IMMUNE MODULATION OF METABOLISM: THE ROLE OF MACROPHAGES, NKT CELLS AND DENDRITIC CELLS IN THE DEVELOPMENT OF THE METABOLIC DISTURBANCES OF OBESITY

by

Benjamin Stuart Mantell

ScB, Brown University, 2006

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Benjamin Stuart Mantell

It was defended on

September 28, 2011

and approved by

Penelope A. Morel, Professor, Departments of Immunology and Medicine

Jon D. Piganelli, Associate Professor, Departments of Immunology and Pediatrics, Division of Immunogenetics

Martin C. Schmidt, Associate Professor, Department of Microbiology and Molecular Genetics

Donald K. Scott, Associate Professor, Departments of Microbiology and Molecular Genetics and Medicine, Division of Endocrinology and Metabolism

Dissertation Director: Robert M. O'Doherty, Associate Professor, Departments of Microbiology and Molecular Genetics and Medicine, Division of Endocrinology and Metabolism

IMMUNE MODULATION OF METABOLISM: THE ROLE OF MACROPHAGES, NKT CELLS AND DENDRITIC CELLS IN THE DEVELOPMENT OF THE METABOLIC DISTURBANCES OF OBESITY

Benjamin Stuart Mantell, PhD

University of Pittsburgh, 2011

It is now established that adipose tissue macrophages, which accumulate in obese individuals and rodents, play a key role in the generation of a low-grade, chronic inflammation, as well as the development of steatosis and insulin resistance, when faced with chronic overnutrition. The hypothesis of this dissertation is that other cells of the immune system and inflammatory responses within other insulin sensitive organs are also involved in the metabolic regulation of nutrient utilization. Specifically, we tested the responses of dendritic cells (DC), Kupffer cells (KC, the resident macrophages of the liver) and Natural Killer T (NKT) cells in the liver, as well as in adipose tissue.

To address this hypothesis, we developed two model systems to artificially alter the immune system while monitoring changes within the liver and/or adipose tissue. First, we established an *in vitro* co-culture model of the liver with mouse hepatocytes and KC to study interactions between the two cell types. Second, we established an adoptive transfer protocol to exogenously increase the numbers of macrophages and various types of DC in mice consuming a high fat diet. Finally, we utilized the $CD1d^{-/-}$

mouse line to clarify an important controversy regarding the involvement of NKT cells in the metabolic disturbances of obesity.

We found that KC decrease hepatocyte palmitate oxidation and identified prostaglandin E_2 (PGE_2) as a potential mediator of this effect. Furthermore, the liver and adipose tissue of mice receiving weekly injections of immature bone marrow-derived DC are skewed towards a predominantly T_H2, or anti-inflammatory, phenotype. Additionally, these mice accumulate less fat and are partially protected against diet-induced insulin resistance. Also, we illustrate that removal of NKT cells does not alter insulin resistance or steatosis when consuming a high fat diet (HFD).

In conclusion, we have demonstrated that responses of immune cells within the liver alter lipid metabolism and can induce changes consistent with the development of steatosis. Additionally, DC play an integral role in the determination of body composition and insulin sensitivity when challenged with a HFD. And finally, NKT cells may not be involved in the development of the metabolic disturbances of obesity.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would like to sincerely thank Drs. Penelope Morel and Michael Turner (Department of Immunology, University of Pittsburgh School of Medicine) for helping to establish BMDC cultures as well as their assistance with the Luminex assays and ongoing discussions and collaborations. I greatly appreciate the assistance of the lab of Dr. Timothy R. Billiar (Department of Surgery, University of Pittsburgh School of Medicine) in helping me to establish primary mouse hepatocyte cultures in our lab. I would also like to thank Drs. Michael Thomas and Russell Salter (Department of Immunology, University of Pittsburgh School of Medicine) for helping me establish BMDM cultures in our lab. I would particularly like to thank my thesis committee, Drs. Penelope Morel, Jon Piganelli, Martin Schmidt, Donald Scott, and Robert O'Doherty, for their guidance and assistance throughout this entire process. Their intellectual support and insightful comments helped to shape my dissertation and ultimately the scientist I will become in the future. Finally, I would like to thank my Thesis Advisor, Dr. Robert O'Doherty, for all his support and mentoring over the past few years.

"A man who dares to waste one hour of time has not discovered the value of life."
- Charles Darwin

“A man should never be ashamed to own he has been in the wrong, which is but saying, in other words, that he is wiser today than he was yesterday." – Alexander Pope

“Destitutis ventis, remos adhibe” – Latin Proverb

1.0 CHAPTER 1 – INTRODUCTION

1.1 OBESITY, DYSLIPIDEMIA, INSULIN RESISTANCE AND TYPE II DIABETES MELLITUS

Obesity and type 2 diabetes mellitus (T2DM) are two diseases that are growing in prevalence to epidemic proportions. The World Health Organization recently reported that over 1.5 billion adults worldwide are overweight, and of those, 500 million are obese [1]. In the United States alone, the Center for Disease Control reports that over one-third of the adult population is obese [2]. Insulin resistance (IR) and dyslipidemia are hallmarks of obesity that predispose those persons towards the development of T2DM and non-alcoholic fatty liver disease (NAFLD). Notably, T2DM is the leading cause of new blindness among adults age 20-74, the leading cause of kidney failure and the cause of more than 60% of non-traumatic lower-limb amputations. Also, patients with T2DM are at increased risk for heart disease, hypertension, and stroke [3]. Although there has been much progress, the physiological, biochemical and molecular mechanisms linking obesity, dyslipidemia, IR, and T2DM remain ill-defined. Understanding these mechanisms is critical to the development of new treatments and to decreasing the morbidity and mortality associated with these diseases. In this dissertation, I will present work which further implicates an involvement of the innate

immune system in the pathogenesis of the metabolic disturbances of obesity and describe a novel mechanism for these changes.

1.2 INFLAMMATION AND DYSREGULATED METABOLISM IN OBESITY

Many potential mechanisms have been suggested as a link between obesity and insulin resistance. Inflammation is one of the earliest reported of such mechanisms. Studies as far back as the 1800s by Professor Wilhelm Ebstein demonstrated an association between inflammation and obesity/T2DM when he showed that high dose sodium salicylate (a related compound to the anti-inflammatory medication acetylsalicylic acid, a.k.a. aspirin) dramatically reduces glycosuria in diabetic patients [4]. Indeed, epidemiologic reports of increased inflammation in patients with obesity and T2DM had been made for almost 50 years, when it was discovered that the serum of diabetics and obese patients exhibit lower fibrinolytic activity, a process closely linked with inflammation that protects against atherosclerosis [5-7]. Further correlative studies reported that obese and T2DM individuals have increased circulating levels of pro-inflammatory cytokines. Hotamisligil and colleagues demonstrated that tumor necrosis factor α (TNFα) is elevated in the plasma and adipose tissue of obese patients. They also noted that weight loss significantly improves insulin resistance, as well as lowers the concentrations of TNFα in these subjects [8,9]. These studies were followed by those of Straczkowski et al., which showed that Interleukin (IL)-8 concentrations in the bloodstream correlates with fat mass and waist-to-hip ratio, both metrics of obesity [10]. Interestingly, monocyte chemoattractant protein 1 (MCP-1), a chemokine known to

recruit monocytes to areas of inflammation, is also elevated in the adipose tissue of obese mice [11]. Additionally, the studies of Creely et al. [12] provided some insight as to how this inflammation arises. They described that circulating endotoxin (bacterial lipopolysaccharide, LPS), which is elevated in the plasma of obese and T2DM individuals, causes cultured human adipocytes to release the pro-inflammatory cytokines TNFα and IL-6. Further, they noted that treatment of diabetic patients with rosiglitazone, an insulin sensitizing thiazolidinedione, decreases both fasting hyperinsulinemia and endotoxemia [12]. While these studies all indicate an association between elevated inflammation and metabolic dysfunction in diabetic and obese subjects, there have since been a number of reports showing a causative relationship between the inflammatory responses and metabolism.

Work by Cani et al. [13,14] expanded on the findings of Creely et al. [12] to show that exogenous and endogenous LPS from the gram-negative bacteria in the gut contribute to endotoxemia in the presence of a high fat diet (HFD). Thus, a continuous infusion of LPS in mice induces obesity and diabetes [13]. Furthermore, they showed that a HFD alters the gut microbiota [13], that by experimentally altering the constituents of the bacterial population of the gut, they could reduce endotoxemia and protect against the development of diabetes [14] and that HFD increases the permeabilization of the gut to LPS [14].

While the groundwork for the concept of immune modulation of metabolism (i.e. cells of the immune system altering nutrient utilization), may have been established by the work of Ebstein [4], the field truly had its inception in the mid-1990s, when Hotamisligil and colleagues demonstrated that an infusion of a neutralizing antibody

against the pro-inflammatory cytokine TNFα, protects Zucker fatty rats from insulin resistance [15]. Furthermore, mice lacking the TNFα gene gain similar amounts of weight as wild-type animals, but are protected against insulin resistance when placed on a HFD [16]. Additionally, bone marrow transplant of wild type bone marrows into TNFα knock-out mice, re-instates the insulin resistance caused by high fat feeding [17]. The work of Shoelson, Shulman and colleagues expanded on Ebstein's initial findings to show that salicylates substantially improve insulin sensitivity and glucose tolerance, and decrease fasting serum triglyceride and free fatty acid levels in genetic and diet-induced models of obesity [18], as well as improving insulin sensitivity in lipid-infused rats [19] and diabetic humans [20]. They also implicated the pro-inflammatory NF-κB pathway in these effects, when they demonstrated that enhancing the action of NF-κB or IKKβ promotes insulin resistance [21], and disrupting IKKβ activity protects against genetic and diet-induced models of insulin resistance [18,19,21]. Notably, activation of NF-κB results in the production of the pro-inflammatory cytokines TNFα, IL-1, and IL-6 [22,23]. Salicylates, contrary to most other NSAIDs, which primarily target the cyclooxygenase (COX) proteins COX-1 and COX-2, also inhibit the action of IKKβ and subsequently decrease TNFα production [24]. Arkan and colleagues provided further evidence that inflammation and immune cells can drive metabolic changes, by reporting that a myeloid-specific knock out of the IKKβ gene imparts protection from diet-induced insulin resistance [25].

Over the past decade, a number of reports have linked increased inflammation with obesity, IR, and dyslipidemia [26-33]. There is now believed to be a causal relationship between inflammation and the metabolic disturbances seen in obesity.

While the work of Cani and Creely implicate endotoxin as an activating molecule, we have been interested in ascribing a role to another molecule known to be highly elevated in the bloodstreams of obese patients: fatty acids.

1.3 FATTY ACID EFFECTS ON THE TISSUES

1.3.1 Physiologic handling and signaling of lipids

When our body ingests a lipid-rich meal, gastric and pancreatic lipases start the digestion process to break dietary fats, usually in the form of triglycerides (TG), into its component parts of fatty acids and glycerol. Once they reach the small intestine, bile salts, produced in the liver and stored in the gall bladder, emulsify the fats to increase the surface area to volume ratio, making the lipolytic process more efficient. The fatty acids and glycerol are transported into the enterocytes of the small intestine, where they are re-esterified into TG, before being packaged into chylomicrons and dumped into the lymphatics for delivery to the tissues (Figure 1). We know from multiple reports that the concentrations of plasma free fatty acids (FFA) are elevated in obese and T2DM patients [34-36] and, in addition to LPS, we are beginning to understand that these high levels of lipids in the bloodstream may contribute to sequelae of these diseases.

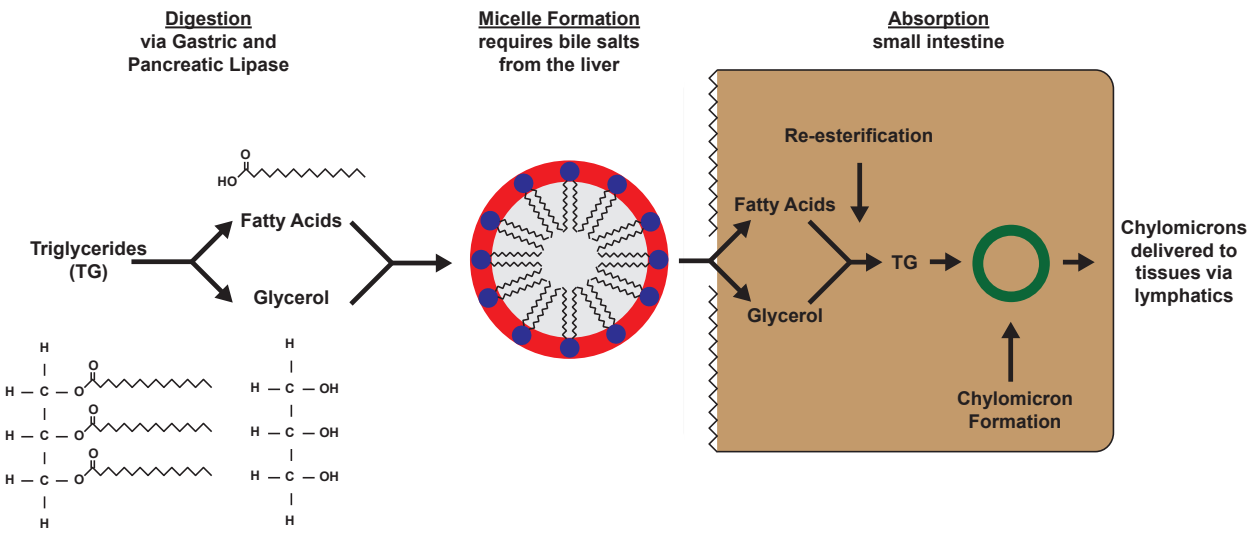


Figure 1 - Physiologic handling of lipids[1]

Interestingly, Toll-like receptor 4 (TLR4), the receptor for LPS, has also been implicated as the receptor for fatty acids [37-39]. Toll-like receptors are a family of receptors known for their involvement in innate immune responses, recognizing conserved, antigenic molecular patterns, such as those present in bacterial LPS and CpG, viral DNA and fungal zymosan [40-42]. TLR4 first signals through the adaptor protein MyD88 and then causes activation of NF-κB, which includes translocation to the nucleus, binding to DNA and subsequently altering gene transcription. Lee and colleagues demonstrated that saturated fatty acids, but not unsaturated fatty acids, activate NF-κB and induce gene expression of COX-1 in macrophages, a process requiring the presence of functional TLR4, MyD88, IκBα, and the PI-3K/Akt signaling

[1] Adapted with permission from Fauci AS, Kasper DL, Braunwald E, Hausler SL, Longo DL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine*, 17th Edition, http://www.accessmedicine.com.

pathway [37,43]. Furthermore, lipid infusion causes NF-κB to bind to DNA in wild type mice, but not in TLR4 knock out adipose tissue, and TLR4 deficiency protects against skeletal muscle insulin resistance [38]. Additionally, mice lacking TLR4 are protected against insulin resistance and steatosis when placed on a HFD ([39] and O'Doherty, unpublished observations), although they are not protected against the weight gain and insulin resistance caused by a diet with elevated trans fat [44]. Interestingly, even though saturated FFA and LPS both activate TLR4, the effects of these two molecules can be quite different, as I will describe in more detail in Chapter 2.

Fatty acids have also been shown to signal through AMP-activated protein kinase (AMPK). AMPK is a nutrient sensor within the cell that monitors the energy status (by detecting the AMP:ATP ratio) and modulates cellular processes to adapt to the current environment [45]. AMPK is also a potent activator of fatty acid oxidation through its inhibition of the lipogenic enzyme acetyl-CoA carboxylase (ACC) [45,46]. Both the saturated fatty acid palmitate and the poly-unsaturated fatty acid linoleate activate AMPK in L6 myotubes, and a dominant-negative form of AMPK completely blocks the fatty acid induced increases in β-oxidation [47].

1.3.2 Effects of fatty acids on insulin response

Studies in rodents [48,49] and humans [50-52] have demonstrated that an acute lipid infusion reduces insulin-stimulated glucose uptake. Additionally, chronic exposure to elevated fatty acids causes the accumulation of lipids in non-adipose tissues, which interferes with normal functioning, including the suppression of insulin secretion from pancreatic β-cells [53], a concept referred to as lipotoxicity. These changes are

apparently caused by activation of protein kinase Cθ (PKCθ) [49,54], which then phosphorylates serine/threonine residues on the insulin receptor, dampening the response to insulin. Chronic elevations of fatty acids also impair insulin sensitivity in rodents, but less evidence exists in larger animals and humans [55].

Together with the liver and skeletal muscle, adipose tissue is one of the three major insulin responsive organs in the body. White adipose tissue, once thought to be simply a repository for the fat stores of the body, is now recognized as a significant player in total body fat metabolism and nutrient homeostasis via the production of adipokines, cytokines, and chemokines [56]. In response to insulin, adipose tissue stores lipid in the form of triglyceride (TG), thus decreasing the levels of circulating plasma FFA postprandially, and maintaining the energy stores for when they are required during periods of fasting. Insulin is a potent inhibitor of lipolysis, such that in states of insulin resistance, lipolysis may not be suppressed, leading to a vicious cycle of FFA causing insulin resistance, and insulin resistance resulting in the release of more FFA into the bloodstreams of obese and T2DM patients. Along these lines, elevated FFA decrease insulin responsiveness and increase TNFα production in cultured 3T3L1 adipocytes [57]. Treatment of rat adipocytes with high concentrations of FFA also decreases insulin-stimulated glucose transport [58].

Skeletal muscle accounts for approximately 90% of the insulin-stimulated glucose removal after a meal [59]. In binding to its receptor, insulin causes a tyrosine kinase cascade, ultimately activating PI3K and Akt, resulting in the translocation of GLUT4 glucose transporters to the cell surface [60]. Accordingly, elevations in fatty acids have also been implicated in skeletal muscle insulin resistance. In response to an

acute challenge with FFA, myocytes exhibit decreased rates of insulin-stimulated glucose uptake, decreased phosphorylation of key signaling proteins downstream of the insulin receptor [52] and decreased insulin-stimulated glycogen synthesis [61]. Notably, activation of AMPK reverses this blockage [61]. Additionally, lipid infusions abolish IRS-1-associated PI-3-kinase activity in both humans [62] and mice [63]. Moreover, mice lacking TLR4 are protected against this decrease [63].

The liver is a critical modulator of systemic lipid and carbohydrate metabolism [64]. It is a primary target of insulin action, is positioned anatomically as the first post-absorptive organ to process most nutrients and gut endotoxin and processes most of the FFA released from the adipose tissue in the fasted state. In the obese state, the liver becomes engorged with fat [i.e. non-alcoholic fatty liver disease (NAFLD)]. NAFLD, now considered the hepatic manifestation of the metabolic syndrome [65], and the profound effects of hepatic insulin resistance on systemic metabolism, exemplify the importance of liver dysfunction in obesity. In healthy, lean subjects, decreases in plasma FFA concentrations decrease gluconeogenesis, but also increase glycogenolysis, such that the endogenous glucose production from the liver remains constant. Accordingly, increases in plasma FFA increase gluconeogenesis and decrease glycogenolysis at equal rates [50,66-68], however the mechanism of these changes are currently unknown. In states of insulin resistance, elevated serum FFA cause an increase in gluconeogenesis, however the liver is unable to coordinately modulate glycogenolysis, thus leading to hyperglycemia [69,70]. Importantly, the liver has its own extensive, resident immune system, which includes macrophages (Kupffer

cells), T cells, Natural Killer T (NKT) cells, Natural Killer (NK) cells and dendritic cells [71].

The interplay between inflammation and insulin resistance is now widely accepted. Particularly in the past decade, significant effort has been devoted to defining the mechanisms and cell types involved [30,32].

1.4 B AND T CELLS IN OBESITY

B and T cells are the major effector cells of the adaptive immune system. B cells classically serve as the producers of antibodies and the generation of humoral immunity. T cells act to destroy host cells that have become infected with pathogens ($CD8^+$ cytotoxic T cells, T_C) or act to assist other cells in enhancing their function through the production of cytokines ($CD4^+$ helper T cells, T_H) [72]. Of particular interest to the work described here, helper T cells have two predominant subsets, T_H1 and T_H2, as well as a newly recognized third subset, T_H17, which are all defined by the cytokines they produce. T_H1 cells secrete their quintessential cytokine, IFNγ [73], which is classically pro-inflammatory, [74] and drive macrophages down the “classical activation” or M1 pathway [75]. On the other hand, T_H2 cells secrete their signature cytokines IL-4, IL-5, IL-10 and IL-13 [73], are classically anti-inflammatory [74], and drive M2 or “alternative activation” of macrophages [75]. T_H17 cells produce IL-17 and IL-22 and are most abundant at mucosal surfaces such as the intestinal lamina propria [76]. These cells have recently been ascribed an important role in mediating anti-inflammatory responses and may play a role in the metabolic imbalance of obesity [77].

Furthermore, another type of T cell, regulatory T cells (Treg), have also recently been implicated in both rodent models and human obesity [78]. Nishimura and colleagues [79] demonstrated that $CD8^+$ T cells play a role in the development of the metabolic disturbances of obesity when they showed mice depleted of $CD8^+$ are protected against insulin resistance and adipose tissue inflammation when placed on a HFD.

In addition to producing antibodies, activated B cells also have some phagocytic and antigen-presenting capabilities. Thus, they can modulate T cell responses to specific antigens [72]. B cells have recently been shown to promote insulin resistance when mice are placed on a HFD through both the production of pathogenic antibodies as well as their interactions with T cells [80].

While recognizing the importance of B and T cells to the pathogenesis of obesity and insulin resistance, this work is primarily aimed at determining the roles that three different immune cell types, macrophages, dendritic cells and NKT cells, play in the development of the metabolic disturbances found in obesity. Below, I describe the role of each of these cell types as is accepted in the field at this point in time.

1.5 MACROPHAGES IN OBESITY

Macrophages are a heterogeneous population of myeloid-derived mononuclear cells that are a critical component of the innate immune response. They are the “garbage collectors” of the immune system: they phagocytose pathogens, degrade them, then present the antigens complexed with MHC molecules to activate and differentiate T cells with the complementary receptor [72]. A classic pro-inflammatory activator of

macrophages is the LPS component of endotoxin, acting through TLR4. More recently, saturated FFA, which are abundant in the plasma of obese patients, have been reported to act on macrophages via a similar mechanism [37,43]. Macrophages are the best studied of the immune cells in terms of their responses in metabolism: a number of seminal studies [81-84] have established that adipose tissue macrophages (ATM) are a contributing factor to the insulin resistance and metabolic deregulation seen in obese individuals.

In 2003, two papers described that, in both humans and mouse models of obesity, macrophages infiltrate into white adipose tissue (WAT) and the degree of ATM accumulation correlates with adiposity and measures of IR [84,85]. Furthermore, these macrophages are key contributors to the increased inflammatory status of obese individuals. Of note, mice with a reduced capacity to recruit macrophages (MCP-1 or CCR2 null) demonstrate reduced inflammation in fat, protection from HFD-induced IR and reduced ATM content [83,86], while stimulation of macrophage recruitment induces IR [87]. Notably, ATMs in obese animals tend to be M1 or “classically activated”, which produce pro-inflammatory cytokines, such as TNFα, IL-6, and IL-12 and reactive oxygen species via iNOS. They also promote T_H1 polarization of the T cells they activate. The macrophages in lean WAT tend to be M2 or “alternatively activated”, which produce anti-inflammatory cytokines, such as IL-10 and IL-1R decoy, and promote T_H2 polarization. Subsequent work further characterized the ATMs, the inflammatory pathways involved and the role of macrophages in the development of IR, dyslipidemia and obesity [25,82-85,87,88]. Together, these seminal studies clearly demonstrate a role for ATMs in the pathogenesis of IR and dyslipidemia in obesity. Macrophages,

however, are also particularly enriched in tissues, such as the liver, that are frequently exposed to exogenous and endogenous antigens and toxins. The potential role of liver macrophages [Kupffer cells (KC)] in the development of these metabolic abnormalities is largely unknown, although recent studies have implicated such a role [89,90].

Our lab has shown that rats depleted of KC with gadolinium chloride injection are resistant to the development of insulin resistance and steatosis [91]. In addition, an *in vitro* co-culture system in rats showed that incubation of activated KC with hepatocytes lowers palmitate oxidation and IRS-1 associated PI3K activity, and increases palmitate esterification and TG accumulation [91]. Furthermore, similar to the numbers of macrophages in the adipose tissue, the numbers of KC increased when mice were placed on a long-term HFD (Stefanovic-Racic et al., submitted).

As I will describe in Chapter 2 and Appendix A, I have developed an *in vitro* system with mice to study interactions between hepatocytes and KC. Similar to the rat system, we show that mouse KC cause a decrease in hepatocyte palmitate oxidation (Appendix A). Additionally, we have identified the eicosanoid prostaglandin E_2 (PGE_2) as a potential mediator of this response (Chapter 2). Thus, KC play an integral part in the development of NALFD in obese subjects, at least in part, by impairing the capacity of hepatocytes to break down lipid.

1.6 DENDRITIC CELLS IN OBESITY

Dendritic cells (DC) are bone marrow-derived, professional antigen presenting cells (APC) that play a key role in the development of innate and adaptive immune

responses, as well as in mediating tolerance to self antigens [92-94]. Dendritic cells are more efficient APCs than B cells or macrophages at activating naïve T cells, because B cells and macrophages must be activated before they can present antigen and they do so with a limited number of MHC molecules. Also, immature DC are poised to capture antigen, while mature DC constitutively express high levels of MHC and the co-stimulatory molecules required to activate T cells [72,92]. Similar to macrophages, DC can polarize an immune response in a T_H1 or T_H2 fashion, and the bacterial endotoxin, LPS, is the classical stimulatory molecule that allows DC to polarize T cells towards a T_H1 phenotype. LPS causes DC maturation and activation via TLR4 [95], and saturated FAs have also been shown to activate DC through TLR4 [95].

Much less is known at this point about the responses of DC in obesity and T2DM. Macia and colleagues [96] described an elevation of DC numbers in the auricular epidermis of leptin deficient *ob/ob* mice. Furthermore, bone marrow-derived DC (BMDC) from *ob/ob* mice do not activate T cells as readily as those from wild type animals [96]. Recently, two studies [97,98] described a novel population of monocytes that increase in obesity and, in addition to expressing the classical macrophage markers F4/80 and CD11b, express the CD11c marker ("triple positive cells"). This is an important observation since CD11c is a classical DC marker, suggesting that DC may be increased in obesity. Recently, Patsouris and colleagues reported on a transgenic mouse model that was depleted of all $CD11c^+$ cells [99]. The authors demonstrate that depletion of triple positive cells results in improved insulin sensitivity. Notably, only half of all the $CD11c^+$ cells that were depleted are triple positive. Thus, the majority of the cells that are still $CD11c^+$, but are not triple positive cells, are likely DC and could also

explain the observed improvement in insulin sensitivity. Furthermore, Nguyen and colleagues [100] demonstrated that triple positive BMDC produce higher levels of pro-inflammatory cytokines than bone marrow-derived macrophages (BMDM), which only express F4/80 and CD11b, at baseline and in response to FFA. Interestingly, the response to FFA was not seen with TLR2/TLR4 double knock out BMDC. This discrepancy has major implications for the timely generation of therapeutics, since work has already been devoted to investigating the use of DCs for vaccine and oncological purposes.

Our lab has found that mice placed on a long-term HFD have increased numbers of DC in both the WAT and liver, and that the changes in the liver are an early and reversible change, after only 3 weeks on the HFD (Stefanovic-Racic, et al., submitted). Furthermore, our collaborators have described that non-obese diabetic (NOD) mice, a mouse model of type I diabetes mellitus, injected with BMDC are protected against the development of diabetes [101].

As addressed in Chapter 3, we sought to expand on these findings to determine if injection of BMDC could protect against the development of T2DM. We generated immature BMDC (iDC), LPS-activated BMDC (LPS-DC), and BMDM and found that iDC, but not LPS-DC or BMDM, decreased weight gain and improved glucose tolerance. Furthermore, injection of iDC caused immune cell infiltration into liver and WAT, and these cells were skewed towards a T_H2 phenotype.

1.7 NKT CELLS IN OBESITY

Natural killer T (NKT) cells are a sub-population of lymphocytes that are proposed to serve as a link between the adaptive and innate immune systems [102-104] and have receptor expression characteristics of both NK cells (they are $NK1.1^+$) and T cells [they express a T cell receptor (TCR)]. Notably, the NKT TCR, rather then being activated by peptide antigens, is activated by glycolipid antigens through the MHC class I-like molecule, CD1d [102-104], while an alternative activation pathway is dependent on cytokine signaling from activated dendritic cells [103]. Irrespective of the method, activation of NKT cells results in rapid cytokine production (within hours), which may be of mixed, T_H1 or T_H2, dominance depending on the microenvironment to which the NKT cells are exposed [104].

Given the abundance of circulating FFA in obese patients, and the fact that NKT cells recognize glycolipid antigens, NKT cells are a logical potential mediator for the effects of lipid rich diets on metabolism. However, there is currently controversy in the field regarding the involvement of NKT cells in the development of the metabolic disturbances of obesity. Studies have indicated that NKT cells are more T_H1 polarized in obesity [105], and administration of the NKT activator α-galactosylceramide exacerbates glucose intolerance and adipose tissue inflammation [106], suggesting a pathogenic role of NKT cells in obesity. Conversely, other studies indicate that NKT cell numbers are decreased in the liver (the predominant site of NKT cells) [107-109], and adoptive transfer of NKT cells improves glucose tolerance and steatosis in *ob/ob* mice [110], which suggests a protective role for NKT cells in the development of the metabolic abnormalities of obesity. Furthermore, one group reported that mice lacking

NKT cells are protected from HFD-induced adipose tissue inflammation and glucose intolerance, although importantly, the mouse model used in this study also lacks $CD8^+$ T cells [106].

As we detail in Chapter 4, mice that specifically lack NKT cells are not protected against weight gain, adiposity, insulin resistance, or steatosis when placed on a HFD [107]. Thus, research efforts designed to ameliorate the sequelae of obesity should concentrate on cell types other than NKT cells.

1.8 STATEMENT OF THE PROBLEM

When I first undertook these projects, the field contained a preponderance of research describing a pathogenic role for macrophages in adipose tissue inflammation and the subsequent metabolic changes observed in obesity. We believe that other insulin sensitive organs, such as the liver, may also play a role in the generation of the metabolic disturbances of obesity. Furthermore, other cells of the immune system may be involved in the generation of the inflammation and metabolic abnormalities present in overnutrition. Our overarching hypothesis was that DC, KC and NKT cells are also integral in the metabolic changes of obesity. Specifically, throughout the work described, we aimed to manipulate these immune cell populations individually and measure known parameters of metabolism, *in vivo and in vitro*, to determine any metabolic changes in response to each.

The goal of this work was not necessarily to discover a way to prevent obesity and T2DM, but rather to discover mechanisms by which the sequelae of these diseases

arise, such that even in the face of these diseases, we can develop new treatments to reduce the associated morbidity and mortality. The research detailed below is novel in that we look not only at responses in adipose tissue, but also in other insulin sensitive organs such as the liver. Additionally, we detail a direct effect of dendritic cells in the development of the metabolic disturbances of obesity.

In our proposed model of the immune modulation of metabolism (Figure 2), FFA, which are elevated in the bloodstream of obese and T2DM patients, bind to TLR4 on the surface of adipocytes, hepatocytes and the resident immune cells of the liver and adipose tissues, such as KC. This activates a signaling cascade that ultimately leads to the expression of various chemokines in these cells. At the same time, DC in the tissues phagocytose a currently unknown antigen, become activated, travel to the lymph nodes, and present the antigen, complexed with an MHCII molecule, to T cells with the cognate T cell receptor (TCR). This MHCII-antigen:TCR interaction initiates the process of T cell activation, however, these T cells are not yet committed to a specific polarization. The production of chemokines in the tissues recruits DC, macrophages and T cells, where the presence of activated DC and a pro-inflammatory cytokine milieu influences the incoming T cells to assume a T_H1 polarization, which further adds to the pro-inflammatory status of the tissues. Recruited and resident macrophages also produce PGE_2, which acts directly on the hepatocyte to alter β-oxidation. Furthermore, the pro-inflammatory cytokines, produced by the DC, macrophages and T cells, act directly on the tissues to decrease insulin responsiveness.

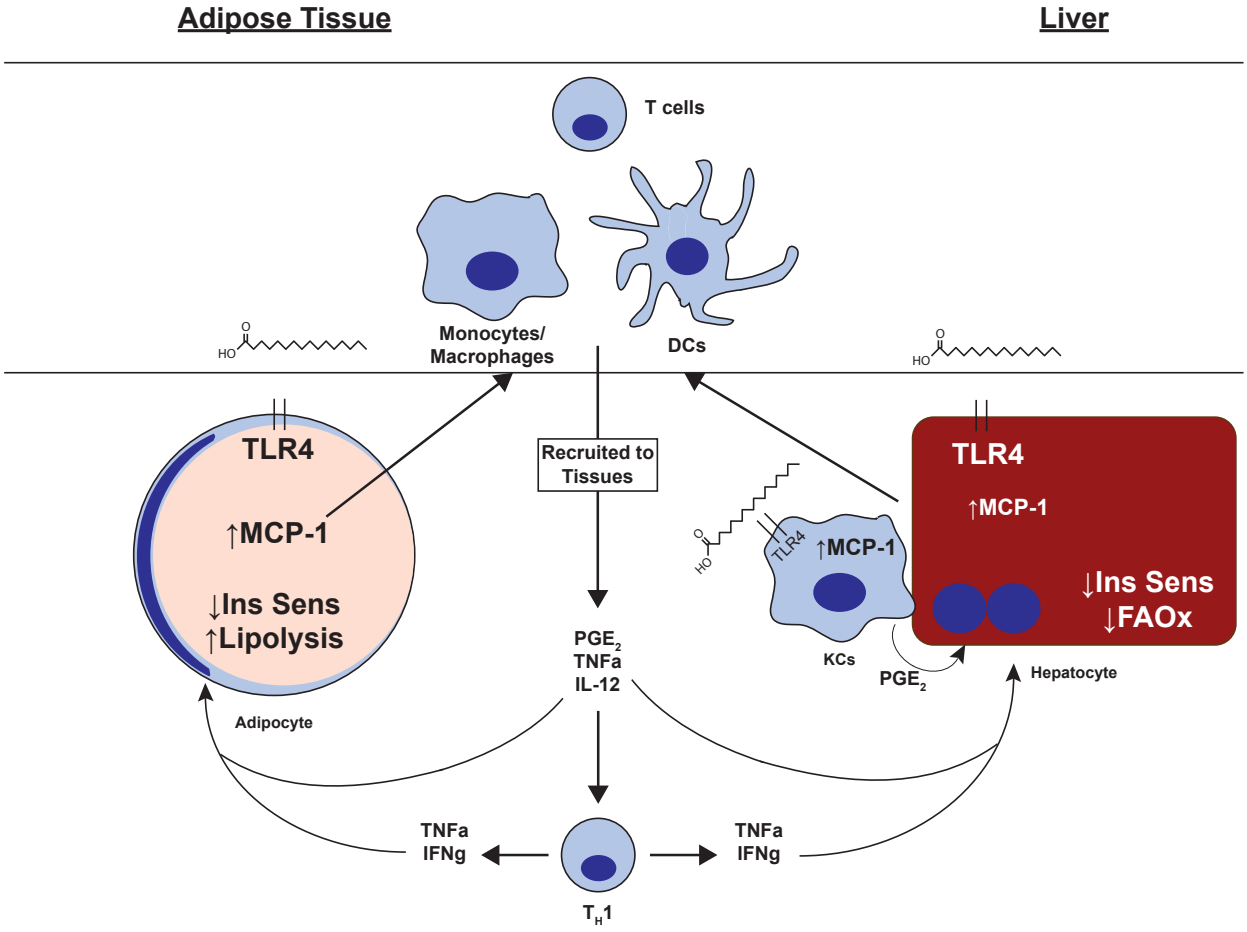


Figure 2 - Proposed model of immune modulation of metabolism

Elevated circulating levels of FFA, as seen in obese patients, activate TLR4 signaling in the parenchymal cells of the liver and adipose tissue, as well as resident immune cells, such as KC. This signaling cascade ultimately leads to the production of MCP-1, which recruits cells of the monocyte/macrophage and DC lineages to the tissues. The cytokine milieu produced by these cells along with T cells, which are recruited and activated upon recognition of a currently unknown antigen, skew the tissues towards a T_H1 phenotype. The pro-inflammatory factors produced reduce insulin sensitivity in these tissues resulting in decreased lipid oxidation in hepatocytes and increased lipolysis in adipoctyes. Abbreviations used: FFA, free fatty acids; TLR4, toll-like receptor 4; KC, Kupffer cells; MCP-1, monocyte chemoattractant protein-1; DCs, dendritic cells; PGE_2, prostaglandin E_2; TNFα, tumor necrosis factor α; IL, interleukin; IFNγ, interferon γ; Ins Sens, insulin sensitivity; FAOx, fatty acid oxidation.

