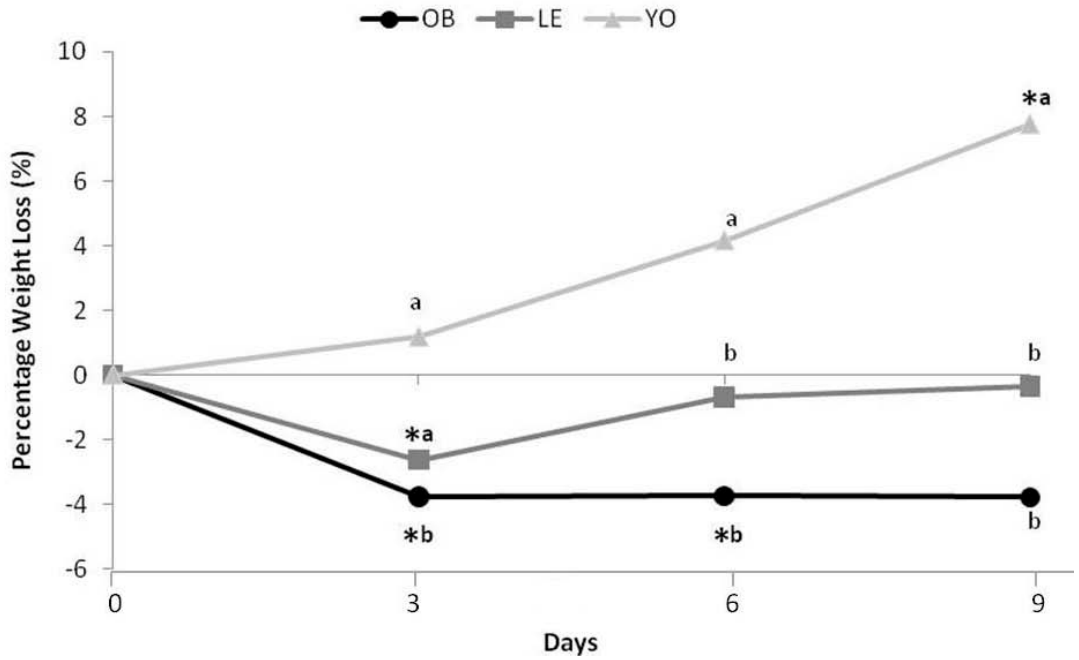

Figure 4.1 Infection stress after infection with *E. chaffeensis*.
Weight loss as a percentage of initial weight was calculated at 3, 6 and 9 days post infection in obese (OB), age-matched lean (LE) and young lean (LE) mice. * $p<0.05$ compared to zero. [a,b] values with different letters are statistically different between groups at different time points at $p<0.05$.

All Mice had an increase in inflammatory cytokines till study termination

Circulating inflammatory cytokines, TNF-α and IL-6 were measured during the course of the infection to assess the magnitude of systemic inflammation after experimental challenge. All mouse groups had a significant increase in both cytokines at day 9 compared to baseline values (Table 4.1). No difference was detected in overall fold change from baseline between all groups for IL-6 (1.5 ± 0.5, 13.5 ± 15.2 and 3.7 ± 1.3 in YO, LE and OB groups respectively, $p>0.05$ Table 4.1) and TNF-α (5.1 ± 1.2, 13.9 ± 7.1 and 14.1 ± 5.7 in YO, LE and OB groups respectively, $p>0.05$, Table 4.1). As expected, leptin levels were highest in the obese group compared to both LE and YO groups at all times ($p<0.01$, Table 4.1). All groups experienced a significant decrease in absolute leptin levels at day 9 compared to baseline values (Table 4.1) but the fold change from baseline was not different between groups (0.6 ± 0.4, 0.5 ±0.2, 0.5 ± 0.3, in YO, LE and OB mice respectively, $p>0.05$**,** Table 4.1). This decrease cannot be explained by

weight loss since LE regained their lost weight and YO mice actually gained weight by the end of the study.

Table 4.1 Serum levels of leptin, IL-6 and TNF-α at days 0, 3, 6 and 9 post E. chaffeensis infection in obese (OB), lean (LE) and young (YO) mice.

