Role of Inflammation in Diet-Induced Obesity: A Dissertation

Sophia Kogan
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ROLE OF INFLAMMATION IN DIET-INDUCED OBESITY

A Dissertation Presented

By

SOPHIA KOGAN

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

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(MARCH 26, 2013)

MD/PHD PROGRAM
ROLE OF INFLAMMATION IN DIET-INDUCED OBESITY
A Dissertation Presented
By
Sophia Kogan

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Abstract

Obesity results from expansion of white adipose tissue. The inability of white adipose tissue to adequately store lipids leads to ectopic deposition of lipids in non-adipose tissue that can lead to systemic insulin resistance. It is well known that insulin resistance correlates with inflammation of adipose tissue in obese animals and humans. Decreasing inflammation in the adipose tissue has been proven as a therapeutic strategy for improvement of insulin sensitivity in vivo. Numerous factors secreted by immune cells, including macrophages, have been suggested as regulating adipose tissue insulin sensitivity.

In the first part of my thesis, I describe the role of one such factor, CD40 in adipose tissue inflammation. The CD40-CD40L dyad acts as co-stimulation in the interaction of antigen-presenting cells, such as macrophages and dendritic cells, with effector cells, such as T cells, in adaptive immunity. We found that CD40 knockout mice were smaller but surprisingly more insulin resistant and glucose intolerant compared to wild-type mice when fed a high fat diet. Consistent with their metabolic phenotype, knockout mice displayed increased adipose tissue inflammation with infiltration of immune cells including macrophages and T cells. Consistent with increased inflammation, CD40 knockout adipose tissue displayed decreased lipid storage. Deficiency of CD40 also led to increased lipid deposition in liver, which may be due to increased lipid release into circulation from the adipose tissue as well as increased lipid synthesis in the liver. CD40 knockout mice had increased hepatic insulin resistance and increased gluconeogenesis.
despite decreased hepatic inflammation. These findings suggest that CD40 is a novel regulator of adipose tissue inflammation in diet-induced obesity.

In the second part of this thesis we examined perivascular adipose tissue and brown adipose tissue for the presence of inflammation. In contrast to visceral adipose tissue, macrophage infiltration was absent in perivascular and brown adipose tissue as defined by reduced F480+ cells by flow cytometry and immunohistochemistry. We also found that perivascular adipose tissue was similar to brown adipose tissue as shown by gross morphology and gene expression pattern. Inflammatory gene expression was not increased in brown or perivascular adipose tissue in obese mice as determined by microarray gene expression analysis. These findings suggest that perivascular adipose tissue is more similar to brown adipose tissue than white adipose tissue and that both perivascular and brown adipose tissue are resistant to inflammation.

We conclude that, (1) CD40 protects against adipose tissue inflammation in diet-induced obesity, (2) the CD40 knockout mouse is an interesting model of hepatic steatosis with decreased inflammation and (3) perivascular adipose tissue is almost identical to brown adipose tissue in obese mice and that both are resistant to inflammation.
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<td>Adipose tissue macrophage</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>cidea</td>
<td>Cell death-inducing DFFA-like effector a</td>
</tr>
<tr>
<td>CLS</td>
<td>Crown-like Structure</td>
</tr>
<tr>
<td>CCR2/CCL2</td>
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<td>CVD</td>
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<td>Diet-induced obesity</td>
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<td>Fat-specific protein 27</td>
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<td>Hepatocellular carcinoma</td>
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<td>High fat diet</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Insulin resistance/resistant</td>
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<td>Insulin sensitivity/sensitive</td>
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<td>Knockout</td>
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<td>Lipoprotein lipase</td>
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<td>MACE</td>
<td>Microarray computational environment</td>
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<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<td>Mcp-1/Ccl2</td>
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<td>NAFLD</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
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<td>PPARγ</td>
<td>Peroxisome proliferator activated receptor gamma</td>
</tr>
<tr>
<td>PPARδ</td>
<td>Peroxisome proliferator activated receptor delta</td>
</tr>
<tr>
<td>PRDM16</td>
<td>PR domain containing 16</td>
</tr>
<tr>
<td>PTT</td>
<td>Pyruvate Tolerance Test</td>
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<tr>
<td>PVAT</td>
<td>Perivascular adipose tissue</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust multi-array average</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element binding protein 1c</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>UCP-1</td>
<td>Uncoupling protein 1</td>
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<tr>
<td>WAT</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>VAT</td>
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</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
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</table>
Preface

The work presented in this thesis was done in collaboration with the following individuals:

CHAPTER II
Chang-An Guo

CHAPTER III
Timothy Fitzgibbons performed experiments for Figures 3.1, 3.2 and 3.3. Figure 3.4 was done by Greg Hendricks; Figures 3.6 and 3.7 were done by the Morphology Core and the microarray hybridizations by the Genomics Core of the Diabetes and Endocrine Research Center (DK-32520). Jeurg Straubhaar uploaded the microarray data to MACE and performed detailed statistical analyses.
Copyright Information

Chapter II of this dissertation has appeared in:


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*These authors contributed equally to this work
CHAPTER I: INTRODUCTION

Obesity and insulin resistance

There are more than one billion overweight adults worldwide at least 300 million of whom are obese and these numbers are rapidly growing in the developed world [1]. Advances in technology and food production have led to a society with a plethora of high calorie food and a decreased need for physical labor, both of which fuel the obesity epidemic. Obesity is a complex disorder caused by an imbalance between energy consumption and expenditure and it increases the risk of developing complications such as type 2 diabetes, hypertension, atherosclerosis, coronary artery disease and cancer [2]. The global incidence of type 2 diabetes is projected to reach 350 million cases by the year 2030, with expenditure attributed to diabetes estimated to hit 132 billion dollars in the US [3]. Public health efforts have largely been geared at preventing obesity on an individual, community and global level [4] while basic science has focused on reaching a better understanding of the mechanisms involved in the progression from simple obesity to metabolic dysfunction.

Metabolic Syndrome describes obesity in the context of comorbidities that increase the risk of type 2 diabetes and cardiovascular disease. The clinical criteria include at least 3 of the following: elevated waist circumference, elevated serum triglycerides (TGs), reduced serum high density lipoprotein (HDL), elevated blood pressure and elevated fasting glucose. Numerous studies have shown that as adipose tissue expands with increasing obesity, it becomes
dysfunctional, but lipodystrophic mice and humans who lack adipose tissue also suffer from metabolic complications [5], highlighting the importance of properly functioning adipose tissue. Given the scope and magnitude of the obesity epidemic, it is particularly important to study the role of adipose tissue in physiological and pathological conditions.

Insulin is a hormone produced by the pancreatic β cells and is central to regulating carbohydrate and lipid metabolism to keep blood glucose within a normal range. The liver, muscle and adipose tissue normally respond to insulin by taking up glucose and storing it as glycogen in the muscle and liver or TGs in adipocytes. Insulin also inhibits glucose production by the liver. To maintain metabolic health, adipocytes must balance the storage and release of lipids in response to the energy state. In a fasted state, adipocytes hydrolyze triglycerides into free fatty acids (FFA) through a process called lipolysis. In a fed state, adipocytes reesterify fatty acids for storage as TG [6].

A healthy organism maintains serum glucose levels through a balance between the influx into the circulation from that stored in tissues and from food and the output from the circulation by uptake and utilization by tissues. Diabetes is a disorder of glucose homeostasis and is defined as fasting blood glucose >125 mg/dL and it is commonly associated with obesity. Type I diabetes is an absence of insulin due to primary failure of insulin-producing β cells, while type II diabetes is a lack of sufficient insulin to maintain normoglycemia in the face of insulin resistance in the peripheral tissues such as muscle, liver and adipose
tissue [7]. With increasing obesity, the muscle, liver and adipose tissues’ capacity to respond to insulin is diminished, and the β cells of the pancreas produce more insulin to maintain appropriate glucose levels in the blood. Such individuals are hyperinsulinemic but metabolically healthy because their blood glucose levels are still normal. A second event, failure of the β cells, is required for progression to insulin resistance and type 2 diabetes [8].

The actions of insulin on adipose, muscle and liver, the main insulin-target tissues, are mediated through the insulin receptor (IR). The IR is a transmembrane tyrosine kinase that undergoes a conformational change upon stimulation, and autophosphorylation at multiple tyrosine residues [9-11]. The phosphorylated tyrosines recruit scaffolding proteins, called insulin receptor substrate (IRS) [12] proteins that further recruit downstream effector molecules which ultimately lead to phosphorylation and activation of Akt/Protein Kinase B on serine 473 by PI3K [13]. Akt/PKB plays an important role in the regulation of glucose and fat metabolism [14] and phosphorylates many substrates including Bcl-2-associated death promoter (BAD), which is important for cell survival, Glycogen synthase kinase 3 β (GSK3β), which regulates glycogen synthesis, and Forkhead box O-class (FOXO1), which controls gene expression and cell survival [12]. In its unphosphorylated state, FOXO1 is localized in the nucleus where it activates transcription of target genes. When FOXO1 is phosphorylated, it is excluded from the nucleus, which inhibits gluconeogenesis and adipocyte differentiation [15, 16]. Insulin can also activate extracellular-signal regulated
kinases (ERKs), which are involved in the regulation of mitogenesis. Insulin promotes lipid storage in adipocytes and inhibits lipolysis by promoting fatty acid reesterification, and TG synthesis, as well as inhibiting fatty acid transport [14].
White adipose tissue

White adipose tissue (WAT) is specialized for the storage of energy as TGs and is capable of synthesizing TGs *de novo* from glucose and importing fatty acids from the blood [3]. Fatty acids are toxic to cells and in early stages of overfeeding, efficient storage of TGs in adipose tissue protects other tissues from inappropriate lipid deposition and is key to maintaining insulin sensitivity [3]. As energy consumption continues to exceed use, adipose tissue grows by hypertrophy and hyperplasia [17]. An increase in the expression of enzymes involved in TG synthesis is observed early in obesity, consistent with an increased ability to store lipid [18], but eventually, the adipocytes' ability to store lipids is perturbed and lipids are deposited in other tissues such as the liver and muscle, which contributes to systemic insulin resistance. WAT is also the largest endocrine organ, required for normal secretion of hormones called adipokines [3].

In humans, WAT is located throughout the body with major intra-abdominal depots around the omentum, intestines and perirenal areas as well as in subcutaneous areas such as the thighs, buttocks and abdomen [19]. There are minor white adipose tissue depots in other areas such as the retro-orbital space, extremities and in the bone marrow [19]. White adipose tissue contains adipocytes and the stromal vascular fraction (SVF), which contains immune cells, endothelial cells, fibroblasts, preadipocytes and other non-adipocyte cells. Adipocyte function differs with location. In humans, distribution of adipose tissue
affects one’s metabolic state. Central or “apple-shaped” obesity with excess visceral fat is associated with increased risk of diabetes and metabolic syndrome whereas pear-shaped obesity with excess subcutaneous adipose tissue (SAT) is not [2]. Subcutaneous adipocytes release more leptin than visceral deposits [20] and several studies have shown that gene expression is significantly different in these WAT depots in mice [21] and humans [22] when compared to SAT. These depots are also anatomically distinct as visceral fat is drained by the portal system so secreted factors (adipokines, FFAs) go directly to the liver and could affect metabolism.

In addition to the subcutaneous and visceral depots described above, WAT is also present surrounding the coronary arteries, aorta, microvasculature of the brain, skeletal muscle and kidney [23]. Like most WAT depots, this perivascular adipose tissue (PVAT) becomes inflamed in obese humans [24]. However, PVAT also has characteristics of brown adipose tissue (BAT), [25, 26] described in the next section.
Brown adipose tissue

Brown adipose tissue (BAT) is a thermogenic tissue that burns energy to produce heat. In humans, BAT is found in the cervical, suprascapular and paravertebral space [27-29]. Brown adipocytes contain mitochondria that express uncoupling protein-1 (UCP-1), a 32 kDa protein which uncouples proton pumping and ATP production in oxidative phosphorylation. The proton leak produces heat in a process called nonshivering thermogenesis [30, 31]. The dissipation of the electron gradient, along with high rates of mitochondrial electron transport and fuel oxidation, result in the generation of heat and expenditure of large amounts of energy [30, 31]. Brown adipose mitochondria from UCP-1 KO mice are fully coupled, have decreased ATP synthesis, and do not recruit other UCP homologues for nonshivering thermogenesis and KO mice are cold-intolerant and lean [32]. Figure 1.1 illustrates the function of UCP1.
Figure 1.1: Molecular basis of UCP1 uncoupling in BAT. UCP1 is expressed at high levels in the inner membrane of BAT mitochondria. Normally, a proton gradient is established by the electron transport chain with a higher proton concentration in the intermembrane space. Passage of a proton down its concentration gradient is coupled to the production of ATP by ATP synthase. UCP1 allows the transfer of protons down their concentration gradient without producing ATP, thereby uncoupling oxidative phosphorylation with ATP production.
UCP-1-positive brown adipocytes have also been identified interspersed in skeletal muscle of mice, suggesting that ectopic brown adipocytes may also play a role in energy balance [33]. Histologically, brown adipocytes have a multilocular appearance with multiple lipid droplets, which contrasts with white adipocytes, which are unilocular in appearance with one lipid droplet that occupies the cytoplasm of the cell (Figure 1.2) [28]. BAT is most obvious in human infants and rodents because small animals have a larger surface area to volume ratio and a higher risk of hypothermia. Brown fat tissue provides heat to the bloodstream in response to sympathetic nerve stimuli and is, consistent with this function, comprised mainly of brown adipocytes, abundant vessels and nerves [25].
Figure 1.2: Histochemical morphology of BAT versus WAT
Top panel: Brown adipose tissue (L) and white adipose tissue (R) specimens that have been stained with hematoxylin and eosin. Brown adipocytes have multiple lipid droplets while white adipocytes are spherical with one lipid droplet. Both images are 25x.
Sophia Kogan, unpublished.
BAT was linked to obesity when it was noted that genetically obese ob/ob mice had less BAT and suppressed UCP-1 expression, making them cold-sensitive and decreasing energy expenditure [34]. Activity of human BAT tissue is stimulated by mild cold exposure [28] and is inversely correlated with obesity [27, 29]. The ablation of BAT or UCP-1 causes mice to be obese and insulin resistant [35-37] and deletion of the TNF-\(\alpha\) receptor 1 (TNFR1) protects against obesity by increasing thermogenesis [38], suggesting that inflammation could play a role in the decline of BAT function in obesity. It has been observed that brown adipose depots "whiten" with increasing obesity and brown adipocytes have been shown to take on the gross morphology of white adipocytes when they atrophy [39].