2.0 CHAPTER 2 – PROSTAGLANDIN E_2, PRODUCED BY PALMITATE-ACTIVATED KUPFFER CELLS, DECREASES HEPATOCYTE LIPID OXIDATION

2.1 ABSTRACT

Kupffer cells (KC), the resident macrophages of the liver, have recently been implicated in the generation of the inflammation and subsequent metabolic changes observed coincident with chronic diseases such as obesity and type II diabetes mellitus. In this study, we describe a mechanism of KC-mediated alterations in hepatocyte metabolism involving the inflammatory lipid mediator prostaglandin E_2 (PGE_2). We hypothesized that PGE_2, produced by Kupffer cells, will decrease hepatocyte lipid oxidation leading to the development of steatosis and insulin resistance.

To address this question, primary mouse hepatocytes and Kupffer cells were isolated and co-cultured using a trans-well system. Lipid metabolism was assessed in co-cultures and hepatocyte monocultures in response to PGE_2 treatment. Additionally, mice lacking the microsomal PGE_2 synthase-2 gene (Ptges2 KO) were exposed to a high fat diet, and weight gain, food intake, body composition, metabolic rate and insulin sensitivity were measured.

KC inhibited palmitate oxidation in cultured mouse hepatocytes. Treatment of co-cultures with indomethacin completely abrogated this decrease in oxidation. PGE_2 is

produced by palmitate-activated KC and exogenous treatment of hepatocytes with increasing doses of PGE_2 decreased palmitate oxidation in the absence of KC. KC also inhibited palmitate esterification, although PGE_2 does not replicate this effect. Ptges2 KO mice gained more fat mass than their age-matched wild type controls, without stark changes in insulin sensitivity.

These data implicate PGE_2 in the metabolic disturbances of obesity. Specifically, PGE_2 may be necessary for the KC depression of hepatocyte palmitate oxidation, which is consistent with the development of steatosis and insulin resistance in conditions of overnutrition, such as obesity and high fat feeding.

2.2 INTRODUCTION

Obesity and type II diabetes mellitus coincide with a state of chronic, low-grade inflammation, which has been implicated in the pathogenesis of insulin resistance and dyslipidemia. Mounting evidence points to a role of adipose tissue macrophages in the development of this inflammatory state and the metabolic disturbances of obesity [81-86]. Notably, macrophages are increased in number and activity in both human obesity [81,82] and rodent models of diet induced obesity [84,85]. Interest persists, however, in exploring the effects of the immune system on other insulin sensitive organs. Indeed, the resident macrophages of the liver, Kupffer cells (KC), have recently been implicated in the generation of steatosis and insulin resistance when mice consume a high fat diet (HFD) [89,90]. We have found that KC are increased in the livers of mice that have been placed on a HFD for 26 weeks, by which time stark steatosis has set in

(Stefanovic-Racic, et al., submitted). Furthermore, we have shown, in rats, that depletion of KC protects against the development of steatosis in the setting of a HFD [91]. The mechanisms by which KC may influence the development of steatosis remain to be elucidated. KC produce a number of inflammatory mediators, some of which have been implicated in altering metabolism and insulin sensitivity. Chief among these have been cytokines, such as TNFα, IL-6 and IL-8 [10,15,111]. Macrophages, however, also produce lipid-derived molecules that serve as potent inflammatory signals. One class of such molecules is the prostaglandins, one member of which, prostaglandin E_2 (PGE_2), has previously been implicated in the regulation of hepatic metabolism.

PGE_2 is a lipid mediator derived from arachidonic acid (AA). It has been classically described as a pro-inflammatory molecule, is produced acutely, in large amounts, in inflammatory states and causes the cardinal signs of inflammation: rubor (redness – erythema), tumor (swelling – edema), calor (heat – fever) and dolor (pain) [112]. AA is first oxidized to PGH_2 by the cyclooxygenase enzymes (COX-1 and COX-2). PGH_2 is then further oxidized by one of the three terminal PGE_2 synthases (cPGES, mPGES-1 or mPGES-2) to generate PGE_2 [113]. PGE_2 signals primarily through four E-prostanoid receptor subtypes, designated EP1-EP4 [114]. These receptors are coupled to different G-proteins (EP1 to G_q, EP3 to G_i, and EP2 and EP4 to G_s), which likely account for the wide variety of effects attributed to PGE_2, often within the same tissue [115,116].

Importantly, PGE_2 has also been shown to have various metabolic functions. Studies have implicated PGE_2 in glucose metabolism, demonstrating both stimulation of glycogenolysis [117-119] and inhibition of glucagon-stimulated glycogenolysis [120-123]

depending on the receptors engaged and signaling pathways involved [119]. Furthermore, PGE_2 causes an increase in glycogen synthesis [124] and gluconeogenesis [118]. PGE_2 has also been implicated in lipid metabolism, as it inhibits VLDL secretion [125] and glucagon-stimulated β-oxidation [126]. Also, KC-derived PGE_2 inhibits lipid synthesis in cultured rat hepatocytes [127]. However, any direct effects of PGE_2 on hepatocyte lipid oxidation have yet to be determined. The current study was undertaken to address this issue. To accomplish this goal, we developed a co-culture system to measure lipid oxidation in primary mouse hepatocytes in the presence or absence of primary KC. The data indicate that KC, activated by the saturated fatty acid palmitate, produce PGE_2, which directly acts on hepatocytes to decrease lipid oxidation. Finally, mice lacking the gene for mPGES-2 become more obese than their wild type counterparts when placed on a high fat diet.

2.3 MATERIALS AND METHODS

2.3.1 Hepatocyte isolation and cell culture

Primary mouse hepatocytes were isolated using a non-recirculating *in situ* collagenase perfusion technique, modified as previously described [128]. A buffered salt solution containing 0.95g/L EGTA was perfused, *in situ*, through the inferior vena cava, exiting the portal vein (retrograde to blood flow), to remove any blood contained in the liver. Subsequently, a 0.2mg/mL collagenase solution (type IV; Sigma C-5138, St. Louis, MO) was perfused to release hepatocytes and immune cells from the connective tissue.

Livers were agitated to release cells, then hepatocytes were separated from other cells using a series of 40 x g spins and a 30% percoll gradient. Resulting hepatocytes were counted, assessed for viability using Trypan Blue exclusion, resuspended at $0.5x10^6$ live hepatocytes/mL, then plated on type I collagen coated 6- or 24-well plates (Falcon 35-3046 or 35-3047, respectively; collagen = Sigma C-8919) at a density of $1.2x10^6$ hepatocytes/6-well well or $2.5x10^5$ hepatocytes/24-well well. Non-attached cells were removed after 1 hour (45 min if in 24-well plates), by washing with RPMI 1640 containing 10% heat inactivated FBS, 4.5g/L glucose, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 100U/mL penicillin, and 100ug/mL streptomycin [high glucose (HG) cRPMI]. Treatment media, containing fatty acid plus or minus 10μM indomethacin, 1mM carbachol, various doses of PGE_2, or 500ng/mL LPS, was prepared in HG cRPMI, and replaced the wash at the start of culture.

2.3.2 Kupffer cell isolation and cell culture

Liver mononuclear cells were isolated, modified as previously described [91]. The supernatants from the non-percoll hepatocyte centrifugations were centrifuged at 40 x g for 5 min, and then the resultant supernatants were centrifuged at 375 x g. The cell pellet containing non-parenchymal cells was resuspended in 2.7mL cRPMI (as above but with 2.0g/L glucose) and mixed with 7.3mL 30% Histodenz (Sigma D-2158). The resulting suspension was split into two aliquots of 5mL and layered under 5mL of cRPMI, and centrifuged at 1425 x g for 20 min at 22°C with no brakes. Mononuclear cells remaining at the interphase between the two layers were collected and plated in cRPMI on 6-well plates (Falcon) or on trans-well inserts, as needed. Kupffer cells (KC)

were allowed to adhere to plates or inserts for 1 hour, then plating media was removed, together with non-adherent mononuclear cells, and replaced with treatment media. Additionally, control inserts plated with only cRPMI were also placed in the incubator for 1 hour, at the end of which the cRPMI was replaced with treatment media.

2.3.3 Hepatocyte and KC co-culture

Liver mononuclear cells were plated in Transwell Permeable Supports (Corning, Corning, NY) at a density 1/3 that of the hepatocytes (thus, $4x10^5$ in 6-well inserts or $8.3x10^4$ in 24-well inserts). Once KC attach, 2mL HG cRPMI containing 0.4mM palmitate or BSA vehicle control was added to the insert, and 2mL treatment media was added directly to the hepatocytes (200uL to the insert and 800uL directly to hepatocytes in 24-well plates). Hepatocytes and KC were co-cultured for 19 hours, then analyzed for palmitate oxidation, PGE_2 production, or RT-PCR.

2.3.4 Mouse primary hepatocyte palmitate oxidation

After 14 hours in culture in the presence or absence of 0.4mM palmitate and the presence or absence of 500ng/mL LPS, 2.5uCi/mL palmitic acid [9,10-^{3}H(N)] (Perkin Elmer, Boston, MA) and palmitate to a level of 0.4mM were added to each well for 5 hours. Palmitate oxidation was assessed by measuring the quantity of tritiated water released into the medium, as previously described [129]. The monolayers were washed twice with ice-cold PBS and collected in 1N NaOH for determination of protein content.

2.3.5 Quantification of cytokine production

Cell culture supernatants from various cell treatments were collected after 19 hours of culture, and assayed for the presence of 23 cytokines/chemokines (IL-1α, 1β, 2, 4, 5, 6, 10, 12(p40), 12(p70), 13, and 17, TNFα, IFN-γ, IP-10, CXCL1, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, and GM-CSF) using a Milliplex Luminex Kit (Millipore), according to the manufacturer's instructions.

2.3.6 PGE_2 EIA

PGE_2 released into the cell culture medium by co-culture or monoculture was measured using a commercial kit (by EIA; Cayman Chemical, Ann Arbor, MI) according to the protocol provided by the manufacturer.

2.3.7 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For analysis of gene expression, total RNA was isolated from liver or adipose tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was carried out using gene specific primers, SYBR green master mix (Bio-Rad, Hercules, CA) and an Applied Biosystems Prism 7300 Real-Time PCR System, as previously described [63]. Fold change in mRNA expression was determined using the $\Delta\Delta$cT method, with all genes normalized to β-actin.

2.3.8 Western blot

Hepatocytes plated in 6-well plates were cultured in the presence of 0.4mM palmitate in the presence or absence of KC, or the presence or absence of PGE_2. After 18 hours in culture, cell lysates were harvested and prepared using standard Western blotting procedures [anti-acetyl-CoA carboxylase (Cell Signaling #3662) and anti-phospho-acetyl-CoA carboxylase (Ser79) (Cell Signaling #3661)]. Immunoblots were quantified by densitometry using ImageJ Software.

2.3.9 Mouse primary hepatocyte palmitate esterification and triglyceride accumulation

Hepatocytes plated in 6-well plates were cultured in the presence of 0.4mM palmitate in the presence or absence of KC, or the presence or absence of PGE_2. After 16 hours of culture, 2.5uCi/mL palmitic acid [9,10-^{3}H(N)] was added to the esterification plates for two hours. Hepatocyte monolayers were washed twice with an excess of ice-cold PBS, then treated briefly with 12.5% trypsin-EDTA, and harvested in 1mL of ice cold PBS. Cells were pelleted, and analyzed for lipids and protein, as previously described [130].

2.3.10 Animal care and maintenance

Male Ptges2$^{-/-}$ (KO, lacking mPGES-2) and age matched wild type C57BL/6 (WT) mice were obtained from Jackson Laboratories. KO mice were generated using 129S embryonic stem cells, injected into C57BL/6 blastocysts, and the resulting chimeras

were backcrossed to pure C57Bl/6 mice for 12 generations. Prior to the experiments, mice were maintained on a constant 12-h light:12-h dark cycle with free access to water and *ad libitum* fed with a standard chow diet. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

2.3.11 *In vivo* experimental design

Ptges2$^{-/-}$ (KO) and age-matched wild type (WT) control mice (n=8 in each group) were fed *ad libitum* with a high fat diet (HFD; 44% calories from fat – 19% lard, 1% corn oil) from Harlan Teklad for 10 weeks. Mice and food were weighed weekly during the course of the experiment. Also, mice underwent an analysis of insulin sensitivity using glucose tolerance, metabolic rate using the CLAMS (Comprehensive Laboratory Animal Monitoring System, OH), and body composition using a Lunar PIXImus densitometer (Lunar, Madison, WI). Whole-body scans, with exclusion of the head, were analyzed for fat and lean masses using the manufacturer's software. At the conclusion of this period, blood and tissues were isolated, flash frozen and stored at –80°C until analysis.

2.3.12 Glucose tolerance tests (GTT)

For the GTT, 6-hour fasted mice were injected i.p. with 1.5 g/kg glucose. Blood was sampled from the tail vein every 15 minutes for 2 hours post-injection and glucose was measured using an Ascensia Elite glucometer (Bayer, Mishawaka, IN).

2.3.13 Tissue and plasma measurements

Liver and plasma triglycerides (TG) were determined as previously described [48,130]. Fasting blood glucose represents t=0 of the GTT (prior to injection of glucose).

2.3.14 Statistical analysis

Data are expressed as means ± SE. Statistical significance was determined by t-test and, where appropriate, analysis of variance (repeated measures or one-way ANOVA; Bonferroni's post-hoc test) was performed using the PASW Statistics program (Chicago, IL). Statistical significance was assumed at $p \leq 0.05$.

2.4 RESULTS

2.4.1 Palmitate-activated Kupffer cells (KC) decrease hepatocyte palmitate oxidation

Previous studies have reported decreases in rat hepatocyte palmitate oxidation when co-cultured with KC [91]. We first sought to confirm these findings in mice, as proof of principle, and then extend these observations, to determine which genes in the KC may be required for the effects seen. Consistent with previous studies, there was a decrease in hepatocyte palmitate oxidation when co-cultured with KC (Figure 3A). This effect was only present when cells were co-cultured overnight in the presence of

palmitate, not LPS (Figure 3A). Additionally, there was no difference in palmitate oxidation between hepatocytes alone and when TLR4-null KC were co-cultured with hepatocytes (Figure 3B).

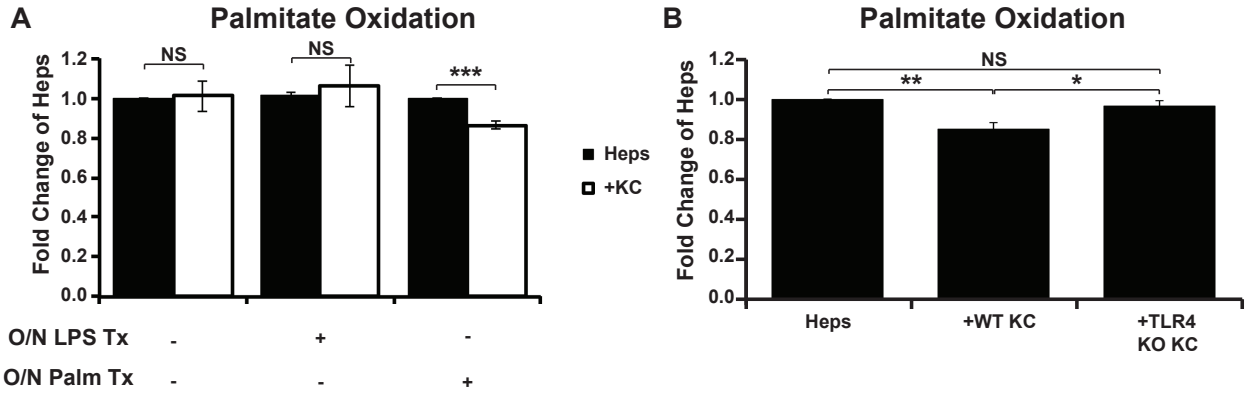


Figure 3 - Palmitate-activated Kupffer cells decrease hepatocyte palmitate oxidation.

(A) Primary hepatocytes from C57Bl/6 mice were cultured overnight in the presence of either 0.4mM palmitate or 500ng/mL LPS and the presence or absence of KC. (B) Alternatively, wild type hepatocytes were cultured in the presence of wild type or TLR4 knock out KC. Palmitate oxidation was measured via tritiated water exchange, as described in Methods. Results are presented as the means±SE for five (Panel A) or four (Panel B) experiments performed in triplicate. *p<0.05, **p<0.005, ***p<0.0005.

2.4.2 Cytokine production from KC and hepatocytes in the presence of palmitate.

Given that KC can alter hepatocyte palmitate oxidation in a co-culture setting, the responsible agent must be a soluble factor that is passed between the two cell types. Our first class of candidate molecules was cytokines, as they are soluble proteins produced in large quantities by macrophages upon activation, and act in autocrine and paracrine fashion [131]. To determine if cytokines were altered by the presence of palmitate, we treated KC or hepatocytes in monoculture with 0.4mM palmitate or vehicle control, and performed Luminex analysis on the supernatants 18 hours later. While we saw a robust response after LPS treatment, overnight culture in the presence of palmitate did not change KC production of the pro-inflammatory cytokines TNFα or IFN-

γ, nor the production of the anti-inflammatory cytokine IL-10 (Figure 4A-C). There was, however, a slight increase in the production of IL-1β (Figure 4D) in response to palmitate, without a concomitant increase in IL-6 (data not shown). Furthermore, there was in increase in KC production of MCP-1 (Figure 4E), and a small, but significant, increase in hepatocyte production of the chemokine CXCL1 (Figure 4F).

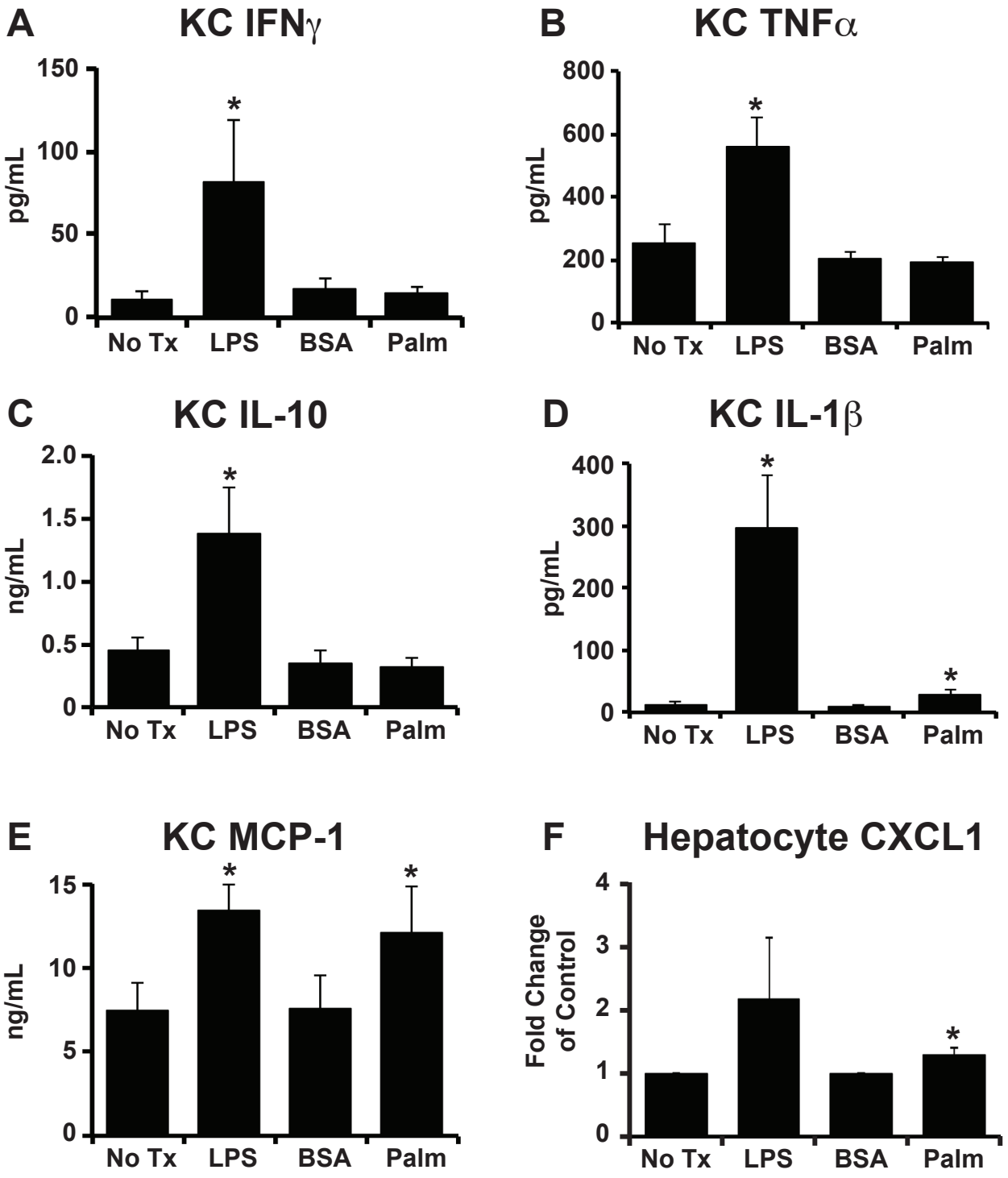


Figure 4 - Cytokine production by Kupffer cells (KC) and hepatocytes in the presence of palmitate.

Kupffer cells or hepatocytes were cultured overnight in the presence or absence of 0.4mM palmitate or the presence or absence of LPS. Cell culture supernatants were collected and analyzed by Luminex. Results are presented as the means±SE for an n=4 in KC and an n=3 in hepatocytes. *$p<0.05$

2.4.3 KC produce prostaglandin E_2 (PGE_2) in response to palmitate

Our search for the soluble factor responsible for the detrimental effects of KC on hepatocyte β-oxidation led to another suitable candidate, which has the added bonus of being a small lipid (and thus particularly pertinent in the setting of obesity), namely the eicosanoid PGE_2. In the presence of palmitate, but not in the vehicle control (BSA), the co-culture of hepatocytes and KC exhibits a roughly three-fold increase in the production of PGE_2 (Figure 5A), which is derived almost completely from the KC (Column #6). We next tested whether PGE_2 in the culture media was sufficient to cause a decrease in hepatocyte palmitate oxidation. We treated hepatocyte monocultures with increasing doses of PGE_2 and saw a decrease with as little as 10pM PGE_2 (Figure 5B). Importantly, the decrease in palmitate oxidation was not due to a loss of cell viability as measured by Alamar Blue fluorescence (Figure 5C). Additionally, we performed a time course experiment to determine the length of PGE_2 exposure time required in order to cause this decrease in palmitate oxidation. Our data indicate that this effect is still present with as little as 5 hours of PGE_2 treatment (Figure 5D).

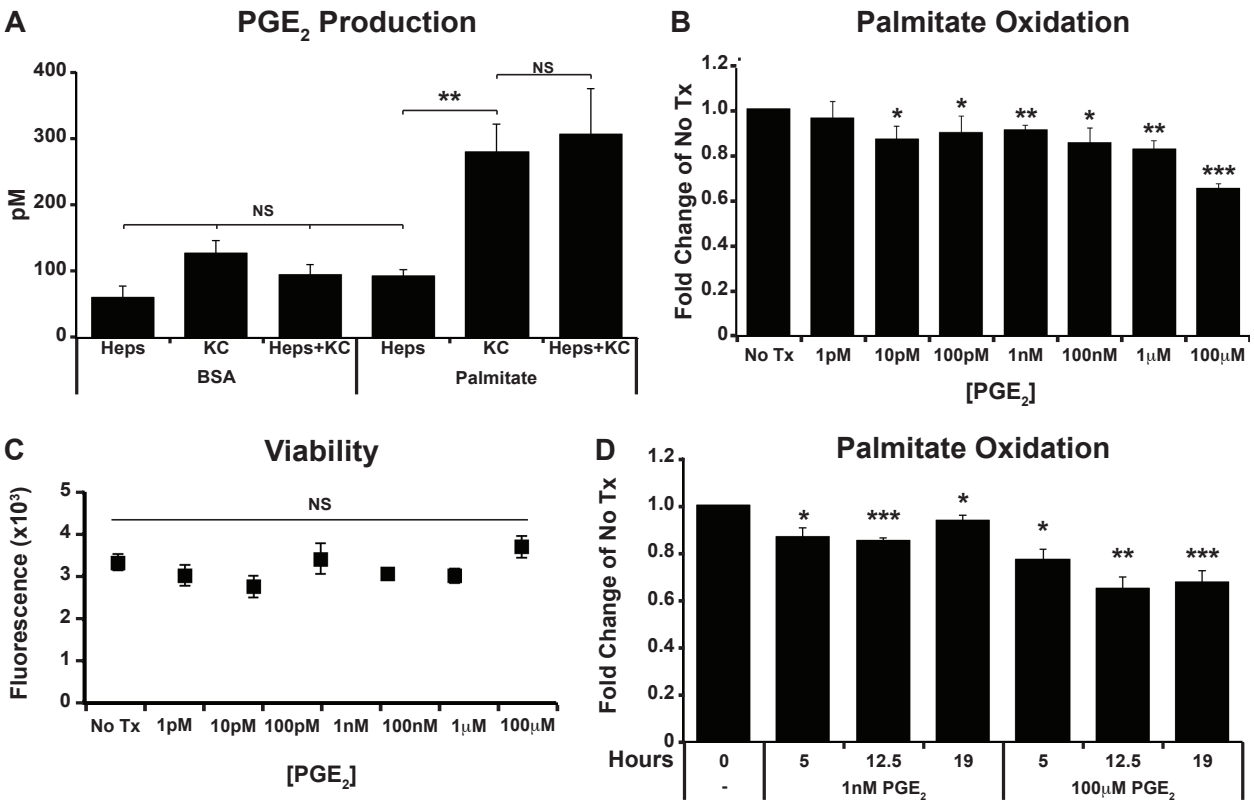


Figure 5 - Prostaglandin E_2 (PGE_2) effects on hepatocyte metabolism.

(A) Hepatocytes (Heps) and KC were cultured separately or co-cultured overnight, as described in Methods, after which cell culture media was collected and analyzed for PGE_2 concentration. (B&C) Hepatocyte monocultures were treated with increasing doses of PGE_2 and analyzed for palmitate oxidation (Panel B) or cell viability (Panel C). (D) Hepatocytes were cultured overnight in the presence of 0.4mM palmitate, and then treated with 1nM PGE_2 or 100mM PGE_2 for the times indicated. They were subsequently analyzed for palmitate oxidation. Results are presented as the means±SE for a minimum of 3 experiments performed in triplicate. *p<0.05, **p<0.005, ***p<0.0005.

2.4.4 EP receptor subtype expression and mechanism

Given that PGE_2 is sufficient to cause a decrease in hepatocyte palmitate oxidation, we next asked which receptor might be responsible for this action. First, we performed RT-PCR to determine which of the EP receptor subtypes are expressed in mouse hepatocytes and found EP1 to be the most highly expressed of the PGE_2 receptor subtypes (Figure 6A), although primer efficiencies are required before this strict

comparison can be made. To test whether action downstream of EP1 is involved in the interaction between KC and hepatocytes, we treated hepatocyte monocultures with carbachol, a pan-muscarinic receptor agonist, which increases the intracellular concentrations of calcium via the M1, M3, & M5 receptors, to determine whether an increase in intracellular calcium will decrease β-oxidation, thus mimicking action through EP1. As the data shows, 1mM carbachol is able to replicate this effect (Figure 6B).

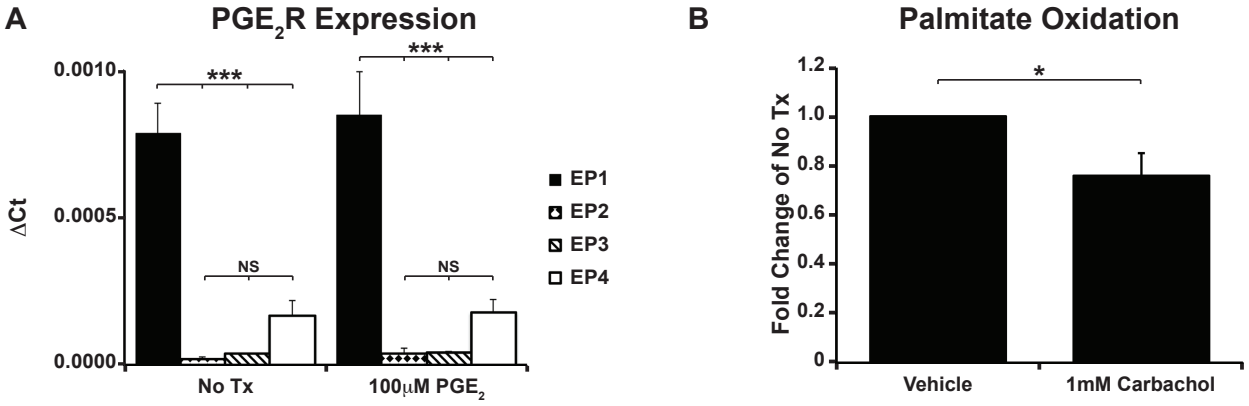


Figure 6 - E-prostanoid receptor expression and involvement of calcium signaling.

(A) Primary mouse hepatocytes were cultured overnight in the presence or absence of 100mM PGE_2. RNA extracts were prepared and RT-PCR was performed, as described in Methods. (B) Primary mouse hepatocytes were cultured in the presence of 0.4mM palmitate and the presence or absence of 1mM carbachol. Results are presented as the means±SE for a minimum of three experiments performed in triplicate. *p<0.05, ***p<0.0005.

2.4.5 Inhibitors of PGE_2 action alter palmitate oxidation

We next tested the ability of indomethacin, a non-selective COX-1/COX-2 inhibitor, to block the effect of KC on hepatocyte metabolism. 10μM indomethacin is able to reverse the decrease in oxidation caused by the presence of KC (Figure 7A). We also attempted to test the case where KC were unable to produce PGE_2 by utilizing KC from

mice lacking the gene encoding microsomal prostaglandin E_2 synthase-2 ($Ptges2^{-/-}$). Interestingly, in this circumstance, we did not see the same protection against the decrease in hepatocyte palmitate oxidation (Figure 7B); however, we discovered that these cells were still producing PGE_2 in response to palmitate (Figure 7C).

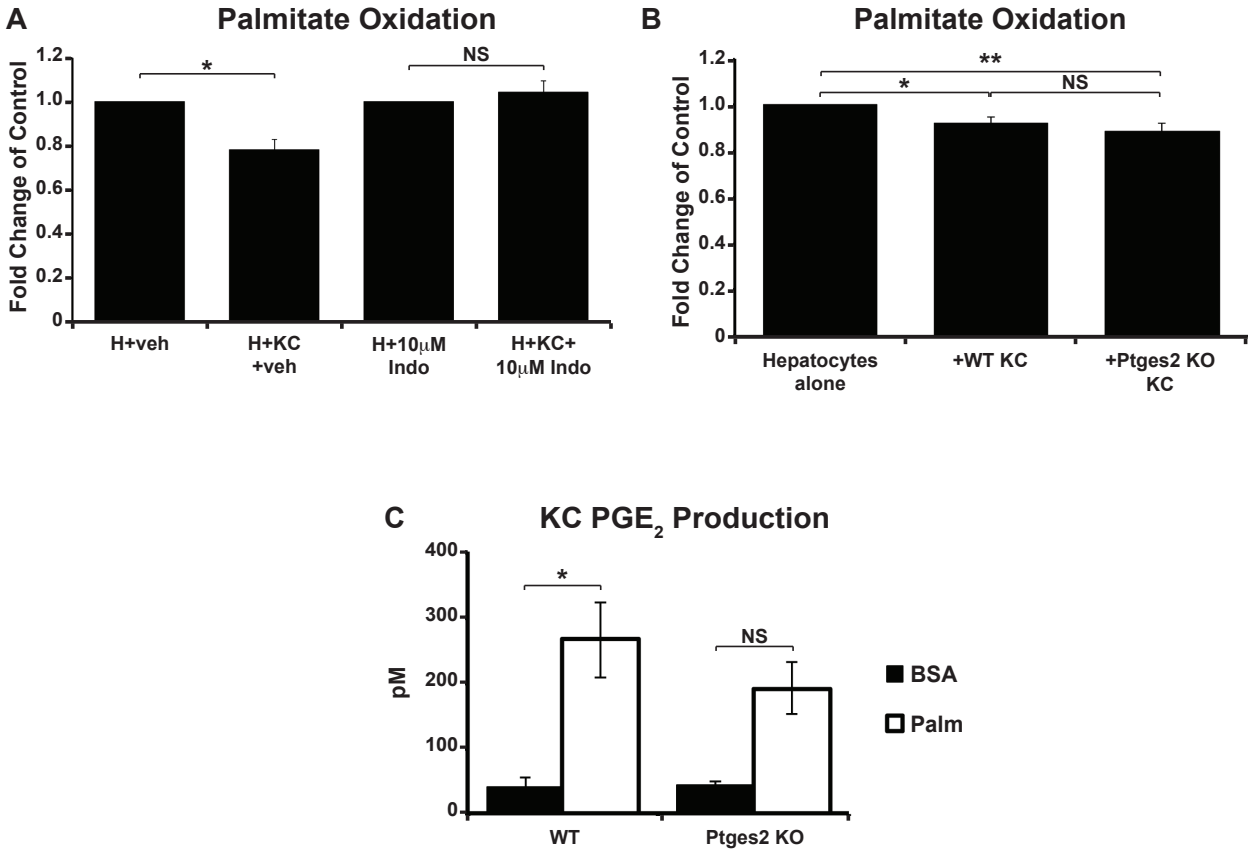


Figure 7 - Inhibitors of PGE_2 alter hepatocyte metabolism.