	Day	Young (YO)	Lean (LE)	Obese (OB)	p-Value*
Leptin, ng/ml	0	3 ± 0.6	8.2 ± 2.7	28 ±4.8	0.0001
	3	2 ± 0.3	5.6 ± 1.6	21.2 ±4.6	0.0001
	6	1 ± 0.2	4.7 ± 1.1	15.8 ± 2.9	0.0001
	9	1.5 ± 0.2#	4.6 ± 1.6#	9.5 ± 3.3#	0.01
Fold change from baseline$		**0.6 ± 0.4**	**0.5 ± 0.2**	**0.5 ± 0.3**	**NS**
IL-6, pg/ml	0	6.8 ± 1.7	5.6 ± 0.7	6.1 ± 0.8	NS
	3	9.4 ± 3.3	10.9 ± 1.5	14.3 ± 1.8	NS
	6	9.3 ± 1.3	13.8 ± 0.9	15.5 ± 2.2	0.04
	9	10.9 ± 4	24.6 ± 3.8#	19 ± 1.0#	NS
Fold change from baseline		**1.5 ± 0.5**	**13.5 ± 15.2**	**3.7 ± 1.3**	**NS**
TNF-α, pg/ml	0	1.8 ± 0.3	2.6 ± 0.9	1.4 ± 0.2	NS
	3	3 ± 0.5	3 ± 0.7	4.3 ± 1.2	NS
	6	7.9 ± 1.2	13.2 ± 1.1	8.9 ± 0.9	0.004
	9	8 ± 1#	19.4 ± 0.7#	15.5 ± 2.8#	0.004
Fold change from baseline		**5.1 ± 1.2**	**13.9 ± 7.1**	**14.1 ± 5.7**	**NS**

* p-value from ANOVA analysis between groups. #Different from value at time zero within the same group, p>0.05. NS not significant

Tissue Bacterial Load was not Different between Obese and Age-Matched Lean Controls

To test whether obese mice had impaired resistance to *E. chaffeensis* infection, we examined bacterial load in spleen, liver, epididymal fat and in peritoneal exudate cells by qRT-PCR looked at different organs and quantified the amount of bacteria by qRT-PCR. There was no difference in percentage fold change in bacteria transcript levels between OB and LE groups when compared to the YO group (p>0.05, Table 4.2) or when compared to each other (data not showed). In WAT, PEC and lungs, there was a lower percentage of bacteria transcript levels in both LE and OB compared to YO (Table 4.2). Nevertheless, we believe that none of the groups totally cleared the infection.

Table 4.2 Percentage fold change in mRNA transcript levels in lean (LE) and obese (OB) mice with the young (YO) as the reference group using the Pfaffl (Pfaffl 2001) method for calculations. Data are presented as Median (range).

	Spleen		Lungs		WAT		PEC	
	Day 6	Day 9	Day 6	Day 9	Day 6	Day 9	Day 6	Day 9
Lean (LE)	97 (41-132)	58 (10-227)	53 (33-192)	15 (8-74)*	29 (6-75)*	29 (10-370)	83 (70-121)	34 (9-64)*
Obese (OB)	100 (23-164)	145 (28-251)	75 (38-174)	15 (7-64)*	27 (13-268)	10 (4-11)**	51 (20-92)*	17 (6-62)**

*Significantly different from 100% as per one sample t-test, $p<0.05$
** Significantly different from 100% as per one sample t-test, $p<0.01$

Macrophages isolated from infected obese mice have a compromised phagocytic activity in vitro that was corrected by leptin supplementation

Previous studies have reported defective phagocytic activity in obese mice (Amar, Zhou et al. 2007). Therefore, we assessed macrophage phagocytosis of *E. chaffeensis* from OB, LE and YO mice. Peritoneal macrophages were isolated from the three groups at day 9 after experimental challenge. "Obese" macrophages had a significantly lower percentage phagocytosed FITC labeled *E. chaffeensis* bacteria compared to YO mice $p<0.05$ (Figure 4.2). However, no difference was detected between the LE mice and the other two groups.