To generate heat for thermogenesis, BAT first uses stored lipids as substrate. This early phase of thermogenesis corresponds to norepinephrine released from sympathetic nerves activating the release of FFAs from lipid droplets. The stored lipids in BAT can only sustain thermogenic activity for a short time and the remaining fatty acids are imported into the cell and combusted [25]. Bartelt et al., performed a study to test whether cold exposure can improve fat tolerance by performing a fat tolerance test in normal or cold mice. In normal mice, TG levels rose following an oil bolus, but in cold mice, TG levels remained low due to massively increased clearance by the BAT [40]. It has been estimated that as little as 50 g of BAT could account for 20% of an adults' daily expenditure if maximally stimulated [25]. Interestingly, transplantation of BAT reverses type 1
diabetes in mice, and euglycemia is still maintained 6 months after transplant [41].

These observations, taken together, suggest that stimulation of BAT could be a tool in the treatment of metabolic disease.
Role of the liver

Glucose metabolism

The liver is a main site of sugar storage, in the form of a polymer called glycogen. During fasting or strenuous exercise, glycogen is broken down by glycogen phosphorylase and glucose is released into circulation via the insulin-independent glucose transporter, GLUT2 [42]. Following a large meal, glucose reaches the liver via the portal vein and is shuttled into the cell by GLUT2. Glucose is then phosphorylated by glucokinase to glucose-6-phosphate (G6P), a central intermediate in hepatic glucose metabolism. G6P can be degraded during glycolysis to make ATP and NADH and the product, pyruvate, can be further broken down in the tricarboxylic acid cycle [42]. Alternatively, G6P can be diverted to the pentose phosphate shunt, which provides NADPH for de novo lipogenesis [42]. Therefore, in physiological conditions, the liver responds to dietary and hormonal cues to regulate both glucose and fat metabolism.

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a result of the accumulation of TGs in the liver and is usually associated with obesity. One population study found the prevalence of steatosis to be 34%, more than 90% of which was attributed to NAFLD [43]. The importance of metabolic parameters in NAFLD risk has been shown in population studies in which the prevalence of NAFLD
increased from 16.4% in normal weight individuals to 75.8% among the obese [44] and with increasing hyperglycemia [45].

NAFLD is the major cause of abnormal liver function in the western world and a risk factor for non-alcoholic steatohepatitis (NASH), fibrosis and hepatocellular carcinoma (HCC) [43]. Simple steatosis refers to steatosis in the absence of histological evidence of hepatocellular damage, whereas NASH is steatosis in the context of inflammation and hepatocyte damage. The prevalence of NAFLD patients who develop NASH is only 2% but one third of those patients progress to fibrosis within 3 years and half progress within 6, a relatively large number of people given the high prevalence of NAFLD [43]. There is significant controversy regarding what factors are involved in NAFLD progression but evidence suggests that inflammation, oxidative stress and mitochondrial dysfunction may all contribute [46-48]. Though reductions in TG synthesis improve hepatic steatosis, there is evidence that they may worsen liver damage, indicating that lipid accumulation may be protective in early stages of obesity [49-51].

Once in the liver, fatty acids are esterified into TGs, which are stored in lipid droplets (LDs) or secreted in very low density lipoproteins (VLDL) into the circulation. There are four factors that contribute to NAFLD in the context of obesity and IR (summarized in Figure 1.3): (1) increased FFA delivery due to increased lipolysis from obese WAT as well as dietary FFA, (2) decreased FFA oxidation, (3) increased de novo lipogenesis and (4) decreased export via VLDL.
FFA delivery to the liver accounts for nearly two thirds of this accumulation [52] but lipid export from the liver might also be impaired in people with NAFLD because of defective incorporation of TG into apolipoprotein carrier proteins [53].

Treatments of NAFLD are mostly aimed at modifying existing risk factors. Weight loss by diet and exercise or surgery has been shown to improve liver histology although rapid weight loss can exacerbate liver inflammation and fibrosis [54, 55]. Furthermore, agents that improve insulin sensitivity, such as metformin, pioglitazone and rosiglitazone, have been shown to improve features of NAFLD in observational studies [56-58].
Figure 1.3: Mechanisms contributing to lipid accumulation in the liver.
At least 4 factors contribute to fat accumulation in the liver in the context of obesity. (1) increased FFA delivery as the result of overnutrition and increased FFA liberation from WAT due to increased lipolysis. (2) Decreased FFA oxidation by the liver. (3) Increased hepatic de novo lipogenesis and (4) decreased export via VLDL (Adapted from Tilg 2008 [59])
Lipogenesis

Lipogenesis is a metabolic pathway occurring mainly in the liver that converts excess glucose into fatty acids, which can be esterified into TGs. A diet rich in carbohydrates, along with high insulin levels, stimulates lipogenesis whereas fasting inhibits it. The fractional contribution of lipogenesis to steatosis is 2-5% in lean humans but can reach 26% in the context of obesity [52, 60]. Lipogenesis is insulin and glucose-dependent and under the control of specific transcription factors. Sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) are transcription factors that regulate lipogenesis and are activated by insulin and carbohydrates, respectively. Insulin activates SREBP-1c by a mechanism initiated in the endoplasmic reticulum [61] and SREBP cleavage is also induced by endoplasmic reticulum (ER) stress [62].

Hepatic insulin resistance

The livers of obese or lipodystrophic animals are characterized by a phenomenon known as selective insulin resistance (summarized in Figure 1.4, below). This describes livers that are severely insulin resistant leading to glucose overproduction, which exacerbates hyperglycemia. Interestingly, insulin-dependent lipogenesis is highly active. Hepatic levels of SREBP-1c are inappropriately high in the livers of obese animals [63]. The inability of insulin to suppress gluconeogenesis while lipogenesis is activated could reflect a
differential sensitivity to insulin of the signaling pathways that control these processes [63]. When insulin binds to its receptor, it activates Insulin receptor substrates 1 and 2 (IRS-1 and -2). IRS-1 and IRS-2 both activate PKB/Akt and it is hypothesized that lipogenesis activation is controlled by sterol-regulatory element-binding protein-1c (SREBP-1c) via mTORC1, while inhibition of gluconeogenesis is via phosphorylation and inactivation of Forkhead Box-O1 (FOXO1), which normally activates gluconeogenesis by downregulating glucose-6-phosphatase (G6Pase), a key gluconeogenic enzyme. It has been shown that insulin-stimulated expression of SREBP-1c is downregulated by rapamycin, an inhibitor of mTORC1, but this treatment has no effect on the insulin-mediated suppression of gluconeogenic genes [64].
Figure 1.4: Selective insulin resistance in the liver.
In an insulin sensitive liver (A), insulin signals through IRS-1, Akt and mTORC1 to stimulate SREBP-1c and, therefore, lipogenesis. At the same time, insulin signals through IRS-2, Akt and FOXO1, inhibiting gluconeogenesis by suppressing transcription of gluconeogenic genes such as G6Pase. In an insulin resistant liver (B), hyperinsulinemia decreases the expression of IRS-2, precluding the inhibition of gluconeogenesis. IRS-1 is not affected, allowing for lipogenesis.
Adapted from Ferre, 2010 [63].
Role of PPARγ in adipose and liver

The differentiation of adipocytes from precursor cells with adipogenic potential is a complex process called adipogenesis. Peroxisome Proliferator-activated receptor-γ (PPARγ) is an essential transcriptional regulator of adipogenesis and is required for the development, survival, and maintenance of adipose tissue [65-67]. PPARγ drives the expression of many key adipocyte genes such as lipoprotein lipase, perilipin and CIDEA, which ultimately direct adipocytes towards FFA storage in lipid droplets. Disruption of PPARγ decreases TG storage in adipocytes, increasing lipid distribution to skeletal muscle and liver, which contributes to systemic insulin resistance [3]. Thiazolidinediones (TZDs) are PPARγ agonists that increase insulin sensitivity in type II diabetes mellitus but their clinical use is limited because they increase the risk of cardiovascular mortality [68]. PPARγ plays a central role in the differentiation of white and brown fat. Mice with the dominant-negative P465L mutation in PPARγ had deficits in brown but not white adipose tissue [69], suggesting that brown fat may be more dependent on PPARγ than white, or that PPARγ regulation of these two tissues occurs via different mediators.

In addition to its role in adipogenesis, PPARγ also regulates TG homeostasis in the liver, contributing to steatosis. Ablation of liver PPARγ reduces TG content but exacerbates hyperlipidemia and muscle IR [70] and deficiency of PPARγ in ob/ob obese mice worsens insulin resistance [71], partly through its action on Fat-specific protein 27 [72]. Interestingly, despite its role in
increasing steatosis, correlative observations suggest that PPARγ may protect against fibrosis [73] and may have a beneficial role for TZDs, PPARγ agonists, in patients with NAFLD [74, 75].
Adipose tissue as an endocrine organ: role of adipokines

Adipose tissue is also a source of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein 1 (MCP-1). These molecules increase immune cell trafficking into WAT and interfere with adipocyte function. As adipocytes hypertrophy with increasing obesity, MCP-1 is secreted by adipose tissue [76]. MCP-1 functions to increase macrophage infiltration into adipose tissue and likely contributes to the pro-inflammatory state of obese adipose tissue. However, the MCP-1 knockout mouse is still insulin resistant in diet-induced obesity, and when MCP-1 knockout monocytes are injected into obese mice, trafficking is reduced by only 40%, suggesting that other factors are also involved [77, 78]. These factors may be any of the wide variety of inflammatory molecules found in obese WAT including tumor necrosis factor-α, interleukin 6 and transforming growth factor-β [79-81]. Adipose tissue also secretes a number of adipokines such as leptin and adiponectin, which play an important role in energy balance but will not be discussed here.
Inflammation and metabolic disease

The observation that obese humans and mice have increased TNF-α provided the first link between inflammation and obesity [81, 82]. Since then, many studies have shown a correlation between systemic inflammation and insulin resistance in humans [83, 84]. Deletion of TNF-α or its receptor, TNFR, protected against diet-induced obesity and insulin resistance in mice [85, 86] and treatment with infliximab, a TNF-α antibody improved insulin sensitivity in humans, who relapsed after cessation of treatment [87, 88].

Numerous studies have shown an increase in macrophages in obese insulin resistant mice [89-91] as well as lipodystrophic mice [92]. Lean adipose tissue is 5-10% macrophages while in obese subjects macrophage content can be as high at 50% and macrophages are likely both a cause and consequence of local and chronic inflammation present in obesity [93-96]. Due to increased cytokines and fatty acids released from obese and/or dying adipocytes [79-81] macrophages infiltrate obese adipose tissue and phagocytose dying adipocytes and fatty acids forming crown-like structures (CLS) [97]. Macrophages are the most abundant immune cell present in obese adipose tissue and are a major source of proinflammatory molecules secreted [98]. ATMs of mice fed a high fat diet have a pro-inflammatory M1 phenotype while lean ATMs have an M2 anti-inflammatory bias [90, 99] partly due to changes in recruitment [100] and this bias reverses when obese mice are switched back to a normal diet [101]. Interestingly, this phenomenon has also been observed in human adipose tissue
before and after gastric bypass surgery [103, 104]. However, physiologically, macrophages exist in a spectrum of activated states which are influenced by factors including PPARγ [99, 105], CCR2[106], TNFα [107] and others. Obese adipocytes and adipose tissue macrophages secrete MCP-1, TNF-α and other cytokines such as IL-1β [79-81]. These cytokines increase lipolysis, which further increases macrophage recruitment [80] and decrease TG synthesis, which leads to increased serum FFAs, which are taken up by the muscle and liver, impairing their insulin sensitivity. Though most studies suggest a detrimental role for macrophages in obese adipose tissue, a few studies suggest that ATMs can behave like foam cells [90] and help adipocytes more effectively sequester lipids and may attenuate lipolysis [97].

In addition to macrophages, T cells are also involved in altering adipose tissue function in the context of obesity by increasing macrophage infiltration and activation [108, 109]. T cells can be broadly divided into two categories: pro and anti-inflammatory. The physiological state of immune cell responses in vivo is much more nuanced, but CD4+ T regulatory cells and CD4+ Th2 T cells are typically anti inflammatory and CD8+ cytotoxic T cells, CD4+ Th1 T cells and CD4+ Th17 cells are pro-inflammatory in obese adipose tissue. Table 1.1 summarizes the normal functions of T cells involved in adipose tissue inflammation.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Main function in immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD8+ Cytotoxic T cell</strong></td>
<td>Kills virus-infected cells</td>
</tr>
<tr>
<td><strong>CD4+ T&lt;sub&gt;H1&lt;/sub&gt; cells</strong></td>
<td>Activates macrophages; provide help to B cells for antibody production</td>
</tr>
<tr>
<td><strong>CD4+ T&lt;sub&gt;H2&lt;/sub&gt; cells</strong></td>
<td>Provide help to B cells for antibody production, especially switching to IgE</td>
</tr>
<tr>
<td><strong>CD4+ T&lt;sub&gt;H17&lt;/sub&gt; cells</strong></td>
<td>Enhance neutrophil response</td>
</tr>
<tr>
<td><strong>CD4+ T regulatory cells</strong></td>
<td>Suppress T cell response</td>
</tr>
</tbody>
</table>

Table 1.1: The roles of effector T cells in immune responses
T cells affect adipose tissue physiology in lean and obese states with an increase in proinflammatory CD8+, Th1 and Th17 cells and a decrease in anti-inflammatory CD4, Th2 and Tregs observed in the adipose tissue of mice and humans as they go from a lean to obese state [108, 110-114]. Rag -/- mice, which lack functional B and T cells, gain more weight and are more glucose and insulin intolerant when fed HFD than wild-type mice. Reconstitution of CD4+ cells improves their glucose tolerance because these cells take on an anti-inflammatory Th2 phenotype [108]. Other papers have shown that IL-17 producing Th17 and interferon-γ-producing Th1 subsets are detrimental to insulin sensitivity because they decrease insulin signaling and adipogenesis while increasing lipolysis and IL-17-deficient mice are obese but insulin sensitive on HFD [115-118]. These papers, taken together, suggest that when adipose tissue mass expands, Tregs and Th2 cells fail to inhibit the actions of the CD8+, Th17 and Th1 population, increasing inflammation and adipose tissue dysfunction.