(A) Primary mouse hepatocytes were co-cultured with KC overnight in the presence or absence of 10uM indomethacin and palmitate oxidation was measured. (B) Hepatocytes were cultured in the presence of wild type or Ptges2 knock out KC. Palmitate oxidation was measured. Results are presented as the means±SE for four experiments performed in triplicate. (C) Wild type (WT – n=3) or Ptges2 knock out (KO – n=2) KC were cultured overnight in the presence or absence of palmitate. Cell culture media was collected and analyzed for PGE_2 concentration. *$p<0.05$, **$p<0.005$.

2.4.6 Intracellular mechanisms of altered metabolism

We postulated that the changes occurring within the hepatocyte in response to PGE_2 that resulted in decreased palmitate oxidation could be a gene transcription change, a protein translation change, or a biochemical change, such as protein phosphorylation. First, we performed RT-PCR on hepatocytes treated with PGE_2, and found that these effects are not due to alteration of gene expression of key lipid metabolism genes (PPARα, PGC-1α, SREBP1c, SCD-1, DGAT1, Foxo1, ACC1; Figure 8A). Interestingly, the Western Blot of total and phosphorylated ACC (Figure 8B) and densitometry quantification (Figure 8C) indicate that phosphorylation of ACC, in the presence of palmitate, decreases. ACC is activated in the dephosphorylated state; therefore, this key lipogenic enzyme could be more active in response to treatment with PGE_2, consistent with the finding of decreased lipid oxidation (a lipolytic process).

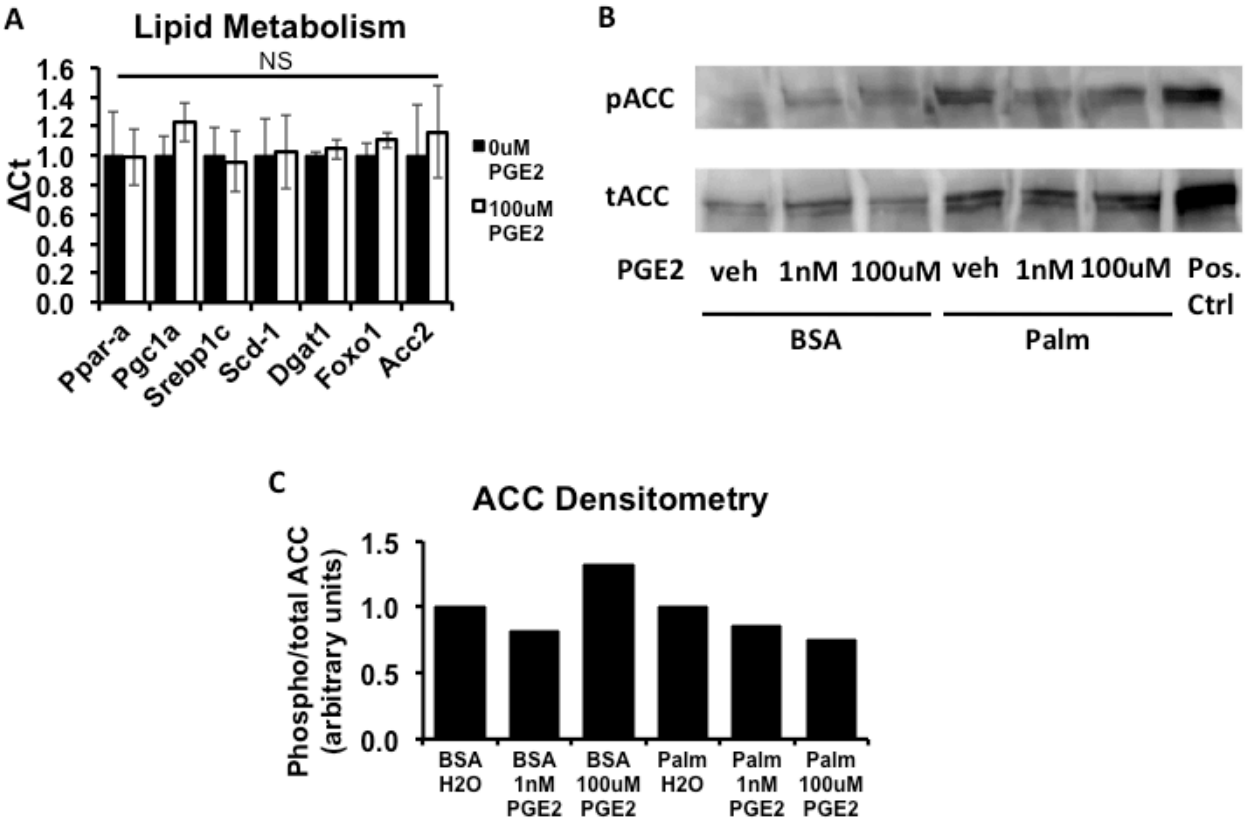


Figure 8 - Gene expression and ACC activation in response to PGE_2.

*(*A) Primary mouse hepatocytes were co-cultured with KC overnight in the presence or absence of 100uM PGE_2. RNA extracts were prepared and RT-PCR was performed, as described in Methods. (B) Western blots of total and phosphorylated ACC in the presence or absence of 0.4mM palmitate and the presence or absence of 1nM or 100mM PGE_2. (C) Densitometry of Western blots shown in (B).

2.4.7 KC and PGE_2 effects on lipid buildup

Given the changes we see in lipid catabolism, we became interested in how co-culture with KC and administration of PGE_2 would affect the opposing processes of lipid synthesis. We tested both palmitate esterification (Figure 9A&B) and triglyceride (TG) accumulation (Figure 9C&D) in mouse hepatocytes. Surprisingly, treatment with PGE_2 did not alter the esterification (Figure 9B), and co-culture of KC with hepatocytes acts to decrease palmitate esterification (Figure 9A), contrary to our expectations given our

ACC phosphorylation data. Additionally, TG accumulation within the hepatocyte trends towards increasing in response to both PGE_2 treatment and incubation with KC.

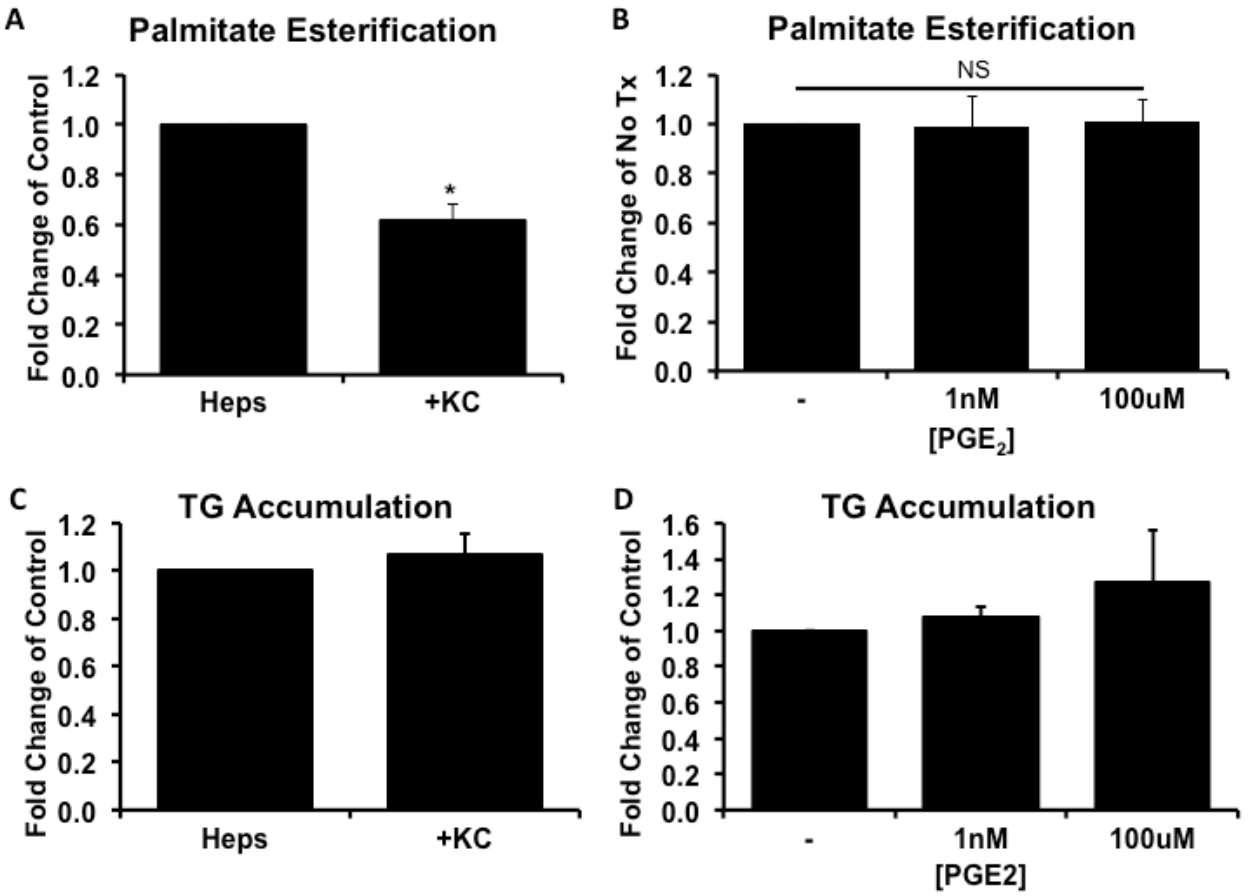


Figure 9 - PGE_2 and KC effects on palmitate esterification and TG accumulation.

(A) Primary mouse hepatocytes were co-cultured with KC overnight in the presence of 0.4mM palmitate or mono-cultured in the presence or absence of PGE_2. Esterification of fatty acids was measured for an n=3 experiments performed in duplicate (PGE_2 treatment) or triplicate (KC co-culture), as described in Methods. (B) Primary mouse hepatocytes were co-cultured with KC overnight in the presence of 0.4mM palmitate or mono-cultured in the presence or absence of PGE_2. Accumulation of TG within the hepatocytes was measured for an n=2 experiments performed in duplicate (PGE_2 treatment) or triplicate (KC co-culture), as described in Methods. *$p<0.05$.

2.4.8 Knockout of prostaglandin E_2 synthase-2 exacerbates the metabolic disturbances of obesity

Given the effects of PGE_2 on hepatocyte metabolism, we became interested in whether a mouse unable to produce PGE_2 upon challenge, would be protected from the metabolic disturbances of obesity. We placed Ptges2$^{-/-}$ and wild type controls on a HFD for 10 weeks and measured weight gain (Figure 10A), adiposity (Figure 10B), glucose tolerance (Figure 10C&D), fasting blood glucose (Figure 10E) and liver triglyceride buildup (Figure 10F). Weight gain was identical for much of the duration of the diet, however at 6 weeks, a gradual deviation started to occur, which did not reach statistical significance (Figure 10A). Additionally, whole body knock out of Ptges2 caused a substantial elevation in the fat mass and percent fat (Figure 10B). Glucose tolerance, fasting blood glucose and liver triglycerides were not altered in these animals. Importantly, analysis of serum of WT and Ptges2 mice demonstrated that Ptges2 mice have lower concentrations of circulating PGE_2 (data not shown).

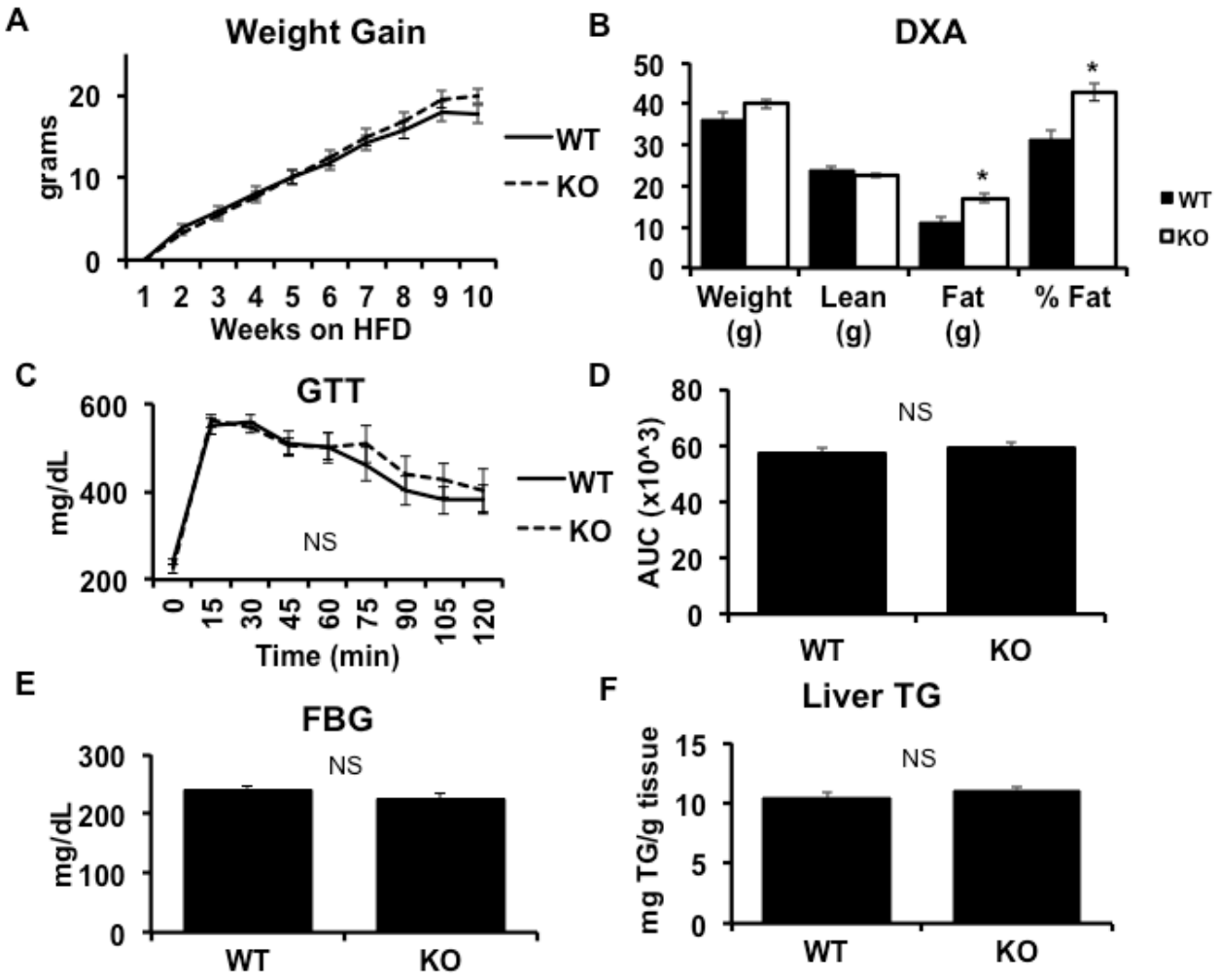


Figure 10 - Ptges2 KO mice gain more fat mass on a HFD.

Wild-type (WT) and Ptges2 null (KO) mice were placed on a high fat diet (HFD) and weight gain was monitored weekly (Panel A). After 8 weeks on HFD, mice underwent glucose tolerance tests (GTT and Area Under the Curve, Panels C&D). After 1 week for recovery, body composition was measured by DXA scan (Panel B). Fasting blood glucose (Panel E) and liver triglycerides (Panel F) were assessed in WT and KO mice as described in Methods. Results are presented as the means±SE for at least 7 animals in each group. *$p<0.05$.

2.5 DISCUSSION

The major goal of this study was to address the potential role of Kupffer cells (KC) in the pathogenesis of one of the hallmarks of overnutrition, obesity, and the metabolic

syndrome, namely the accumulation of fat in the liver. A number of novel observations are presented which expand on the known KC-mediated effects on hepatocyte metabolism and address the impact of the immune system on nutrient utilization. The data demonstrate that 1) hepatocytes exhibit lower rates of palmitate oxidation when co-cultured in the presence of KC, 2) co-culturing these cells in the presence of indomethacin abrogates this effect, 3) KC are activated by palmitate to produce PGE_2 and 4) genetic deletion of the constitutive PGE_2 synthase (mPGES-2, the *Ptges2* gene product) may exacerbate adiposity when mice are placed on a HFD. We were motivated by previous reports that have suggested an involvement of the pro-inflammatory cytokine TNFα, as well as the eicosanoids in metabolism. These studies have demonstrated that TNFα is elevated in the adipose tissue of human obesity and insulin resistance [9,15], mediates the induction of insulin resistance in 3T3-L1 adipocytes [57] and contributes to the development of insulin resistance in the Zucker fatty rat model of obesity [15]. Additionally, prostaglandins have been implicated in nutrient metabolism. Specifically, PGE_2 influences glycogenolysis [119,121,122] and glycogen synthesis [124], as well as gluconeogenesis [118]. Of particular interest to the work described here, PGE_2 inhibits lipid synthesis [127] and also inhibits hormone-stimulated β-oxidation [126]. Importantly, no previous studies have been undertaken which analyzed mouse primary hepatocyte lipid oxidation in response to co-culture with KC, nor have other studies described the response of cultured mouse hepatocytes to PGE_2.

A number of seminal studies have now established that macrophage numbers and activation increase in the hypertrophic adipose tissue abundant in obesity

[82,84,85]. To date, however, much less is known about the overnutrition response of macrophages in other insulin sensitive tissues. In the liver, resident Kupffer cells account for over 5% of the total cell population and are located in the sinusoids where they are well positioned to sample nutrients and other inflammatory mediators delivered to the liver directly from both the gut (via the portal vein) and systemic circulation (via the hepatic artery) [132]. Thus, KC are among the first cells to recognize and respond to potential macrophage-activating signals.

The nature of these signals in the setting of overnutrition is still not fully understood. We have demonstrated previously that co-culture of rat primary hepatocytes with LPS-activated KC decreases palmitate oxidation [91]. Indeed, a number of recent studies have demonstrated that an increase in circulating LPS may mediate the effects of overnutrition [12-14]. Thus, circulating LPS is elevated in obese patients and can cause the release of pro-inflammatory cytokines from cultured human adipocytes [12]. Additionally, a continuous infusion of LPS to levels reported in obesity induces steatosis, insulin resistance and obesity [13], while treatment of high fat fed mice with antibiotics to lower endotoxemia improves insulin sensitivity and steatosis [14]. Of note, however, in the present study we have demonstrated that LPS is not required to induce a reduction in hepatocyte palmitate oxidation when co-cultured with KC; i.e., the presence of the saturated fatty acid itself is sufficient to start the cascade resulting in decreased lipid breakdown. Furthermore, we have presented data that reveal a number of differences between the response to LPS and the response to saturated fatty acids. As shown in Figure 3A, only when palmitate is present overnight did the addition of KC to hepatocytes lower the rate of β-oxidation. Additionally, LPS

induced TNFα and IFNγ production from mouse KC, but palmitate did not. These data are particularly interesting in light of the proposed mechanism by which these two molecules exert their functions on effector cells of the immune system.

Toll like receptor (TLR) 4, the classical receptor for LPS, has also recently been described as the receptor for saturated free fatty acids [37-39]. Specifically, saturated fatty acids activate NF-κB and induce the expression of COX-1 in macrophages [37,43]; TLR4 deficient mice are protected against insulin resistance and steatosis when placed on a HFD [39]; and knock out of TLR4 protects mice against the skeletal muscle insulin resistance induced during acute lipid infusion [38,63]. Furthermore, we have determined that TLR4 is required in the KC to decrease hepatocyte palmitate oxidation (Figure 3B). It is important to note that we have not ruled out LPS being involved in the modulation of hepatocyte lipid metabolism, but rather have presented evidence that saturated fatty acids or, potentially, a combination of LPS and fatty acids may be responsible for KC mediated alterations in lipid breakdown in the setting of obesity.

The current study expands on recent reports that implicate KC in the modulation of hepatocyte lipid metabolism [89-91]. These other studies have all linked the pro-inflammatory, M1 or 'classical' activation of macrophages in the pathogenesis of the metabolic disturbances of obesity. Thus, mice with a myeloid-specific depletion of PPARδ, a transcription factor integral in the M2 or 'alternative' activation of macrophages (i.e. traditionally thought of as anti-inflammatory), exhibit higher levels of inflammation, steatosis, and insulin resistance [89,90]. Furthermore, hepatocytes from these animals exhibit elevated expression of genes involved in lipid synthesis, while the expression of genes involved in oxidation remains unchanged [89]. Our group has

recently shown that rats depleted of KC by injection of gadolinium chloride ($GdCl_3$) are protected against the development of insulin resistance and steatosis when placed on a HFD. Additionally, M1 polarized KC induce alterations in metabolic pathways in rat hepatocytes which promote the development of steatosis (decrease palmitate oxidation, and increase palmitate esterification and triglyceride accumulation); an effect which appears to require the pro-inflammatory cytokine TNFα [91]. Notably, in the current study, which utilized a mouse hepatocyte/KC co-culture system, we recapitulated the KC-mediated decrease in hepatocyte fatty acid oxidation, but we did not observe the same increase in TNFα as was seen in the rat system. It is possible that factors such as the animal model utilized (mouse versus rat), stimulus employed (LPS versus saturated fatty acid) or materials used (6- versus 24-well plates or trans-well inserts, as detailed as possible in Appendix A) may have masked changes. However, a more likely explanation is that the Kupffer cells mediate alterations in hepatocyte metabolism by signals other than cytokines. Indeed, the studies reviewed above do not discuss other potential mediators of inflammation and the effects those molecules may exert on hepatocyte metabolism. The current study explores the hypothesis that another inflammatory mediator, namely the eicosanoid PGE_2, is also involved in the regulation of metabolism.

Previous studies have generated a rather controversial picture of the effects of PGE_2 on nutrient metabolism. According to some studies, PGE_2 inhibits glucagon-mediated glycogen phosphorylase activity [119,133], and therefore decreases glycogenolysis [121] by inhibiting glucagon stimulated elevations in cAMP concentrations within the hepatocyte [126]. Utilizing the same system, others have

found that PGE_2 also stimulates glycogen phosphorylase activity, and therefore enhances glycogenolysis, via calcium signaling [117,134,135]. These differences were later ascribed to differences in receptor activation; the glycogenolytic effect resulting from engaging the EP1 receptor and subsequent G_q activation, and the anti-glycogenolytic effect resulting from EP3 receptor activation with downstream effects due to G_i action [119]. Neyrinck and colleagues [127] have shown that removal of KC-derived PGE_2, via $GdCl_3$ injection into rats, increases lipid accumulation within the hepatocytes of liver sections. Furthermore, they demonstrate that rates of lipid synthesis decrease, esterification does not change and oxidation increases in cultured rat hepatocytes in response to 48 hour PGE_2 treatment. They, therefore, conclude that PGE_2 serves a protective role against the development of steatosis. Conversely, Brass and colleagues [126] demonstrated that PGE_2 decreases glucagon-stimulated palmitate oxidation and Björnsson and colleagues [125] determined that PGE_2 inhibits VLDL secretion, both supporting a pathogenic role for PGE_2 in the development of steatosis. These apparently contradictory results are exemplified by findings that PGE_2, classically the quintessential pro-inflammatory lipid mediator, is now attributed both pro- and anti-inflammatory functions [115].

The current study implies a role for the eicosanoid PGE_2 in the pathogenesis of the deregulated lipid metabolism caused by overnutrition. Thus, PGE_2 is produced by KC in response to elevated saturated fatty acid, treatment of hepatocyte/KC co-cultures with indomethacin restores oxidative capacity to levels comparable to those seen with hepatocytes in the absence of KC, and exogenous treatment of hepatocytes with PGE_2 decreases the rate of lipid oxidation. Our findings that PGE_2 decreases palmitate

oxidation are in opposition to the findings of Neyrinck et al. [127]. This may be due to differences in the model system used (mouse versus rat), exposure time to PGE_2 (19 hours versus 48 hours), or method of oxidation measurement ($^{14}CO_2$ versus $^{3}H_2O$ quantification). Further studies would be required to discern the cause of this discrepancy. Similar to Neyrinck et al. [127], we found no alteration of palmitate esterification in response to PGE_2 treatment, however we did observe a decrease in response to the addition of KC, indicating that the KC-mediated regulation of esterification may occur by a mechanism distinct from PGE_2 or cytokines. In conclusion, these data clearly demonstrate that PGE_2 is involved in the KC effects on hepatocyte palmitate oxidation.

3.0 CHAPTER 3 – THE ROLE OF EXOGENOUS ADMINISTRATION OF DENDRITIC CELLS ON THE METABOLIC DISTURBANCES OF OBESITY

3.1 ABSTRACT

Dendritic cells (DC), the professional antigen presenting cells in the body and the cells responsible for directing both innate and adaptive immunity, have recently been implicated in the generation of inflammation and the subsequent metabolic changes seen in type II diabetes mellitus and obesity. In this study, we describe a previously unknown role for DC in the determination of body composition and insulin sensitivity in the setting of overnutrition.

To address this question, bone marrow-derived dendritic cells (BMDC) were generated and injected weekly into syngeneic C57Bl/6 mice consuming a high fat diet (HFD). Weight gain and caloric intake were measured weekly, and adiposity, metabolic rate, activity, insulin sensitivity, steatosis and immunophenotype were assessed at the end of the seven-week diet.

Injection of immature BMDC (iDC) decreased adiposity of mice consuming a HFD, but bone marrow-derived macrophages (BMDM) and LPS-activated BMDC did not. Basal metabolic rate and activity were unchanged between the groups. iDC injection caused infiltration of mononuclear cells predominantly in the white adipose

tissue, an effect that was independent of diet. This infiltrate was skewed towards the anti-inflammatory T_H2 phenotype. iDC injection did not alter liver triglyceride buildup or plasma triglyceride levels; however, mice receiving iDC exhibited improved insulin sensitivity by glucose tolerance test.

These data implicate DC in the development of the metabolic disturbances of obesity. Specifically, DC may be involved in determining how our bodies respond to caloric challenge in terms of depositing fat and preventing insulin resistance.

3.2 INTRODUCTION

Obesity and type II diabetes mellitus coincide with a state of chronic, low-grade inflammation, which has been implicated in the pathogenesis of insulin resistance and dyslipidemia. Many studies have implicated elevations of adipose tissue macrophages (ATM) in the development of this inflammatory state and the concomitant systemic changes seen in obesity [81-85]; however, there continues to be interest in determining the potential role of other immune cells in altering metabolism. For example, $CD8^+$ T cells [79], $CD4^+$ T cells [136], regulatory T cells [78] and B cells [80] have all been shown to play a role in the development of the metabolic disturbances of obesity. In this regard, dendritic cells (DC) are bone marrow-derived professional antigen presenting cells (APCs) that are key in the initiation and modulation of both innate and adaptive immune responses [92-94]. Studies have shown that cells expressing the classical DC marker, CD11c, increase in the adipose tissue of obese mice [97,100]; however, this has been in the context of describing elevations in ATM content. Our group has

recently discovered that a substantial proportion of the $CD11c^+$ cells in adipose tissue were DC and that these cells promoted the accumulation of macrophages in obese adipose tissue (Stefanovic-Racic, et al., submitted). We have also found that Flt3L knock out mice, which lack DC, are completely protected from weight gain, insulin resistance and steatosis when placed on a high fat diet (O'Doherty, unpublished observations). While these findings indicate that DC are associated with the elevated inflammatory state and metabolic disturbances of obesity, they do not assign a causal role to DC in these processes. The current study tests the hypothesis that DC are directly involved in the development of obesity and the subsequent metabolic changes when mice are placed on a HFD. Our data demonstrate that exogenous administration of immature bone marrow-derived DC (BMDC), but not LPS-activated BMDC or BMDM, decreases the weight gain and improves the insulin sensitivity of mice eating a HFD.

3.3 MATERIALS AND METHODS

3.3.1 Animal care and maintenance

Male wild type C57BL/6 mice were obtained from Jackson Laboratories. Prior to experiments, mice were maintained on a constant 12-h light:12-h dark cycle with free access to water and *ad libitum* fed with a standard chow diet. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

3.3.2 Experimental design

At 7 weeks of age, wild type C57BL/6J mice received their first injection i.p. and were started on an *ad libitum* fed high fat diet (HFD; 44% calories from fat, 19% Lard, 1% Corn Oil) from Harlan Teklad or kept on a standard chow (SC) diet (n=7 in SC groups, n=10-12 in HFD groups). During the course of the experiment, mice received a single injection of $1x10^6$ bone marrow-derived dendritic cells (BMDC) or bone marrow-derived macrophages (BMDM), every week for 6 weeks. Mice and food were weighed weekly over the course of the experiment. During this period, mice underwent an analysis of insulin sensitivity using glucose tolerance tests, metabolic rate using the CLAMS (Comprehensive Laboratory Animal Monitoring System, OH), and body composition using a Lunar PIXImus densitometer (Lunar, Madison, WI). Whole-body scans, with exclusion of the head, were analyzed for fat and lean masses using the manufacturer's software. At the end of this period, one full week after the sixth injection, blood and tissues were isolated for flow cytometric analysis or triglyceride measurements, or flash frozen and stored at –80°C until analysis.

3.3.3 Generation and purification of BMDC

BMDC were prepared, with modifications as previously described [101]. In brief, a single cell suspension of bone marrow cells was depleted of $I\text{-}A^+$ cells, B cells and T cells via incubation with monoclonal antibodies and complement treatment. Remaining cells were cultured in serum free AIM-V medium (Gibco 12055) containing 5ng/mL GM-CSF for 5 days. On day two, 50% of the medium was replaced with fresh AIM-V with

GM-CSF. On day 5, half of the cultured cells were treated with 1ug/mL LPS (Sigma L-3012) for two hours to generate activated BMDC (LPS-DC). Untreated, immature BMDC (iDC) and LPS-DC were purified using CD11c magnetic beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions.

3.3.4 Generation and culture of bone marrow-derived macrophages (BMDM)

BMDM were generated from the culture of bone marrow progenitors, as previously described [137]. Briefly, bone marrows, isolated from femur and tibia, were depleted of red blood cells using Red Blood Cell Lysing Buffer (Sigma R7757). Cells were plated at a density of $1x10^7$/15cm dish (day 0) in 20mL DMEM supplemented with 20% heat-inactivated FBS, 1mM sodium pyruvate, 2mM L-glutamine, 100U/mL penicillin, 100ug/mL streptomycin and 30% L-cell supernatant, until confluent. Differentiation media was replaced 3-4 days after initial plating, then used for injections on day 7.

3.3.5 Glucose tolerance tests (GTT)

For the GTT, 6-hour fasted mice were injected i.p. with 1.5 g/kg glucose. Blood was sampled from the tail vein every 15 minutes for 2 hours post-injection and glucose was measured using an Ascensia Elite glucometer (Bayer, Tarrytown, NY).

3.3.6 Tissue collection and generation of single mononuclear cell suspensions

One week after the 6th injection (7 weeks total on HFD), the mice were sacrificed, as follows: blood was collected by cardiac puncture, then 10mL PBS was perfused through the liver from the proximal IVC, one of the smaller lobes of the liver was then removed and flash frozen for triglyceride measurements. Subsequently, 5mL of a 1mg/mL solution of collagenase (type IV; Sigma C-5138, St. Louis, MO) was perfused to release hepatocytes and non-parenchymal cells from the surrounding connective tissue. Spleen and epididymal and renal fat pads were collected as well. Livers were agitated to release cells, then incubated at 37°C 5% CO_2 for 30 min. Resulting digest was passed over a 70um cell strainer, hepatocytes were removed with one 45 x g centrifugation, and mononuclear cells in the supernatants were pelleted, then isolated on an 8% Histodenz gradient. Fat pads were combined and digested in a 1mg/mL collagenase solution, then passed over a 100um cell strainer. Red blood cells were lysed twice using Red Blood Cell Lysing Buffer (Sigma R7757). Splenocytes were isolated by forcing spleens through a 40um strainer and subsequently lysing the red blood cells using Red Blood Cell Lysing Buffer (Sigma R7757).

3.3.7 Quantification of cytokine production

Tissue mononuclear cells were plated at a density of 1x10^6/mL in 96-well plates (Falcon 35-3077) and treated with PMA/ionomycin (PMA = 10ng/mL, ionomycin = 500ng/mL). Cell culture supernatants were collected after 24 hours of treatment, and assayed for the presence of 23 cytokines/chemokines (IL-1α, 1β, 2, 4, 5, 6, 10, 12(p40), 12(p70),

13, 17, TNFα, IFN-γ, IP-10, CXCL1, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, and GM-CSF) using a Milliplex Luminex Kit (Millipore), according to the manufacturer's instructions.

3.3.8 Flow cytometry

Cell suspensions (2 x 10^6 cells/sample) from liver, MLNs, spleen and white adipose tissue (WAT) were pre-incubated in FACS buffer with anti-CD16/32 (Fc "blocking" antibodies [Abs], clone 93) for 15 min at 4^{o}C, then stained with either fluorescent-labeled primary Abs or IgG isotype controls for 30 min at 4^{o}C. The following Abs were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (clone M1/70) and anti-CD25; peridinin cholorophyll-a protein (PerCP)-conjugated CD4; V450-conjugated FoxP3; PB-conjugated anti-F4/80 (clone BM8); allophycocyanin (APC)-conjugated anti-CD11c (N418) and CD8; and APC-Alexa 780-conjugated CD3. PerCP-conjugated Abs were purchased from BD Biosciences, whereas all other Abs were from eBiosciences (San Diego, CA). Cells were gently washed twice in FACS buffer and re-suspended in 4% paraformaldehyde (Fisher) prior to analysis using a FACSCalibur flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA). A minimum of 50,000 cells were analyzed for each sample and a proportion of up to 1% false positive events were accepted in the isotype control samples.