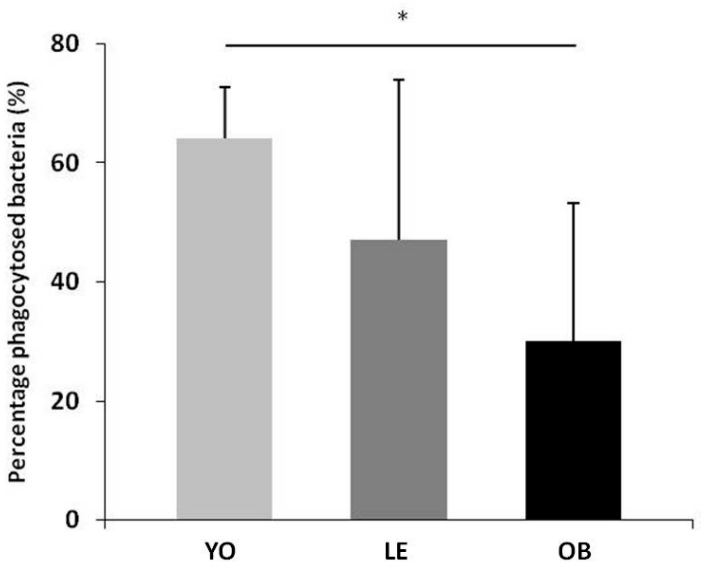

Figure 4.2 OB macrophages exhibit a low phagocytic index compared to YO and LE macrophages.
Percent phagocytosis of FITC-labeled *E. chaffeensis* from *E. chaffeensis* infected obese (OB), age-matched lean (LE) and young (YO) mice at day 9 post-infection (n=6-9 per group). * $p<0.05$.

To determine if leptin affected phagocytosis, we repeated our phagocytosis assays with exogenous leptin. "Obese" macrophages had a significant increase in the percentage phagocytosis when supplemented with 500 ng/ml rm-Leptin ($p<0.05$). This restored phagocytosis to the phagocytic levels of the YO mice. Higher concentration of leptin (5000ng/ml) had no added effect (Figure 4.3). YO macrophages did not respond to leptin supplementation and had the same phagocytic efficiency with or without leptin ($p>0.05$, Figure 4.3).

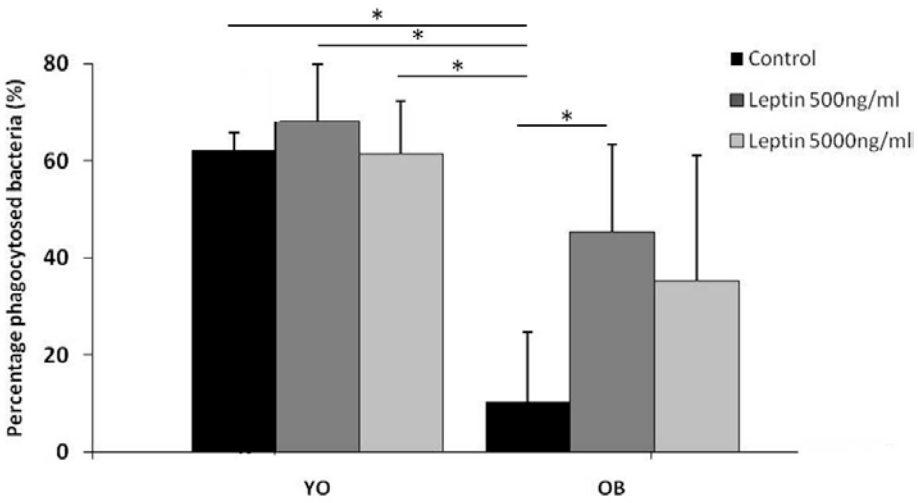

Figure 4.3 Leptin supplementation restored the phagocytic index of OB macrophages to that of the YO.
Percentage phagocytosis of FITC-labeled E, chaffeensis in peritoneal macrophages isolated from E. chaffeensis infected obese (OB) and young (YO) mice at day 9 post-infection supplemented in vitro with 0, 500ng/ml or 5000ng/ml rm-leptin (n=3 per supplementation group). * $p<0.05$.