Other immune cells also play a role in adipose tissue inflammation. Natural killer T (NKT) cells in adipose tissue exacerbate insulin resistance in obese mice but may be protective in ND-fed animals [119-121]. B cells also play a pathogenic role in diet-induced adipose tissue inflammation. They accumulate in VAT in HFD mice in early stages of obesity and mice lacking B cells have equal adiposity but are insulin sensitive on HFD due to decreased macrophage and T cell activation and an absence of pathogenic IgG antibodies [122]. Eosinophils protect against insulin resistance activating macrophages towards an
anti-inflammatory M2 phenotype [123] while mast and neutrophils cells exacerbate adipose tissue inflammation [124-126]. In addition to their role in inflammation, there is also evidence that immune cells become insulin resistant, which in turn contributes to global insulin resistance [127]. Table 1.2 summarizes the actions of immune cells in WAT in obesity.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number in adipose tissue with increasing obesity</th>
<th>Effect on metabolic disease</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>↑ in M1:M2 ratio ↑ in total number</td>
<td>Exacerbate insulin resistance through the secretion of proinflammatory cytokines (TNF-α, IL1β, etc.)</td>
<td>[90, 91, 100, 102, 128]</td>
</tr>
<tr>
<td>T cells</td>
<td>↑ in CD8+:CD4+ ratio ↑ in CD8+ ↑ in Th1 ↑ Th17 ↓ in Treg ↓ in Th2</td>
<td>CD8+, Th1 and Th17 exacerbate insulin resistance and promote adipose tissue inflammation. Tregs and Th2 cells decrease adipose tissue inflammation and decrease in number with increasing obesity.</td>
<td>[108, 109, 111-114, 117, 114, 117, 129]</td>
</tr>
<tr>
<td>NKT cells</td>
<td>↑ activation</td>
<td>Exacerbate macrophage infiltration and adipose tissue inflammation in DIO. May protect against insulin resistance under ND conditions.</td>
<td>[119-121]</td>
</tr>
<tr>
<td>B cells</td>
<td>↑ in number</td>
<td>Promote insulin resistance by activating proinflammatory macrophages and T cells and production of pathogenic IgG antibodies.</td>
<td>[122]</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>↓ in number</td>
<td>Protect against insulin resistance by promoting M2 activation of macrophages. Increase in eosinophils enhances glucose tolerance of obese mice.</td>
<td>[123]</td>
</tr>
<tr>
<td>Mast cells</td>
<td>↑ in number</td>
<td>Exacerbate insulin resistance by contributing to angiogenesis and apoptosis via IL-6 and IFN-γ production.</td>
<td>[124]</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>↑ in number transiently in early obesity</td>
<td>Exacerbate insulin resistance by increasing macrophage infiltration and promote inflammation through secretion of elastase.</td>
<td>[125, 126]</td>
</tr>
</tbody>
</table>

Table 1.2: Immune cells found in adipose tissue and their roles in metabolic disease
Immune cells affect adipose tissue metabolism partly through the secretion of factors that alter insulin signaling. Tumor necrosis factor alpha (TNF-α) is secreted by obese adipose tissue and has been shown to downregulate several steps in the insulin signaling cascade, leading to decreases in insulin receptor tyrosine kinase activity and IRS phosphorylation [130]. TNF-α stimulates lipolysis and subsequent release of FFAs from adipose tissue, which could induce insulin resistance in other organs such as the liver and skeletal muscle [131-133]. Interferon-γ (IFNγ) is produced by Th1 and CD8+ cytotoxic T cells. IFNγ increases lipolysis, downregulates adipogenesis and exacerbates insulin resistance. Depletion of IFNγ improves insulin sensitivity in HFD-fed mice [116, 134]. IL17 is secreted by Th17 cells and impairs adipogenesis and glucose uptake by adipocytes, which further exacerbates metabolic disease [117]. Anti-inflammatory cytokines such as IL10 and IL4 are also expressed in obese adipose tissue. These cytokines function to improve insulin sensitivity by inhibiting macrophage activation and TNF-α action [135-137]. Many other cytokines and chemokines are known to be expressed in obese adipose tissue and new ones are constantly being characterized. The integration of these signals maintains the metabolic state of the adipose tissue in healthy individuals but goes awry with increasing obesity.
Role of CD40 in Inflammation

An organism's health depends on its ability to regulate lymphocyte activation; inappropriate lymphocyte activation plays a role in the development of metabolic disease. Obese adipose tissue is characterized by acute and chronic inflammation with the involvement of a broad range of immune cells including T cells, macrophages, mast cells, eosinophils and others. These cells, and their interactions with each other and adipocytes have been shown to alter adipose tissue physiology in the context of obesity. T cells have been shown to be of particular importance in this process [108, 109, 111, 112].

T cells require three signals to be activated. The first signal confers specificity and is the interaction of the histocompatibility (MHC) complexed to an antigen for which the T cell receptor (TCR) is specific. The second and third signals confer effector function and are co-stimulation by antigen presenting cells (APCs) and inflammatory cytokines. In the absence of these additional signals, lymphocytes fail to respond and enter a permanent deactivated state called anergy or fail to develop optimal effector functions [138, 139]. Three major families provide the co-stimulatory signal: the B7:CD28 superfamily, the tumor necrosis factor (TNF) subfamily that lacks death domains, and the CD2 superfamily. Please see Figure 1.5 for a schematic of T cell activation by APCs.
Figure 1.5: Major receptor ligand pairs involved in T cell activation.
T cells recognize peptides presented on MHC class II on APCs with adhesion molecules strengthening the interaction and providing co-stimulation. APC, antigen presenting cell; Ag, antigen; MHC II, major histocompatibility complex class II; TCR, T cell receptor; (Adapted from Banchereau et al., 1994 [138].)
CD40 is a 45-48 kDa type I transmembrane glycoprotein cell surface receptor that belongs to the TNF receptor family, which includes TNFR1, TNFRII, CD95, CD27, CD30, OX40, and 4-1BB [140]. Its action is summarized in Figure 1.6. The gene spans 16.3 Kb of DNA and is encoded by 1.5 Kb mRNA transcribed from a gene on human chromosome 20 or murine chromosome 2. CD40 was discovered as a factor that stimulates the transition of B cells through the cell cycle [141] but is now known to also be expressed on the surface of antigen-presenting cells (APCs) such as macrophages/monocytes and dendritic cells (DCs) and is an important regulator of the production of inflammatory mediators, cell survival and antigen presentation [138]. It is also expressed in a large variety of cell types such as endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, keratinocytes and platelets [142, 143] (Table 1.3). Consistent with its broad expression, antibodies blocking CD40 signaling have been shown to be beneficial in several disease models including transplantation, autoimmunity and infectious diseases [144, 145].
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Functional consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre B cells</td>
<td>Proliferation, CD23 expression</td>
</tr>
<tr>
<td>Naïve mature B cells</td>
<td>Proliferation, differentiation, isotype switch</td>
</tr>
<tr>
<td>Germinal center B cells</td>
<td>Proliferation, differentiation, Fas expression, selection</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>IL-6 production</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>Cytokine secretion, NO production, Metalloproteinase production Monocyte procoagulant activity and tissue factor expression Triggering CD4+ T cell-dependent activation IL-12 expression</td>
</tr>
<tr>
<td>Synovial monocytes</td>
<td>TNF secretion</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>High-level CD40 expression in sarcoidosis</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Growth and survival proliferation and differentiation Expression of costimulatory molecules MHC class II, CD25, CD58, CD80 (B7-1), CD86 (B7-2), CD154 Enhanced cytokine/chemoattractant production TNF-α, IL-8, IL-10 and IL-12 MIP-1α, MIP-1β, RANTES</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>Same as dendritic cells</td>
</tr>
<tr>
<td>CD34+ precursors</td>
<td>Proliferation Development dendritic cells</td>
</tr>
<tr>
<td>T cells</td>
<td>Proliferation, CD25 expression cytokine production</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Enhanced survival GM-CSF production</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
</tr>
<tr>
<td>Human umbilical vein</td>
<td>Uregulation of CD54, CD62E, CD106 Increase tissue factor/thrombomodulin expression and procoagulant activity T cell costimulation Increased production LIF, IL-6, GM-CSF expression of adhesion molecules such as E-selectin, VCAM-1, ICAM-1</td>
</tr>
<tr>
<td>endothelial cells (HUVEC)</td>
<td></td>
</tr>
<tr>
<td>Microvascular endothelial cells</td>
<td>CD40 expression increased by HIV</td>
</tr>
<tr>
<td>Vascular endothelial cells</td>
<td>Increased IL-1, IL-6, IL-8 production</td>
</tr>
<tr>
<td><strong>Epithelial cells</strong></td>
<td><strong>Kidney epithelial cells</strong></td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Thymic epithelial cells</td>
<td>GM-CSF production co-stimulation for clonal expansion of CD4+ thymocytes</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>Enhanced expression CD54, Bcl-x IL-8 secretion</td>
</tr>
<tr>
<td>Carcinomas and transformed epithelial cells</td>
<td>Growth inhibition/apoptosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Fibroblasts</strong></th>
<th><strong>Fibroblasts</strong></th>
<th><strong>Fibroblasts</strong></th>
<th><strong>Fibroblasts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Synoviocytes</td>
<td>Proliferation Cytokine production (GM-CSF, MIP1α)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung fibroblasts</td>
<td>Increased IL-6, IL-8, NF-κB Increased Cox-2, PGE2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermal fibroblasts</td>
<td>Increased IL-6, IL-8, NF-κB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid fibroblasts</td>
<td>Increased IL-6, IL-8, NF-κB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gingival fibroblasts</td>
<td>Increased IL-6, IL-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular dendritic cells</td>
<td>Growth CD54 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular smooth muscle cells</td>
<td>Increased MMP-1, MMP-3, MMP-9, MMP-2 Activation ICE (IL-1β converting enzyme) Induction stromelysin-3 (MMP-11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Induction of Fas-L expression</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3: Cells expressing CD40 and functional consequences of in vitro activation** (Modified from van Kooten, 2000 [146]).
Murine CD40 ligand (CD40L, CD154, gp39, T-BAM, TRAP) is a type II transmembrane protein encoded by a 2.3 Kb mRNA which is translated into a 261 amino-acid protein. CD40L exists in both a soluble and a transmembrane form. The transmembrane form has a molecular weight of 39 kDa. The soluble form lacks the intracellular, transmembrane and part of the extracellular domains and has a molecular weight of 18 kDa. CD40L is expressed on activated mature T cells but not on resting T cells and is primarily restricted to CD4+ T cells. It is also expressed on dendritic cells, B cells, endothelial cells, epithelial cells, macrophages and others [138]. The expression of CD40L on activated T cells is transient and tightly regulated with expression reported as soon as 5 minutes after activation [147].

CD40 can be found on a cell’s surface as a monomer, dimer or trimer. Only the dimeric and trimeric forms are sufficient for signal transduction, the latter sending an enhanced signal [148, 149]. Binding of CD40L to CD40 causes trimerization of CD40 in lipid raft domains between interacting cells in order to propagate the signal (Figure 1.6 below). Ligation of CD40 leads to the activation of kinases, the expression of genes related to cell stress, the expression of new surface molecules, the increase or decrease of apoptosis and the further differentiation of immune cells [150-154]. Activation of CD40 signaling stimulates cells to produce cytokines and chemokines, to express adhesion molecules, costimulatory molecules and enzymes such as matrix metalloproteinases (MMPs). These molecules, summarized in Table 1.3, are involved in local
inflammatory and chronic inflammatory processes [142]. Expression of CD40 is observed starting 6-12 h following stimulation, peaks after 24 hours and persists for 24-72h. In contrast, CD40L expression is more transient[155]. Induction of CD40 expression is controlled at the transcriptional level, but inhibition by certain cytokines, such as TNF-β, occurs via enhanced degradation of CD40 mRNA [156]
Figure 1.6: Potential mechanism of CD40 signaling.
Binding of CD40 on the extracellular surface of the cell causes trimerization. The intracellular signal is propagated via TRAF proteins. Adapted from Schonbeck, 2001 [155].
CD40 signaling is essential for activation of B cells by T cell-dependent antigens, germinal center formation, immunoglobulin class switching, affinity maturation of B cells and the development of plasma and memory B cells. This is illustrated by Hyper-IgM syndrome, an X-linked immunodeficiency resulting from a mutation of CD40 or CD40L. Patients with this disease have a reduced ability to generate T cell dependent antibody responses, have a lack of circulating IgA, IgE and IgG and are unable to generate memory B cells [157].

CD40 can be activated by ligands other than CD40L. Human C4b binding protein (C4BP) binds to CD40 on human B cells at a different site from the one used by CD40L. C4BP induces proliferation and isotype switching in a CD40-dependent manner in human cells in vitro [158]. CD40 can also be stimulated by the 70 kDa mycobacterial heat shock protein HSP70, which stimulates mononuclear cells to release chemokines [159]. The in vivo significance of these interactions has yet to be determined. Not only can CD40 be activated by ligands other than CD40L, CD40L can also mediate inflammation independently of CD40 through interaction with Mac-1/CD11b [160].
**CD40 role in disease processes and obesity**

The discovery that hyper IgM syndrome was due to an alteration in the CD40L gene demonstrated the critical role of the CD40-CD40L dyad in humoral immunity [161]. CD40 and CD40L-deficient mice had a comparable phenotype [162-164]. Since then, CD40 signaling has been demonstrated to be important in both humoral and cellular immunity and not surprisingly, has been a target for many chronic inflammatory and autoimmune diseases such as neurodegenerative disorders, graft versus host reactions and neoplasias [155].