3.3.9 Statistical analysis

Data are expressed as means ± SE. Statistical significance was determined by t-test and, where appropriate, analysis of variance (repeated measures or one-way ANOVA; Bonferroni's post-hoc test) was performed using the PASW Statistics program (Chicago, IL). Statistical significance was assumed at $p \leq 0.05$.

3.4 RESULTS

3.4.1 Injection of immature BMDC diminishes body composition gains when consuming a high fat diet

Our lab's previous data showed that lack of DC completely protects against obesity. Additionally, DC increased in number in the liver and white adipose tissue (WAT) when animals are placed on a HFD (Stefanovic-Racic, et al., submitted) and our collaborators' work indicates that a single injection of BMDC can prevent the onset of diabetes in a NOD mouse [101]. All these findings sparked our interest in determining whether an injection of BMDC could prevent the onset of type II diabetes in a diet induced obesity model. We started animals on either a high fat (HFD) or standard chow (SC) diet and gave them an injection of $1x10^6$ immature BMDC (iDC), LPS-treated BMDC (LPS-DC), or BMDM, once per week for 6 weeks. Animals on SC showed no differences in weight gain (Figure 11A) or adiposity (Figure 11B) between those animals receiving an injection of PBS or iDC. While animals placed on a HFD did not exhibit any differences

in weight (Figure 11A), animals receiving an injection of iDC each week showed a significant drop in fat mass and %fat compared to control animals (Figure 11B). Notably, this is not due solely to the fact that these animals are receiving an injection of mononuclear cells because injection with LPS-activated DC or BMDM do not afford the same protection (Figure 11C&D). Furthermore, this difference was not attributable to changes in caloric intake (Supplemental Figure 1).

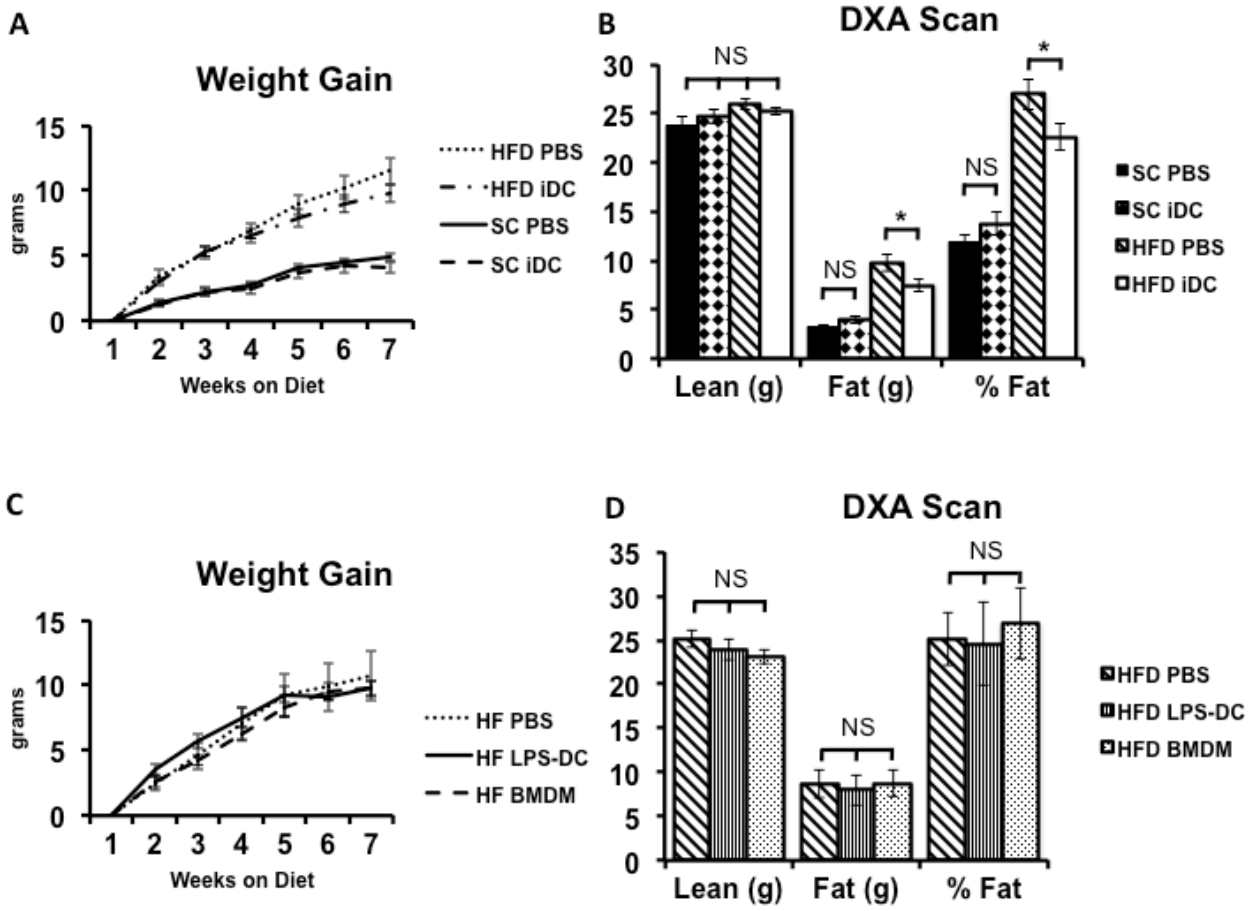


Figure 11 - Injection of immature bone marrow-derived dendritic cells alters body composition.

Wild-type C57Bl/6 mice were placed on a SCD or HFD and injected once per week with either PBS, $1x10^6$ immature BMDC (iDC, Panels A & B), $1x10^6$ LPS treated BMDC (LPS-DC, Panels C&D) or $1x10^6$ bone marrow-derived macrophages (BMDM, Panels C&D). Weight gain was assessed, as described in Methods (Panels A & C). After 5 weeks on the diet, all mice underwent dual x-ray absorbitometry (DXA) as described in Methods (Panels B & D). Results are presented as the means±SE for a minimum of six animals in each group. *$p<0.05$.

3.4.2 Exogenous BMDC administration does not change metabolic phenotype

Given these animals have altered accumulation of fat, we next wanted to test whether the metabolic rate or activity of these animals differ from their controls. Using the CLAMS system, we assessed the metabolic rate, as demonstrated by VO_2 (Figure

12A&B), and activity (Figure 12C&D) and found no differences based on diet or injection.

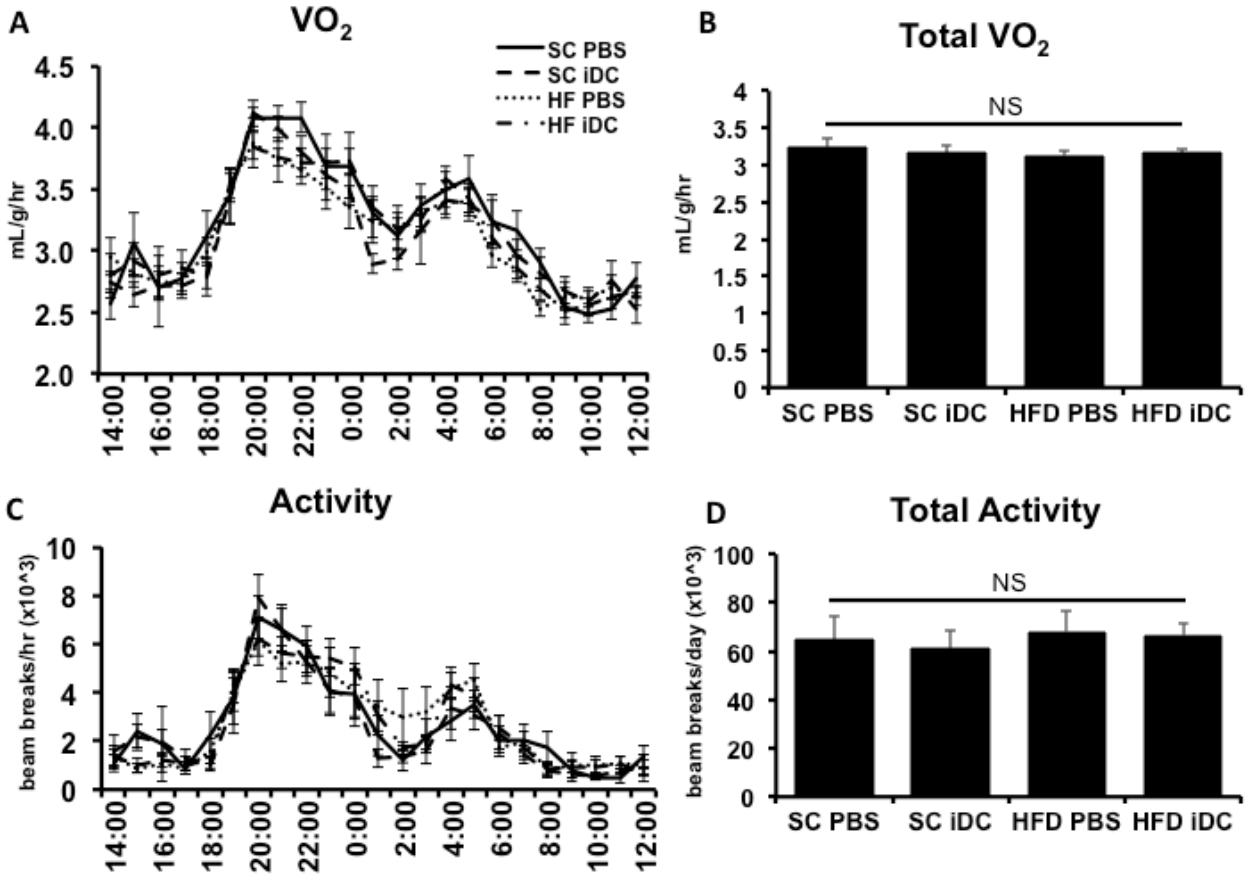


Figure 12 - Metabolic analysis using CLAMS (Comprehensive Laboratory Animal Monitoring System).

Standard chow (SC) and high fat fed (HFD) wild-type mice injected with either PBS or iDC underwent CLAMS analysis, as described in Methods. Data for VO_2 (Panels A and B), and ambulatory activity (Panel C & D) are presented. Results are presented as the means±SE for a minimum of seven animals in each group.

3.4.3 Injection of BMDC alters the immunophenotype of WAT

There is now a seminal body of work implicating the T_H1, pro-inflammatory phenotype in the development of the metabolic disturbances of obesity. Given the changes in body

composition, we were very interested in discovering how the immunophenotype of these animals is altered in response to exogenous administration of BMDC. Grossly, iDC injection causes an influx of immune cells into the WAT of mice, as measured by total mononuclear cell (MNC) fraction isolated from the tissues, regardless of diet (Figure 13A). There is also a trend towards increased MNC in the liver, but this did not reach statistical significance. More specifically, the numbers of $CD4^+$ T-helper cells (as defined by cells expressing CD3 and CD4), regulatory T cells (as defined by $CD3^+CD4^+FoxP3^+$ cells), dendritic cells (the total $CD11c^+$ population) and macrophages ($CD11b^+CD11c^-$ cells) increase in the adipose tissue after iDC injection (Figure 13B). These increases were not observed in liver (Supplemental Figure 2) or spleen (Supplemental Figure 3). Importantly, the cytokine profile of the MNC isolated from both WAT (Figure 13C and Supplemental Figure 4) and liver (Supplemental Figure 5) demonstrated that the cells from these tissues were predominantly T_H2 polarized, as indicated by a decrease in the ratio of TNFα to IL-4 (T_H1 to T_H2).

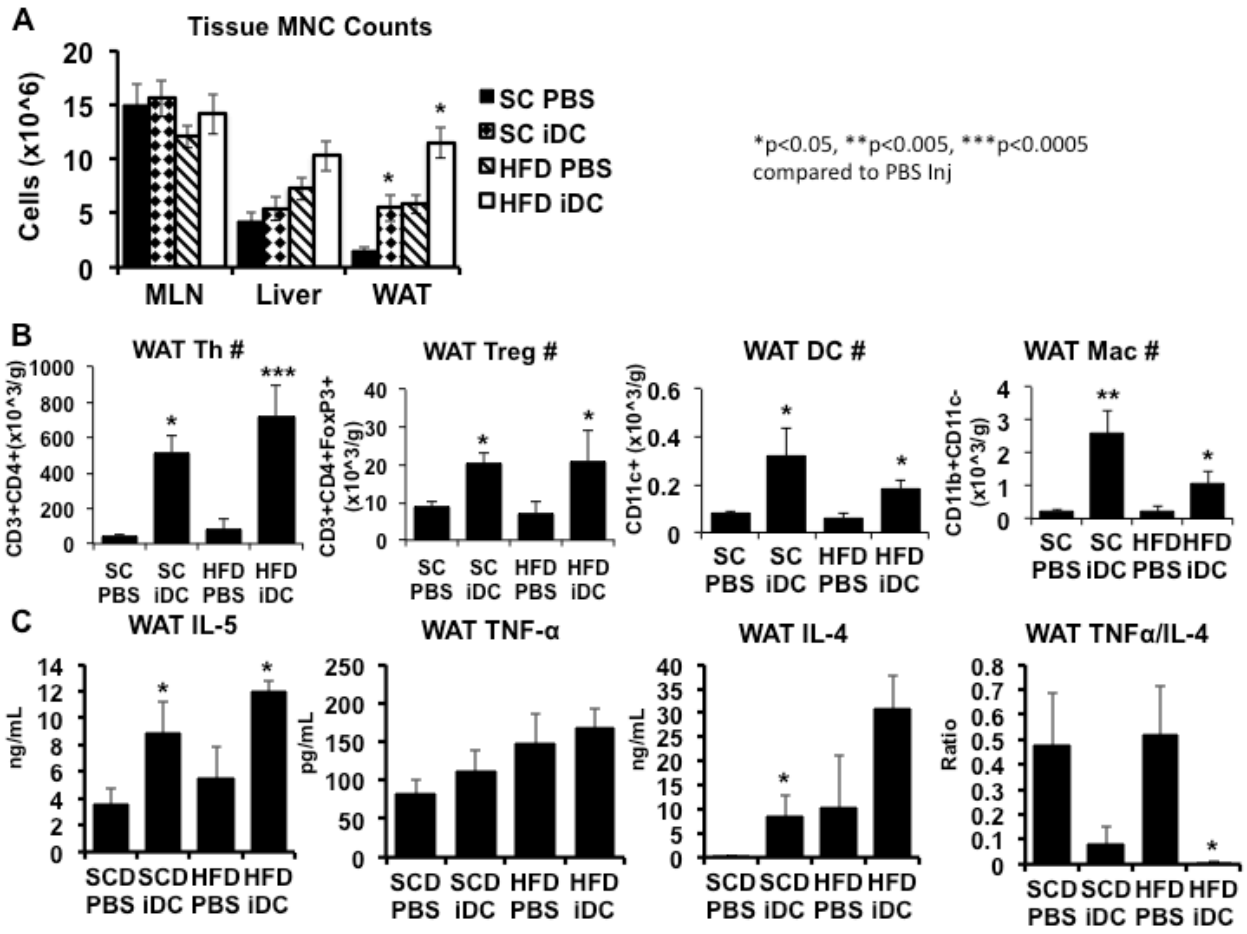


Figure 13 - Immunophenotype of iDC injected mice.

White adipose tissue (WAT), mesenteric lymph nodes (MLN) and liver were collected from SC, HFD, PBS injected, and iDC injected mice, immune cells were isolated, counted (Panel A) and analyzed by FACS (Panel B), as described in Methods. Additionally, immune cells were kept in culture overnight in the presence of 10ng/mL PMA and 500ng/mL ionomycin, and supernatants underwent Luminex analysis (Panel C), as described in Methods. Results are presented as the means±SE for a minimum of seven animals in each group.

3.4.4 Injection of BMDC partially protects against glucose intolerance but not steatosis

While the immunophenotype suggests that iDC injection is protective against the long-term sequelae of obesity, we were interested in testing specific metabolic parameters of the metabolic syndrome: namely insulin resistance, glucose intolerance, and steatosis.

Animals on a HFD had worse glucose tolerance than their SC counterparts, as indicated by higher GTT curve tracing (Figure 14A) and higher AUC (Figure 14B). Additionally, animals on a HFD that received weekly injections of iDC demonstrated significantly improved glucose tolerance as indicated by the lower AUC (Figure 14B).

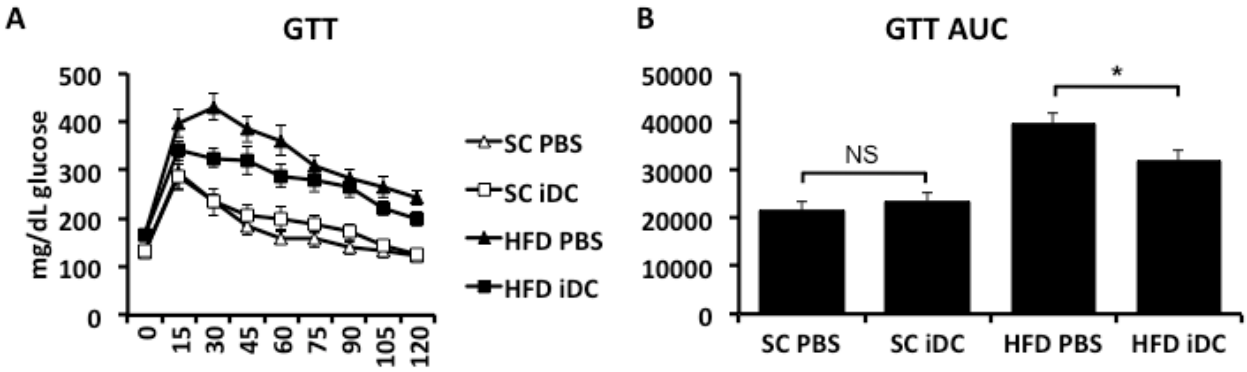


Figure 14 - Glucose tolerance in iDC injected mice.

Standard chow and high fat fed wild-type mice injected with either PBS or iDC underwent glucose tolerance tests (GTT), as described in Methods. Results are presented as the means±SE for a minimum of seven animals in each group. *p<0.05.

While iDC injection protected against glucose intolerance, the fasting blood glucoses (FBG) of these animals remained unchanged to that of controls (Figure 15A). Furthermore, liver and plasma TG were not different between the iDC and PBS injected groups (Figure 15B&C).

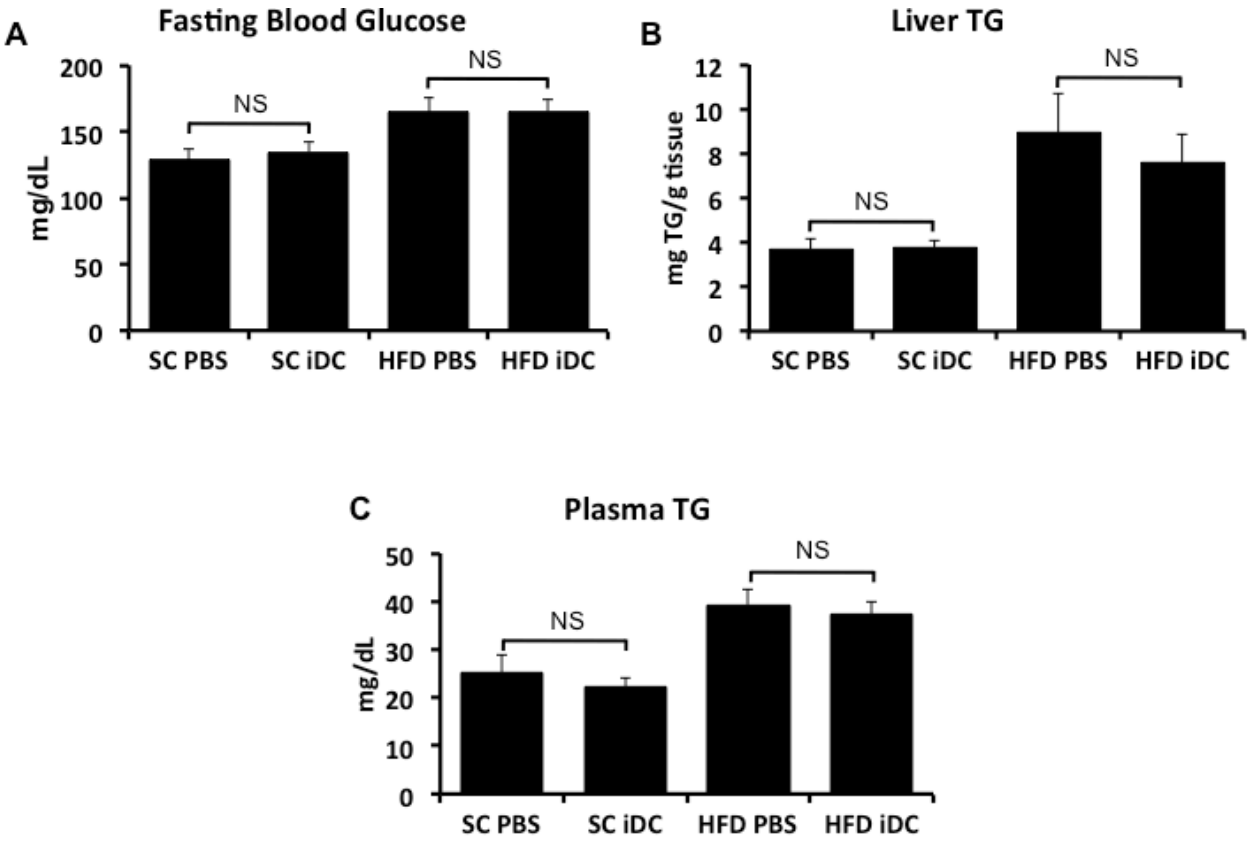


Figure 15 - Fasting blood glucose and liver and plasma triglycerides in iDC injected mice.

Fasting blood glucose (Panel A), and liver and plasma triglycerides (Panels B & C, respectively) were assessed in PBS or iDC injected mice, as described in Methods. Results are presented as the means±SE for a minimum of seven animals in each group.

3.5 DISCUSSION

The purpose of the current study was to address the potential role of DC in the pathogenesis of the metabolic disturbances of obesity. A number of novel observations have been presented. Namely, C57Bl/6 mice that receive weekly injections of iDC while consuming a HFD 1) have predominantly T_H2 polarized immune cells within the adipose tissue and liver, 2) accumulate lower amounts of fat mass and 3) are protected from diet-induced insulin resistance. We were motivated by previous reports postulating

involvement of DC, or cells expressing the classical DC marker CD11c, in the development of inflammation and insulin resistance in response to overnutrition. These studies have indirectly noted that triple positive ($CD11b^+CD11c^+F4/80^+$) cells are elevated in the WAT of obese individuals [97,98], have established that these cells are responsible for the elevated pro-inflammatory status of obese [98,100] or have directly attributed elevations in $CD11c^+$ populations in the WAT and liver to DC (Stefanovic-Racic, et al., submitted). Furthermore, Patsouris and colleagues [99] demonstrated that removal of all CD11c-expressing cells protects against diet-induced insulin resistance and steatosis.

While it is now established that macrophages are elevated in number and activation in obesity [82,84,85], and that the triple positive cells account for much of the inflammation present in obese individuals, few studies have specifically studied the responses of DC in response to overnutrition. Utilizing cultured cells, Nguyen et al. [100], describe that BMDC, which express CD11c, are more pro-inflammatory than BMDM, which do not express CD11c. Furthermore, they show that BMDC become activated after exposure to free fatty acids, which are elevated in the circulation of obese individuals [35] and are proposed to exert many effects leading to the sequelae of obesity [138]. Our group has recently found that the increases in triple positive cells in the WAT of obese mice were due to increases in DC, not macrophages as was originally surmised (Stefanovic-Racic, et al., submitted). We also discovered that DC were directly involved in the recruitment of macrophages, to the adipose tissue.

In the current study, we set out to investigate the role of DC in the development of obesity-associated metabolic changes, by administering weekly injections of bone

marrow-derived DC to mice eating a HFD. We were first intrigued by the prospect of utilizing DC to alter metabolic fate when referred to studies which show BMDC injection protecting against the development of diabetes in non-obese diabetic (NOD) mice, a model of type I diabetes mellitus [101]. Furthermore, injection of BMDC has already been translated into the clinic for applications in the cancer [139] and transplant [140] arenas. It is this prior knowledge that will help transform any potential therapeutics into clinical applications for obesity. As DC have long been known to play a crucial role in the initiation and modulation of innate and adaptive immune responses by recruitment and activation of B and T cells [92], we postulated that DCs may be responsible for the onset of inflammation and the initiation of a T_H1 response in the setting of overnutrition.

A pro-inflammatory, T_H1 polarized immune system is associated with worse metabolic outcomes in obesity, such as hepatic steatosis and insulin resistance [141]. Thus, T_H1 polarized T helper cells infiltrate WAT while consuming a HFD [136]. Also, M1 polarized macrophages, which promote the skewing towards a T_H1 immunophenotype, are more prevalent in obese adipose tissue [84,85]. Additionally, mice with a reduced capacity to recruit these macrophages exhibit lower levels of inflammation and are protected from HFD-induced insulin resistance [83,86] and the M2 polarization of macrophages, which promotes the T_H2 skewing of T cells, has been associated with improved metabolic outcomes. Mice that are impaired in their ability to develop an M2 response, PPARδ knock out mice, exhibit increased adiposity and steatosis while consuming a HFD [90]. This is exemplified by the different susceptibility of various strains to diet-induced obesity: Balb/c mice which mount a strong T_H2 response, are resistant to weight gain and steatosis, while C57Bl/6 mice, which mount a

strong T_H1 response, are highly susceptible to obesity, insulin resistance and steatosis when consuming a HFD [142]. Here, we have demonstrated that iDC injection causes recruitment of macrophages, T cells and dendritic cells to WAT, regardless of diet (Figure 13), and notably, the infiltrating cells were polarized towards a T_H2 phenotype. While this result is consistent with the current belief that T_H2 skewing is beneficial for the prevention of the metabolic disturbances of obesity, it brings up an interesting contradiction with the work of Patsouris and colleagues [99]: namely that both exogenous administration of BMDC and removal of all DC result in improved insulin sensitivity. A recent report from Li et al. [143], however, may shed some light on this apparent conundrum. The authors describe that triple positive cells exhibit phenotypic plasticity, i.e. these cells switch from a T_H1 polarizing phenotype to a T_H2 polarizing phenotype after mice are placed on a standard chow diet following consumption of a HFD for 20 weeks. After maintaining the standard chow diet for three weeks, mice have decreased weight gain, adiposity and steatosis, as well as improved insulin sensitivity, compared to animals remaining on HFD, but still have the triple positive cells. It, therefore, follows logically that the cells themselves do not entirely explain the link between inflammation and insulin resistance, but rather the cytokine milieu that those cells produce play an important role, as well. Thus, identifying the inflammatory landscape may, in fact, be the more important defining criterion for metabolic outcomes.

One particular observation worthy of note is the improvement of glucose tolerance in animals receiving iDC injections. Other metabolic readouts such as liver TG accumulation (i.e. steatosis) and fasting blood glucose were unchanged between iDC injected and control animals. This would indicate that basal levels of

gluconeogenesis from the liver are similar in these animals, suggesting that liver insulin action is unaltered. The differences in the GTT AUC may instead reflect differences in the action of insulin at the level of the skeletal muscle. And, although the gold-standard test to distinguish liver versus total body insulin resistance would be hyperinsulinemic euglycemic clamp studies, we do not believe such experiments are warranted at this time.

In conclusion, we have demonstrated that DC are involved in the determination of body composition and insulin resistance that develops in the setting of overnutrition. Specifically, the anti-inflammatory T_H2 polarized immunophenotype caused by injection of immature BMDC is protective against adiposity and insulin resistance. The mechanisms which cause these effects have yet to be determined; however, given that DC are known to modulate immune responses by activation and differentiation of T cells, studies are currently ongoing to describe the involvement of T cells in the protection afforded by iDC.

4.0 CHAPTER 4 – THE ROLE OF NKT DELETION ON THE METABOLIC DISTURBANCES OF OBESITY[2]

4.1 ABSTRACT

The contribution of natural killer T (NKT) cells to the pathogenesis of metabolic abnormalities of obesity is controversial. While the combined genetic deletion of NKT and $CD8^+$ T cells improves glucose tolerance and reduces inflammation, interpretation of these data has been complicated by the recent observation that the deletion of $CD8^+$ T cells alone reduces obesity-induced inflammation and metabolic deregulation, leaving the issue of the metabolic effects of NKT cell depletion unresolved. To address this question, CD1d null mice ($CD1d^{-/-}$), which lack NKT cells but have a full complement of $CD8^+$ T cells, and littermate wild type controls (WT) on a pure C57BL/6J background were exposed to a high fat diet, and glucose intolerance, insulin resistance, dyslipidemia, inflammation, and obesity were assessed. Food intake (15.5±4.3 vs 15.3±1.8 kcal/mouse/day), weight gain (21.8±1.8 vs 22.8±1.4g) and fat mass (18.6±1.9 vs 19.5±2.1g) were similar in $CD1d^{-/-}$ and WT, respectively. As would be expected from these data, metabolic rate (3.0±0.1 vs 2.9±0.2 ml O_2/g/h) and activity (21.6±4.3 vs

[2] Adapted from 107. Mantell BS, Stefanovic-Racic M, Yang X, Dedousis N, Sipula IJ, et al. (2011) Mice Lacking NKT Cells but with a Complete Complement of CD8 T-Cells Are Not Protected against the Metabolic Abnormalities of Diet-Induced Obesity. PloS one 6: e19831.

18.5±2.6 beam breaks/min) were unchanged by NKT cell depletion. Furthermore, the degree of insulin resistance, glucose intolerance, liver steatosis (12.5±2.2 mg TG/g tissue vs 8.4±2.0 mg TG/g tissue), and adipose and liver inflammatory marker expression (TNFα, IL-6, IL-10, IFN-γ, MCP-1, MIP1α) induced by high fat feeding in CD1d$^{-/-}$ were not different from WT. We conclude that deletion of NKT cells, in the absence of alterations in the CD8^{+} T cell population, is insufficient to protect against the development of the metabolic abnormalities of diet-induced obesity.

4.2 INTRODUCTION

Obesity coincides with a state of chronic, low-grade inflammation that has been implicated in the pathogenesis of insulin resistance and dyslipidemia. In particular, it has been demonstrated that macrophages, CD8^{+} T cells and regulatory T cells in adipose tissue and liver play a role in mediating the detrimental effects of inflammation on metabolism [28,81,82,84,85,144]. However, there continues to be interest in determining the potential role for other immune cells in altering metabolism. In this regard, NKT cells are a sub-population of lymphocytes that are proposed to serve as a link between the adaptive and innate immune systems [102-104]. NKT cells have receptor expression characteristics of NK cells (they are NK1.1^{+}) and T cells (they express a T cell receptor (TCR)). Notably, the NKT TCR, rather then being activated by peptide antigens, is activated by glycolipid antigens through the MHC class I-like molecule, CD1d [102-104], while an alternative activation pathway is dependent on cytokine signaling from activated dendritic cells [103]. Irrespective of the method,

activation of NKT cells results in rapid cytokine production (within hours), which may be of mixed, T_H1 or T_H2 dominance depending on the microenvironment to which the NKT cells are exposed and/or different functional subsets of NKT cells [104].

The potential role of NKT cells in altering metabolism has received attention, but the data are inconclusive and somewhat contradictory. Of relevance to the current study are the observations that diet-induced obese mice [105,145] and *ob/ob* mice [108] have a larger proportion of T_H1 polarized liver NKT cells than non-obese mice [105], and that administration of the NKT activator α-Galactosylceramide to DIO mice exacerbates glucose intolerance and adipose tissue inflammation [106], suggesting that NKT may play a pathogenic role in the metabolic abnormalities associated with these models. Conversely, obesity reduces overall NKT cell numbers in liver [105,108,109], while an agonist of NKT cells, glucocerebroside [146], or adoptive transfer of NKT cells [110] improves liver steatosis and glucose tolerance in *ob/ob* mice, suggesting a protective effect of NKT cells. Clearly, an approach that would clarify some of these issues would be to ablate NKT cells and address the consequences of this manipulation on the development of metabolic deregulation in obesity. Indeed, a recent study [106] demonstrated that mice lacking NKT cells have reduced adipose tissue inflammation and improved glucose tolerance compared to wild-type mice when exposed to a high fat diet. However, the animals used in this study were also depleted of $CD8^+$ T cells, a vitally important caveat, since a recent study demonstrates a role for $CD8^+$ T cells in driving obesity-related inflammation and deregulated glucose homeostasis [79]. Thus, the metabolic and inflammatory effects of NKT cell deletion alone remain unknown. The current study was undertaken to address this issue. To accomplish this goal, mice

lacking CD1d were used. These animals are depleted of NKT cells but maintain a normal complement of $CD8^+$ T cells [147,148], making it possible to demarcate the effects of NKT cell deletion alone from the effects of combined NKT/$CD8^+$ T cell deletion.

4.3 MATERIALS AND METHODS

4.3.1 Animal care and maintenance

$CD1d^{-/-}$ mice on a Balb/c background (C.129S2-*Cd1*^{tm1Gru}/J) were obtained from Jackson Laboratories. Homozygous mutant mice are deficient in both the *Cd1d1* and *Cd1d2* genes and as a result lack the natural killer T subset[147,148]. These mice were bred with C57Bl/6 mice to obtain N1F1 heterozygotes, which were subsequently backcrossed to pure C57Bl/6 mice. The gender of the mice in the backcross was alternated with each generation. CD1d heterozygotes of at least the N7 generation (>99% Bl/6 background) were mated to obtain $CD1d^{-/-}$ and wild type littermate controls. Prior to experiments, mice were maintained on a constant 12-h light:12-h dark cycle with free access to water and *ad libitum* fed with a standard chow diet. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

4.3.2 Experimental design

CD1d$^{-/-}$ (n=9) and wild type littermate control mice (n=11) were fed *ad libitum* with a standard chow (SC) or high fat (HF) diet (44% calories from fat – 19% Lard, 1% Corn Oil) from Harlan Teklad for 26 weeks. Mice and food were weighed weekly for the course of the experiment. During this period, mice underwent an analysis of insulin sensitivity using glucose tolerance and insulin tolerance tests, metabolic rate using the CLAMS (Comprehensive Laboratory Animal Monitoring System, OH), and body composition using a Lunar PIXImus densitometer (Lunar, Madison, WI). Whole-body scans, with exclusion of the head, were analyzed for fat and lean masses using the manufacturer's software. At the end of this period, blood and tissues were isolated, flash frozen and stored at –80°C until analysis.