When we assayed phagocytosis of macrophages from normal mice, no differences were detected in phagocytic activity in macrophages coming from any group (Figure 4.4) and leptin supplementation had no effect on any macrophage group; not even those from OB mice (data not shown).

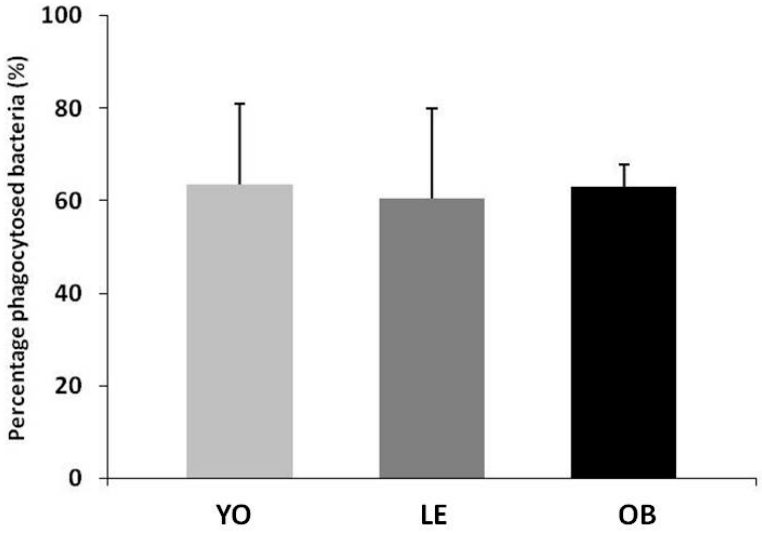

Figure 4.4 Naïve OB, LE and YO macrophages exhibit the same phagocytic index.
Percentage phagocytosis of FITC-labeled E, chaffeensis in peritoneal macrophages isolated from non-infected obese (OB), age-matched lean (LE) and young (YO) mice at day 9 post-infection (n=6-9 per group).

Discussion

Obesity is associated with an increased risk of infection, impaired pathogen clearance and a higher sepsis-related mortality and morbidity (Falagas and Kompoti 2006; Desruisseaux, Nagajyothi et al. 2007). The mechanisms for these compromised immune responses is not well understood, but leptin may play a role in this process (Mancuso, Gottschalk et al. 2002; Shapiro, Khankin et al. 2010). We hypothesized that upon infection, leptin levels fail to increase in obesity, hence negatively affecting macrophage response to bacterial pathogen and delaying bacterial clearance. We used a model of DIO mice followed by an i.p. injection with a suspension of live *E. chaffeensis* bacteria and monitored their systemic leptin and cytokine levels and bacterial clearance for nine days.

We assessed the ability of obese mice to clear an experimental challenge of *E. chaffeensis*. We measured no differences between obese and age-matched lean mice in bacterial loads in mice at 6 or 9 days after challenge. This contrasts studies that showed that DIO mice were less capable of clearing bacterial infections compared to lean controls (Amar, Zhou et al. 2007; Zhou, Leeman et al. 2009). It is possible that the differences are due to the types of bacteria used for the challenge. *E. chaffeensis* is an obligate intracellular bacterium whereas *P.gingivalis* is an LPS-expressing Gram-negative pyogenic bacterium. Although both bacteria stimulate innate resistance through toll-like receptors, *E.chaffeensis* requires T-cells for most effective clearance. *P. gingivalis* LPS is recognized by macrophages through Toll-Like receptor 2 (TLR2). Activation of this receptor leads to chronic inflammatory reactions conferring host protection against these bacteria. In obesity, TLR2 signaling was impaired explaining the higher susceptibility of DIO mice to *P. gingivalis* infection (Zhou, Leeman et al. 2009). The receptor involved in *E. chaffeensis* recognition is yet to be identified and the exact mechanism involved in the clearance of this pathogen is still to be completely defined but is believed to require both innate and adaptive immune mechanisms (Ganta, Cheng et al. 2007).

We detected no difference between obese and lean mice in circulating inflammatory cytokines. Groups had a significant increase in both serum IL6 and TNF-α throughout the study period. This would be consistent with their equal ability to cure infections. However, our results are in disagreement with those who reported DIO mice had a blunted circulating cytokine response following infection with *P. gingivalis*.