Both CD40 and its ligand have also been implicated in atherosclerosis, a chronic disease that is often associated with obesity. The cells types present in atherosclerotic plaques--endothelial cells, macrophages, and smooth muscle cells can all express CD40 and/or CD40L and are similar to the types of cells present in the SVF (the non-adipocyte portion of adipose tissue). Activation of CD40 signaling in these cells activates the expression of proinflammatory cytokines, adhesion molecules, matrix metalloproteinases and procoagulants, all of which participate in decreasing plaque stability. Atherosclerotic mice that are genetically deficient in CD40L or treated with an anti-CD40L antibody have decreased atherosclerosis on a high cholesterol diet as well as features associated with increased plaque stability [165, 166]. CD40L inhibition has also been shown to retard the progression of atherosclerotic lesions [167, 168]. Soluble CD40L (sCD40L) serum levels are associated with cardiovascular
disease [169]. There is evidence that the role of CD40L in vascular disease is not entirely mediated by CD40 [160, 170, 171].

In chronic and inflammatory disease, CD40-CD40L signaling polarizes T cells towards a pro-inflammatory Th1 phenotype, suggesting that CD40 signaling may have a detrimental effect in obesity. Lim et al. measured plasma sCD40L levels in obese humans and found that they were higher in diabetic patients and that statin and aspirin use was associated with a decrease in sCD40L [172]. Cipollone et al. also found that sCD40L was higher in diabetic humans but also showed that this was associated with an increase in the inflammatory state of endothelial cells and monocytes [173], factors that could be relevant to CD40-CD40L signaling in obese adipose tissue. In other studies, levels of soluble CD40L are significantly associated with metabolic syndrome and waist circumference [174], while CD40 mRNA levels in adipose tissue are correlated with BMI [175] and sCD40L levels decreased after gastric bypass surgery in obese men [176]. These correlative studies in humans all suggest that the CD40-CD40L dyad may play a role in the pathophysiology of diet-induced obesity.

Given the detrimental role of inflammation in obesity, and the role of the CD40/CD40L dyad in inflammation, one may hypothesize that a mouse deficient in CD40 or CD40L would have improved insulin sensitivity due to decreased inflammation in adipose tissue. Wolf et al. showed that CD40L-deficient mice on HFD have decreased adipose tissue B cells, macrophages and adipose tissue expression of MCP-1 with a concomitant increase in Tregs. Interestingly,
knockout mice were more glucose intolerant and insulin resistant after 8 weeks of HFD despite decreased inflammation in adipose tissue. Knockout mice were also hypoinsulinemic and had a large increase in hepatic accumulation of TGs and cholesterol [177]. This study is consistent with one done by Villeneuve et al which also showed increased steatosis in CD40L deficient mice fed an olive-oil-rich diet due to impaired VLDL secretion and increased lipogenesis as a result of increased endoplasmic reticulum stress [178]. This consistency is particularly remarkable because they used two completely different mouse strains (Wolf et al used mice on a C57BL/6J background and Villeneuve et al used BALB/c mice) and different diets (Wolf et al used 45% fat and Villeneuve used and olive oil enriched diet). A third paper by Poggi et al [179] reached a different conclusion. Using CD40L/-/- on a C57BL/6J background fed a diet of 45% fat, the same mice and diet that Wolf et al used, they found that knockout mice had decreased hepatic steatosis and improved insulin sensitivity compared to controls. They also reported decreased macrophage and T cell presence in epididymal adipose tissue as well as a general decrease in adipose tissue inflammation [179].

In summary, obesity is associated with chronic adipose tissue and systemic inflammation. Correlative studies have suggested that CD40-CD40L may have a role in metabolic disease in humans but studies involving CD40L knockout mice have been unable to elucidate the role that CD40-CD40L signaling plays in diet-induced obesity in mice.
The CD40 knockout mouse

Though human studies have taught us a lot about obesity and diabetes, they are confounded by genetic and lifestyle differences that cannot be fully accounted for and are observational in nature. Numerous mouse models of insulin resistance have been described with phenotypes ranging from embryonic lethality to hypoglycemia [180]. The CD40 knockout mouse was first described by Kawabe et al in 1994 as a mouse that could only mount an IgM response (no IgG, IgA or IgE) and had defective germinal center formation [164]. The original experiments, and many subsequent immunological studies describing the CD40 KO mouse were done on a mouse on the BALB/c background, which is protected against diet-induced obesity and insulin resistance and therefore rarely used for metabolic studies. The CD40 KO mouse has been used primarily to study the role of CD40 in immunological processes such as virus infection [181] and cellular and humoral immunity [182-184] so little is known about its metabolic phenotype.
**Specific aims**

It is well-established that obesity is associated with increased systemic and adipose tissue-specific inflammation. Inflammation relies on cell-cell interactions that are mediated by many different molecules, one of which is the CD40/CD40L dyad. It has been observed that sCD40L is increased in human obesity, metabolic syndrome and other inflammatory diseases and CD40 expression is increased in obese insulin resistant adipose tissue. Studies using CD40L knockout mice have yielded inconsistent results regarding the role of CD40-CD40L signaling in metabolic disease.

It has also been shown that different adipose depots confer a beneficial or detrimental risk of type 2 diabetes in the context of obesity. Unlike WAT, which is specialized for energy storage, BAT burns energy to produce heat by uncoupling oxidative phosphorylation via a protein called UCP-1 while PVAT surrounds vessels. The ability of a small amount of BAT to burn a lot of energy has made it an attractive target for the treatment of metabolic disease. Brown fat activity is inversely correlated with obesity in humans and BAT ablation or genetic disruption of UCP-1 both lead to obesity in mice. Studies have shown that brown adipocytes can atrophy in response to inflammatory cytokines, such as TNF-α, but no one has shown whether or not local inflammation in brown adipose tissue plays a role in type 2 diabetes.
In Chapter II of this dissertation we aimed to test the hypothesis that CD40-deficient mice will be more insulin sensitive due to lower local and systemic inflammation.

The specific aims of this study were:

To compare glucose and insulin tolerance in CD40-/- mice challenged with HFD;

To determine whether CD40-/- mice exhibited decreased adipose tissue inflammation; and

To determine whether CD40 deficiency will increase or decrease hepatic steatosis.

In Chapter III of this dissertation we tested the hypothesis that brown adipose tissue and perivascular adipose tissue become inflamed with increasing obesity and that BAT inflammation plays a role in metabolic disease.
CHAPTER II: CD40 IN MICE EXACERBATES OBESITY-INDUCED ADIPOSE TISSUE INFLAMMATION, HEPATIC STEATOSIS AND INSULIN RESISTANCE

Disclaimer: Experiments were performed in collaboration with Chang-An Guo.

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Abstract

The pathophysiology of obesity and type 2 diabetes in rodents and humans is characterized by low-grade inflammation in adipose tissue and liver. The CD40 receptor and its ligand, CD40L, initiate immune cell signaling promoting inflammation, but conflicting data on CD40L null mice confound its role in obesity-associated insulin resistance. Here we demonstrate that CD40 receptor deficient mice on a high fat diet display the expected decrease in hepatic cytokine levels, but paradoxically exhibit liver steatosis, insulin resistance and glucose intolerance compared to their age-matched wild type controls. Hyperinsulinemic-euglycemic clamp studies also demonstrated insulin resistance in glucose utilization by the CD40 null mice compared to wild type mice. In contrast to liver, adipose tissue in CD40 deficient animals harbors elevated cytokine levels and infiltration of inflammatory cells, particularly macrophages and CD8$^+$ effector T-cells. In addition, ex vivo explants of epididymal adipose tissue from CD40$^{-/-}$ mice display elevated basal and isoproterenol-stimulated lipolysis, suggesting a potential increase of lipid efflux from visceral fat to the liver. These findings reveal that, 1) CD40 null mice represent an unusual model of hepatic steatosis with reduced hepatic inflammation, and 2) CD40 unexpectedly functions in adipose tissue to attenuate its inflammation in obesity, thereby protecting against hepatic steatosis.
Introduction

Obesity is often associated with type 2 diabetes, hepatic steatosis, non-alcoholic steatohepatitis [185], cardiovascular complications [186, 187] and certain cancers [188, 189]. When nutrient supply exceeds demand, adipocytes expand by hypertrophy and hyperplasia to store excess fat in the form of triglycerides. As adipocytes increase in size, their ability to store fat is diminished and fatty acids are ectopically deposited in the liver and muscle [190, 191]. This transition of lipid handling is thought to cause lipotoxicity in these peripheral tissues, disrupting insulin signaling. Glucose uptake by skeletal muscle in response to insulin is impaired in this condition and hepatic gluconeogenesis is elevated, leading to glucose intolerance.

Adipose tissue in obese rodents and humans has the hallmarks of chronic inflammation, including the involvement of T cells, macrophages and other immune cells [110, 112, 119, 192, 193]. During the progression to obesity, immune cells and the factors they secrete interact with adipocytes and alter the ability of adipose tissue to store fat in response to insulin. Inflammation has been described in obese fat [194], liver [195] and muscle [196], the three key insulin target tissues. Importantly, it has been shown that decreasing inflammation with either genetic manipulation in rodents or pharmacologic inhibition of key mediators of inflammation in humans [197, 198], improves insulin sensitivity. Although the association between obesity and inflammation is extensively
described, the exact mechanisms for initiation of inflammation are unknown and the spatial and temporal actions of the cells involved are unclear.

The CD40-CD40L costimulatory dyad plays a critical role in regulating innate and adaptive immune responses. CD40 is a 48-kDa transmembrane glycoprotein cell surface receptor that belongs to the tumor necrosis factor receptor (TNFR) superfamily and is activated by binding to its ligand CD40L [138]. Various immune cells including macrophages, B lymphocytes, T cells, dendritic cells and mast cells, as well as smooth muscle cells, endothelial cells and activated platelets express CD40 [199]. Recent evidence indicates that the CD40-CD40L complex is also a potential mediator of chronic inflammation in obesity and its related metabolic disorders [175, 200-202], suggesting a broader role of CD40 in cell biology. Obese and diabetic individuals have higher levels of active soluble CD40L (sCD40L) in the circulation compared to lean healthy subjects [203] and CD40 mRNA levels in white adipose tissue were found to positively correlate with BMI [175]. Interruption of CD40 signaling has been shown to limit experimental autoimmune diseases in mice such as arthritis, lupus nephritis, multiple sclerosis and thyroiditis and treatment of hyperlipidemic mice with an anti-CD40L antibody reduced the number of macrophages, T cells and inflammatory markers in atherosclerotic lesions [166]. Currently, targeting CD40-CD40L signaling is considered to be a promising strategy for effecting plaque stabilization in the treatment of atherosclerosis [204].
Based upon the above considerations, we anticipated that suppression of the CD40-CD40L signaling cascade would also reduce the deleterious inflammation and metabolic effects associated with obesity. In fact, the CD40-CD40L axis has recently been implicated in the pathogenic complications of obesity [177-179]. However, various research groups have reported conflicting roles of CD40L in diet-induced obesity in mice. For example, two studies showed that CD40L deficiency aggravated hepatic steatosis in obesity [177, 178] while another study indicated that the absence of CD40L attenuated diet-induced steatosis [179]. Furthermore, in one study, CD40L deficiency improved insulin resistance [179], while in another study, lack of CD40L in mice did not ameliorate high fat diet-induced insulin resistance [177]. A caveat in these studies is that CD40L can mediate inflammation through CD40-independent mechanism by interacting directly with Mac-1/CD11b, which is expressed abundantly on macrophages and monocytes [160]. We therefore designed the present studies to specifically evaluate the role of the CD40-CD40L dyad in adipose tissue inflammation, insulin resistance and hepatic steatosis associated with diet-induced obesity. Here we evaluated the effects of depletion of CD40 itself in mice, which has not been yet reported. Surprisingly, we found that CD40 knockout (CD40−/−) mice paradoxically displayed significantly higher levels of adipose tissue inflammation, impaired glucose tolerance and remarkable hepatic steatosis without liver inflammation. These results suggest an unexpected primary role for CD40 in attenuating immune cell recruitment to the visceral
adipose tissue during the progression of diet-induced obesity. This in turn reduces adipocyte lipolysis, thereby protecting the liver from increased influx of lipid from adipose tissue and enhancing whole-body glucose tolerance.
Materials and Methods

Animal studies

Male CD40$^{-/-}$ (B6.129P2-Cd40$^{Im1Kik}/J$) and control C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The background strain of B6.129P2-Cd40$^{Im1Kik}/J$ mice in this study is C57BL/6Ncr, which is maintained by Jackson Laboratory via sibling mating (34 generations as of Nov, 2008). Since the C57BL/6Ncr mouse line from NIH have been separated from the B6.129P2-Cd40$^{Im1Kk}/J$ mice for many generations, we therefore created littermate control animals for this study to avoid potential genetic drift from various C57BL/6Ncr sub strains or colonies. We bred B6.129P2-Cd40$^{Im1Kik}/J$ mice with C57BL/6J mice and then their F1 hybrids were used to generate knockout and wild-type littermates. All animals were fed standard chow diet (LabDiet PicoLab 5053, Purina Mills, St. Louis, MO) until 8 weeks of age and then divided into two groups; one was fed chow diet and the other group fed high fat diet (TD.93075, 55/Fat, Harlan Teklad, Madison, WI, fatty acid profile as % total fat: 28% saturated, 30% trans, 28% monounsatuated cis, 14% polyunsaturated cis). Animals were housed in the University of Massachusetts (UMass) Medical School Animal Medicine facility with a 12-hour light/dark cycle and given *ad libitum* access to food and water. Mice and food were weighed weekly over the duration of the study.
Intraperitoneal glucose tolerance test (GTT), insulin tolerance test (ITT) and pyruvate tolerance test (PTT) was performed as previously described [205]. Composition of total fat and lean mass was assessed by 1H-MRS-based body composition analysis (EchoMRI-3in1™, EchoMRI, Houston, TX). Measurements of energy expenditure, respiratory exchange ratio, indirect calorimetry and physical activity using metabolic cages (TSE Systems, Bad Homburg, Germany) were done by the UMass Mouse Metabolic Phenotyping Center. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees (IACUC) at UMass Medical School.