4.3.3 Glucose tolerance tests (GTT), insulin tolerance tests (ITT), and pyruvate tolerance tests (PTT)

For the GTT, 6-hour fasted mice were injected i.p. with 1.5 g/kg glucose. Blood was sampled from the tail vein every 15 minutes for 2 hours post-injection and glucose was measured using an Ascensia Elite glucometer (Bayer, Mishawaka, IN). For the ITT, non-fasted animals were injected i.p. with 1 unit/kg of human recombinant insulin (Humulin R, Lilly, Cincinnati, MO). Blood was sampled from the tail vein every 15 minutes for 2 hours post-injection and glucose was measured using an Ascensia Elite glucometer. For the PTT, 6-hour fasted mice were injected i.p. with 2 g/kg Sodium Pyruvate. Blood was sampled from the tail vein every 15 minutes for 2 hours post-

injection and glucose was measured using an Ascensia Elite glucometer (Bayer, Mishawaka, IN).

4.3.4 Tissue collection and generation of single mononuclear cell suspensions for flow cytometric analysis

At the end of the 26 week diet, blood was collected by cardiac puncture, then 10mL PBS was perfused through the liver from the proximal IVC, one of the smaller lobes of the liver was then removed and flash frozen for triglyceride measurements. Subsequently, 5mL of a 1mg/mL solution of collagenase (type IV; Sigma C-5138, St. Louis, MO) was perfused to release hepatocytes and non-parenchymal cells from the surrounding connective tissue. Spleen and epididymal and renal fat pads were collected as well. Livers were agitated to release cells, then incubated at 37°C 5% CO_2 for 30 min. Resulting digest was passed over a 70um cell strainer, hepatocytes were removed with one 45 x g centrifugation, and mononuclear cells in the supernatants were pelleted, then isolated on an 8% Histodenz gradient. Fat pads were combined and digested in a 1mg/mL collagenase solution, then passed over a 100um cell strainer. Red blood cells were lysed using Red Blood Cell Lysing Buffer (Sigma R7757) twice.

4.3.5 Flow cytometry

Cell surface staining was performed in PBS containing 2% FBS with the following antibodies: anti-CD3-FITC, anti-CD3-PB, anti-CD69-PE, anti-CD8-PerCP, and anti-

NK1.1-PE/Cy7. Stained cells were analyzed on an LSR II (BD Biosciences) using FACSDiva software (BD Biosciences).

4.3.6 Tissue and plasma measurements

Liver triglycerides were determined as previously described [48,130]. Plasma insulin levels were measured using a commercial kit (by ELISA; ALPCO, Salem, NH) according to the protocol provided by the manufacturer.

4.3.7 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For analysis of gene expression, total RNA was isolated from liver or adipose tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was carried out using gene specific primers, SYBR green master mix (Bio-Rad, Hercules, CA) and an Applied Biosystems Prism 7300 Real-Time PCR System, as previously described [63]. Fold change in mRNA expression was determined using the $\Delta\Delta$cT method, with all genes normalized to β–actin.

4.3.8 Statistical analysis

Data are expressed as means ± SE. Statistical significance was determined by t-test and, where appropriate, analysis of variance (repeated measures or one-way ANOVA; Bonferroni's post-hoc test) was performed using the PASW Statistics program (Chicago, IL). Statistical significance was assumed at $p < 0.05$.

4.4 RESULTS

4.4.1 Early and reversible decrease in liver NKT cells in response to a high fat diet

Previous studies have reported decreases in liver NKT cells in response to a high fat diet. We first sought to confirm and extend these observations, and to assess the effects of diet in other relevant tissues of C57BL/6J mice. In agreement with previous studies [105,108,109], there was a selective decrease in the NKT cell population and the NKT activation marker, CD69 in the liver (Figure 16A&B) after exposure to a high fat diet. Conversely, the NKT cell population was enriched in adipose tissue (Figure 16A). NKT populations were not altered in the spleen or mesenteric lymph nodes (MLN). To assess the time-course and reversibility of high fat diet-induced alterations in NKT cells, mice were placed on a high fat diet for 3 weeks, and then a proportion of these mice were placed back on a standard chow diet for a further 3 weeks. In response to a 3-week high fat diet, the proportion of NKT cells was reduced to a similar extent as that observed with a prolonged diet. Furthermore, reversion to a standard chow diet for 3 weeks was sufficient to reverse the alterations in NKT cells (Figure 16C). Additionally, there was no change in NKT cell numbers in the spleen at 3 weeks of HFD or when the animals were subsequently placed on a standard chow diet for a further 3 weeks (Figure 16D).

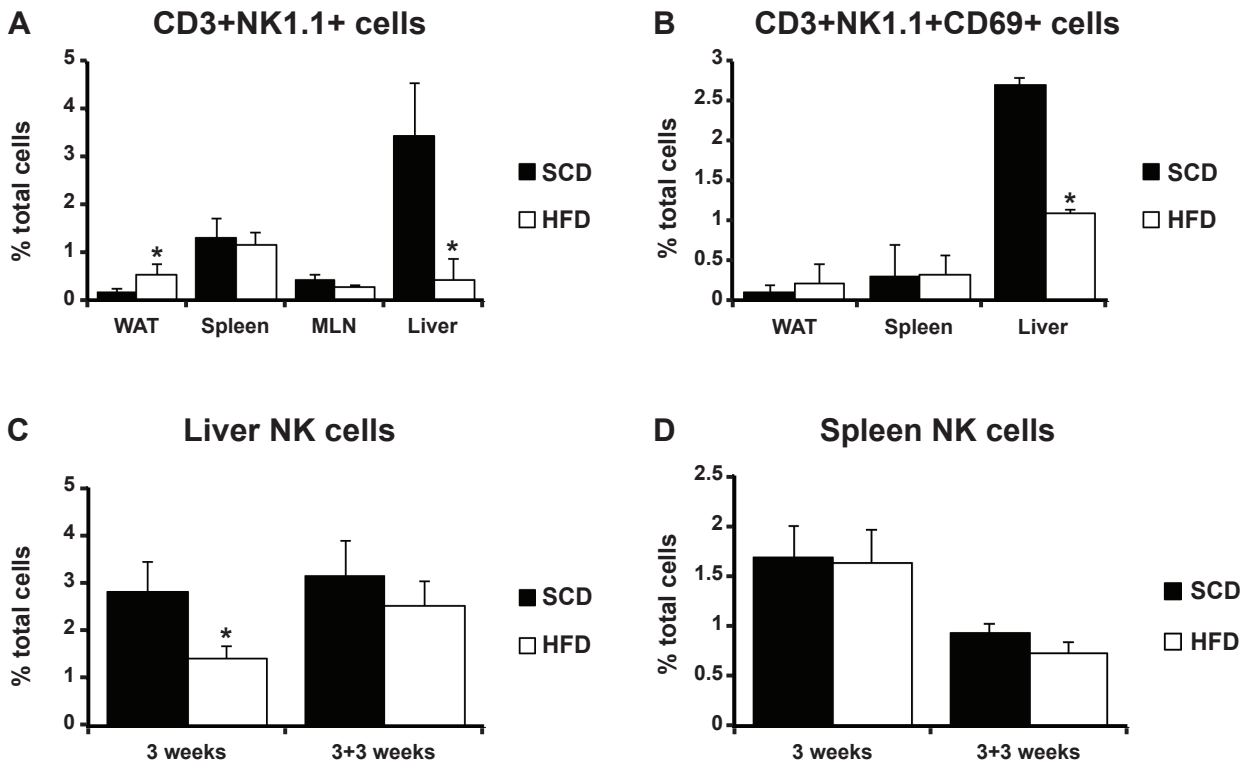


Figure 16 - Alterations in tissue NKT cell composition in the setting of high fat feeding.

Wild-type C57Bl/6 mice were placed on a SCD or HFD for 26 weeks (Panels A and B), 3-weeks (Panel C and D), or 3 weeks followed by a 3-week standard chow diet (Panel C and D). White adipose tissue (WAT), spleen, mesenteric lymph nodes (MLN) and liver were collected from lean and obese mice, immune cells were isolated and underwent FACS, as described in Methods. Results are presented as the means±SE for a minimum of five animals in each group. *p<0.05.

4.4.2 NKT depletion does not alter weight gain, food intake, adiposity, or energy expenditure

The $CD1d^{-/-}$ null mouse lacks NKT cells but has a normal complement of $CD8^{+}$ T cells ([147,148] and Figure 17E). $CD1d^{-/-}$ mice and littermate wild-type controls (WT), on a pure C57BL/6J background, were exposed to a high fat diet, and a number of metabolic variables were assessed. Weight gain, caloric intake, and body composition were unaffected by NKT cell deletion (Figure 17A-D). Furthermore, indirect calorimetry

demonstrated that metabolic rate and activity were similar in CD1d$^{-/-}$ and WT mice (Figure 18A-C), as would be expected given the lack of an effect on NKT depletion on body composition and caloric intake.

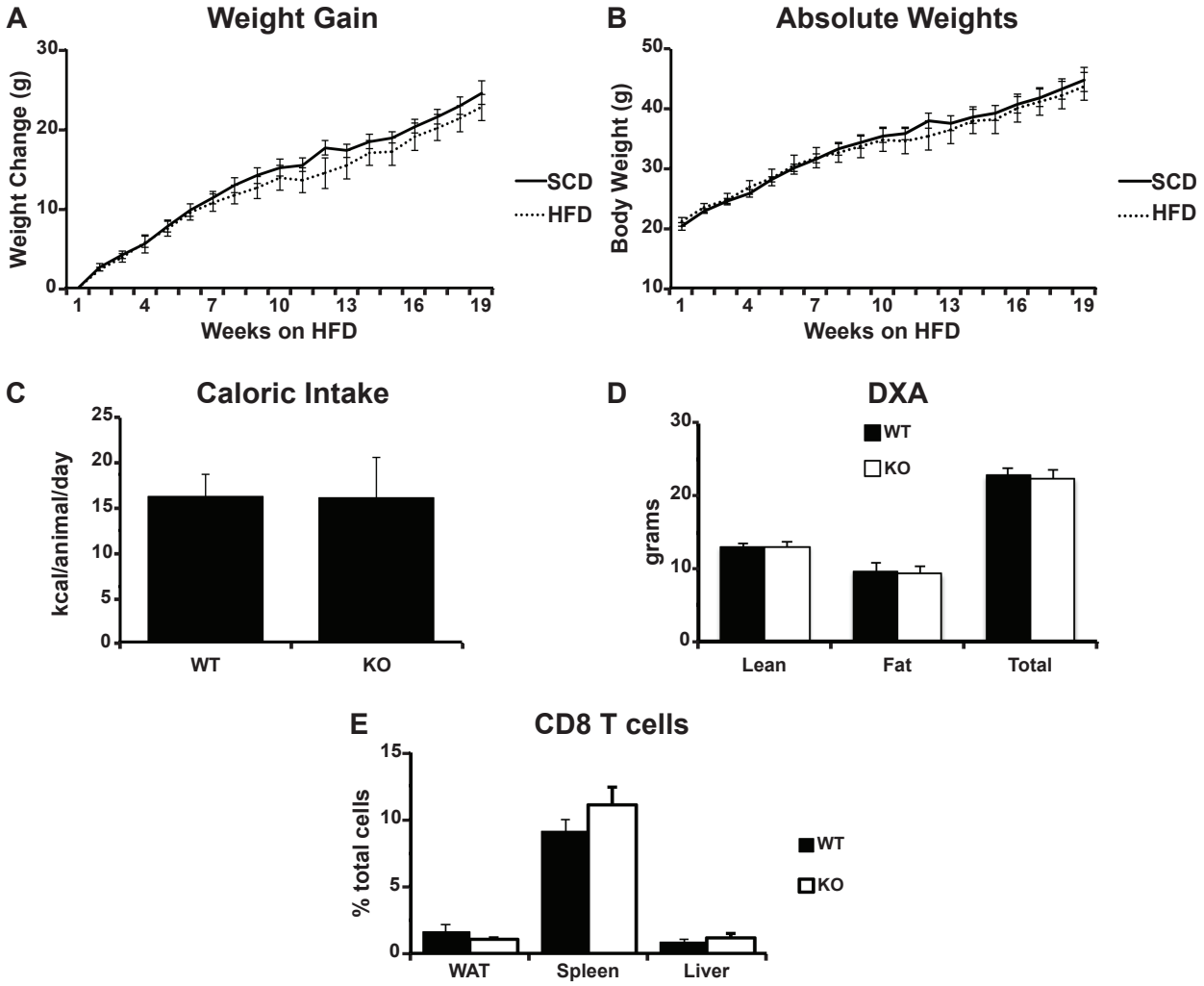


Figure 17 - Weight gain and body composition of wild type (WT) and CD1d null (KO) littermate mice on a high fat diet.

Weight gain and caloric intake were assessed in high fat fed wild-type and CD1d knock out mice, as described in Methods (Panels A-C). All mice underwent dual x-ray absorbitometry (DXA) as described in Methods (Panel D). CD8^{+} T cells were assessed by FACS (Panel E). Results are presented as the means±SE for a minimum of five animals in each group.

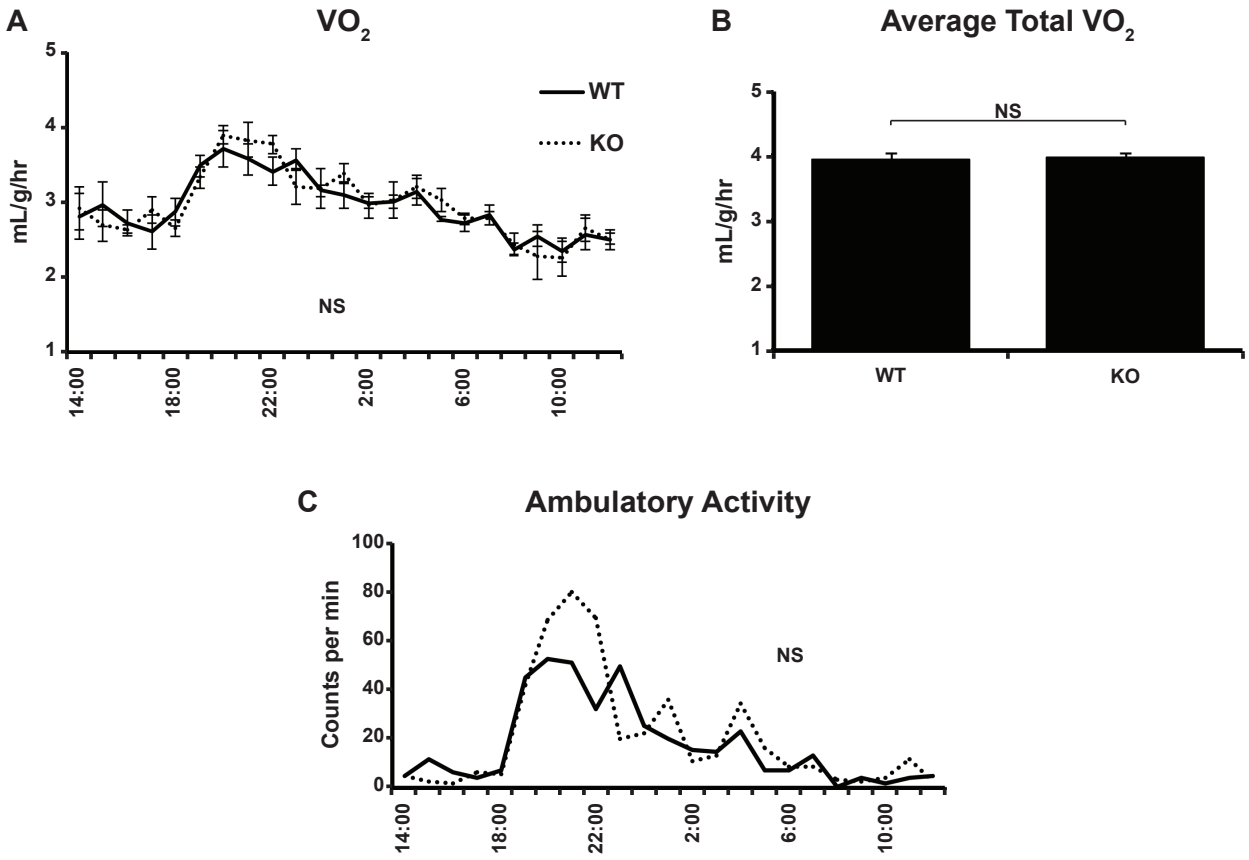


Figure 18 - Metabolic analysis using CLAMS (Comprehensive Laboratory Animal Monitoring System).

High fat fed wild-type (WT) and CD1d null (KO) mice underwent CLAMS analysis as described in Methods. Data for VO_2 (Panels A and B), and ambulatory activity (Panel C) are presented. Results are presented as the means±SE for four animals in each group.

4.4.3 Insulin sensitivity and glucose tolerance are unaffected by NKT cell deletion

We next assessed the effects of NKT deletion on obesity-induced insulin resistance (Figure 19), using insulin (ITT) and glucose tolerance tests (GTT). As Figure 19, Panels A&B show, the GTT and ITT demonstrate that the degree of insulin resistance in obese $CD1d^{-/-}$ mice was similar to obese WT mice. This is also indicated by the area under the curve for the GTT and ITT (Figure 19C and 19D, respectively). Fasting blood

glucose concentrations were similar between WT and CD1d$^{-/-}$ mice, as were the fasting insulin concentrations and HOMA values (Figure 20A-C). Liver triglyceride levels were also measured and again, no difference between the groups was observed (Figure 20D). Additionally, Pyruvate Tolerance Test (Figure 20E) indicated no difference between the two groups.

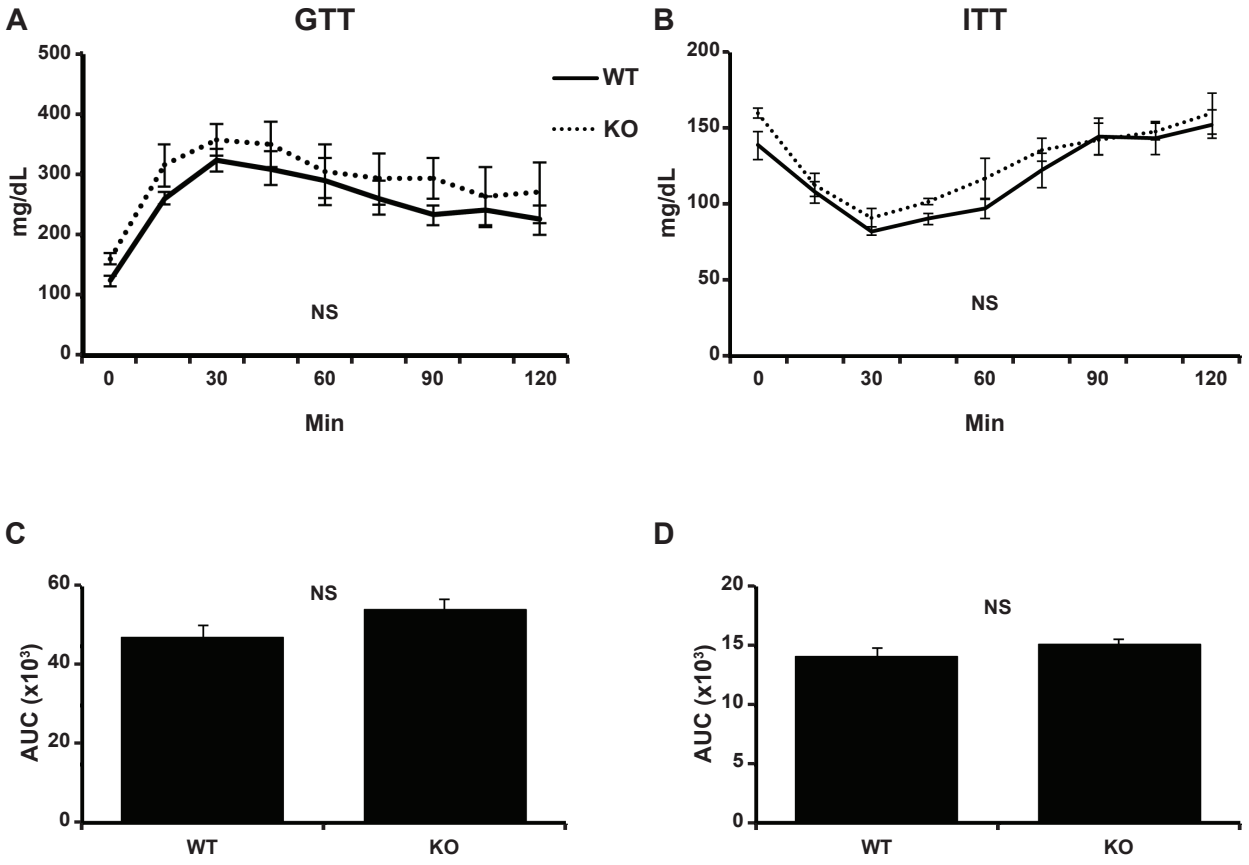


Figure 19 - Glucose and insulin tolerance in wild-type and CD1d-null mice.

High fat fed wild-type (WT) and CD1d null (KO) mice underwent glucose tolerance tests (GTT) as described in Methods. After 1 week for recovery, all mice underwent insulin tolerance tests (ITT) as described in Methods. Results are presented as the means±SE for a minimum of five animals in each group.

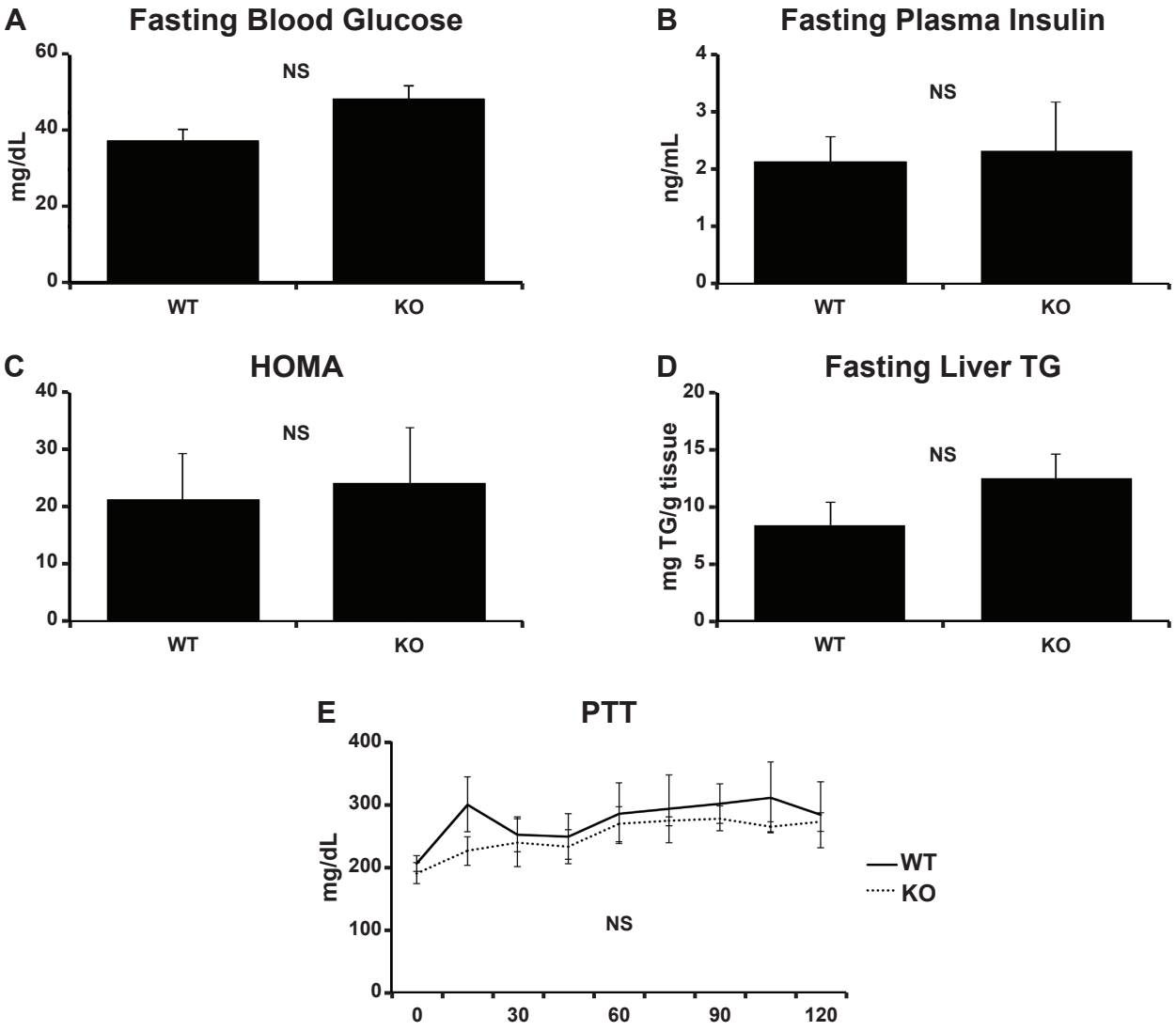


Figure 20 - HOMA, Hepatic glucose output and liver triglycerides in wild-type and CD1d null mice.

Fasting blood glucose (Panel A) and plasma insulin (Panel B) concentrations, Pyruvate Tolerance Test (Panel E) and liver triglycerides (Panel D) were assessed in WT and KO mice as described in Methods. Results are presented as the means±SE for a minimum of five animals in each group.

4.4.4 Deletion of NKT cells does not alter expression of inflammatory markers

The inflammatory response in obesity is well described and involves increased expression of T_H1 cytokines and macrophage infiltration/activation into adipose tissue and liver [15,82,84,85,91]. A previous study [106] demonstrated that the combined

deletion of $CD8^+$ T cells and NKT cells reduced adipose tissue inflammation in obesity. However, the effects of NKT deletion alone are unknown. Thus, we next assessed the effects of NKT deletion on the expression of inflammatory markers in WAT and liver of obese $CD1d^{-/-}$ and WT mice. As Figure 21 shows, qRT-PCR analysis of TNFα, IL-6, IL-10, IFN-γ, MCP-1 and MIP1α demonstrated that expression of these genes was similar in $CD1d^{-/-}$ and WT animals. These data, taken with the data presented above demonstrate that deletion of NKT cells alone is not sufficient to alter the inflammatory status of obesity.

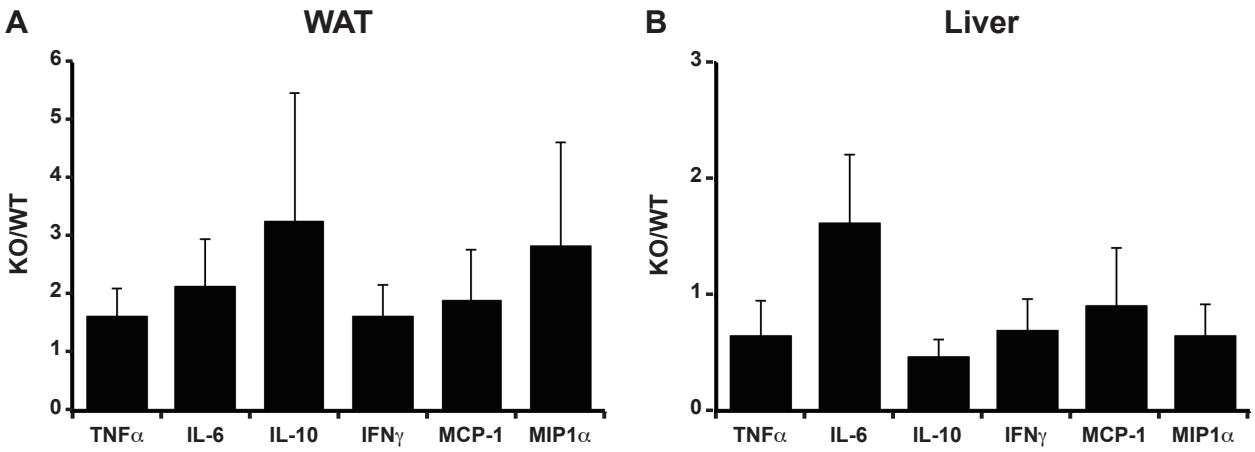


Figure 21 - Inflammatory marker expression in adipose tissue and liver of wild-type and CD1d null mice.

qRT-PCR was used to assess the expression of indicated genes in liver and adipose tissue from high fat fed wild-type (WT) and CD1d null (KO) mice. Results are presented as ΔΔCt (KO/WT) ±SE for a minimum of five animals in each group.

4.5 DISCUSSION

The major goal of this study was to address the contribution of NKT cells to the development of the metabolic abnormalities of obesity, motivated by previous reports that have suggested metabolic and inflammatory effects of altered NKT activity. These studies have either indirectly inferred roles for NKT cells [105,108,109], altered the activity/numbers of NKT cells in pre-existing obesity models [106,110,145,146,149], or have deleted other cell types i.e. $CD8^+$ T cells, in addition to NKT cells [106], that have been implicated in mediating increased inflammation and metabolic deregulation in obesity. Importantly, no previous studies have been undertaken in models with a specific deletion of NKT cells.

To investigate the role of NKT cells in the development of obesity-related metabolic disturbances in the absence of alterations in $CD8^+$ T cells, we utilized the CD1d knockout mouse line, which lacks NKT cells, but has a full complement of $CD8^+$ T cells, as opposed to the β2-microglobulin knockout mouse used in a previous study, which lacks both NKT and $CD8^+$ T cells [106]. Furthermore, and again unlike the study of Ohmura et al. [106], the current study controlled for the genetic background of the mice by utilizing wild-type littermates of the C57Bl/6J $CD1^{-/-}$ mice. The Ohmura study [106] demonstrated that deletion of both NKT and $CD8^+$ T cells results in an improvement in the glucose intolerance and inflammation resulting from exposure of the animals to a high fat diet. The authors conclude that it is unlikely that $CD8^+$ T cells contribute to this phenotype. However, given the more recent study of Nishimura et al.

[79], which demonstrates a role for $CD8^+$ T cells in mediating the inflammation and metabolic abnormalities of obesity, and the data obtained in the current study, a more likely explanation is that much, if not all, of the improvements in metabolic function and inflammation observed in the β2-microglobulin null mouse are due to the absence of $CD8^+$ T cells.

While we did not expect a lack of NKT cells to effect gross body composition, we next sought to discover any changes in metabolic phenotype of these animals. We used a Comprehensive Laboratory Animal Monitoring System (CLAMS) to simultaneously measure energy expenditure, caloric intake and activity levels in four WT and four KO mice. We found no difference in energy expenditure, activity, respiratory exchange ratio, or caloric intake between the two groups of animals. As a point of interest, we looked at these data in different ways, including normalizing for total body weight and lean body mass. When incorporating to body weight, differences between the two groups become statistically significant, however, as Butler and Kozak suggest [150], this can be misleading due to small (<10%) differences in the weights of the animals that were run in the CLAMS. We posited that the most appropriate way to present the VO_2 and heat data would be as per kg animal, especially considering that the body composition of the two groups of animals was not statistically different.

A number of studies have addressed the effects of obesity on NKT cell populations and activity [105,109,145] and the effects of interventions that alter NKT cell activity or number [106,110,145,146,149] on the metabolic abnormalities of obesity. By putting the current observations in the context of these studies, it is possible to conjecture on the various and somewhat contradictory conclusions reached in these

reports. The issue of the contribution of $CD8^+$ T cells to the metabolic phenotype of β2-microglobulin null mouse has been dealt with above. Turning to the effects of obesity on NKT cell populations and activity, it is now well established that obesity reduces liver NKT cell numbers [105,108,109], and that the remaining NKT cells are T_H1 polarized [109,149]. However, although these studies are suggestive and are useful for hypothesis generation, by themselves they do not demonstrate a causative role for NKT cells in development of the metabolic or inflammatory abnormalities of obesity. More direct studies have attempted to manipulate the activity or number of NKT cells *in vivo*. One approach has been to administer glycolipids, which are NKT cell agonists, to obese animals and determine the metabolic consequences of this intervention. Thus, Ohmura et al. report that administration of α-Galactosylceramide (αGC), which is reported to expand the iNKT population [106], to DIO or *ob/ob* mice exacerbates adipose tissue inflammation (DIO and *ob/ob*) and glucose intolerance (DIO). However, while the effects of αGC on inflammatory status in this study are substantial, the effects on glucose tolerance are small, although they are statistically significant. Administration of glucocerebroside (β-Glucosylceramide) is reported to improve liver steatosis and glucose intolerance in *ob/ob* mice[146], although the effects of this glycolipid on NKT cell activity is unclear. Unfortunately, NKT cell activity was not assessed in this study. However, it is possible that these two glycolipids have differential effects on NKT cell activity or expand different subsets of NKT cells, although these issues have not been addressed. Finally, adoptive transfer of NKT cells improves liver steatosis and glucose tolerance in *ob/ob* mice [110]. Unfortunately, polarization of the transferred cells was not assessed in this study, so again we are left to conjecture that perhaps the beneficial

effects are the result of the specific NKT cell subset used in this study and/or their polarization. Putting these studies in the context of the current study, we can say that ablation of NKT cells does not appear to protect against or contribute to the metabolic abnormalities of obesity. However, interventions that alter specific NKT populations or their activity in a pre-existing state of obesity appear to result in alterations in inflammatory status and metabolic function, the direction of which may be dependent on the specific NKT population targeted or the effects of the intervention on NKT polarization.

Two observations worthy of note are the rapidity with which nutrient composition-induced alterations in the liver NKT cell population occurs, and the different responses of the liver and adipose tissue NKT populations in obesity. We demonstrated that as little as 3 weeks of high fat diet exposure was sufficient to induce alterations in the liver NKT population. Furthermore, these changes were rapidly reversed when mice were returned to a low fat chow diet. Together, these data indicate that the liver microenvironment is very sensitive to altered nutrient states, and as for longer term diets, these changes correlate with hepatic metabolic and inflammatory alterations, since it is well-established that liver steatosis and insulin resistance and inflammation are present in rodent models within a 1-3 week exposure to a high fat diet. We analyzed multiple tissues of obese C57Bl/6 mice to extend previous observations of decreases in liver NKT cells. As others have shown, we observed a decrease in the percentage of NKT cells in the liver [105,149], however we also found an increase in NKT cells in the white adipose tissue. As opposed to Miyazaki et al. [145], but similar to Li et al.[105], we did not see a difference in the NKT populations in the spleen. In

conclusion, the current study emphasizes the complex relationship between NKT cells and metabolism and suggests that the simple removal of NKT cells does not influence metabolic regulation in obesity.

5.0 SUMMARY AND CONCLUSIONS

5.1 KNOWLEDGE GAPS IN THE FIELD

The seminal papers by Xu et al. and Weisberg et al. in 2003 [84,85], inspired significant amounts of research investigating the role of the immune system in the development of the metabolic disturbances of obesity. Much of this subsequent research has centered on responses of macrophages in the white adipose tissue of obese individuals. The work presented throughout this dissertation has sought to define additional aspects of how our immune system responds to and affects nutrient metabolism in the setting of overnutrition.