All mice had decreases in serum leptin levels following *E. chaffeensis* infection. Serum leptin levels have been shown to decrease (Hultgren and Tarkowski 2001), increase (Karlsson, Sheridan et al. 2010) or remain unchanged (Smith, Sheridan et al. 2007) depending on the infectious agent (Smith, Sheridan et al. 2007; Karlsson, Sheridan et al. 2010). In the study looking at influenza viral infection, leptin levels were decreased 3 days after infection in DIO mice whereas they were maintained in the lean group. The decrease in the obese group was correlated with a higher infection rate (Smith, Sheridan et al. 2007). Leptin levels significantly increased after influenza virus infection in lean mice but this response failed to occur in obese mice (Karlsson, Sheridan et al. 2010). It is possible that leptin changes were due to change in weight (Maffei, Halaas et al. 1995). However, despite the fact that DIO mice lost significant weight during our study, the weight loss does not totally account for the decrease in leptin levels since the young group actually gained weight but also had a significant drop in leptin levels.

Macrophages isolated from either obese humans or mice have an impaired phagocytic and bactericidal activity (Krishnan, Trost et al. 1982; Mancuso, Gottschalk et al. 2002). We showed that macrophages isolated from infected obese mice have a decreased phagocytic activity *in vitro* compared to control macrophages and that leptin supplementation was able to restore the phagocytic index back to normal. Leptin supplementation in macrophages isolated from starved mice (low leptin levels) or leptin deficient mice have been shown to increase their pahgocytic activity back to regular levels (Mancuso, Huffnagle et al. 2006). This can be attributed to leptin activation the JAK/STAT signaling pathway, leading to stimulation of phagocytosis, production of oxygen and nitrogen reactive species, and secretion of pro-inflammatory cytokines by macrophages (Shirshev and Orlova 2005). Leptin supplementation had no effect on lean macrophages probably because these macrophages exhibited optimal level of phagocytosis at baseline.

In conclusion, our *in vivo* data showed no difference in serum leptin and circulating cytokines or in bacterial clearance was detected in our study between DIO and age-matched lean mice; we believe that the use of a longer experimental period along with more traditional gram negative bacteria would have yielded different results. Nevertheless, we show that infected obese macrophages have compromised phagocytic activity *in vitro,* which can be corrected by leptin supplementation.

Chapter 5 - Epilogue

Obesity and metabolic disease are the epidemic diseases of this century. The implication of the immune system and different immune cells in the pathology of obesity has led to the emergence of a new area of research known as “Metaflammation” or “Immunometabolism”. Dissecting the different pathways and mechanisms in this elaborate metabolic-immune interaction is of primary importance and ongoing research is gradually unraveling this intricate interplay of these two component systems. Leptin is investigated as the link that might explain the activation of the immune system in the context of obesity. The role of leptin is investigated in the context of diet-induced obesity on both adipose tissue inflammation and infection susceptibility. An *in vitro* model is generated to study the leptin-obesity-immunity system further.

The major project (Chapter 2) **“Leptin Receptor Contributes to the Metabolic and Inflammatory Profile of High Fat Fed Mice”** examines the role of leptin in exacerbating the inflammatory phenotype in obese adipose tissue. Obesity is characterized by an increased recruitment of pro-inflammatory macrophages to the adipose tissue contributing to systemic inflammation and metabolic disease. The study hypothesis states that leptin, the hormone secreted by adipocytes, is a major player in the enhanced macrophage recruitment in obesity. The main research objective is to investigate whether the lack of leptin signaling on bone marrow cells prevents the recruitment of pro-inflammatory macrophages to the adipose tissue of DIO mice. Consequently, these mice will display an improved metabolic and inflammatory profile. To test this hypothesis, wild type (WT) mice were irradiated and transplanted with either leptin receptor deficient or WT bone marrow and were randomized to either a high fat (HFD) or normal fat diet (NFD) for sixteen weeks. Weight and food intake were measured weekly. Fasting blood sugar, insulin, leptin, MCP-1, IL-6, and TNF-α were measured at 0, 8 and 16 weeks. Adipose tissue morphology was assessed and liver steatosis was determined. Adipocytes gene transcript levels of key metabolic and inflammatory genes were assessed and adipose tissue macrophage phenotype - resident versus recruited pro-inflammatory- and quantification of crown-like structures, a measure of adipose tissue inflammation, were assessed. Mice reconstituted with leptin receptor-deficient bone marrow were resistant to HFD-induced weight and fat mass gain.