**Hyperinsulinemic-euglycemic clamp studies**

The clamp study was performed at the UMass Mouse Metabolic Phenotyping Center. Mice fed HFD for 12 weeks were subject to an overnight fast (~15 hours) and a 2-hour hyperinsulinemic-euglycemic clamp was conducted in awake mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 2.5 mU/kg/min; Humulin; Eli Lilly). During the clamp, 20% glucose was infused at variable rates to maintain euglycemia [206]. Whole body glucose turnover was assessed with a continuous infusion of [3-³H] glucose and 2-deoxy-D-[1-¹⁴C]glucose (PerkinElmer, Waltham, MA) was administered as a bolus (10 µCi) at 75 min after the start of clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the study, mice were anesthetized, and tissues were taken for biochemical analysis.
**Hepatic triglyceride analyses**

Hepatic triglyceride content measurement was performed as previously described (11). Mice were fasted for 4 hours; total lipids were extracted from liver samples (50 mg) using a 2:1 mixture of chloroform and methanol. The organic layer was dried overnight and reconstituted in a solution containing 60% butanol and 40% of a 2:1 mixture of Triton-X114 and methanol. Colorimetric analyses were used to measure total triglyceride (Wako Diagnostics, Richmond, VA).

**Histology**

Liver, pancreas and epididymal white adipose tissue were dissected and fixed by immersion in 10% neutral buffered formalin (Sigma, St. Louis, MO) for 12 hours, dehydrated, cleared and then embedded in paraffin. Sections (7µm) were stained by hematoxylin and eosin to assess morphology. Pancreatic islets were stained with insulin antibody (Cell Signaling, Danvers, MA). Oil red O (Sigma) was used to stain neutral lipids in frozen liver sections.

**Real-Time quantitative RT-PCR**

RNA was extracted from homogenized liver and adipose tissue using the TRizol Reagent according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For real-time PCR, synthesized cDNA,
forward and reverse primers along with the iQ SYBR Green Supermix were run on the CFX96 real-time PCR system (Bio-Rad). Primer sequences are available on request. Fold change in mRNA expression was determined using the $2^{-\Delta\Delta CT}$ method [207], with all genes normalized to the ribosomal mRNA 36B4.

**Western Blotting**

Tissue pieces were homogenized in S-50 protein lysis buffer (20 mM Tris [pH 7.2], 1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 25mM sodium fluoride, 1 mM sodium orthovanadate, 1mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL of aprotinin and leupeptin) in gentleMACS M tubes (Miltenyi Biotec, Germany). Protein was quantified using the BCA protein assay kit (Thermo Scientific, Waltham, MA), resolved on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% non-fat milk in TBST (0.05% Tween 20 in Tris-buffered saline), washed with TBST and incubated with primary antibody overnight. The blots were washed with TBST and a horseradish peroxidase secondary antibody was applied. Proteins were visualized using Western Lightening Plus ECL (PerkinElmer, Waltham, MA). Primary antibodies used were Phospho-Akt (Ser473) and total Akt (Cell Signaling, Danvers, MA).

**Ex-vivo Lipolysis Assay in epididymal adipose tissue- explants**

Epididymal fat pads were surgically removed from male mice and washed with ice-cold PBS. Fat pads (~100mg, n=4/mouse) were preincubated for 1 hour in
140 µl DMEM (Life Technologies) containing 2% fatty acid free serum albumin (Sigma-Aldrich). Subsequently, fat pads were incubated in 250 µl KRH buffer (125 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2.6 mM MgSO₄, 5 mM Hepes, pH 7.2) +2% BSA (fatty acid free) with or without the presence of isoproterenol (10 µM) for 2 hours at 37°C. Free glycerol content was quantified for each sample in the medium using the Free Glycerol Determination Kit (Wako Diagnostics). Glycerol release from each sample was normalized to the weight of each fat pad.

Flow cytometry

Epididymal adipose tissue was isolated from mice and treated with 2 mg/mL collagenase (Sigma) for 45 minutes at 37°C. Digested tissue was filtered through a 200 µM nylon mesh and then centrifuged at 1000 rpm for 10 min. Pelleted stromal vascular fraction of adipose tissue was stained with fluorescently conjugated primary antibodies according to manufacturers’ instructions. Primary antibodies used were CD4-FITC (BD Pharmingen cat 553046), CD3-APC-Cy7 (BD Pharmingen 1452C11), CD25-APC (eBiosciences cat 17-0251-81), CD8a-PerCP-Cy5.5 (cat 551162), CD90-PE-Cy7 (BD Pharmingen cat 561558), CD11b-PerCP-Cy5.5 (BD Pharmingen cat 562127), F4/80-APC (ABD Serotec), NK1.1-APC (eBiosciences Cat 17-5941), CD19-Alexa 405 (BD Pharmingen cat 560375), FoxP3-PE (eBiosciences 12-4774). Samples were run on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed in FlowJo (TreeStar).
ELISA assay

Blood was obtained from the tail veins of mice or via cardiac puncture and allowed to clot. Serum was isolated by centrifugation at 1000g for 10 min. Mouse insulin ELISA kit (EMD Millipore, Billerica, MA) and Mouse sCD40L Platinum ELISA kit (eBioscience, San Diego, CA) were used to measure serum insulin and sCD40L as recommended by the manufacturer.

Statistical Analysis

Differences between groups were examined for statistical significance using the two-tailed Student's test or analysis of variance (ANOVA) followed by post hoc Bonferroni tests. The data are presented as the means ± S.E.M. P-values ≤ 0.05 are considered significant.
**Results**

**CD40-/- mice have decreased weight, food intake and physical activity.**

To investigate the role of CD40 in diet-induced obesity, CD40−/− mice and wild type (WT) controls were put on feeding regimens consisting of a high fat diet (HFD) or a normal diet (ND). The CD40 null mice were found to be approximately 2 grams lighter than control mice on either diet throughout most of a 14-week feeding period (Figure 2.1 A). These data are consistent with a previous study on CD40L−/− mice (32), suggesting a common role for CD40 and CD40L in the regulation of energy homeostasis. Food intake of CD40−/− mice was significantly lower on HFD (Figure 2.1B), potentially explaining in part the decreased body weight (Figure 2.1A). Physical activity measurements on mice fed the HFD over a 24-hour period indicated that CD40−/− mice were significantly less active than WT mice at 8 and 14 weeks of age (0 and 6 weeks on HFD) (Figure 2.1C). However, respiratory exchange ratio (RER) and energy expenditure were not significantly different between the two groups of mice (Figure 2.1.2).
Figure 2.1: CD40 deficiency alters energy homeostasis.
(A) Growth curve of mice fed either normal diet (ND) or high-fat diet (HFD) for 14 weeks (n = 12), * indicates statistically significant difference between WT ND vs CD40−/− ND groups or WT HFD vs. CD40−/− HFD groups. (B) Daily food intake of mice (n = 12) fed ad libitum on ND or HFD for 13 weeks. (C) Ambulatory activity of mice on ND or HFD for 13 weeks. (D) Glucose infusion rate (GIR) in mice on WT HFD or CD40−/− HFD for 20 weeks. (E) Glucose uptake in different tissues (WAT, Skeletal Muscle, BAT, Heart) of mice on WT HFD or CD40−/− HFD for 20 weeks. 

* indicates statistically significant difference between groups.
as estimated by infrared beam breaks over a 24-hour period for mice on HFD for 0, 6, 12 or 16 weeks (n = 6). (D) Steady-state glucose infusion rate (GIR) for WT and CD40−/− mice on HFD for 16 weeks to maintain euglycemia during hyperinsulinemic-euglycemic clamps (n = 10). (E) 14C-2-deoxy-glucose uptake in visceral white adipose tissue (WAT), gastrocnemius muscle (Skeletal Muscle), intrascapular brown adipose tissue (BAT) and heart. The data presented are mean ± S.E.M. Statistically significant differences are indicated (* p<0.05, ** p<0.01 vs. control).
Figure 2.1.2: RER and heat are not altered in the absence of CD40.
(A) Respiratory exchange ratio and (B) heat.
CD40 deficiency exacerbates diet-induced insulin resistance

Decreased weight, food intake and activity in CD40−/− mice suggested a role for CD40 in whole-body metabolism. To characterize this role, we did a series of metabolic analyses on these mice including a hyperinsulinemic-euglycemic glucose clamp study. CD40−/− mice on HFD showed a reduced steady-state glucose infusion rate (GIR) (Figure 2.1 D), suggesting that absence of CD40 aggravates systemic insulin resistance in obesity. We also observed decreased 14C-2-deoxy-glucose uptake in the brown adipose tissue (BAT) of CD40−/− mice with no changes of glucose uptake in the white adipose tissue or muscle (Figure 2.1 E). The morphology of pancreatic islets, fasting serum insulin, serum sCD40L, serum triglyceride (TG) and free fatty acids (FFA) were similar in both CD40−/− and WT mice on HFD (Figure 2.2.2). To further analyze the metabolic phenotype of the CD40−/− mice, we subjected them to a glucose tolerance test (GTT) as well as an insulin tolerance test (ITT). Despite the reduced body weight of CD40−/− mice on HFD, they surprisingly exhibited glucose intolerance (Figure 2.2 A and 2.2 E) and insulin resistance (Figure 2.2 B and 2.2 F) compared to WT controls. To assure these phenotypes we observed are due to CD40 deficiency and not due to the potential differences in mouse colonies or substrains, we created CD40−/− mice and WT littermate control mice by breeding the F1 hybrids of CD40−/− (B6.129P2-Cd40tm1Kik/J) and C57BL6/J parental mice. These cohorts were also subjected to GTT (Figure 2.2 C and 2.2 G) and ITT tests (Figure 2.2 D and 2.2 H). Similar to our original findings, the
mice were significantly more glucose intolerant and insulin resistant than littermate control animals.
Figure 2.2: CD40−/− mice fed a high fat diet are more glucose intolerant and insulin resistant.

(A) Glucose tolerance test (GTT) for CD40−/− and C57BL/6J control mice (30 weeks old) on HFD for 22 weeks (n=8). Animals were fasted overnight and glucose was injected intraperitoneally at a dose of 1g/kg of body weight. (B) Insulin tolerance test (ITT). 31 weeks old CD40−/− and C57BL/6J control mice on HFD for 23 weeks (n=8) were fasted for 4 hours and injected with insulin at a dose of 1U/kg. (C) Glucose tolerance test (GTT) for CD40−/− and littermate control mice (18 weeks old) on HFD for 10 weeks (n=4). Animals were fasted overnight and glucose was injected intraperitoneally at a dose of 1g/kg of body weight. (D) Insulin tolerance test (ITT). 18 weeks old CD40−/− and littermate control mice on
HFD for 10 weeks (n=4) were fasted for 4 hours and injected with 1U/kg insulin. (E) Area under the curve (AUC) for A. (F) AUC for B. (G) AUC for C. (H) AUC for D. Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40⁻ HFD are indicated (*p < 0.05, ** p < 0.01, ***p < 0.001).
Figure 2.2.2: CD40 deficiency does not alter pancreatic islet morphology, serum insulin, serum sCD40L, serum Triglycerides or serum FFA under HFD conditions.

(A) Light microscopy image of pancreatic tissue treated with an anti-insulin primary antibody. (B) Serum insulin. (C) Serum sCD40L. (D) Serum TG. (E) Serum FFA.
CD40−/− mice develop hepatic steatosis and increased *de novo* lipogenesis in the liver.

Recent literatures have implicated CD40L as a protective factor against hepatic steatosis (43, 46), which has a strong association with insulin resistance (29). Since we found impaired systemic insulin sensitivity in CD40−/− mice, we evaluated the hepatic steatosis and hepatic insulin sensitivity in these mice. No difference was observed in lipid content of livers of CD40−/− mice fed ND (Figure 2.3 C) compared to WT controls on the same diet (Figure 2.3A). CD40−/− mice on HFD, however, displayed clearly steatotic livers, as observed both macroscopically and microscopically by H&E or Oil-red O staining (Figure 2.3 D vs 2.3 B). To quantify this increased steatosis, we measured total hepatic triglycerides (TG) levels and found a 59% increase in CD40−/− mice (Figure 2.3 E). Elevated expression of two lipid droplet proteins, cell death-inducing DFFA-like effector A (CIDEA) and Fat Specific Protein 27 (FSP27) (34, 35), accompanied the increase in total TGs in the liver (Figure 2.3 F). These lipid droplet proteins are specifically expressed in steatotic livers and absent in lean livers (Figure 2.3 F). Increased hepatic steatosis could be mediated by different pathways including increased dietary lipid absorption, increased *de novo* lipogenesis, increased influx of non-esterified free fatty acids (FFA) from hypertrophied adipose tissue or decreased very low density lipoprotein (VLDL) secretion and fat oxidation (7, 9). As no difference in plasma FFAs, respiratory exchange ratio (RER) and heat production was observed between CD40 null and WT mice.
(Figures 2.2.2 and 2.1.2), a role for increased dietary fat absorption or impaired fat oxidation is unlikely the cause for the hepatic steatosis in CD40<sup>-/-</sup> mice. Therefore, we measured the expression of transcription factors that promote hepatic lipogenesis as well as genes that encode enzymes contributing to lipogenesis. Peroxisome proliferator-activated receptor gamma (PPAR<sub>γ</sub>) and sterol-regulatory element-binding protein-1c (SREBP1-c), two key regulators of hepatic lipogenesis were both significantly elevated in the CD40<sup>-/-</sup> mice fed the HFD (Figure 2.3 G). Acetyl-CoA carboxylase 2 (ACC2), Long-chain-fatty-acid-CoA ligase 1 (ACLS1) are genes both involved in <i>de novo</i> lipogenesis and were both increased in CD40<sup>-/-</sup> mice (Figure 2.3 H). These data suggest that the hepatic steatosis observed in CD40<sup>-/-</sup> mice is probably due to an increased <i>de novo</i> lipogenesis.
Figure 2.3: CD40 protects against diet-induced hepatic steatosis and decreases hepatic lipogenesis. (A-D) Macroscopic (upper left inlays) and microscopic examination of WT and CD40\(^{-/-}\) mouse livers on ND or 24-week HFD. Representative sections are stained with hematoxylin-eosin or Oil red O (bottom left inlays) (Magnification: 200X). (E) Hepatic total triglycerides in WT and CD40\(^{-/-}\) mice fasted for 6 hours (n=6). (F) The gene expression of lipid droplet proteins Cidea and FSP27 in the livers of WT and CD40\(^{-/-}\) mice (n=6) was measured by qRT-PCR. (G) The expression of genes that encode lipogenic transcription factors and coactivators (C/ebp\(\alpha\), C/ebp\(\beta\), Ppary, Sreb1c, Chreb1b and Lxr) in the livers of HFD fed WT and CD40\(^{-/-}\) mice (n=6). (H) The expression of genes that encode enzymes (Fasn, Acc1/2, SCD1, Acs1/4, Dgat1/2, Gyk,
Mttp) that promote lipogenesis in the livers of HFD fed WT and CD40^{−/−} mice (n=6). Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40^{−/−} HFD are indicated (^p < 0.05, **p< 0.01).
**CD40⁻/⁻ mouse livers are insulin resistant despite decreased inflammation**