I have proposed that in response to elevations in circulating free fatty acids, parenchymal cells of the liver and adipose tissue (i.e. hepatocytes and adipocytes, respectively) will increase expression of factors that recruit macrophages and DC, causing immune cell infiltration into these tissues, as illustrated in Figure 2. I have shown evidence that macrophages are, then, capable of producing PGE_2, which, in turn, decreases hepatocyte lipid oxidation rates. Furthermore, I have described a situation in which DC induce a T_H2 phenotype in liver and adipose tissue, thus protecting against diet-induced insulin resistance. While it is now established that immune cells are linked to obesity, we still need to understand the mechanism of these

changes to drive the rational design of therapeutics and appropriate prevention of disease, in the clinical arenas, as well as advancement of scientific knowledge and to generate new questions, in the basic science arenas. Prior to the work described in the preceding chapters, much preliminary work had been done ascribing a role for inflammation and the immune system in the determination of the metabolic disturbances of obesity; however, a number of key questions remain.

As described earlier, leading up to the work performed in completion of this dissertation, we have seen many associative studies and glimpses into the importance of the immune system in determining metabolism under the challenge of overnutrition. Thus, migration of macrophages into WAT is necessary for diet-induced insulin resistance and steatosis [83,86], and the pro-inflammatory M1 and T_H1 phenotypes of macrophages and T cells, respectively, are detrimental to maintaining insulin responsiveness in comparably healthy individuals [141]. Additionally, we have seen the importance of the NF-κB pathway and the involvement of the pro-inflammatory cytokine TNFα [28,141]. Furthermore, there was controversy over the role of NKT cells in determining the metabolic changes during overnutrition [107] and there were only hints that DCs were involved at all [99,100].

This preliminary work in the field of immune modulation of metabolism was fundamental in establishing a previously unrecognized method of maintaining nutrient homeostasis, but, at the same time, raised many questions. From these studies, we understand that macrophages play a major role in determining insulin resistance and steatosis in the setting of obesity; however, the mechanism by which macrophages cause these changes was unknown. Furthermore, there have been no definitive studies

to date determining whether the immune cell infiltration and inflammation occurs prior to the development of metabolic abnormalities, or vice versa. As early as two days on a HFD, macrophage infiltration can be measured in the liver in addition to steatosis [151], while another group has demonstrated that the same occurs in the adipose tissue as early as three days after starting a HFD [152]. Also, the controversy over NKT cell involvement and the lack of definitive studies investigating the role of DC begs the question of whether, or not, each of these cell types plays a part in determining the metabolic disturbances of obesity. The results presented in this dissertation have provided some insight into these questions, which have implications for the field at large.

5.2 IMPLICATIONS OF THE WORK DESCRIBED

The first obvious implication of the work described is the development of a new technology: the use of mouse hepatocyte/KC co-cultures for metabolic studies. We are one of the first groups to utilize mouse hepatocytes in co-culture with KC for studies of lipid metabolism. Prior to this innovation, only rat hepatocytes and KC had been used for co-culture metabolic studies [91]. This approach has the limitation of only investigating cell responses in their normal/wild type state, while the use of mouse cells introduces a multitude of potential manipulations. The flexibility of the mouse system is demonstrated in Figure 3B, where TLR4 knock out KC are utilized in combination with wild type hepatocytes to show that TLR4 is required in the KC to decrease hepatocyte palmitate oxidation. Interestingly, although TLR4 is required (Figure 3B), and palmitate

and LPS both activate TLR4 [37], the cells seem to respond differently to the two ligands (Figure 3A). This could represent differences in the signaling of each of these molecules, either upstream or downstream of TLR4, or may suggest a role of accessory receptors in mediating the effects.

This is not to say, however, that the mouse system is without limitations itself. Indeed, as is the case with any *in vitro* model system, the mouse co-culture system is a simplification of the physiological case that actually occurs in the liver. Thus, many more than just two cell types interact within the liver and they do not only interact by passing soluble factors to each other. Signals that require direct cell-to-cell contact may also alter the metabolic status of an animal. With that said, though, the mouse co-culture system has helped to define one potential mechanism of macrophage-mediated alterations in metabolism.

The experiments performed in Chapter 2 implicate PGE_2 in paracrine crosstalk between hepatocytes and KC resulting in decreased lipid breakdown. Our receptor expression data (Figure 6) point towards an involvement of EP1 or EP4 in mediating the effects of PGE_2 on hepatocyte metabolism. Interestingly, all four subtypes of the PGE_2 receptor are G-protein coupled receptors, but to different G-proteins. Specifically, EP1 is linked to G_q, thus it acts to increase the intracellular concentration of calcium in a cell via phospholipase C and calcium release from the endoplasmic reticulum. EP4 is linked to G_s, and thus acts to increase cAMP production via activation of adenylate cyclase (AC). Given that PGE_2 decreases palmitate oxidation (Figure 5), we thought that it would be unlikely that cAMP was involved, because glucagon, a hormone that activates AC in the fasted state (opposite to insulin), acts to increase β-oxidation. This, however,

raises the question of whether all receptors linked to G_q can inhibit palmitate oxidation in hepatocytes. In this regard, carbachol, a pan-muscarinic agonist, activates the M1, M3 and M5 receptors, which are all linked to G_q, and subsequently raises intracellular calcium concentrations. Even though carbachol is a somewhat non-specific mediator used to raise calcium concentrations, the result that it can recapitulate the PGE_2 effect of lowering oxidation rates provides added confidence that PGE_2 is exerting its effects through EP1. A more definitive test for the involvement of EP1 would be to use an EP1 inhibitor such as SC-51322. We would expect to block the KC-mediated decreases in hepatocyte palmitate oxidation with the inclusion of SC-51322. Furthermore, if PGE_2 is exerting these effects via elevation of calcium within the cell, we would expect to block this effect with the addition of a calcium channel blocker, such as verapamil. Another way we aimed to demonstrate the involvement of PGE_2 in the effects of KC on hepatocyte metabolism was by preventing its production (Figure 7).

Perhaps the most convincing piece of evidence that PGE_2 is involved in metabolic crosstalk between hepatocytes and KC is that indomethacin, a generic COX-1/COX-2 inhibitor, completely blocks KC-induced decreases in hepatocyte palmitate oxidation. Unfortunately, indomethacin is impractical for use in human studies because of the GI side effects, but the development of small molecules capable of more specific targeting may circumvent this issue. We also attempted to utilize KC which were defective in producing PGE_2 in our co-culture system. The *Ptges2* gene product, microsomal PGE_2 synthase (mPGES)-2, is the constitutively active PGE_2 synthase, however, as demonstrated in Figure 7C, *Ptges2* knock out KC still produce significant amounts of PGE_2 in response to palmitate. A more appropriate model may be to use

KC from mPGES-1 knock out mice. mPGES-1, as opposed to mPGES-2, is an inducible form of PGE_2 synthase, which has been shown to become activated in peritoneal macrophages in response to LPS [143]. If we find that mPGES-1 knock out KC still produce PGE_2 in response to palmitate, it would constitute yet another difference between LPS's and palmitate's action on KC. Alternatively, if mPGES-1 knock out KC do not produce PGE_2, we would be able to test more specifically whether PGE_2 from the KC is acting on hepatocytes to decrease palmitate oxidation. Furthermore, another strategy we could use to block the effects of PGE_2 would be to use hepatocytes from mice that lack the EP1 receptor. To further investigate the effects of PGE_2, we recently started testing other endpoints of lipid metabolism beyond palmitate oxidation: namely, palmitate esterification and the accumulation of triglycerides (TG) with hepatocytes.

Esterification of lipids and TG accumulation are both anabolic processes, i.e. building up components of lipid. Under normal circumstances, these processes oppose β-oxidation. It was, therefore, somewhat perplexing when we found that esterification also decreased when adding KC to the hepatocytes (Figure 9). We did see trends towards increasing TG accumulation with both the addition of KC and the addition of PGE_2, however this represents an n=2, so repetition will be required to confirm this result. In light of decreased oxidation, the most likely mechanism for triglyceride buildup is an increase in lipid esterification. However, cells can still accumulate TG in the absence of an increase in esterification. For this to occur, another process, such as lipid trafficking, would need to be altered in addition to the routine mechanism of anabolic and catabolic activities on lipids. For example, if VLDL secretion was inhibited

by the addition of KC or PGE_2, as demonstrated by Björnsson et al. [125], then TG could accumulate within the hepatocyte, even in the absence of a concordant increase in palmitate esterification. There are a number of enzymes involved in the coordination of these processes. We have begun to investigate one such enzyme: acetyl-CoA carboxylase (ACC).

ACC is a key lipogenic enzyme which becomes activated in the fed state, a time when we want to store fats. It catalyzes the formation of malonyl-CoA, which inhibits the opposing process of lipid breakdown (β-oxidation). ACC is activated by dephosphorylation; therefore, if ACC activation is responsible for the decrease in β-oxidation, we would expect that ACC phosphorylation decreases when we treat our hepatocytes with PGE_2. At this point in time, we only have one experiment completed, but there is a trend towards decreasing ACC phosphorylation in response to PGE_2 treatment when palmitate is present (Figure 8 B&C). This result may be indicating a mechanism by which PGE_2 exerts its effects; however, repetition is needed to confirm these results, and additional co-culture experiments are required to determine if this is also occurring in response to KC. Furthermore, ACC activation in response to PGE_2 treatment would predict an increase in esterification in addition to the increase in TG accumulation and decrease in oxidation. Whether or not ACC is responsible for PGE_2's effects on lipid metabolism, the increase in TG accumulation and decrease in oxidation are changes consistent with the development of steatosis. A wholesale removal of PGE_2, however, was bound to prove more complicated than simply protecting against the development of steatosis, since many different functions have been attributed to PGE_2 within the body.

Intriguingly, PGE_2 has been shown to have multiple, and apparently conflicting functions on a given target tissue [116]. PGE_2 inhibits T_H1 responses [153]; therefore, a whole body knock out of *Ptges2* may benefit the hepatocyte (since oxidation levels will remain elevated and TG accumulation will remain low), but may, in fact, worsen the immunological phenotype. Thus, these animals had the potential to exhibit either an improved or worse metabolic phenotype than their WT counterparts when placed on a HFD. We discovered that the *Ptges2* KO animals exhibited greater fat mass gain, but no worsening of insulin resistance or steatosis beyond the level of the WT animals (Figure 10). Given the substantially elevated fat mass, we would expect steatosis to have been worsened in these animals; however, liver triglycerides were unchanged between the WT and KO groups. This could either be an indication that the lower levels of PGE_2 were actually protective against the development of steatosis, or the steatosis caused by a ten week HFD could be masking the effects of PGE_2 removal. Another possibility speaks to one of the limitations of this system described earlier; namely that our *in vitro* system is an isolated system, which only investigates interactions between two cell types and any soluble factors that are passed between the two. The complexity of the *in vivo* setting, which involves many cell types and both soluble signals and direct cell-contact signals, may be another explanation for the lack of difference between the two groups. It is also possible that the multitude of signals *in vivo* results in normalized oxidation rates in the liver of KO animals. Unfortunately, these studies did not assess oxidation rates in the livers of the WT and KO animals, so we are left to conjecture the cause of this result. Additionally, it would be an extraordinary stretch to conclude that PGE_2 produced by KC is the only mechanism by which immune cells alter metabolism.

Indeed, the experiments performed in Chapter 3 would indicate that not only KC are involved in metabolic regulation, but DC are as well.

Throughout Chapter 3, I elucidated a role for DC in the determination of body composition and insulin resistance when mice are challenged with a HFD; however, the mechanism(s) for these effects remain unknown. The injection of iDC could result in changes within liver and/or WAT and/or skeletal muscle to alter these processes. Furthermore, the injected iDC could be acting directly on these target tissues, could be activating other immune cell types which act on target tissues or, even, endogenous DC could be processing the injected iDC, presenting antigen from the iDC, and either acting on the target tissues or activating other cells. Regardless of mechanism, the data presented offer a significant amount of new information. In particular, we can now conclude that some factor specific to iDC, or at least not present in LPS-DC or BMDM, is protective against the accumulation of fat tissue in the setting of consuming a HFD (Figure 11). If the injected cells are directly causing the effect (or indirectly via altering the activity of other cell types), this could represent a functional difference between the various cell types tested, e.g. antigen processing and presentation, expression of co-stimulatory molecules or cytokine production. Alternatively, if endogenous DCs are processing the injected DC, an antigen specific to iDC could potentially be causing this result. Again, however, irrespective of the mechanism, injection of iDC alters the immunophenotype in the WAT and liver of these animals (Figure 13).

Our data, indicating that injection of iDC promotes T_H2 polarization, parallel the sentiments of the field that a T_H1 skewed immunophenotype is detrimental to the sequelae of obesity, while a T_H2 skewed immunophenotype may be protective.

Importantly, injection of iDC overrides the influence of obesity, which, by itself, causes infiltration to the WAT of a predominantly T_H1 skewed phenotype. However, it is currently unclear why injection of iDC would predominantly cause infiltration of immune cells into WAT. There is no obvious anatomical or physiological reason that cells injected intraperitoneally (i.p.) ought to cause mononuclear cell infiltration into epididymal and peri-renal fat pads, and our attempts at tracking experiments were inconclusive (data not shown). Importantly, we cannot explicitly rule out the possibility that the increase in DC numbers observed within the adipose tissue was not a direct measurement of the injected cells. The mice were sacrificed one week after the final injection, by which time other studies have suggested the injected cells are undetectable, but a number of important caveats prevent direct correlation to the present study. The present study injected DCs i.p., whereas many other studies inject BMDC subcutaneously or intravenously [154]. Additionally, the majority of studies which track i.p. injected cells do not report findings past 48 hours-post-injection. Indeed, Shinomiya et al. demonstrate that BMDC are detectable in the spleen, liver, gut, pancreas and kidney 24 hours after i.p. injection [155], and Eggert and colleagues use the same method to determine these cells are not present in these tissues after 48 hours [156]. Notably, they show that most of the injected cells remain at the site of injection, but again, no information on cell migration after 48 hours was presented. Creusot et al. [157] measured DC migration to a multitude of tissues at a number of different time points and demonstrate that BMDC injected i.p. mostly disappear from the tissues of recipient mice by day 5-6, although they did not test C57Bl/6 animals and there were notable differences between the two strains they did test (NOD and FVB),

indicating these results may not hold true for C57Bl/6 mice. Intriguingly, Creusot and colleagues also demonstrate that 3 days after i.p. injection, BMDC migrate to the omentum, which is predominantly composed of adipose tissue. While, migration to epididymal or peri-renal adipose tissue was not reported in any of the above studies, these latter findings suggest that i.p. injection of iDC may track to fat depots. If our iDC injections home to the epididymal and peri-renal fat pads, it is quite possible they are directly responsible for recruiting the T cells, DCs and macrophages presented in Figure 13. Interestingly, not only was immunophenotype altered by iDC injection, but metabolic readouts of the sequelae of obesity were also modulated.

We found that the glucose tolerance of iDC injected mice consuming a HFD was improved over control mice also on a HFD. As opposed to the *Ptges2* KO mice, where fat mass and glucose tolerance appeared unlinked, the iDC injected mice follow the more predictable pattern of decreased fat mass accompanying improved glucose tolerance. Glucose tolerance was not improved all the way to lean values, but this does not rule out the possibility that altering the experimental design could improve the metabolic readouts even further and more closely approximate the lean values. As was discussed in Chapter 3, we see a trend towards decreased liver TG in the iDC injected animals (Figure 15B), thus the possibility remains that incremental alterations in the experimental design could show a difference here as well. Options would include maintaining the diet and injections for longer than six weeks, starting the injections prior to the start of the diet, or making the animals obese prior to intervention with iDC injections. While allowing the mice to become obese and then starting iDC injections would be most analogous to any potential clinical application, this may also prove the

most difficult to induce differences, and thereby may not be informative of mechanism. In addition to introducing a new mechanism for macrophage-mediated alterations in metabolism (Chapter 2) and introducing a novel effector cell in the immune modulation of metabolism (Chapter 3), we also clarified an important scientific question regarding the involvement of NKT cells in the metabolic abnormalities of obesity (Chapter 4).

Through the experiments performed in Chapter 4, we definitively showed that removal of NKT cells does not alter the development of the metabolic disturbances of obesity. As explained previously, there was a controversy in the literature over whether NKT cells protected against, or exacerbated, insulin resistance and steatosis while consuming a HFD. This controversy came to a head when Ohmura and colleagues published their results indicating that the β_2-microglobulin (β_2m) knock out mice were protected against diet-induced inflammation and insulin resistance [106]; however, as previously discussed, the β_2m knock out mice also lack $CD8^+$ T cells. Our data, indicating specific removal of NKT cells does not alter weight gain, metabolic rate, inflammation or insulin resistance caused by HFD exposure, can now be put into context given the work of Nishimura et al., demonstrating that $CD8^+$ T cells are involved in these processes [79]. Notably, removal of CD1d prevents the development of the invariant type NKT cells. Different subsets of NKT cells may account for the results of other studies discussed in more detail in Chapter 4.

5.3 FUTURE DIRECTIONS

In addition to the new experiments discussed above, which naturally followed from obtained results, this research has also led to some unanticipated new questions for future investigation. In Chapter 2, I discussed how KC can lower hepatocyte lipid oxidation, however it is unknown whether other immune cells besides KC can also exert this effect. We could easily test dendritic cells, T cells, B cells or other types of macrophages, e.g. BMDM, utilizing the co-culture system. Furthermore, since TLR4 KO KC do not decrease hepatocyte oxidation, it would be interesting to measure PGE_2 production from TLR4 KO KC in response to palmitate, and if these cells do not produce PGE_2, the next logical progression would be to determine what signaling cascade links TLR4 to PGE_2. Another question that arises in light of the responses seen to palmitate is whether other fatty acids have the same effect. For example, do other saturated fatty acids longer, or shorter, than 16 carbons also induce PGE_2 production from KC. Alternatively, do unsaturated fatty acids also have this effect? Results discussed in Appendix A (Figure 23) would point towards PGE_2 production in response to shorter, but potentially not longer, fatty acids. Taking another step further, is PGE_2 the only mediator of these effects? The Luminex data presented in Figure 4 indicate that palmitate does not induce the production of TNFα, IFNγ or IL-10; however, this does not rule them out as also having effects on lipid metabolism. By adding recombinant cytokines to mouse hepatocytes, we can screen many potential mediators of these effects. We might predict that pro-inflammatory cytokines, such as TNFα, IL-6, IL-1, IFNγ and IL-12 would produce changes consistent with the development of steatosis,

while anti-inflammatory cytokines, such as IL-4 and IL-10, would oppose these changes. To obtain a more detailed analysis of how each of these cytokines may be involved, we can use a similar technique as described with the TLR4 KO KC co-culture experiment with cells obtained from, for example, TNFα or TNFα receptor knock out animals.

We also do not know what changes occur within the hepatocyte to result in lowered oxidation and potentially increased TG accumulation. Beyond the involvement of ACC, are these results a function of alterations in CPT1 activity or a lipid shuttling mechanism upstream from CPT1? Since CPT1 is the rate-limiting step of β-oxidation, using octanoic acid, which bypasses this step, can help answer this question. If PGE_2 does not decrease rates of octanoate oxidation, as it does in the case of palmitate, which is shuttled into the mitochondria by CPT1, we would conclude that PGE_2 reduces palmitate oxidation at the level of CPT1. We can also directly measure the CPT1 activity in hepatocytes that have been treated with PGE_2. Furthermore, we could utilize transcription and translation inhibitors to determine whether these processes are required or phosphatase and kinase inhibitors to determine whether kinase cascades are involved. Lastly, with regards to questions that could be addressed with our co-culture system, I have shown that 5 hours of PGE_2 treatment is sufficient to inhibit palmitate oxidation when palmitate is present overnight (Figure 5D); however, KC do not inhibit oxidation when palmitate is absent overnight (Figure 3A). We interpret this result as a demonstration that palmitate is necessary to activate the KC overnight, and subsequently cause PGE_2 production, but there is no evidence that the palmitate has no effect on the hepatocytes themselves. Therefore, does a five-hour treatment of PGE_2 still cause a decrease in hepatocyte palmitate oxidation if palmitate is absent overnight?

A number of questions have also surfaced in response to our findings in Chapter 3. The major question that remains is that we still do not know the mechanism of protection afforded by iDC. Are the injected iDC acting directly on parenchymal cells of the liver and WAT? Are iDC recruiting other immune cells to exert effects on the tissues? Are endogenous DC phagocytosing the iDC and presenting some unknown antigen to recruit other immune cells and affect tissue function? Since DC have long been known to alter innate and adaptive immune responses via activation and recruitment of T and B cells [92], we have recently started to probe the involvement of T cells. The DC-T cell interaction is classically mediated by the interaction of an MHC class II (MHCII)-antigen complex on the DC and the T cell receptor (TCR) on $CD4^+$ T cells. By injecting iDC generated from MHCII-null mice, we are testing the case where this interaction does not occur. If injection of MHCII-null iDC does not afford the same protection as WT iDC, we can conclude that the injected DCs are interacting with endogenous T cells to exert the effects observed. If, on the other hand, the MHCII-null iDC show the same effects as WT iDC, then either the endogenous DC are processing the injected DC or the injected DC are exerting their effects by a mechanism distinct from an MHCII-TCR interaction. An alternative method would be to inject WT iDC into RAG knock out mice. RAG knock outs do not produce T or B cells, thus if the injected cells were working through T or B cells, injection of iDC would not afford any protection in these animals. Importantly, RAG knock out mice do become obese when placed on a HFD [136]. Additionally, we could utilize the flt3L KO mice to investigate the involvement of endogenous DC. If endogenous DC are processing the injected DC, injection of iDC into flt3L KO mice, which do not have any endogenous DC, would not

respond to the injections with immune cell infiltration into WAT or a predominant T_H2 polarization of the tissues.

A separate question related to immunotherapy is that we tested two types of DC and BMDM, but do any other cell types recapitulate the effects of iDC? Other cell types that have been shown to affect insulin resistance, which could easily be tested, are $CD4^+$ T cells [136], $CD8^+$ T cells [79] and B cells [80]. As discussed previously, the goal of injecting these different cell types would be to attempt to induce not only a T_H2 immune response, as was seen with the iDC, but also a T_H1 response, to determine if polarization is indeed the key factor in determining protection from diet-induced obesity, insulin resistance and steatosis. We had hoped that the LPS-treated DC would induce a T_H1 response, but the immunophenotype of liver, WAT and spleen all demonstrated a drop in the TNFα/IL-4 ratio in these animals similar to the animals receiving iDC injections (Supplemental Figure 6). Given that animals receiving injections of LPS-DC were not afforded the same protection as those animals receiving iDC, we can again postulate that the determination of body composition and insulin sensitivity in response to immunotherapy is multi-factorial. Finally, once all the cells are recruited and the cytokine milieu is determined, how do these factors actually alter the metabolic responses of the liver and adipose tissue? One conjecture would be that a T_H1 polarized environment leads to the inhibitory serine phosphorylation of the insulin receptor in liver and WAT, thus dampening the insulin responsiveness of these tissues. Alternatively, a T_H2 polarization could alter kinase cascades within the parenchymal cells to maintain lipid handling procedures in spite of increased substrate.

5.4 FINAL THOUGHTS

In summary, as originally proposed in Figure 2, we believe that elevated circulating levels of FFA may be the initial factor that sets off a cascade of orchestrated changes leading to the sequelae of obesity. These fatty acids activate signaling mechanisms within the parenchymal cells of the liver and adipose tissue, presumably via engaging TLR4, which promote the recruitment of macrophages and dendritic cells to these tissues. Subsequently, FFA activate these recruited cells, which ultimately leads to alterations in the nutrient utilization procedures of hepatocytes and adipocytes. Based on our results from Chapter 3, DC are instrumental in directing the recruitment of many different cell types to the liver and adipose tissue. In the setting of obesity, once present, the T cells and macrophages cooperate to create a T_H1 polarized, pro-inflammatory cytokine milieu, which promotes the steatosis, adiposity and insulin resistance, while certain subsets of DC may act to prevent this by driving a T_H2 response. Concurrently, in the liver, FFA induce COX-1 & COX-2 within the KC to produce PGE_2 with mPGES-1, which acts directly on the hepatocyte through the EP1 receptor, to reduce β-oxidation rates and increase TG accumulation; changes which promote the development of steatosis and non-alcoholic fatty liver disease. An important step in generating new treatments to combat the metabolic disturbances of obesity will be to more fully understand the potential influence of a T_H2 polarized immunophenotype on nutrient metabolism and what actions can be taken to drive this response.

APPENDIX A - DEVELOPMENT OF MODEL SYSTEMS

A.1 INTRODUCTION

The use of animal model systems is a necessary alternative to the ethical conundrums posed by experimentation on humans. Our model system of choice, house mice Mus musculus, are smaller, have a faster metabolism, and shorter breeding and life cycles than humans, in addition to providing the opportunity to work with knock outs which lack a specific gene of interest. This allows us to ask very specific mechanistic questions and is an essential component of determining the inner workings of our cells' physiology. Even while using mice, however, yield of certain cell types is very low, limiting their potential for use in certain circumstances. Kupffer cells (KC), the macrophages of the liver, are one such cell type. Thus, one model system we sought to explore was the use of bone marrow-derived macrophages (BMDM) to serve as a surrogate for Kupffer cells. Additionally, previous work from the lab has implicated dendritic cells (DC) in the development of the metabolic disturbances of obesity, thus we set out to investigate the use of bone marrow-derived dendritic cells (BMDC) as another surrogate.

We also aimed to establish a model system to mimic the liver microenvironment in order to study the effects of immune cells on liver metabolism. The liver has its own extensive resident immune system, consisting of B and T cells, NK cells, NKT cells, dendritic cells, and KC [71]. Our lab has previously set up a trans-well system of rat liver to investigate these interactions [91]. This allows us to maintain primary

hepatocytes in close proximity to, but preventing direct contact with, mononuclear cells; thus measuring changes due solely to the effects of soluble factors. While metabolic studies of hepatocyte β-oxidation have been described in rats [91], the adaptation of this method for use with mouse hepatocytes would prove advantageous to ask directed mechanistic questions. Until this point in time, however, a number of differences between rat and mouse hepatocytes have made this adaptation difficult. Particularly, the trans-well inserts, used to co-culture the cells, themselves alter mouse hepatocyte metabolism in the absence of immune cells but not in rat hepatocytes. Further, turnover of tritiated water decreases differences between experimental groups over longer tracer incubation times.

In the following subsections, I describe the two main model systems I have been instrumental in developing during the course of my PhD training; namely a method of co-culturing mouse hepatocytes and immune cells to measure hepatocyte lipid metabolism, and the injection of bone marrow-derived immune cells into mice to acquire insight into the implications of exogenous administration of dendritic cells or macrophages.

A.2 MATERIALS AND METHODS

A.2.1 Hepatocyte isolation and cell culture

Primary mouse hepatocytes were isolated using a non-recirculating *in situ* collagenase perfusion technique, modified as previously described [128]. A buffered salt solution

containing 0.95g/L EGTA was perfused, *in situ*, through the inferior vena cava, exiting the portal vein (retrograde to blood flow), to remove any blood contained in the liver. Subsequently, a 0.2mg/mL collagenase solution (type IV; Sigma C-5138, St. Louis, MO) was perfused to release hepatocytes and immune cells from the connective tissue. Livers were agitated to release cells, then hepatocytes were separated from other cells using a series of 40 x g spins and a 30% percoll gradient. Resulting hepatocytes were counted, assessed for viability using Trypan Blue exclusion, resuspended at $0.5x10^6$ live hepatocytes/mL, then plated on type I collagen coated 6- or 24-well plates (Falcon 35-3046 or 35-3047, respectively; collagen = Sigma C-8919) at a density of $1.2x10^6$ hepatocytes/6-well well or $2.5x10^5$ hepatocytes/24-well well. Non-attached cells were removed after 1 hour (45 min if in 24-well plates), by washing with RPMI 1640 containing 10% heat inactivated FBS, 4.5g/L glucose, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 100U/mL penicillin, and 100ug/mL streptomycin [high glucose (HG) cRPMI]. Treatment media containing free fatty acid was prepared in HG cRPMI, and replaced the wash at the start of co-culture.

A.2.2 Kupffer cell isolation and cell culture

Liver mononuclear cells were isolated, modified as previously described [91]. The supernatants from the non-percoll hepatocyte centrifugations were centrifuged at 40 x g for 5 min, then the resultant supernatants were centrifuged at 375 x g. The cell pellet containing non-parenchymal cells was resuspended in 2.7mL cRPMI (as above but with 2.0g/L glucose) and mixed with 7.3mL 30% Histodenz (Sigma D-2158). The resulting suspension was split into two aliquots of 5mL and layered under 5mL of cRPMI, and

centrifuged at 1425 x g for 20min at 22°C with no brakes. Mononuclear cells remaining at the interphase between the two layers were collected and plated in cRPMI on 6-well plates (Falcon) or on trans-well inserts, as needed. Kupffer cells (KC) were allowed to adhere to plates or inserts for 1 hour, then plating media was removed, along with non-adherent mononuclear cells, and replaced with treatment media. Additionally, control inserts plated with only cRPMI were also placed in the incubator for 1 hour, at the end of which, the cRPMI was replaced with treatment media.

A.2.3 Hepatocyte and KC co-culture

Liver mononuclear cells were plated in Transwell Permeable Supports (Corning, Corning, NY) at a density 1/3 that of the hepatocytes (thus, $4x10^5$ in 6-well inserts or $8.3x10^4$ in 24-well inserts). Once KC attach, 2mL HG cRPMI containing 0.4mM palmitate or BSA vehicle control was added to the insert, and 2mL treatment media was added directly to the hepatocytes (200uL to the insert and 800uL directly to hepatocytes in 24-well plates). Hepatocytes and KC were co-cultured for 19 hours, then analyzed for palmitate oxidation, PGE_2 production, or RT-PCR.

A.2.4 Mouse primary hepatocyte fatty acid oxidation

A.2.4.1 Original experimental design

At the start of the co-culture, cells were incubated in the presence of 0.4mM palmitate and 2.5uCi/mL palmitic acid [9,10-^{3}H(N)] (Perkin Elmer, Boston, MA). Hepatocytes alone were compared to hepatocytes co-cultured with KC using a trans-well insert.

After 19 hours in culture, palmitate oxidation was assessed by measuring the quantity of tritiated water released into the medium, as previously described [91,129].

A.2.4.2 Updated experimental design

Hepatocytes and KC were co-cultured in the presence of palmitate, octanoate, stearate, or oleate, but in the absence of the radioactive tracer, overnight. Hepatocytes cultured in the presence of a trans-well insert with no cells added were compared to hepatocytes with KC attached to the insert. After 14 hours in culture in the presence of 0.4mM fatty acid, 2.5uCi/mL tracer, either palmitic acid [9,10-^{3}H(N)] (Perkin Elmer, Boston, MA), octanoic acid n-[8-^{3}H], stearic acid [9,10-^{3}H(N)] or oleic acid [9,10-^{3}H(N)] (American Radiolabeled Chemicals, St. Louis, MO), was added to each well, with the corresponding fatty acid present, for 5 hours. β-oxidation was assessed by measuring the quantity of tritiated water released into the medium. Cell monolayers were washed twice with an excess volume of ice-cold PBS and collected in 1N NaOH for determination of protein content.

A.2.5 Generation and culture of bone marrow-derived dendritic cells (BMDC)

BMDC were generated from the culture of bone marrow progenitors, as previously described [101], originally adapted from the work of Inaba, et al. [158] and Lu, et al. [159]. Briefly, bone marrows, isolated from femur and tibia, were depleted of RBCs using Red Blood Cell Lysing Buffer (Sigma R7757), and of T cells, B cells and MHC class-II expressing cells using monoclonal antibody and complement treatment. The remaining cells were cultured in serum free AIM-V medium (Gibco 12055) with 1mM

sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 100U/mL penicillin, 100ug/mL streptomycin and 0.00035% β-mercaptoethanol (Sigma M7522) containing 5ng/mL GM-CSF for 5 days. On day two, 50% of the medium was replaced with fresh AIM-V with GM-CSF. On day 5, half of the cultured cells were treated with 1ug/mL LPS (Sigma L-3012) for two hours to generate activated BMDC (LPS-DC). Untreated, immature BMDC (iDC) and LPS-DC were purified using CD11c magnetic beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions.

A.2.6 Generation and culture of bone marrow-derived macrophages (BMDM)

BMDM were generated from the culture of bone marrow progenitors, as previously described [137], originally adapted from the work of Davis and Gordon [160]. Briefly, bone marrows, isolated from femur and tibia, were depleted of RBCs using Red Blood Cell Lysing Buffer (Sigma R7757). Cells were plated on 150x15mm petri dishes (VWR 25384-326) at a density of $1x10^7$/dish (day 0) and cultured in DMEM supplemented with 20% heat-inactivated FBS, 1mM sodium pyruvate, 2mM L-glutamine, 100U/mL penicillin, 100ug/mL streptomycin and 30% L-cell supernatant, which is generated by incubating L-cell fibroblasts (CCL-1 from ATCC, gift of Dr. R. Salter) in DMEM supplemented with 10% heat-inactivated FBS, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM L-glutamine, 100U/mL penicillin, and 100ug/mL streptomycin, until confluent. Supernatants were passed through a 0.2um filter and stored at -80°C until preparing media. Differentiation media was changed 3-4 days after initial plating, then subcultured every 4^{th} day thereafter. BMDM were used for

experiments on day 7 for injections after a negative selection for CD11c using MACS beads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions.

A.2.7 Mouse TNFα ELISA

Cells were plated at a density of $4x10^6$ per well in 6-well plates. Cells were cultured for 19 hours in the presence of 0.4mM palmitate or 1.15% BSA (vector control, Sigma A7030). The next morning, cell culture supernatants were collected and stored at -20°C until Mouse TNF-α Quantikine Immunoassay (R&D Systems, MTA00, Minneapolis, MN) was performed, according to the manufacturer's instructions.

A.2.8 Quantification of cytokine production

Cell culture supernatants from various cell treatments were collected after 19 hours of culture, and assayed for the presence of 23 cytokines/chemokines (IL-1α, 1β, 2, 4, 5, 6, 10, 12(p40), 12(p70), 13, and 17, TNFα, IFN-γ, IP-10, CXCL1, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, and GM-CSF) using a Milliplex Luminex Kit (Millipore), according to the manufacturer's instructions.

A.2.9 PGE_2 EIA

PGE_2 released into the cell culture medium by co-culture or monoculture was measured using a commercial kit (by EIA; Cayman Chemical, Ann Arbor, MI) according to the protocol provided by the manufacturer.

A.3 MOUSE CO-CULTURE SYSTEM

A.3.1 Effects of Kupffer cells and trans-well inserts on hepatocyte palmitate oxidation

Previous studies have reported decreases in rat hepatocyte palmitate oxidation when co-cultured with rat KC in the presence of LPS [91]. A direct adaptation of that system, using mouse hepatocytes and KC generated a large artifact (Figure 22A). We discovered that the trans-well insert itself, with no immune cells added, decreased the palmitate oxidation in mouse hepatocytes (Figure 22A). Even with the effect of the insert, there was a reproducible, but small, difference between the Hepatocytes+Insert and Hepatocytes+KC groups. We therefore set out to alter the experimental conditions to increase this difference while maintaining the effect of KC.