Although adipocyte size distribution was consistent with that observed under a HFD feeding, adipocytes from mice reconstituted with leptin receptor-deficient bone marrow had a 50% reduction in the expression of inflammatory genes. Epididymal fat showed significantly fewer crown-like structures compared to WT-reconstituted mice and most of these macrophages expressed Mgl1 and were $CCR2^-$, indicative of an anti-inflammatory phenotype. Systemically, TNF-α and IL-6 levels were not changed from baseline in any of the groups, but leptin-receptor deficient mice on HFD were more insulin sensitive compared to mice reconstituted with WT. In conclusion, leptin through its long receptor contributes to the recruitment of pro-inflammatory macrophages in the adipose tissue of obese mice and the expression of the obese phenotype.

The second project (Chapter 3) is entitled **"Establishment and Characterization of DB-1: A Leptin Receptor Deficient Murine Macrophage Cell Line".** A leptin receptor-deficient macrophage cell line, DB-1 was immortalized and characterized to provide an *in vitro* tool to investigate the link between leptin and innate immunity. The cell line was created using bone marrow cells from leptin-receptor deficient (*db/db*) mice. Bone marrow cells were differentiated into macrophages by culturing them with rmM-CSF, and passaged when confluent. This treatment was maintained for a period of six months. The cells spontaneously immortalized at approximately passage 20. Cells were cloned twice by limiting dilution cloning prior to characterization. The macrophage cell line is diploid and has a three-day doubling time. The cells are MAC-2 and F4/80 positive and have phagocytic activity similar to primary macrophages from WT and *db/db* mice. DB-1 cells respond to stimulation with IFN-γ by increasing Nos2 transcript levels. In addition, DB-1 macrophages are responsive to neither the chemotactic signaling of adipocyte conditioned media nor leptin when compared to primary WT macrophages. As a result, DB-1 cells provide a dependable tool to study the role of leptin or the leptin receptor in obesity-associated inflammation and immune system dysregulation.

In the third study (Chapter 4) entitled: **"Role of Leptin in Obesity-Associated Infection Susceptibility"** the major hypothesis is that leptin response to infection is impaired in obesity, leading to a compromised immune response and defective pathogen clearance. Three groups of mice were experimentally challenged with *Ehrlichia chaffeensis* i.p.: 1) obese 2) age-matched lean and 3) young lean. Serum leptin and IL-6 were measured at 0, 3, 6 & 9 days

post-infection. Bacterial infection was assessed at days 6 and 9 post-infection using qRT-PCR. The effect of leptin supplementation on macrophage phagocytosis in "obese" and "lean" macrophages was assessed *in vitro*. Peritoneal macrophages were isolated from the three groups, incubated with different concentrations of leptin, challenged with FITC-labeled *E. chaffeensis* and phagocytosed bacteria were quantified by FACS. A difference in the infection between lean and obese mice *in vivo* was not detected. This is probably related to the use of these specific bacteria and the short experimental period. Nevertheless, results showed that obese macrophages have a compromised phagocytic activity *in vitro*, which was normalized by leptin supplementation.

The data from the above mentioned studies suggest that leptin contributes to the inflammation of obesity. They also suggest that leptin affects macrophage function in obesity *in vitro* though more studies are required to assess leptin's contribution to infection outcomes *in vivo.* Finally, DB-1 cells provide a dependable tool to study the role of leptin in obesity-associated inflammation and immune system dysregulation further. We believe that results from our experiments will have the potential of elucidating the role of leptin in precipitating the inflammatory state associated with obesity. Further understanding this pathway will provide researchers the scientific background needed to help them design targeted pharmacological and interventional therapies that can help prevent the development of the different obesity-associated morbidities.

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