Since hepatic steatosis is correlated with hepatic insulin resistance in mouse and human obesity, we tested the hypothesis that CD40⁻/⁻ mice on HFD had greater hepatic insulin resistance. We injected mice intraperitoneally with insulin (1mU/g) and measured Akt phosphorylation at Ser-473 as an indicator of hepatic insulin signaling. Compared to WT controls, CD40⁻/⁻ mice on HFD for 24 weeks exhibited 45% lower Ser-473 phosphorylation on immunoblot densitometry analysis (Figure 2.4 A and 2.4 B). Hepatic insulin resistance often results in dysregulated hepatic gluconeogenesis that contributes to glucose overproduction and obesity-related hyperglycemia (9). Indeed, we found that livers of CD40⁻/⁻ mice expressed increased levels of the transcription factor forkhead box protein O1 (FOXO1) (Figure 2.5 A). The expression of glucose-6-phosphatase (G6pc), a key gluconeogenic enzyme, regulated by FOXO1/PGC-1α, was also upregulated in the livers of CD40⁻/⁻ mice (Figure 2.5 A). To test whether gluconeogenesis was increased in HFD-fed CD40⁻/⁻ mice, a pyruvate tolerance test (PTT) was performed with 16-hour fasted CD40⁻/⁻ and WT mice. We found that CD40⁻/⁻ mice displayed significantly higher rate of hepatic glucose output by converting pyruvate into glucose (Figure 2.5 B). These data suggest that hepatic insulin resistance in CD40⁻/⁻ mice leads to elevated levels of hepatic gluconeogenesis. Liver inflammation has been proposed as a link between hepatic steatosis and insulin resistance (39). However, in testing liver inflammation in CD40⁻/⁻ mice, we surprisingly found that nucleotide-binding
domain and leucine-rich-repeat-containing protein-3 (NLRP3), interferon gamma (IFNγ), and interleukin-1 beta (IL-1β) were significantly decreased in CD40⁻/⁻ mice fed HFD (Figure 2.5 C). In accordance with the lower level of inflammatory cytokine secretion in the livers of CD40⁻/⁻ mice, the expression of macrophage marker, F4/80, was downregulated (Figure 2.5 C). These data indicate that the hepatic steatosis and insulin resistance observed in the CD40⁻/⁻ mice on HFD occurs independently of liver inflammation.
Figure 2.4: CD40 deficiency exacerbates hepatic insulin resistance. 
(A) Hepatic Akt activation following acute intraperitoneal insulin injection (1U/kg, 10 minutes). Western blot analysis of liver extracts showing phospho (Ser473) AKT (pAkt) levels under control (PBS, indicated as “-”) and insulin (“+”) treated conditions. (B) Densitometric quantitation of pAkt/Akt ratios (n=6). Data are presented as mean ± SEM. Statistically significant differences between groups WT HFD and CD40−/− HFD are indicated (*p < 0.05, **p < 0.01).
Figure 2.5: CD40−/− mice display elevated gluconeogenesis and decreased hepatic inflammation.

(A) The expression of genes that encode gluconeogenic transcription factors and coactivators (Pgc1α, FOXO1, Hnf4α, Pepck, G6pc) in the livers of HFD fed WT and CD40−/− mice (n=6). (B) Pyruvate tolerance test (PTT) for mice (32-week old) on HFD for 24 weeks (n=8). Animals were fasted overnight and sodium pyruvate was injected intraperitoneally at a dose of 2g/kg of body weight. (C) The expression of genes (Icam, Vcam, Nlrp3, F4/80, CD68, CD3, IFNγ, TNFα and IL-1β) associated with inflammation in the livers of WT and CD40−/− mice (n=6). Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40−/− HFD are indicated (*p < 0.05, **p< 0.01, ***p<0.001).
CD40<sup>−/−</sup> mice exhibit increased basal lipolysis in the adipose tissue.

Lipolysis is a process that releases fatty acids from the adipose tissue through hydrolysis of triglyceride stores. Decreased adipose tissue mass and the associated lipolysis have been correlated with hepatic steatosis (7), thus we examined the adipose tissue mass and lipolytic rate of HFD-fed WT and CD40<sup>−/−</sup> mice. There was no statistically significant difference in the percent of lean (Figure 2.6 A) or fat mass (Figure 2.6 B) between CD40<sup>−/−</sup> mice and WT controls. However, when examined histologically (Figure 2.6 C), epididymal adipose tissue from CD40<sup>−/−</sup> mice had increased numbers of crown-like structures (CLS). CLS are characterized by infiltrated immune cells, especially macrophages, surrounding necrotic adipocytes (28) and are a hallmark of adipose tissue inflammation, which is associated with increased lipolysis. Indeed, higher basal lipolytic activity was observed in the epididymal adipose tissue from CD40<sup>−/−</sup> mice when compared with the same adipose tissue from WT mice (Figure 2.6 D). These data suggest that increased adipose tissue lipolysis providing increased fatty acid flux to the liver could be a mechanism by which CD40<sup>−/−</sup> mice develop hepatic steatosis.
Figure A: Graph showing the change in lean mass (% of total) over time on a high-fat diet (HFD) for WT and CD40⁻/⁻ mice.

Figure B: Graph showing the change in fat mass (% of total) over time on a high-fat diet (HFD) for WT and CD40⁻/⁻ mice.

Figure C: Images showing muscle sections for WT and CD40⁻/⁻ mice under normal diet (ND) and high-fat diet (HFD) conditions. Arrowheads indicate muscle fiber atrophy.

Figure D: Bar graph showing the effect of isoproterenol on free glycerol levels in WT and CD40⁻/⁻ mice on a high-fat diet (HFD).
Figure 2.6: CD40<sup>−/−</sup> mice have increased immune cell infiltration in adipose tissue as well as elevated adipose tissue lipolysis.

(A) lean mass and (B) fat mass in WT and CD40<sup>−/−</sup> mice fed HFD was measured by magnetic resonance imaging (MRI) analysis as a percentage of total mass over an 11-week period (n=10). (C) Representative sections of the epididymal adipose tissue from ND and HFD fed WT and CD40<sup>−/−</sup> mice were stained with hematoxylin-eosin. Arrows indicate crown-like structures. (Magnification: 200X) (D) Ex<em> vivo</em> lipolysis assay on epididymal adipose tissue explants from HFD fed WT and CD40<sup>−/−</sup> mice (n=6). Adipose tissues were incubated in phenol red-free and serum-free KRH buffer. Glycerol content in the medium was assayed as an index of lipolysis. Epididymal fat pads lipolysis was measured at basal or following stimulation with 10µM isoproterenol for 2 hours at 37°C. Data are presented as mean ± SEM. Statistically significant differences between mouse group WT HFD and CD40<sup>−/−</sup> HFD are indicated (*p < 0.05).
**CD40 depletion increases adipose tissue inflammation.**

Since increased lipolysis and CLS in adipose tissue are often associated with increased infiltration of macrophages and other proinflammatory cells, we analyzed epididymal fat pads from HFD-fed mice for the presence of inflammation. First, we measured the presence of cells expressing macrophage surface markers F4/80 and CD11b in the stromal vascular fraction (SVF) of epididymal fat by flow cytometry and did not detect a difference (Figure 2.7 A). However, the process of isolating SVF involves centrifugation to separate adipocytes from the denser SVF cells. Therefore, lipid-laden macrophage foam cells, which are F4/80+ CD11b+, can be excluded from the SVF because they float to the top and are discarded as part of the adipocyte fraction. As such, we measured the expression of macrophage markers in whole adipose tissue by real time qRT-PCR and found that CD11b was significantly increased in the HFD fed CD40-/- mice (Figure 2.7 B). The macrophage population in the epididymal adipose tissue of CD40-/- mice tends to have higher expression of the proinflammatory integrin CD11c (Figure 2.7 B), suggesting an enhanced M1 macrophage polarization in this fat depot (24). Consistently, macrophage infiltration was also increased in the epididymal adipose tissue of CD40-/- mice compared to their WT littermate controls (data not shown).

Since T cells can also infiltrate adipose tissue in HFD-induced obesity and contribute to metabolic dysfunction, we measured T cell content in the adipose tissue of CD40-/- mice. We observed a 57% increase of CD3+CD90+ cells in the
epididymal SVF of HFD fed CD40$^{-/-}$ mice by flow cytometry (Figure 2.7 C). This increase was confirmed by measuring CD3 expression levels by qRT-PCR (Figure 2.7 D). We also analyzed the mRNA expression of inflammatory cytokines to confirm the elevated inflammation in the epididymal fat depot of CD40$^{-/-}$ mice. Tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), two inflammatory cytokines expressed by activated macrophages and T cells, were both significantly increased (Figure 2.7 E). As increased obesity and inflammation are often associated with increased angiogenesis, we measured endothelial markers CD31 and von Willebrand factor (vWF) expression, which were both increased in the CD40$^{-/-}$ mice (Figure 2.7 F). These results suggest that CD40$^{-/-}$ mice have increased adipose tissue inflammation characterized by increased macrophage and T cell content and inflammatory cytokine expression.
Figure 2.7: CD40 protects against adipose tissue inflammation in obese mice.

(A) After 24 weeks on HFD, the stromal vascular fraction (SVF) was isolated from epididymal adipose tissues of WT and CD40−/− mice and analyzed for CD11b and F4/80 expression by flow cytometry. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). (B) Gene expression of macrophage markers (F4/80, CD68, CD11b, CD11c and MGL-1) from whole epididymal adipose tissues (n=6). (C) SVF from epididymal adipose tissue of WT and CD40−/− mice was analyzed for CD3 and CD90 expression by flow cytometry. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). (D) Gene expression of CD3 from whole epididymal adipose
tissues (n=6). (E) Gene expression of inflammatory genes (IFNγ, TNFα, IL-1β, IL-6, CD40, CD154 and SAA2) from whole epididymal adipose tissues (n=6). (F) Gene expression of CD31 and Von Willebrand factor (vWF) from whole epididymal adipose tissue (n=6). Data are presented as mean ± SEM. Statistically significant differences are indicated p < 0.05, **p<0.01 vs control.
Figure 2.7.2: CD40 protects against adipose tissue inflammation in obese mice. CD40⁻/⁻ mice (n=4) on HFD for 10 weeks were compared to littermate controls (n=4). SVF was isolated from epididymal adipose tissue. (A) Tregs were defined as CD4⁺CD25⁺FoxP3⁺. (B) CD3⁺CD8⁺. (C) NK cells were defined as CD3⁻NK1.1⁺. (D) Macrophages were defined as CD11b⁺F4/80⁺.
CD40⁻/⁻ mice have increased cytotoxic T cell content in the adipose tissue

Many different sub-populations constitute the normal repertoire of T cells in the adipose tissue of obese mice and humans. A previous study suggested that T cells contribute to the recruitment of adipose tissue macrophages and increased CD8⁺ effector T cells precede the accumulation of macrophages (30). Therefore, we investigated the proportion of these different T cells populations in the epididymal adipose tissue of CD40⁻/⁻ and WT mice by flow cytometry. Of all CD3⁺CD90⁺ T cells, the percentage of CD4⁺ cells (T helper cells) was decreased by 29% and the percentage of CD8⁺ cells (cytotoxic T cell) in adipose tissue was about doubled in the CD40⁻/⁻ mice (Figure 2.8A, 2.8B and 2.8D). This increase in CD8⁺ cells was also observed when we used WT littermates as the control group (data not shown). The percentage of CD25⁺FoxP3⁺ regulatory T cells (Tregs), a subgroup of CD4⁺ T cells, was increased by 82% in the CD40⁻/⁻ mice (Figure 2.8C), an increase that was also observed when WT littermates were used as the control group (data not shown). In summary, the absence of CD40 increased overall T cell abundance in adipose tissue and skewed the T cell population of adipose tissue towards increased CD8⁺ cells and decreased CD4⁺ cells (Figure 2.8D).
Figure 2.8. Deficiency of CD40 leads to increased CD8:CD4 ratio and increased Tregs in visceral adipose tissue of obese mice.

(A) After 24 weeks on HFD, the SVF was isolated from epididymal adipose tissue of WT and CD40\(^{-/-}\) mice and CD3\(^{+}\)CD90\(^{+}\) cells were analyzed for CD4 and CD8a expression by flow cytometry. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). (B) Gene expression of CD4 and CD8 in the whole epididymal adipose tissue was measured by qRT-PCR (n=6). (C) SVF was isolated from epididymal adipose tissue of WT and CD40\(^{-/-}\) mice and CD3\(^{+}\)CD90\(^{+}\)CD4\(^{+}\) cells were analyzed for the presence of FoxP3 and CD25 by flow cytometry. Representative dot plots are shown on the left and...
summarized in bar graph on the right (n=8). (D). Schematic pie chart showing the composition of total T cells defined as CD3⁺CD90⁺. Within this subset, Tregs are defined as FoxP3⁺CD4⁺CD25⁺. Data are presented as mean ± SEM. Statistical significances are indicated *p < 0.05. **p<0.01. ***p<0.001 vs control.
CD40−/− mice have increased eosinophil, B cell, NK/NKT cell infiltration in the adipose tissue

The roles of macrophages and T cells in adipose tissue inflammation are well described and recently literature has shown that other immune cell types are also present in obese adipose tissue (8, 31, 47). As such, we analyzed epididymal adipose tissue for the presence of eosinophils (Figure 2.9 A), B cells (Figure 2.9 B), NK and NKT cells (Figure 2.9C) and found that all were increased in CD40−/− mice fed HFD. NK cells were also increased in the epididymal adipose tissue of CD40−/− mice when WT littermates were used as the control group (3. 2.7.2).
Figure 2.9: CD40 deficiency leads to increased eosinophil, B cell and NK/NKT cell recruitment into visceral adipose tissue in obese mice. After 24 weeks on HFD, the SVF was isolated from epididymal adipose tissue of WT and CD40−/− mice and analyzed for expression of (A) CD11b, siglec-f, (B) Gr-1, CD19, (C) CD3 and NK1.1. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). Eosinophils were defined as CD11b+Siglec-f+. B cells were defined as Gr-1−CD19+. NK cells were defined as CD3−NK1.1+. NKT cells were defined as CD3+NK1.1+. Data are presented as mean ± SEM. Statistical significances are indicated *p < 0.05. ***p<0.001 vs control.
Discussion

Contrary to the generally accepted concept that disrupting the CD40-CD40L signaling cascade alleviates inflammation [199, 204], we show here that CD40 deficiency in mice unexpectedly aggravates adipose tissue inflammation in obesity. This enhanced adipose inflammation in CD40 null mice includes increased recruitment of macrophages, CD8+ effector T cells and other immune cells including B cells, eosinophils, NK cells, NKT cells and Tregs (Figure 2.7, Figure 2.8 and Figure 2.9). Elevated immune cell infiltration into this tissue in CD40-/- mice creates a highly inflamed adipose depot characterized by increased levels of inflammatory cytokines (Figure 2.7E) and significantly higher basal lipolysis (Figure 2.6 D). The hydrolyzed FFAs from visceral adipose tissue are known to flow into the circulation via the portal vein and are then taken up by hepatocytes. This likely contributes to the development of the remarkable hepatic steatosis we observed in CD40-/- mice fed HFD (Figure 2.3 D), in the face of the decreased liver inflammation, expected when CD40 is deficient (Figure 2.5 C). With combined adipose tissue inflammation and steatotic liver, CD40-/- mice exhibit dramatic glucose intolerance and systemic insulin resistance (Figure 2.1 D and Figure 2.2).