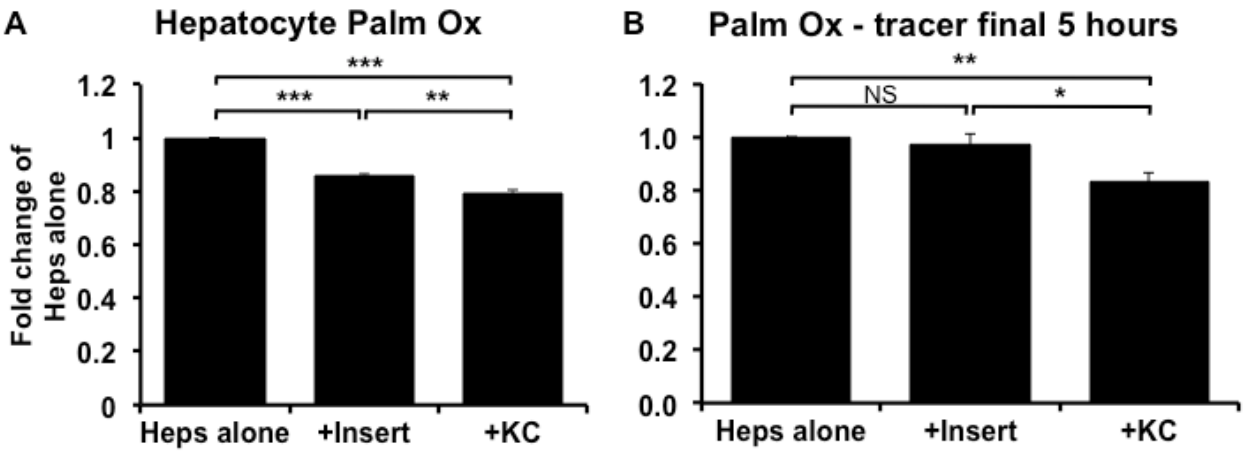


Figure 22 - Palmitate-activated KC and trans-well inserts decrease hepatocyte palmitate oxidation.

(A) Primary hepatocytes (Heps) from C57Bl/6 mice were mono-cultured overnight in the presence of 0.4mM palmitate (column 1), co-cultured with KC that were attached to a trans-well insert (column 3), or cultured in the presence of a trans-well insert with no cells present (column 2), as described in the Original experimental design Mouse Primary Hepatocyte Fatty Acid Oxidation method (A.2.4.1). (B) Alternatively, primary hepatocytes from C57Bl/6 mice were mono-cultured overnight in the presence of 0.4mM palmitate (column 1), co-cultured with KC that were attached to a trans-well insert (column 3), or cultured in the presence of a trans-well insert with no cells present (column 2), as described in the Updated experimental design Mouse Primary Hepatocyte Fatty Acid Oxidation method (A.2.4.2). Results are presented as the means±SE for four experiments performed in triplicate. *p<0.05, **p<0.005, ***p<0.0005.

A.3.2 Determination of optimal tracer exposure time for mouse hepatocyte palmitate oxidation

The radioactive palmitate tracer allows for the total β-oxidation to be measured. Based on the length of time the tracer is present, a certain amount of tracer will be oxidized, transferring the tritium atoms to water, which is captured by evaporative exchange. We tested a number of different exposure times, and found that if we co-cultured the cells for 14 hours and then added the tracer to the culture medium for the final 5 hours of the experiment, we were able to minimize the effect of the trans-well insert, and maximize the effect of the KC (Figure 22B). Oxidation levels when the tracer was present for only

the final 3 hours were still affected by the insert, and statistical differences were not achieved between the +Insert and +KC conditions. Similar results were obtained when testing a 4 hour co-culture with tracer present for the entire time. Additionally, an 8 hour co-culture with tracer present for the entire duration of the culture, showed no difference between the Hepatocytes+Insert and Hepatocytes+KC groups (data not shown).

A.3.3 Effects of KC on fatty acid oxidation

Given our evidence that palmitate, in and of itself, is capable of acting on the Kupffer cells to alter the level of β-oxidation (see also Chapter 2), we wanted to test the ability of other fatty acids to produce similar effects. Using the updated experimental design and utilizing palmitate (C16:0, Figure 23A), octanoate (C8:0, Figure 23B), stearate (C18:0, Figure 23C), or oleate (C18:1 cis-9, Figure 23D) as fatty acid substrates, and the radioactive tracer with the corresponding tritiated fatty acid, we measured β-oxidation levels in the presence or absence of KC. We saw the same effect on β-oxidation when we used octanoate as our substrate as with palmitate. These data were particularly interesting, because octanoate, as a short to medium chain fatty acid, is not shuttled through CPT-1 into the mitochondria. This speaks to how lipids are handled in the presence of KC regardless of the mode of delivery. We also did not find a decrease with the long chain saturated fatty acid stearate, nor the mono-unsaturated fatty acid oleate. Additionally, the effect of palmitate on KC is dependent on TLR4 (see Chapter 2). Taken together, these data would indicate that fatty acids above a certain length may not be able to trigger action through TLR4, thus not inducing the detrimental effects on lipid oxidation.

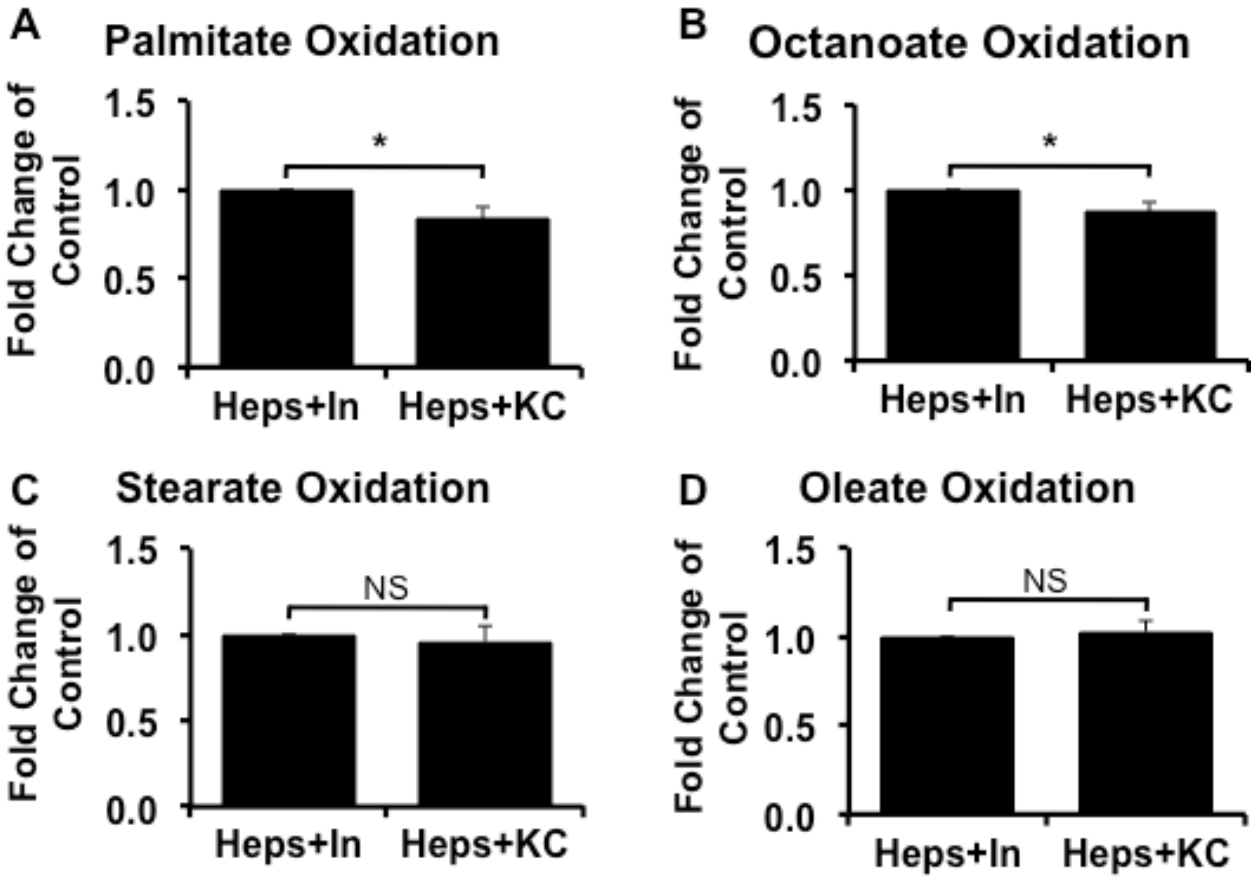


Figure 23 - KC decrease hepatocyte oxidation of palmitate and octanoate, but not oleate or stearate.

Kupffer cells and hepatocytes were co-cultured overnight in the presence of 0.4mM fatty acid utilizing the Updated experimental design as described in Methods (A.2.4.2). Hepatocytes cultured with an empty insert (Heps+In) were compared to hepatocytes co-cultured with KC (Heps+KC). Results are presented as the means±SE for a minimum of five experiments performed in triplicate. *$p<0.05$.

A.4 INJECTION OF BONE MARROW-DERIVED IMMUNE CELLS

A.4.1 Differences between various immune cell lines

In previous chapters, we described the use of various immune cell lines and immune cell primary cultures. KC, BMDC, and BMDM are used most extensively to describe the

effects of immune cells on lipid metabolism and the metabolic disturbances of obesity. We were particularly interested in utilizing the BMDM as a model for KC, since the yield of KC from a mouse liver is extremely small. BMDM are much more abundant and easier to manipulate, therefore, the applications and potential for new experiments would greatly expand. As explored more fully in Chapter 2, we have attributed a role for the prostanoid PGE_2 in the effects of KC on hepatocyte lipid metabolism. We, therefore, started by comparing the ability of KC and BMDM to generate PGE_2 in response to palmitate (Figure 24A). As the data shows, KC generate roughly four times more PGE_2 in the presence of palmitate compared to vehicle (BSA), while BMDM show no difference. Notably, the basal levels of PGE_2 production were similar between the two cell types (KC 80±19 pg/mL; BMDM 101±46 pg/mL; average±SEM). The pro-inflammatory cytokine TNFα has been implicated in the metabolic disturbances of obesity [8,9,91]. We, therefore, were also interested in comparing the TNFα production in these cells in response to palmitate. We found that KC trend towards dropping TNFα production while levels were undetectable in BMDM culture supernatants in the presence or absence of palmitate (Figure 24B). Additionally, as a further control, RAW cells, an immortalized mouse macrophage cell line, exhibited an increase in TNFα production in the presence of palmitate (Figure 24B). Thus, the findings showed that BMDM and KC respond differently to stimulation by palmitate, suggesting BMDM are not the optimal surrogate cell type for KC in this model.

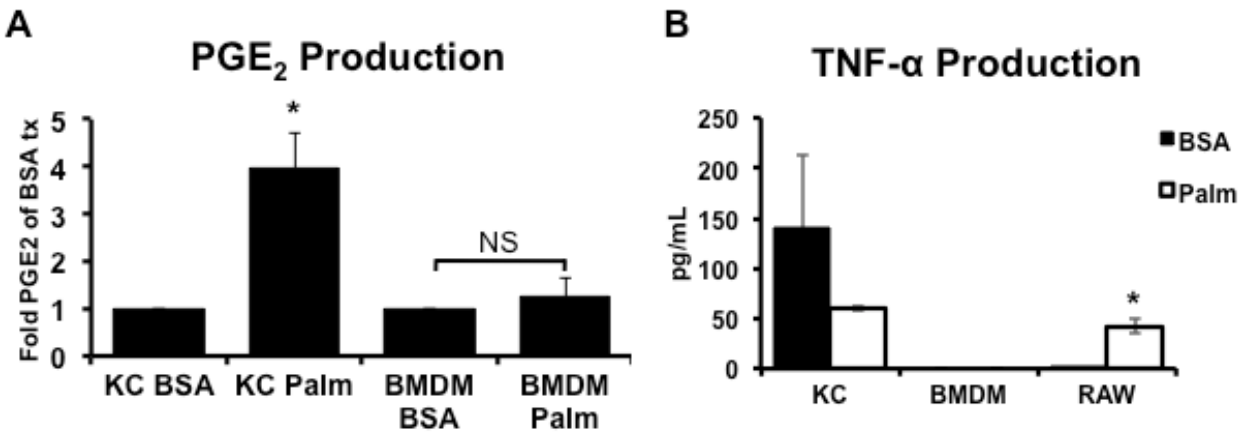


Figure 24 - Macrophage comparison via prostaglandin E_2 (PGE_2) synthesis and TNFα production in response to palmitate.

(A) KC and bone marrow-derived macrophages were cultured overnight in the presence or absence of 0.4mM palmitate, after which cell culture media was collected and analyzed for PGE_2 concentration, as described in Methods. (B) KC, BMDM and RAW cells were cultured overnight in the presence or absence of 0.4mM palmitate, after which cell culture media was collected and analyzed for TNFa concentration, as described in Methods. Results are presented as the means±SE for a minimum of 3 experiments performed in duplicate. *$p<0.05$ compared to BSA.

A.4.2 Immune cells as therapy

In Chapter 3, we used an exogenous cell transfer technique to alter the development of obesity, insulin resistance and steatosis in mice when they are placed on a high fat diet for seven weeks. Additionally, we have used a separate, shorter (one week) experimental design to address changes specifically in the immunophenotype of these animals. Dendritic cells only survive *in vivo* for approximately 3 days [101], therefore we decided to give injections of DC every week for the duration of the experiments (thus six injections during the course of the seven week experiments, starting with the first injection at the time of starting the HFD, and a single injection for the shorter-term experiments). There was always at least one week between the time of the last injection and sacrifice of the animals. In this manner, we could minimize any influence

the physically injected cells may have had on the final analysis. Most of the data from this project was presented in Chapter 3; however, as a first pass, to characterize the cells, we performed Luminex on overnight cultures of the injected cells (Figure 25). Not surprisingly, the LPS-stimulated BMDC showed higher production of the pro-inflammatory cytokines TNFα and IL-6 (Figures 25A&B). Additionally, the BMDMs produced significantly more MCP-1 and significantly less RANTES than either immature or LPS-DC (Figure 25 C&D).

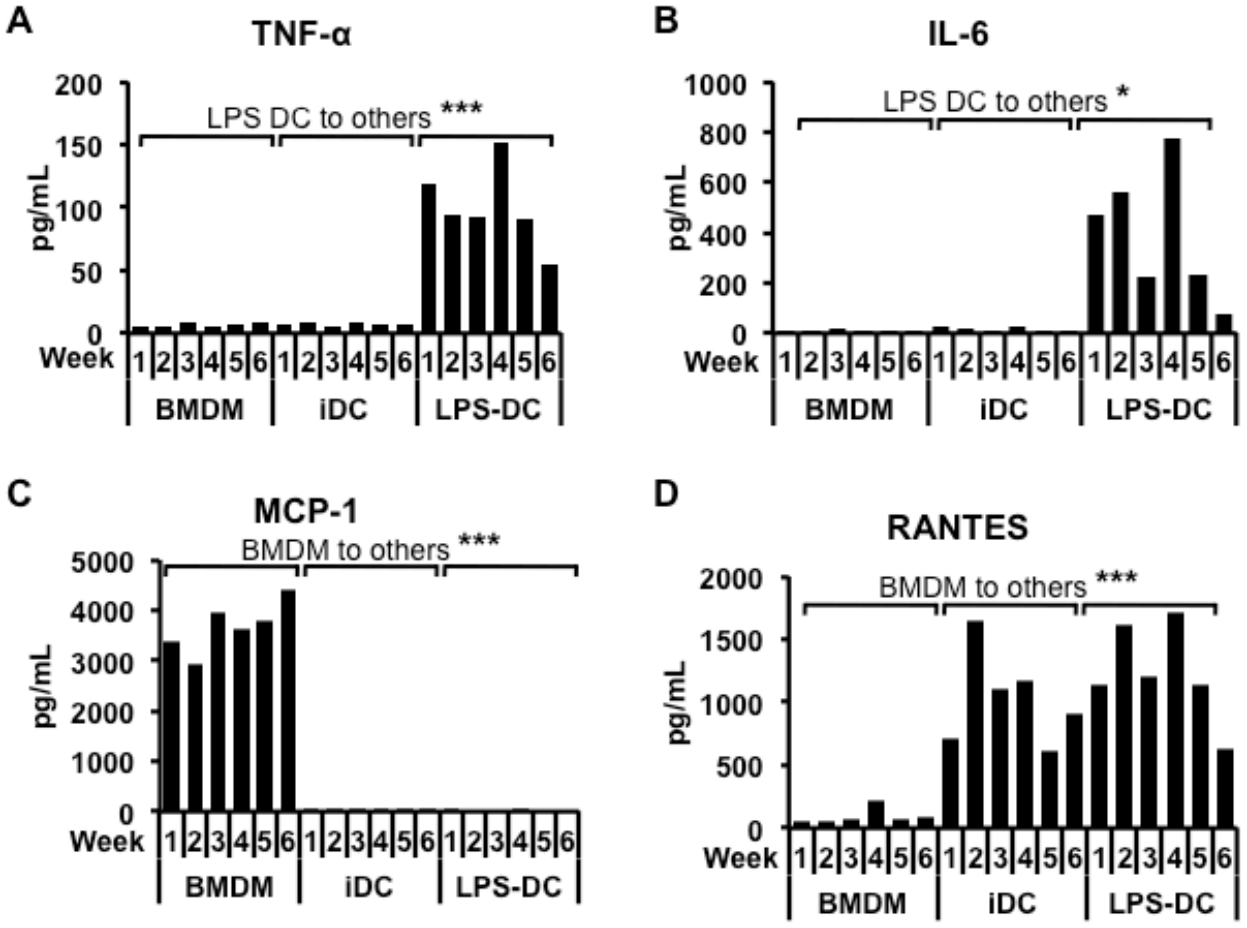
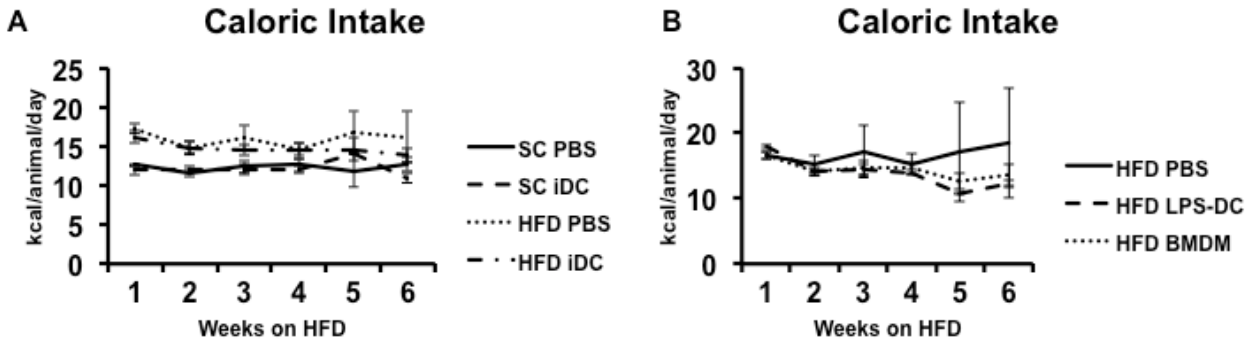


Figure 25 - Cytokine production of bone marrow-derived immune cells.

BMDM, iDC and LPS-DC were generated, as described in Methods. After injection into mice, remaining cells were kept in culture overnight. Supernatants were collected and analyzed by Luminex, as described in Methods. Data is shown for TNFa (A), IL-6 (B), MCP-1 (C), and RANTES (D). Results are presented as the concentration of each cytokine produced by the injected cells from the individual week indicated. *$p<0.05$, ***$p<0.0005$.

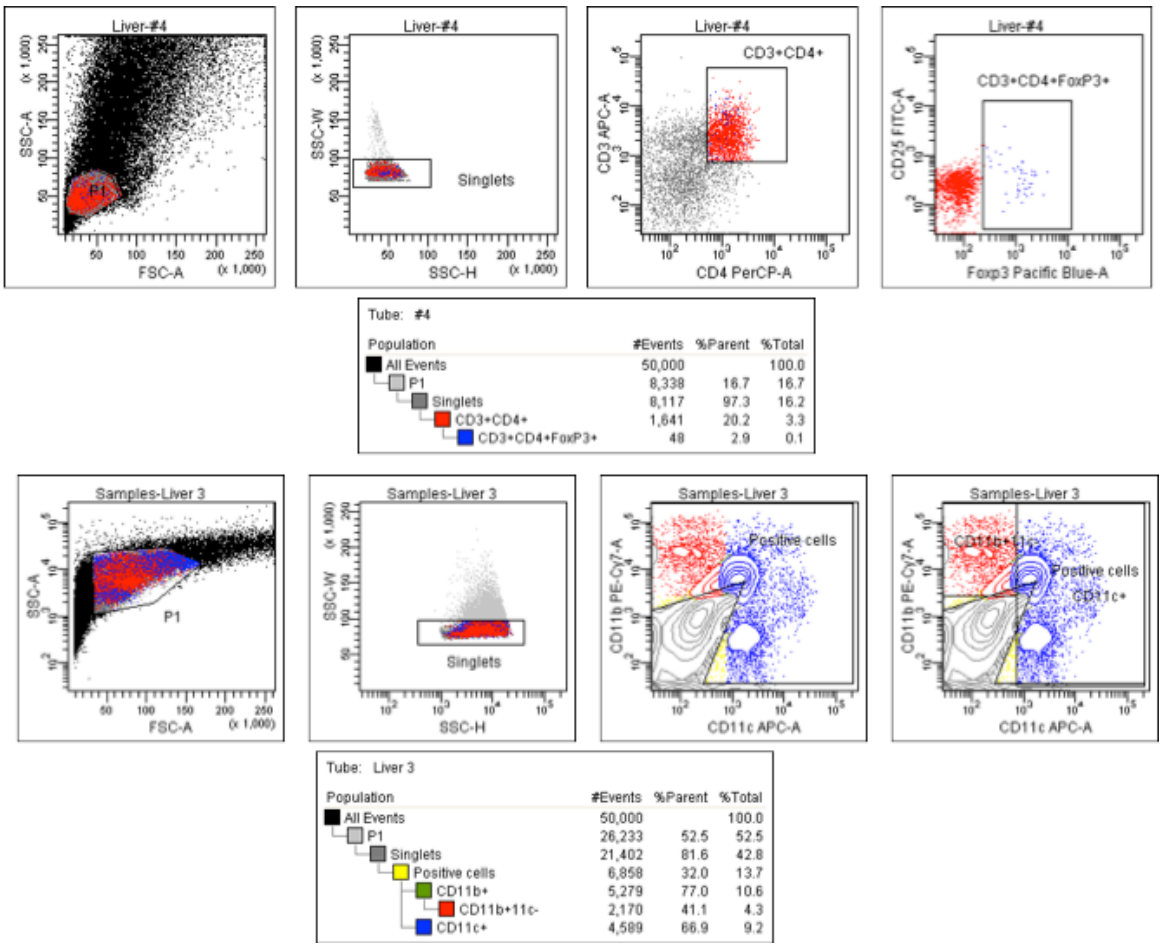
APPENDIX B - SUPPLEMENTAL FIGURES

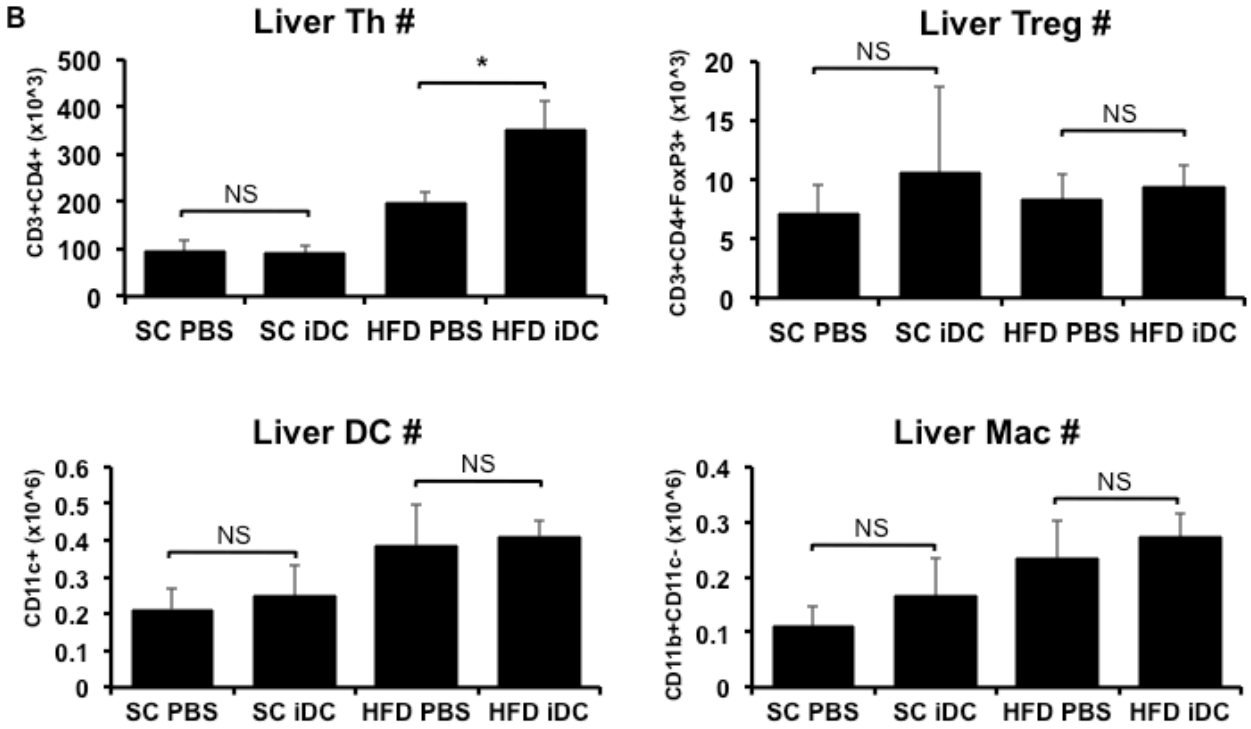


Supplemental Figure 1 - Caloric intake of DC and macrophage injected mice.

Wild-type C57Bl/6 mice were placed on a SCD or HFD for 6 weeks and injected once per week with either PBS, $1x10^6$ immature BMDC (iDC), $1x10^6$ LPS treated BMDC (LPS-DC) or $1x10^6$ bone marrow-derived macrophages (BMDM). Caloric intake was assessed, as described in Methods. Results are presented as the means±SE for an n of 4 (A) or 2 (B) cages in each group.

A

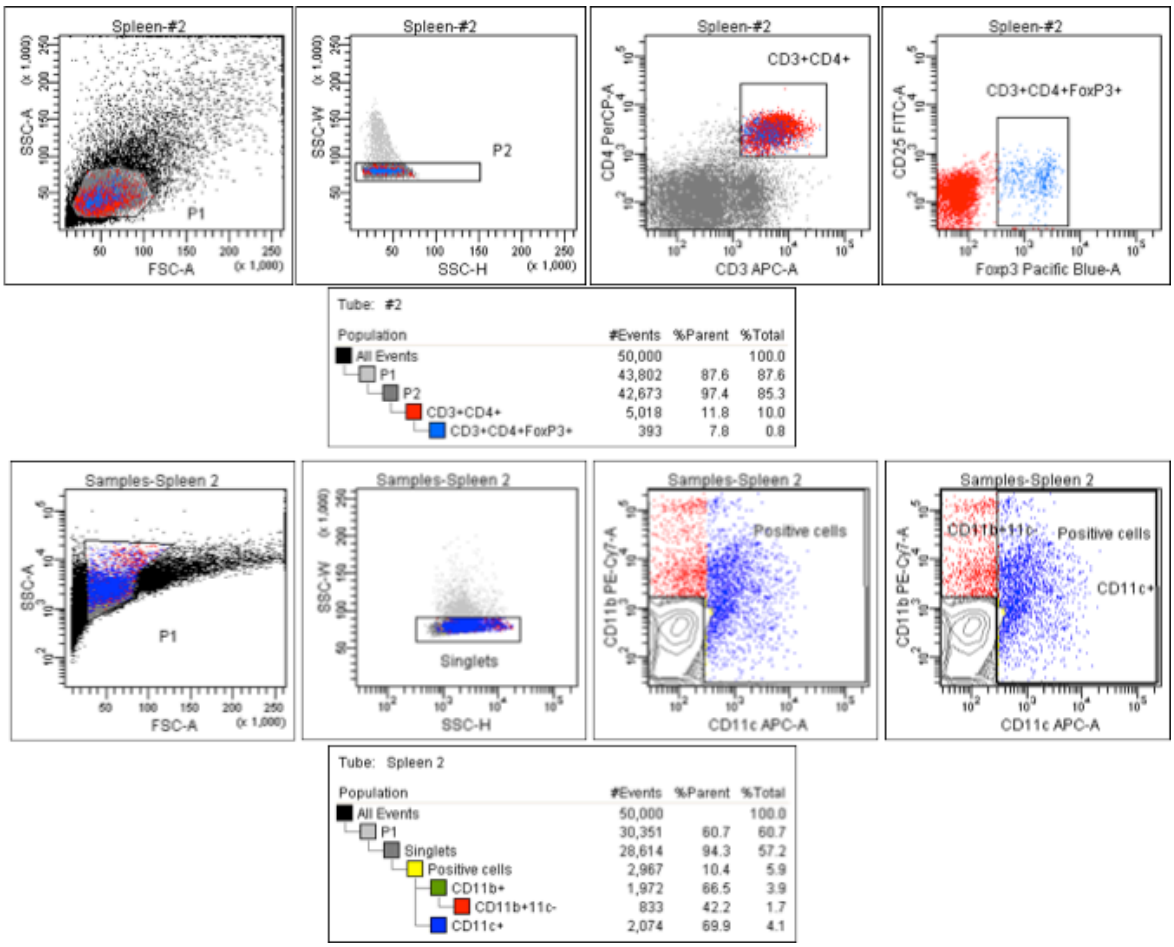

Liver-#4
SSC-A
FSC-A
P1
Liver-#4
SSC-W
SSC-H
Singlets
Liver-#4
CD3+CD4+
CD3 APC-A
CD4 PerCP-A
Liver-#4
CD3+CD4+FoxP3+
CD25 FITC-A
Foxp3 Pacific Blue-A
Tube: #4
Population #Events %Parent %Total
All Events 50,000 100.0
P1 8,338 16.7 16.7
Singlets 8,117 97.3 16.2
CD3+CD4+ 1,641 20.2 3.3
CD3+CD4+FoxP3+ 48 2.9 0.1
Samples-Liver 3
SSC-A
FSC-A
P1
Samples-Liver 3
SSC-W
SSC-H
Singlets
Samples-Liver 3
CD11b PE-Cy7-A
CD11c APC-A
Positive cells
Samples-Liver 3
CD11b PE-Cy7-A
CD11c APC-A
CD11b+11c-
Positive cells
CD11c+
Tube: Liver 3
Population #Events %Parent %Total
All Events 50,000 100.0
P1 26,233 52.5 52.5
Singlets 21,402 81.6 42.8
Positive cells 6,858 32.0 13.7
CD11b+ 5,279 77.0 10.6
CD11b+11c- 2,170 41.1 4.3
CD11c+ 4,589 66.9 9.2

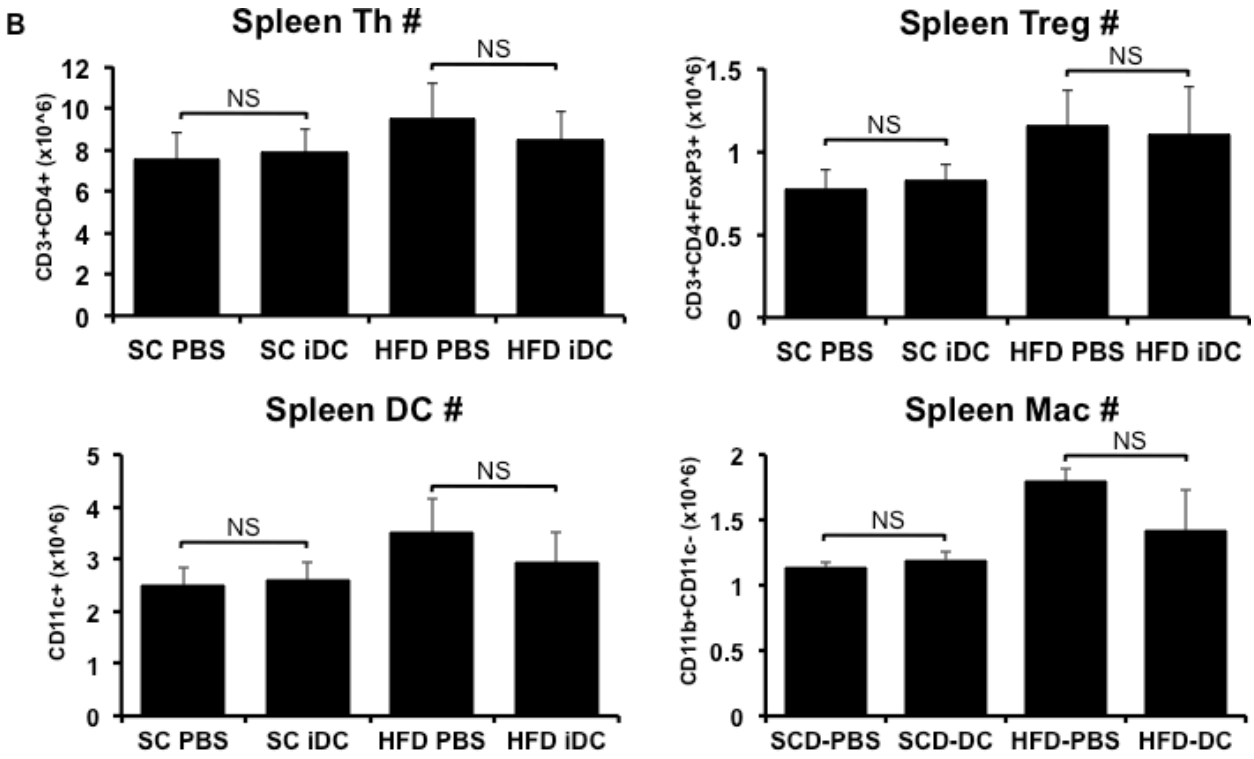


Supplemental Figure 2 - Liver FACS analysis of iDC injected mice.