We found that genetic deficiency of CD40 reduced food intake, which attenuated weight gain (Figure 2.1 B and 2.1 A), observations that were also noted in CD40L deficient animals [179]. These findings imply a common role of the CD40-CD40L axis in the regulation of energy homeostasis. Reduced food
intake and body weight gain often correlate with better insulin sensitivity. Surprisingly, we found aggravated insulin resistance in these CD40^{−/−} mice as assessed by GTT, ITT and hyperinsulinemic-euglycemic clamp measurements (Figure 2.2 and 2.1 D). Of note, age matched C57BL/6J mice were used as wildtype controls for part of this study. These mice are known to be susceptible to diet-induced obesity and display glucose intolerance when fed HFD. The genetic locus underlying this phenotype was mapped to nicotinamide nucleotide transhydrogenase (Nnt) [208, 209]. C57BL/6J mice have a naturally occurring in-frame five-exon deletion in Nnt that removes exons 7-11. Transgenic expression of the entire Nnt gene in C57BL/6J mice rescues their glucose intolerant phenotype [208]. Importantly, the CD40^{−/−} mice used in this study, which are on the C57BL/6Ncr background, having intact Nnt gene, are more insulin resistant than the C57BL/6J controls, an effect that is in the opposite direction of that expected from the genetic background effect, if any exists, suggesting that CD40 plays a critical role in regulating mouse whole body glucose hemostasis.

The decreased steady-state GIR in clamp studies on CD40^{−/−} mice could be caused by reduced efficiency of glucose disposal in tissues (muscle, adipose tissue, liver, heart and brain) as well as elevated hepatic glucose production. Indeed, reduced glucose uptake by brown adipose tissue is at least partly responsible for the overall decreased GIR in CD40^{−/−} mice (Figure 2.1 E). Hepatic insulin resistance (Figure 2.4) in CD40^{−/−} mice accounts for part of their overall systemic insulin resistance phenotype. We observed increased gene expression
of major regulators and enzymes (Figure 2.5 A and 2.5 C) in the
gluconeogenesis pathway and increased hepatic glucose production in CD40⁻/⁻
mice was confirmed by a pyruvate tolerance test (Figure 2.5 B). We were
unsuccessful in detecting elevated hepatic glucose production in CD40⁻/⁻
mice during our glucose clamp study (data not shown), but our study compared
two groups of mice that were on a high fat diet for 16 weeks, therefore even the
control mice were already highly insulin resistant. Thus, suppression of hepatic
glucose output by insulin was already inhibited in the control mice making it
difficult to detect a further significant inhibition for CD40⁻/⁻ mice in the clamp
study.

The infiltration of activated macrophages into obese adipose tissue is
correlated with adipose tissue dysfunction and systemic insulin resistance. CD8⁺
T cells also infiltrate into obese adipose tissue and their depletion improves
systemic insulin sensitivity while adoptive transfer to CD8 null mice aggravates
adipose tissue inflammation [112]. Despite reduced body weight, CD40 deficient
animals on HFD exhibited increased adipose tissue inflammation and lipolysis
(Figure 2.6 C & Figure 2.6 D). In the present study, we observed increased
macrophages and T cells in the adipose tissue of CD40⁻/⁻ mice with T cells
increasingly biased towards CD8⁺ cells (Figure 2.7 and Figure 2.8). Importantly, it
was reported that CD40 deficiency did not affect hematopoietic development or
differentiation; therefore no systemic abnormality in the number and ratio of T
and B cells was detected in these CD40 null mice [164]. Hence, the altered
population of immune cells in the adipose tissue of CD40−/− mice observed here is likely due to changes in the process of immune cell infiltration rather than defects in the development of lymphocytes. In addition to elevated macrophage and T cell infiltration into adipose tissue, we also noted a general increase of other immune cells, including B cells, eosinophils, NK cells and NKT cells (Figure 2.9). Interestingly, Tregs and eosinophils are typically correlated with decreased inflammation in obesity [110, 123]. We believe their presence in the inflamed CD40−/− adipose tissue may play an important compensatory role to neutralize the deleterious effect from the activated macrophage and CD8+ effector T cells. Previous studies on CD40L−/− mice indicated that CD40L deficiency attenuates HFD-induced adipose tissue inflammation [177, 179], which is opposite to what we observed here in CD40−/− mice. However, since CD40L can signal through a non-CD40 pathway [160], differences between CD40−/− and CD40L−/− mouse models are not surprising.

The surprising elevation in the adipose tissue inflammation in CD40−/− mice was accompanied by an expected decrease in hepatic inflammation due to the CD40 deficiency (Figure 2.5 C). The remarkably elevated hepatic steatosis in CD40−/− mice fed a HFD is consistent with two reports on CD40L−/− mice [177, 178] but inconsistent with another [179]. The discrepancy among different studies on CD40L deficient mice could be due to different sources of HFD used in each study. In concert with the morphological changes we observed, the more steatotic livers of CD40−/− mice show significantly higher expression of hepatic
CIDEA and FSP27, two lipid droplet proteins that are not normally expressed in lean livers (Figure 2.3F).

Studies in rodents and humans have revealed that the accumulation of TG observed in hepatic steatosis is mainly due to the increased availability of free fatty acids (FFAs) arising from the visceral adipose tissue through unabated lipolysis as well as increased hepatic lipogenesis. These two pathways appear to account for more than 80% of the fat storage in steatotic livers [191]. In our study, the increased hepatic steatosis in CD40−/− mice may be in part a result of fatty acid overflow from adipose tissue lipolysis (Figure 2.6 D). Chronic exposure of tissues to elevated FFAs is known to induce impaired responsiveness of Akt to insulin and decreased insulin signaling [210]. Indeed, we observed impaired insulin-mediated phosphorylation of Akt (Ser473) in the livers of CD40−/− mice upon acute insulin treatment (Figure 2.4).

How can the deficiency of a proinflammatory receptor protein that normally activates adaptive immunity paradoxically cause elevated inflammation in the adipose tissue of mice? We found increased immune cell infiltration in the adipose tissue of CD40−/− mice but decreased inflammation in the liver, suggesting that the phenotype observed is tissue specific and not due to a global defect of the immune system. One possible mechanism could involve endothelial cells in adipose tissue. Endothelial cells control leukocyte entry into tissues from the vasculature through the expression of adhesion molecules involved in leukocyte rolling and extravasation. CD40 is present on both epithelial and
endothelial cells and different vascular beds express various levels of CD40 [211]. Previous studies demonstrated that the CD40-CD40L dyad affects endothelial cell function and migration during angiogenesis [212, 213]. In our studies presented here, increased expression of endothelial cell markers von Willebrand factor and CD31 in the adipose tissue of CD40−/− mice (Figure 2.7 F) suggests an increased presence of endothelial cells. In contrast, CD40 expression in the vascular beds of the liver is undetectable [211]. Future studies will be needed to test whether adipose endothelial cell disruption of CD40 might lead to the phenotype we observed in CD40 null mice.

In summary, results from the present study indicate that CD40 deficiency exacerbates inflammation in visceral adipose tissue, further promoting the negative metabolic manifestations in obesity, such as insulin resistance and hepatic steatosis. This hepatic steatosis is not associated with localized inflammation. We thus have identified a unique mouse model whereby an apparent primary inflammation and disruption of adipose function leads to hepatic steatosis without liver inflammation. These findings reveal the co-stimulatory signaling CD40-CD40L dyad as an important feature controlling adipose tissue inflammation and its connection to metabolic disease and glucose intolerance.
CHAPTER III: Similarity of Mouse Perivascular and Brown Adipose Tissue and their Resistance to Diet Induced Inflammation

Disclaimer: Experiments were performed in collaboration with the following individuals:

Figures 3.1, 3.2 and 3.3 were performed by Timothy Fitzgibbons. Figure 3.4 which was done by Greg Hendricks; Figures 3.6 and 3.7 were done by the Morphology Core and the microarray hybridizations by the Genomics Core of the Diabetes and Endocrine Research Center (DK-32520). Jeurg Straubhaar uploaded the microarray data to MACE and performed detailed statistical analyses.

Abstract

Thoracic perivascular adipose tissue (PVAT) is a unique adipose depot that likely influences vascular function and susceptibility to pathogenesis in obesity and metabolic syndrome. Surprisingly, PVAT has been reported to share characteristics of both brown and white adipose, but a detailed direct comparison to interscapular brown adipose tissue (BAT) has not been performed. Here we show by full genome DNA microarray analysis that global gene expression profiles of PVAT are virtually identical to BAT, with equally high expression of Ucp-1, Cidea and other genes known to be uniquely or very highly expressed in BAT. PVAT and BAT also displayed nearly identical phenotypes upon immunohistochemical analysis, and electron microscopy confirmed that PVAT contained multilocular lipid droplets and abundant mitochondria. Compared to white adipose tissue (WAT), PVAT and BAT from C57BL6/J mice fed a high fat diet for 13 weeks had markedly lower expression of immune cell-enriched mRNAs, suggesting resistance to obesity induced inflammation. Indeed, staining of BAT and PVAT for macrophage markers (F4/80, CD68) in obese mice showed virtually no macrophage infiltration, and FACS analysis of BAT confirmed the presence of very few CD11b+/CD11c+ macrophages in BAT (1.0%) in comparison to WAT (31%). In summary, murine PVAT from the thoracic aorta is virtually identical to interscapular BAT, is resistant to diet induced macrophage infiltration, and thus may play an important role in protecting the vascular bed from inflammatory stress.
Introduction

The burgeoning prevalence of obesity and diabetes throughout the world threatens to accelerate the incidence of associated cardiovascular diseases, including coronary artery disease, hypertension, and congestive heart failure. Although the mechanism by which obesity causes vascular disease is not fully understood, insulin resistance and the metabolic syndrome are thought to play a major role[2, 8, 186, 214, 215]. In human obesity as well as mouse models of obesity, enlarged adipocytes in visceral adipose tissue become insulin resistant and fail to effectively store excess triglyceride through decreased capacity for lipogenesis and increased lipolysis [2, 8, 214, 216, 217]. These changes in adipose function lead to infiltration of immune cells, which are thought to cause chronic low grade inflammation in obese subjects with the metabolic syndrome [8, 218]. Lipid overload in the face of decreased capacity of adipocytes to store triglycerides leads to ectopic lipid deposition in liver and muscle resulting in systemic insulin resistance [8, 80]. Prolonged obesity with these associated disorders is thought to be the major cause of beta cell failure and type 2 diabetes in humans.

It is now well established that expansion of visceral adipose tissue (VAT), rather than subcutaneous adipose tissue (SAT), confers a high risk for the metabolic syndrome and incident cardiovascular disease [2, 186, 219]. Hypotheses for this observation include: increased expression of angiotensinogen and complement, a greater rate of monocyte infiltration, and
increased IL6 secretion in VAT compared to SAT [220]. It has also been postulated that the elevated rates of lipolysis in VAT compared to SAT render the former less able to sequester triglycerides away from liver and muscle [221]. These and other important differences between VAT and SAT have stimulated interest in the discovery of additional adipose depots, which might have similar or increasingly pathologic functions in obesity. One such adipose depot is perivascular adipose tissue (PVAT), which was recently proposed to be a potential link between diabetes and cardiovascular disease [222-226]. The outermost connective tissue or adventitia surrounding an artery, including PVAT, may be prone to the same inflammation as VAT, and the idea that vascular disease could be impacted and perhaps promoted by factors in the adventitia has long-standing support [227-230].

Initial studies of PVAT from human coronary arteries surprisingly detected expression of some BAT-specific genes (PRDM16, UCP-1, CPT1B) to a degree that appeared intermediate to white and brown adipose tissues [222]. These studies suggested PVAT might be an example of the recently described “brite” adipose tissue--white adipose that retains many of its usual characteristics but also displays some expression of BAT genes, including Ucp-1 [231]. Interestingly, in rodents, PVAT surrounding the abdominal aorta displays characteristics of white adipose tissue, whereas PVAT surrounding the thoracic aorta appears quite different, and is known to express some BAT genes [26, 225]. Although thoracic PVAT showed similarities to BAT in morphology and
expressed *Ucp-1*, a direct detailed genomic comparison to true interscapular BAT has not yet been made [26, 225]. The extent to which thoracic PVAT in mice is more similar to human coronary PVAT, which displays only partial similarity to BAT, or to true interscapular BAT is an important question since mice are frequently used as models of human cardiovascular disease.