Liver was collected from SC, HFD, PBS injected, and iDC injected mice, immune cells were isolated and analyzed by FACS, as described in Methods. (A) Gating scheme for T cell stain (top) and DC/Macrophage stain (bottom). (B) Total numbers of $CD3^+CD4^+$, $CD3^+CD4^+FoxP3^+$, $CD11c^+$, and $CD11b^+CD11c^-$ cells in the liver were quantified. Results are presented as the means±SE for a minimum of seven animals in each group. *$p<0.05$.

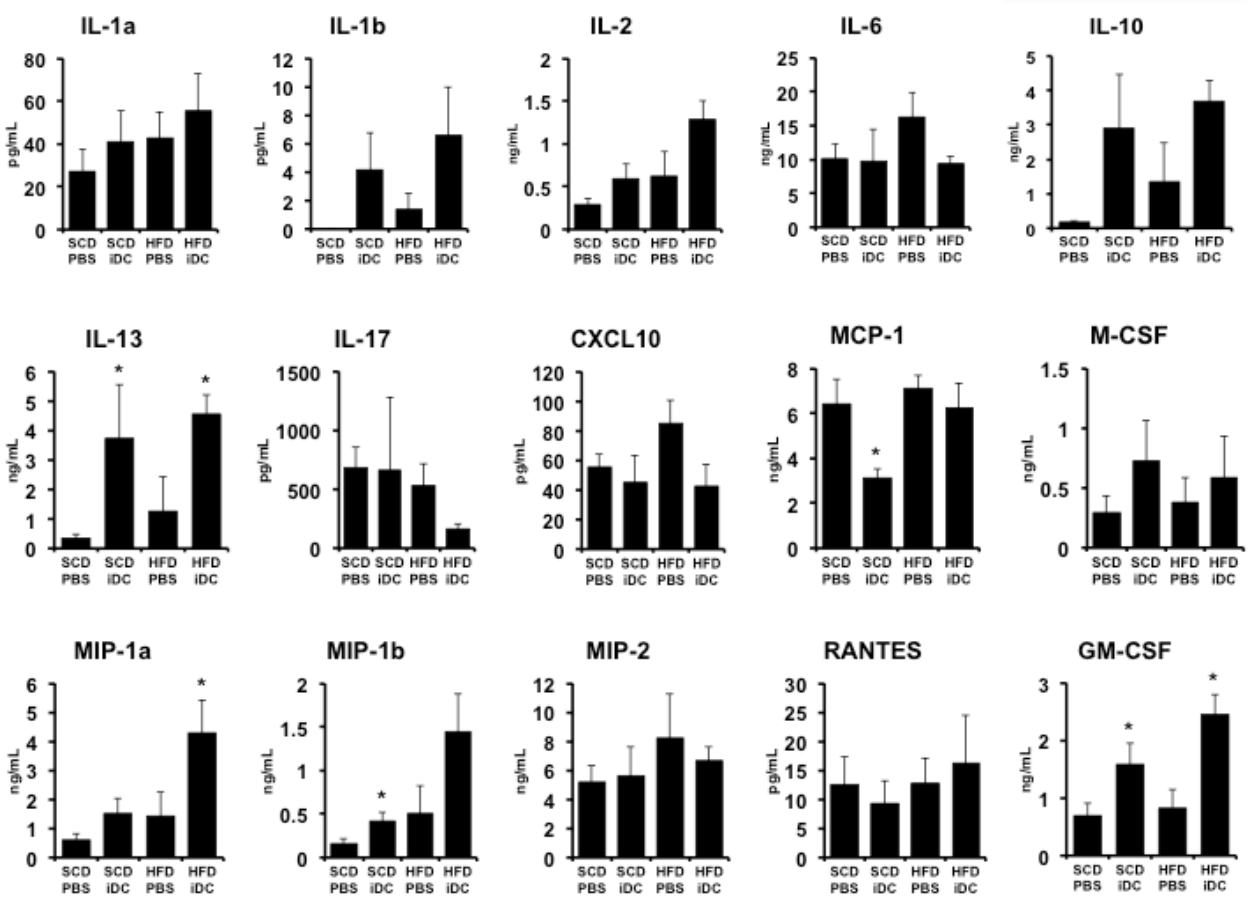
A


Spleen-#2
SSC-A
FSC-A
P1
SSC-W
SSC-H
P2
CD4 PerCP-A
CD3 APC-A
CD3+CD4+
CD25 FITC-A
Foxp3 Pacific Blue-A
CD3+CD4+FoxP3+
Tube: #2
Population #Events %Parent %Total
All Events 50,000 100.0
P1 43,802 87.6 87.6
P2 42,673 97.4 85.3
CD3+CD4+ 5,018 11.8 10.0
CD3+CD4+FoxP3+ 393 7.8 0.8
Samples-Spleen 2
Singlets
CD11b PE-Cy7-A
CD11c APC-A
Positive cells
CD11b+11c-
CD11c+
Tube: Spleen 2
Population #Events %Parent %Total
All Events 50,000 100.0
P1 30,351 60.7 60.7
Singlets 28,614 94.3 57.2
Positive cells 2,967 10.4 5.9
CD11b+ 1,972 66.5 3.9
CD11b+11c- 833 42.2 1.7
CD11c+ 2,074 69.9 4.1



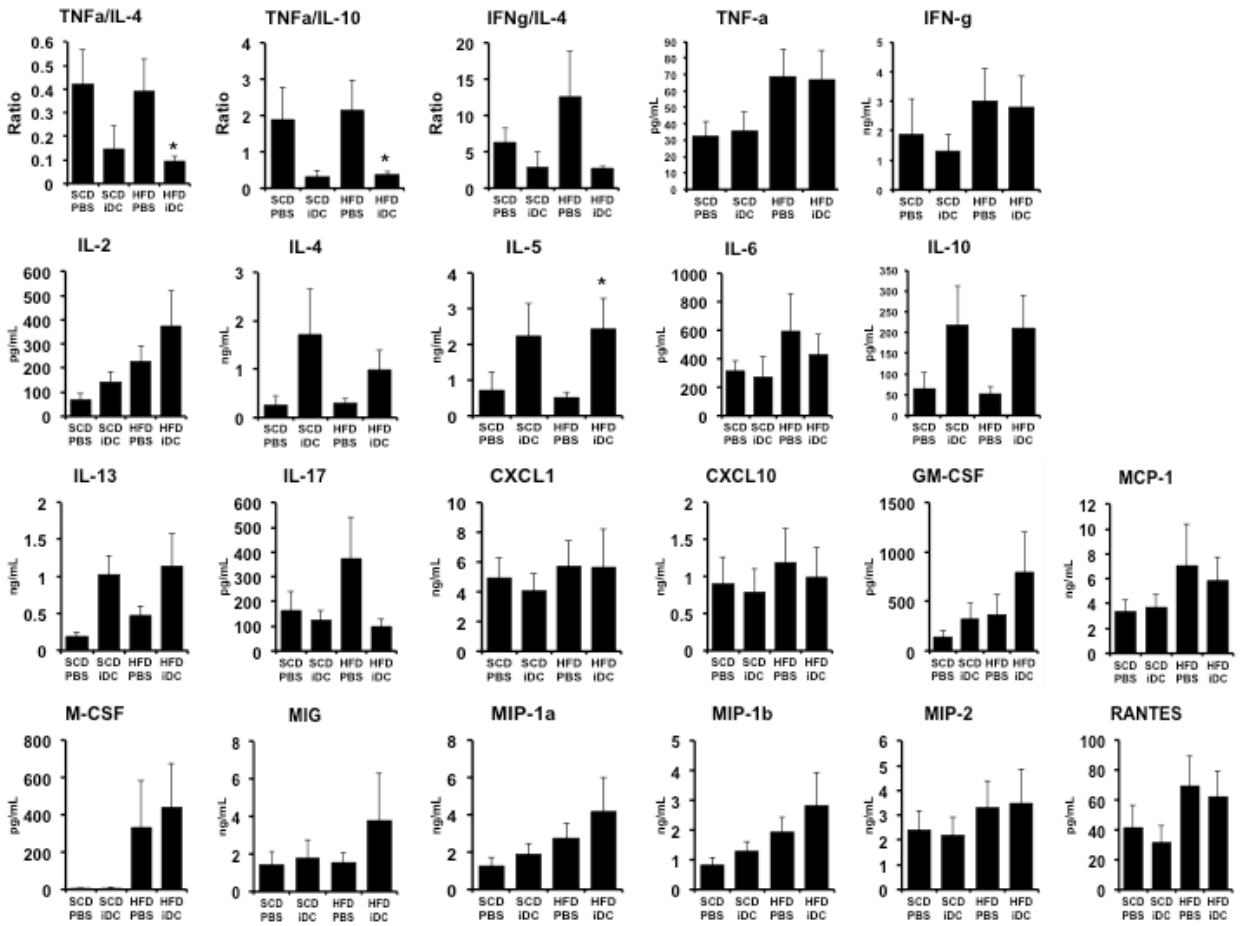
Supplemental Figure 3 - Spleen FACS analysis of iDC injected mice.

Spleen was collected from SC, HFD, PBS injected, and iDC injected mice, immune cells were isolated and analyzed by FACS, as described in Methods. (A) Gating scheme for T cell stain (top) and DC/Macrophage stain (bottom). (B) Total numbers of $CD3^{+}CD4^{+}$, $CD3^{+}CD4^{+}FoxP3^{+}$, $CD11c^{+}$, and $CD11b^{+}CD11c^{-}$ cells in the spleen were quantified. Results are presented as the means±SE for a minimum of seven animals in each group.



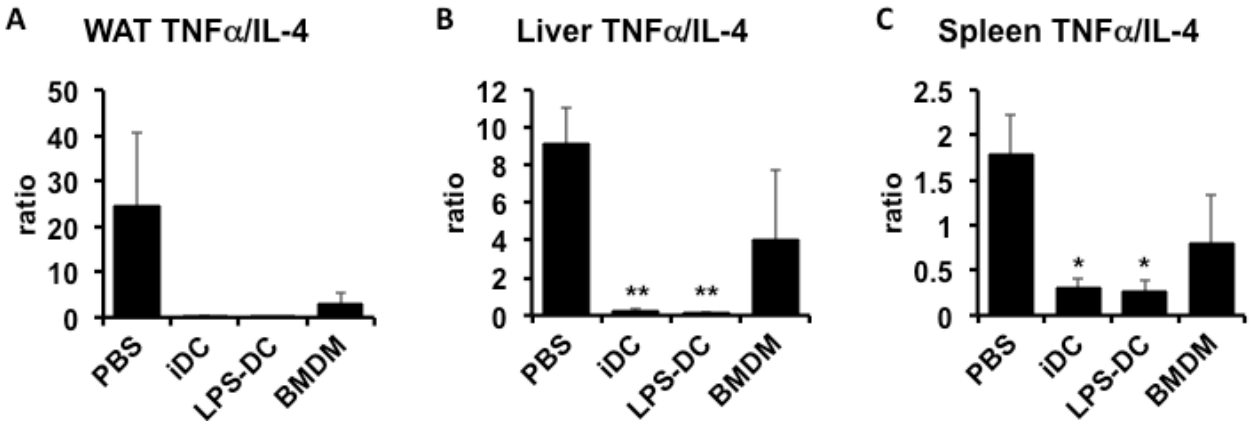
Supplemental Figure 4 - WAT Luminex analysis of iDC injected mice.

Complete Luminex analysis of WAT immune cells (continuation from Figure 13C). Immune cells, isolated from WAT, were kept in culture overnight in the presence of 10ng/mL PMA and 500ng/mL ionomycin. Subsequently, supernatants underwent Luminex analysis, as described in Methods. Results are presented as the means±SE for five-six animals in each group. *p<0.05.



Supplemental Figure 5 - Liver Luminex analysis of iDC injected mice.

Immune cells, isolated from liver, were kept in culture overnight in the presence of 10ng/mL PMA and 500ng/mL ionomycin. Subsequently, supernatants underwent Luminex analysis, as described in Methods. Results are presented as the means±SE for six animals in each group. *p<0.05.



Supplemental Figure 6 - Luminex analysis of DC and macrophage injected mice.

Immune cells, isolated from WAT, liver and spleen, were kept in culture overnight. Subsequently, supernatants underwent Luminex analysis, as described in Methods. Results are presented as the means±SE for five animals in each group. *$p<0.05$, **$p<0.005$.

BIBLIOGRAPHY

1. (2011) Obesity and Overweight Fact sheet. World Health Organization Media centre.

2. Flegal KM, Carroll MD, Ogden CL, Curtin LR (2010) Prevalence and trends in obesity among US adults, 1999-2008. JAMA : the journal of the American Medical Association 303: 235-241.

3. (2011) Diabetes Fact Sheet. Center for Disease Control and Prevention.

4. Ebstein W (1876) Zur Therapie des Diabetes mellitus, insbesondere euber die Anwendung des salicy-sauren Natorns bei demselben (=on the treatment of diabetes mellitus, esp. on the use of salicylic-acid sodium). Klinische Wochenschrift (Berlin) 13: 337-340.

5. Fearnley GR, Chakrabarti R, Avis PR (1963) Blood fibrinolytic activity in diabetes mellitus and its bearing on ischaemic heart disease and obesity. Br Med J 1: 921-923.

6. Fearnley GR, Vincent CT, Chakrabarti R (1959) Reduction of blood fibrinolytic activity in diabetes mellitus by insulin. Lancet 2: 1067.

7. Ogston D, McAndrew GM (1964) Fibrinolysis in Obesity. Lancet 2: 1205-1207.

8. Hotamisligil GS, Spiegelman BM (1994) Tumor necrosis factor alpha: a key component of the obesity-diabetes link. Diabetes 43: 1271-1278.

9. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 95: 2409-2415.

10. Straczkowski M, Dzienis-Straczkowska S, Stepien A, Kowalska I, Szelachowska M, et al. (2002) Plasma interleukin-8 concentrations are increased in obese subjects and related to fat mass and tumor necrosis factor-alpha system. J Clin Endocrinol Metab 87: 4602-4606.

11. Sartipy P, Loskutoff DJ (2003) Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A 100: 7265-7270.

12. Creely SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, et al. (2007) Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab 292: E740-747.

13. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, et al. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56: 1761-1772.

14. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, et al. (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57: 1470-1481.

15. Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259: 87-91.

16. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 389: 610-614.

17. De Taeye BM, Novitskaya T, McGuinness OP, Gleaves L, Medda M, et al. (2007) Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity. Am J Physiol Endocrinol Metab 293: E713-725.

18. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, et al. (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 293: 1673-1677.

19. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, et al. (2001) Prevention of fat-induced insulin resistance by salicylate. J Clin Invest 108: 437-446.

20. Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, et al. (2002) Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. J Clin Invest 109: 1321-1326.

21. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, et al. (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nature medicine 11: 183-190.

22. Miller SI, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. Nature reviews Microbiology 3: 36-46.

23. Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annual review of immunology 12: 141-179.

24. Yin MJ, Yamamoto Y, Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 396: 77-80.

25. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, et al. (2005) IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med 11: 191-198.

26. Hotamisligil GS (2003) Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord 27 Suppl 3: S53-55.

27. Hotamisligil GS (2005) Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes. Diabetes 54 Suppl 2: S73-78.

28. Hotamisligil GS (2006) Inflammation and metabolic disorders. Nature 444: 860-867.

29. Hotamisligil GS, Erbay E (2008) Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol 8: 923-934.

30. Schenk S, Saberi M, Olefsky JM (2008) Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 118: 2992-3002.

31. Shoelson SE (2006) Banking on ATM as a new target in metabolic syndrome. Cell Metab 4: 337-338.

32. Shoelson SE, Lee J, Goldfine AB (2006) Inflammation and insulin resistance. J Clin Invest 116: 1793-1801.

33. Shoelson SE, Lee J, Yuan M (2003) Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance. Int J Obes Relat Metab Disord 27 Suppl 3: S49-52.

34. Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD (1988) Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. Diabetes 37: 1020-1024.

35. Gordon ES (1960) Non-esterified fatty acids in the blood of obese and lean subjects. The American Journal of Clinical Nutrition 8: 740-747.

36. Taskinen MR, Bogardus C, Kennedy A, Howard BV (1985) Multiple disturbances of free fatty acid metabolism in noninsulin-dependent diabetes. Effect of oral hypoglycemic therapy. The Journal of clinical investigation 76: 637-644.

37. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, et al. (2003) Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem 278: 37041-37051.

38. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, et al. (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance. The Journal of clinical investigation 116: 3015-3025.

39. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, et al. (2007) Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 56: 1986-1998.

40. Akira S, Sato S (2003) Toll-like receptors and their signaling mechanisms. Scandinavian journal of infectious diseases 35: 555-562.

41. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.

42. Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signalling. Nature 430: 257-263.

43. Lee JY, Sohn KH, Rhee SH, Hwang D (2001) Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem 276: 16683-16689.

44. Vijay-Kumar M, Aitken JD, Carvalho FA, Ziegler TR, Gewirtz AT, et al. (2011) Loss of function mutation in toll-like receptor-4 does not offer protection against obesity and insulin resistance induced by a diet high in trans fat in mice. Journal of inflammation 8: 2.

45. Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annual review of biochemistry 67: 821-855.

46. Winder WW, Hardie DG (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. The American journal of physiology 277: E1-10.

47. Watt MJ, Steinberg GR, Chen ZP, Kemp BE, Febbraio MA (2006) Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. The Journal of physiology 574: 139-147.

48. Dube JJ, Bhatt BA, Dedousis N, Bonen A, O'Doherty RM (2007) Leptin, skeletal muscle lipids, and lipid-induced insulin resistance. Am J Physiol Regul Integr Comp Physiol 293: R642-650.

49. Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, et al. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes 48: 1270-1274.

50. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, et al. (1996) Mechanism of free fatty acid-induced insulin resistance in humans. The Journal of clinical investigation 97: 2859-2865.

51. Boden G, Jadali F, White J, Liang Y, Mozzoli M, et al. (1991) Effects of fat on insulin-stimulated carbohydrate metabolism in normal men. The Journal of clinical investigation 88: 960-966.

52. Boden G, Chen X (1995) Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes. The Journal of clinical investigation 96: 1261-1268.

53. Zraika S, Dunlop M, Proietto J, Andrikopoulos S (2002) Effects of free fatty acids on insulin secretion in obesity. Obesity reviews : an official journal of the International Association for the Study of Obesity 3: 103-112.

54. Bays H, Mandarino L, DeFronzo RA (2004) Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. The Journal of clinical endocrinology and metabolism 89: 463-478.

55. Bergman RN, Ader M (2000) Free fatty acids and pathogenesis of type 2 diabetes mellitus. Trends in endocrinology and metabolism: TEM 11: 351-356.

56. Kershaw EE, Flier JS (2004) Adipose tissue as an endocrine organ. The Journal of clinical endocrinology and metabolism 89: 2548-2556.

57. Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, et al. (2005) JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. The Journal of biological chemistry 280: 35361-35371.

58. Van Epps-Fung M, Williford J, Wells A, Hardy RW (1997) Fatty acid-induced insulin resistance in adipocytes. Endocrinology 138: 4338-4345.

59. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J (1985) Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. The Journal of clinical investigation 76: 149-155.

60. Samuel VT, Petersen KF, Shulman GI (2010) Lipid-induced insulin resistance: unravelling the mechanism. Lancet 375: 2267-2277.

61. Olsen GS, Hansen BF (2002) AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle. American journal of physiology Endocrinology and metabolism 283: E965-970.

62. Shulman GI (2000) Cellular mechanisms of insulin resistance. The Journal of clinical investigation 106: 171-176.

63. Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM (2008) Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. Diabetologia 51: 336-346.

64. Robbins, Cotran (2005) Pathologic Basis of Disease; Kumar, Abbas, Fausto, editors: Elsevier Inc.

65. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, et al. (2001) Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 50: 1844-1850.

66. Boden G (2002) Interaction between free fatty acids and glucose metabolism. Current opinion in clinical nutrition and metabolic care 5: 545-549.

67. Jenssen T, Nurjhan N, Consoli A, Gerich JE (1990) Failure of substrate-induced gluconeogenesis to increase overall glucose appearance in normal humans. Demonstration of hepatic autoregulation without a change in plasma glucose concentration. The Journal of clinical investigation 86: 489-497.

68. Chen X, Iqbal N, Boden G (1999) The effects of free fatty acids on gluconeogenesis and glycogenolysis in normal subjects. The Journal of clinical investigation 103: 365-372.

69. Wahren J, Ekberg K (2007) Splanchnic regulation of glucose production. Annual review of nutrition 27: 329-345.

70. Radziuk J, Pye S (2002) Quantitation of basal endogenous glucose production in Type II diabetes: importance of the volume of distribution. Diabetologia 45: 1053-1084.

71. Mehal WZ, Azzaroli F, Crispe IN (2001) Immunology of the healthy liver: old questions and new insights. Gastroenterology 120: 250-260.

72. Kindt TJ, Goldsby RA, Osborne BA, Kuby J (2007) Kuby immunology. New York: W.H. Freeman. xxii, 574, A-531, G-512, AN-527, I-527 p. p.

73. Zhu J, Yamane H, Paul WE (2010) Differentiation of effector CD4 T cell populations (*). Annual review of immunology 28: 445-489.

74. Lebre MC, Burwell T, Vieira PL, Lora J, Coyle AJ, et al. (2005) Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells: a role for different mature dendritic cell populations in attracting appropriate effector cells to peripheral sites of inflammation. Immunology and cell biology 83: 525-535.

75. Gordon S (2003) Alternative activation of macrophages. Nature reviews Immunology 3: 23-35.

76. Littman DR, Rudensky AY (2010) Th17 and regulatory T cells in mediating and restraining inflammation. Cell 140: 845-858.

77. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, et al. (2009) Obesity predisposes to Th17 bias. European journal of immunology 39: 2629-2635.

78. Deiuliis J, Shah Z, Shah N, Needleman B, Mikami D, et al. (2011) Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers. PloS one 6: e16376.

79. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, et al. (2009) CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 15: 914-920.

80. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, et al. (2011) B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nature medicine 17: 610-617.

81. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, et al. (2005) Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes 54: 2277-2286.

82. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, et al. (2006) Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes 55: 1554-1561.

83. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, et al. (2006) CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest 116: 115-124.

84. Xu H, Barnes GT, Yang Q, Tan G, Yang D, et al. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112: 1821-1830.

85. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, et al. (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112: 1796-1808.

86. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, et al. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. The Journal of clinical investigation 116: 1494-1505.

87. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, et al. (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J Biol Chem 281: 26602-26614.

88. Hotamisligil GS (2000) Molecular mechanisms of insulin resistance and the role of the adipocyte. Int J Obes Relat Metab Disord 24 Suppl 4: S23-27.

89. Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, et al. (2008) Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. Cell Metab 7: 485-495.

90. Odegaard JI, Ricardo-Gonzalez RR, Red Eagle A, Vats D, Morel CR, et al. (2008) Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. Cell Metab 7: 496-507.

91. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, et al. (2010) Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 59: 347-357.

92. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392: 245-252.

93. Thomson AW, O'Connell PJ, Steptoe RJ, Lu L (2002) Immunobiology of liver dendritic cells. Immunol Cell Biol 80: 65-73.

94. Wu L, Liu YJ (2007) Development of dendritic-cell lineages. Immunity 26: 741-750.

95. Weatherill AR, Lee JY, Zhao L, Lemay DG, Youn HS, et al. (2005) Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. J Immunol 174: 5390-5397.

96. Macia L, Delacre M, Abboud G, Ouk TS, Delanoye A, et al. (2006) Impairment of dendritic cell functionality and steady-state number in obese mice. Journal of immunology 177: 5997-6006.

97. Brake DK, Smith EO, Mersmann H, Smith CW, Robker RL (2006) ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice. Am J Physiol Cell Physiol 291: C1232-1239.

98. Lumeng CN, Bodzin JL, Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 117: 175-184.

99. Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, et al. (2008) Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. Cell Metab 8: 301-309.

100. Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, et al. (2007) A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. The Journal of biological chemistry 282: 35279-35292.

101. Feili-Hariri M, Dong X, Alber SM, Watkins SC, Salter RD, et al. (1999) Immunotherapy of NOD mice with bone marrow-derived dendritic cells. Diabetes 48: 2300-2308.

102. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. Annu Rev Immunol 25: 297-336.

103. Van Kaer L (2007) NKT cells: T lymphocytes with innate effector functions. Curr Opin Immunol 19: 354-364.

104. Godfrey DI, Kronenberg M (2004) Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest 114: 1379-1388.

105. Li Z, Soloski MJ, Diehl AM (2005) Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. Hepatology 42: 880-885.

106. Ohmura K, Ishimori N, Ohmura Y, Tokuhara S, Nozawa A, et al. (2010) Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. Arterioscler Thromb Vasc Biol 30: 193-199.

107. Mantell BS, Stefanovic-Racic M, Yang X, Dedousis N, Sipula IJ, et al. (2011) Mice Lacking NKT Cells but with a Complete Complement of CD8 T-Cells Are Not Protected against the Metabolic Abnormalities of Diet-Induced Obesity. PloS one 6: e19831.

108. Guebre-Xabier M, Yang S, Lin HZ, Schwenk R, Krzych U, et al. (2000) Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. Hepatology 31: 633-640.

109. Yang L, Jhaveri R, Huang J, Qi Y, Diehl AM (2007) Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. Lab Invest 87: 927-937.

110. Elinav E, Pappo O, Sklair-Levy M, Margalit M, Shibolet O, et al. (2006) Adoptive transfer of regulatory NKT lymphocytes ameliorates non-alcoholic steatohepatitis and glucose intolerance in ob/ob mice and is associated with intrahepatic CD8 trapping. J Pathol 209: 121-128.

111. Mohamed-Ali V, Flower L, Sethi J, Hotamisligil G, Gray R, et al. (2001) beta-Adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies. The Journal of clinical endocrinology and metabolism 86: 5864-5869.

112. Ricciotti E, FitzGerald GA (2011) Prostaglandins and inflammation. Arteriosclerosis, thrombosis, and vascular biology 31: 986-1000.

113. Scher JU, Pillinger MH (2009) The anti-inflammatory effects of prostaglandins. Journal of investigative medicine : the official publication of the American Federation for Clinical Research 57: 703-708.

114. Sugimoto Y, Narumiya S (2007) Prostaglandin E receptors. The Journal of biological chemistry 282: 11613-11617.

115. Tilley SL, Coffman TM, Koller BH (2001) Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. The Journal of clinical investigation 108: 15-23.

116. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD (2001) Prostanoid receptors: subtypes and signaling. Annual review of pharmacology and toxicology 41: 661-690.

117. Mine T, Kojima I, Ogata E (1990) Mechanism of prostaglandin E2-induced glucose production in rat hepatocytes. Endocrinology 126: 2831-2836.

118. Busby ER, Cooper GA, Mommsen TP (2002) Novel role for prostaglandin E2 in fish hepatocytes: regulation of glucose metabolism. The Journal of endocrinology 174: 137-146.

119. Puschel GP, Kirchner C, Schroder A, Jungermann K (1993) Glycogenolytic and antiglycogenolytic prostaglandin E2 actions in rat hepatocytes are mediated via different signalling pathways. European journal of biochemistry / FEBS 218: 1083-1089.

120. Brass EP, Garrity MJ (1985) Effect of E-series prostaglandins on cyclic AMP-dependent and -independent hormone-stimulated glycogenolysis in hepatocytes. Diabetes 34: 291-294.

121. Brass EP, Garrity MJ, Robertson RP (1984) Inhibition of glucagon-stimulated hepatic glycogenolysis by E-series prostaglandins. FEBS letters 169: 293-296.

122. Okumura T, Sago T, Saito K (1988) Effect of prostaglandins and their analogues on hormone-stimulated glycogenolysis in primary cultures of rat hepatocytes. Biochimica et biophysica acta 958: 179-187.

123. Okumura T, Sago T, Saito K (1988) Pertussis toxin blocks an inhibition of hormone-stimulated glycogenolysis by prostaglandin E2 and its analogue in cultured hepatocytes. Prostaglandins 36: 463-475.

124. Okumura T, Kanemaki T, Kitade H (1993) Stimulation of glucose incorporation into glycogen by E-series prostaglandins in cultured rat hepatocytes. Biochimica et biophysica acta 1176: 137-142.

125. Bjornsson OG, Sparks JD, Sparks CE, Gibbons GF (1992) Prostaglandins suppress VLDL secretion in primary rat hepatocyte cultures: relationships to hepatic calcium metabolism. Journal of lipid research 33: 1017-1027.

126. Brass EP, Alford CE, Garrity MJ (1987) Inhibition of glucagon-stimulated cAMP accumulation and fatty acid oxidation by E-series prostaglandins in isolated rat hepatocytes. Biochimica et biophysica acta 930: 122-126.

127. Neyrinck AM, Margagliotti S, Gomez C, Delzenne NM (2004) Kupffer cell-derived prostaglandin E2 is involved in regulation of lipid synthesis in rat liver tissue. Cell biochemistry and function 22: 327-332.

128. Stadler J, Curran RD, Ochoa JB, Harbrecht BG, Hoffman RA, et al. (1991) Effect of endogenous nitric oxide on mitochondrial respiration of rat hepatocytes in vitro and in vivo. Archives of surgery 126: 186-191.

129. Huang W, Dedousis N, Bandi A, Lopaschuk GD, O'Doherty RM (2006) Liver triglyceride secretion and lipid oxidative metabolism are rapidly altered by leptin in vivo. Endocrinology 147: 1480-1487.

130. Stefanovic-Racic M, Perdomo G, Mantell BS, Sipula IJ, Brown NF, et al. (2008) A moderate increase in carnitine palmitoyltransferase 1a activity is sufficient to substantially reduce hepatic triglyceride levels. Am J Physiol Endocrinol Metab 294: E969-977.

131. Sherry B, Cerami A (1988) Cachectin/tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses. The Journal of cell biology 107: 1269-1277.

132. Shoelson SE, Herrero L, Naaz A (2007) Obesity, inflammation, and insulin resistance. Gastroenterology 132: 2169-2180.

133. Hespeling U, Jungermann K, Puschel GP (1995) Feedback-inhibition of glucagon-stimulated glycogenolysis in hepatocyte/Kupffer cell cocultures by glucagon-elicited prostaglandin production in Kupffer cells. Hepatology 22: 1577-1583.

134. Athari A, Jungermann K (1989) Direct activation by prostaglandin F2 alpha but not thromboxane A2 of glycogenolysis via an increase in inositol 1,4,5-trisphosphate in rat hepatocytes. Biochemical and biophysical research communications 163: 1235-1242.

135. Hespeling U, Puschel GP, Jungermann K, Gotze O, Zwirner J (1995) Stimulation of glycogen phosphorylase in rat hepatocytes via prostanoid release from Kupffer cells by recombinant rat anaphylatoxin C5a but not by native human C5a in hepatocyte/Kupffer cell co-cultures. FEBS letters 372: 108-112.

136. Winer S, Chan Y, Paltser G, Truong D, Tsui H, et al. (2009) Normalization of obesity-associated insulin resistance through immunotherapy. Nature medicine 15: 921-929.

137. Chu J, Thomas LM, Watkins SC, Franchi L, Nunez G, et al. (2009) Cholesterol-dependent cytolysins induce rapid release of mature IL-1beta from murine macrophages in a NLRP3 inflammasome and cathepsin B-dependent manner. Journal of leukocyte biology 86: 1227-1238.

138. Boden G (2011) Obesity, insulin resistance and free fatty acids. Current opinion in endocrinology, diabetes, and obesity 18: 139-143.

139. Fields RC, Shimizu K, Mule JJ (1998) Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 95: 9482-9487.

140. Thomson AW, Lu L, Murase N, Demetris AJ, Rao AS, et al. (1995) Microchimerism, dendritic cell progenitors and transplantation tolerance. Stem cells 13: 622-639.

141. Baker RG, Hayden MS, Ghosh S (2011) NF-kappaB, inflammation, and metabolic disease. Cell metabolism 13: 11-22.

142. Kunjathoor VV, Wilson DL, LeBoeuf RC (1996) Increased atherosclerosis in streptozotocin-induced diabetic mice. The Journal of clinical investigation 97: 1767-1773.

143. Uematsu S, Matsumoto M, Takeda K, Akira S (2002) Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. Journal of immunology 168: 5811-5816.

144. Olefsky JM, Glass CK (2010) Macrophages, inflammation, and insulin resistance. Annu Rev Physiol 72: 219-246.

145. Miyazaki Y, Iwabuchi K, Iwata D, Miyazaki A, Kon Y, et al. (2008) Effect of high fat diet on NKT cell function and NKT cell-mediated regulation of Th1 responses. Scand J Immunol 67: 230-237.

146. Margalit M, Shalev Z, Pappo O, Sklair-Levy M, Alper R, et al. (2006) Glucocerebroside ameliorates the metabolic syndrome in OB/OB mice. J Pharmacol Exp Ther 319: 105-110.

147. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR (1997) Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity 6: 459-467.

148. Smiley ST, Kaplan MH, Grusby MJ (1997) Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science 275: 977-979.

149. Ma X, Hua J, Li Z (2008) Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 49: 821-830.

150. Butler AA, Kozak LP (2010) A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. Diabetes 59: 323-329.

151. Shiri-Sverdlov R, Wouters K, van Gorp PJ, Gijbels MJ, Noel B, et al. (2006) Early diet-induced non-alcoholic steatohepatitis in APOE2 knock-in mice and its prevention by fibrates. Journal of hepatology 44: 732-741.

152. Lee YS, Li P, Huh JY, Hwang IJ, Lu M, et al. (2011) Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance. Diabetes 60: 2474-2483.

153. Vassiliou E, Jing H, Ganea D (2003) Prostaglandin E2 inhibits TNF production in murine bone marrow-derived dendritic cells. Cellular immunology 223: 120-132.

154. Baumjohann D, Lutz MB (2006) Non-invasive imaging of dendritic cell migration in vivo. Immunobiology 211: 587-597.

155. Shinomiya M, Fazle Akbar SM, Shinomiya H, Onji M (1999) Transfer of dendritic cells (DC) ex vivo stimulated with interferon-gamma (IFN-gamma) down-modulates autoimmune diabetes in non-obese diabetic (NOD) mice. Clinical and experimental immunology 117: 38-43.

156. Eggert AA, Schreurs MW, Boerman OC, Oyen WJ, de Boer AJ, et al. (1999) Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. Cancer research 59: 3340-3345.

157. Creusot RJ, Yaghoubi SS, Chang P, Chia J, Contag CH, et al. (2009) Lymphoid-tissue-specific homing of bone-marrow-derived dendritic cells. Blood 113: 6638-6647.

158. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. The Journal of experimental medicine 176: 1693-1702.

159. Lu L, Rudert WA, Qian S, McCaslin D, Fu F, et al. (1995) Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. The Journal of experimental medicine 182: 379-387.

160. Davies JQ, Gordon S (2005) Isolation and culture of murine macrophages. Methods in molecular biology 290: 91-103.