We addressed this question by directly comparing genome wide expression of thoracic PVAT to brown and white adipose depots from mice fed a normal or high fat diet for 13 weeks. Remarkably, PVAT and BAT had virtually identical gene expression profiles. Furthermore, in comparison to the infiltration of macrophages into VAT of obese mice and the increased expression of macrophage enriched genes in this tissue, thoracic PVAT and BAT were strikingly resistant to inflammation induced by high fat feeding, as shown by microarray, immunohistochemistry, and FACS analysis. We conclude that PVAT surrounding the murine thoracic aorta is virtually identical to interscapular BAT, and like BAT is resistant to inflammation under HFD conditions.
Materials and Methods

**Animal Studies**

Male C57BL6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were fed normal chow until 8 weeks of age. Animals were then divided into two groups; one fed normal chow and one fed high fat diet (45 kcal% fat, D12451 Research Diets) for 13 or 20 weeks. Animals were fed *ad libitum* with free access to water and housed in the UMASS Animal Medicine facility with a 12:12 light dark cycle. Animals were weighed weekly for the duration of the diet study. Intraperitoneal (i.p.) glucose tolerance testing (GTT) was performed as previously described [232]. Area under the curve for the GTT was calculated using the trapezoidal method [233]. At the completion of the high fat diet, mice were fasted for 6 hours and then euthanized with CO2 inhalation and bilateral pneumothorax. SAT (inguinal), VAT (epididymal) and BAT(interscapular) were harvested and snap frozen in liquid nitrogen. Blood was drawn via cardiac puncture into heparin coated tubes and the circulatory system was perfused with ice cold PBS. Thoracic PVAT directly adjacent to the lesser curvature of the aortic arch was then harvested and snap frozen in liquid nitrogen. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee at UMASS Medical School.
**Insulin levels**

Blood was collected via cardiac puncture at the time of euthanasia into heparin coated microcentrifuge tubes and centrifuged at 3000 x g for 15 minutes. Serum was then decanted and stored at -80 °C. Fasting insulin levels were measured in duplicate with 10 µl of serum using a Rat/Mouse Insulin ELISA (Millipore, St. Charles, MO) according to the manufacturers’ instructions.

**Quantitative PCR**

Adipose tissue was isolated as previously described, snap frozen in liquid nitrogen and stored at -80°C. Tissues were homogenized and total RNA was isolated with RNA Mini Lipid kits (Qiagen, Valencia, CA). 250 ng total RNA was reverse transcribed with the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). cDNA was diluted 1:5 and 2.5 µl was used in a 12.5 µl reaction volume; each reaction was performed in duplicate. Real time qPCR was performed with a Bio-Rad C1000 Thermal Cycler and SYBR Green Master Mix (Bio-Rad, Hercules, CA) using the following cycle parameters: 95.0° C for 3 min, 95.0° for 0:10 min, 60.0° C for 0:15 min, 72.0° C for 0:30 min for 40 cycles. Expression was normalized to the reference gene 36b4 and expressed as relative to expression in SAT of normal diet mice using the 2^{−ΔΔct} method [207]. Melt curve analysis and agarose gel electrophoresis was performed to determine the specificity of the PCR reaction products. Primer sequences were: Ucp-1 for AGGCTTCCAGTACCATTAGGT, Ucp-1 rev CTGAGTGAGGCAAAGCTGATT.
**Microarray Analysis**

RNA was isolated from SAT, VAT, BAT, and PVAT as previously described. RNA concentrations were determined using a Nanodrop 2000 Spectrophotometer (Thermo Fisher, Willmington, DE). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with a RNA Integrity Number >7.5 and normal 18 and 28s fractions on microfluidic electrophoresis were used. RNA from two mice per tissue and diet was pooled for a total of 250 ng total RNA template for cDNA synthesis and *in vitro* transcription using the Ambion WT Expression kit (Ambion, Carlsbad, CA). Second strand cDNA was then labeled with the Affymetrix WT Terminal Labeling kit and samples were hybridized to Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). Gene chip expression array analysis for individual genes was performed as previously described [233], filtering for *p* <0.05 and a fold change of >2. Three biological replicate hybridizations per tissue and diet were performed, for a total of 24 hybridizations. Robust multi-

Acrp30 for TGTTCCTCTTAATCCTGCCA and Acrp30 rev CCAACCTGCACAAGTTCCCTT, 36b4 for TCCAGGCTTTGGGCATCA, 36b4 rev CTTTATCAGCTGCACATCAGA, Cidea for ATCACAActGGCCTGGTTACG, Cidea rev TACTACCCGGTGTCACATTcct, PPARγ2 for ATGGGTGAAACTCTGGGAG, and PPARγ2 rev GTGGTCTTCCATCACGGAGA.
array average (RMA) was adopted in the UMASS Microarray Computational Environment (MACE) to preprocess raw oligonucleotide microarray data. The algorithm was implemented as a function of the R package Affy [234], which is part of the Bioconductor project [235] using the statistical computing language R (R Foundation for Statistical Computing, Vienna, Austria). All statistical calculations are performed using the R statistical computing environment and results are stored in a relational database. The preprocessed data are stored as base 2 log transformed real signal numbers and are used for fold-change calculations and statistical tests and to determine summary statistics. Mean signal values and standard deviations are computed for each gene across triplicate experiments and stored in the database. The fold change of expression of a gene in two experiments is the ratio of mean signal values from these experiments and is always a number greater than one. If the ratio is less than one, the negative value of the inverse ratio is stored as fold change. All down regulated genes therefore have a negative fold change value, up regulated genes have a positive fold change. In both cases this value is greater or equal than one.

To determine differential expression of genes in two hybridization experiments MACE internally conducts a Student's t-test with the expression signal values of the two hybridizations for all genes in the set. The t-test value and test p-value are stored in MACE and can be queried through the MACE user interface. The p-values stored and displayed as a result of a query are not
adjusted for multiple testing. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [236] and are accessible through GEO Series accession number GSE28440 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28440).

**Histology and Immunohistochemistry**

Adipose tissue samples (n=3 per group) from normal and high fat diet animals were fixed in 4% formalin for immunohistochemistry. Briefly, samples were embedded in paraffin, sectioned, and stained with a rat anti-mouse F4/80 (ABd Serotec, Raleigh, NC)(1:50 dilution) or rat anti-mouse CD68 primary antibody (ABd Serotec, Raleigh, NC)(1:40 dilution). Staining was visualized with a HRP linked rabbit anti-rat secondary anti-body. Staining with the secondary antibody alone was performed as a negative control. Images were taken with a Zeiss Microscope and PixeLINK SE Software.

**Transmission electron microsopy of adipose tissue samples**

Adipose tissue samples were fixed in 2.5% glutaraldehyde in PBS (pH 7.2). Tissue samples were dissected into 1 mm blocks and the tissues washed three times and left overnight in fresh buffer. The next morning the samples were post-fixed for 1 hr in 1% osmium tetroxide. The fixed adipose tissue samples were then washed again in the PBS and dehydrated through a graded series of ethanol to 100% and transferred through two changes of propylene
oxide and finally into a 50:50 (v/v)mixture of propylene oxide: epoxy resin (SPON 812/Araldite 502) and left overnight to infiltrate. The following morning the adipose tissue samples were processed through two changes of fresh epoxy resin and embedded, allowing the blocks to polymerize 48 hrs at 70°C. Blocks of tissue were then selected, cut out and attached to blank epoxy stubs with a drop of Super Glue. Ultrathin sections were cut on a Reichart-Jung ultratome using a diamond knife. The sections (64 nm thick) were collected and mounted on copper support grids and contrasted with lead citrate and uranyl acetate and examined on a Philips CM 10 transmission electron microscope at 80 Kv accelerating voltage.

**FACS Analysis**

Mice were euthanized and BAT and VAT was removed and treated with 4 mg/mL and 2 mg/mL collagenase respectively in 4% BSA in PBS at pH=7.4 for 1 hour. Samples were filtered through 200µM and 30 µM spectra mesh sequentially and stained with CD31 (BD Biosciences, Franklin Lakes, NJ; 1:1000 dilution), CD11b (BD Biosciences, Franklin Lakes, NJ; 1:200 dilution) and CD11c (BD Biosciences, Franklin Lakes, NJ; 1:200 dilution) according to the manufacturer’s instructions. Samples were run on a BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed in Flow Jo, gating for CD31 negative cells.
**Statistical Analysis**

All values are shown as mean ± SEM. For experiments other than the microarray analyses, the Student’s *t* test for two tailed distributions with equal variances was used for comparison between 2 groups; for comparisons greater of ≥ 3 groups, two way ANOVA followed by the Bonferroni correction was used. Differences less than *p* ≤ 0.05 were considered significant. Data was entered into Microsoft Excel and statistical analyses were performed with Graph Pad Prism 5.
RESULTS

_Cidea and Ucp-1 are highly expressed in BAT and PVAT independent of obesity._

HFD mice (n=12) gained more weight than ND mice (n=12) beginning at 3 weeks and remained significantly more obese until the end of the study (30.9 ± 0.75 vs. 42.08 ± 0.78 gms p<0.001). At the end of 13 weeks, HFD mice were more glucose intolerant as shown by an elevated fasting blood glucose concentration (136.4 ± 1.22 vs. 185.8 ± 21.4 mg/dL p<0.02), elevated fasting insulin levels (2.1 ± 0.64 vs. 5.4 ± 1.03 ng/mL p<0.02), and increased area under the curve for the IPGTT ((min*mg/dL) =29352 ± 753 vs. 38983 ± 1928 p<0.01).

Analysis of mRNA from adipose tissue samples by qPCR showed that _Cidea_ and _Ucp-1_ were highly expressed in PVAT and BAT compared to SAT. Remarkably, the relative expression between these two depots was similar for both genes (Figure 3.1 A, B). Interestingly, _Cidea_ expression was reduced >50% by HFD in both PVAT and BAT; this effect was not observed for _Ucp-1_ (Figure 3.1 A, B). With respect to other markers of adipose differentiation, _PPARγ2_ expression was slightly greater in VAT, PVAT, and BAT than SAT and significantly reduced by HFD in these depots (Figure 3.1 D). Adiponectin (_Acrp30_) expression was significantly greater in VAT and decreased with HFD (Figure 3.1 C); expression of this gene was similar in all other depots. Therefore, PVAT expresses _Cidea_ and _Ucp-1_ to an equivalent degree as BAT, suggesting that it may be more similar to BAT than “brite” adipose tissue. Furthermore, it is
responsive to HFD and not different from other adipose depots with respect to
makers of adipose differentiation, such as PPARγ and Acrp30.
Figure 3.1: Cidea and Ucp-1 are highly expressed in interscapular brown adipose tissue (BAT) and perivascular adipose from the aortic arch (PVAT) independently of obesity. A, B, C, and D: Cidea, Ucp-1, Acrp30, and Ppary2 expression in normal diet (ND) and high-fat diet (HFD) mice. Quantitative PCR was performed on total RNA isolated from inguinal (SAT), epididymal (VAT), BAT, and PVAT. Expression levels were calculated with the 2−ΔΔCt method using 36b4 as the reference gene and normalized to expression in SAT from ND conditions. Ucp-1 expression is shown in log10 scale. AU, arbitrary units. ND: n = 12 white; HFD: n = 12, black. Results are means ± SE. ***P < 0.001 vs. SAT ND; #P < 0.05. ND vs. HFD of the same fat depot using two-way ANOVA and the Bonferonni correction; P < 0.05 vs. SAT ND.
**Mouse Thoracic PVAT is morphologically similar to BAT.**

In order to test the hypothesis that thoracic PVAT is virtually identical to interscapular BAT, we performed microscopic analysis of these adipose depots. As judged by light microscopy on hematoxylin and eosin stained sections of PVAT and BAT under ND and HFD conditions, PVAT appeared very similar to BAT, with round nuclei, and small, multilocular lipid droplets (Figure 3.2 G). The majority of PVAT surrounding the thoracic aorta had the appearance of BAT, with small areas of adipose tissue resembling WAT (Figure 3.2 G). BAT lipid droplet architecture was obviously distorted by 13 weeks of HFD, with enlargement and coalescence of lipid droplets (Fig. 3.2 F). Surprisingly, this effect was not apparent in PVAT, although it did occur with a longer duration of HFD (Figure 3.6 H).

Transmission electron microscopy confirmed the BAT phenotype of thoracic PVAT, and detected some interesting differences compared to interscapular BAT (Figure 3.3). Both PVAT and BAT from mice fed ND were densely packed with mitochondria and had multilocular lipid droplets which were darkly stained with osmium tetroxide (Figure 3.3 A, C). Interestingly, lipid droplets in BAT tended to lose their avidity for osmium when obtained from mice after high fat feeding, mostly appearing white (Fig. 3B). This effect was not observed in PVAT (Figure 3.3 D). High fat feeding resulted in significant mitochondrial swelling and unfolding of cristae in PVAT (Figure 3.3 D), which was not apparent in BAT at this time point, but visible after a longer duration of HFD (Data not
shown). These observations correlated with changes in gene expression. For example, *Fads1* was significantly downregulated (-1.82) in BAT compared to PVAT in HFD conditions. Likewise, a search for alterations in the expression of mitochondrial genes revealed that expression of the proapoptotic gene *Bid* and mitochondrial solute carrier genes *Slc25a35* and *Slc25a37* were upregulated in PVAT compared to BAT in HFD conditions; a third member of this latter gene family, the calcium binding carrier protein *Aralar1 (Slc25a12)*, was significantly upregulated in BAT compared to PVAT (2.58 FC, p=0.002, Supplemental Table S4). Therefore, thoracic PVAT and BAT appear virtually identical upon analysis by both light and electron microscopy, with the exception of subtle differences in lipid droplet and mitochondrial morphology, which correlate with changes in gene expression.
Figure 3.2: PVAT appears morphologically similar to BAT. Fat was harvested from SAT (A and B), VAT (C and D), interscapular BAT (E and F), and PVAT (G–J) from the lesser curvature of the aortic arch from ND and HFD fed mice and then fixed in formalin. Tissues were stained with hematoxylin and eosin and visualized at ×25. Images G and H are low magnification images (×6.3) of I and J. Ao, aortic lumen.
Transmission electron microscopy reveals many similarities between PVAT and BAT. Sections of brown (A and B) and perivascular adipose (C and D) were taken and stained with osmium tetroxide. In ND conditions, brown (A) and perivascular adipose (C) appear very similar with multilocular lipid droplets and abundant mitochondria. Two prominent changes were noted in high-fat feeding conditions; lipid droplets in BAT, but not PVAT, lose their avidity for osmium tetroxide (B), and mitochondria become swollen with unfolded cristae (D); the latter effect was more prominent in perivascular adipose but also observed in brown adipose after a longer duration of HFD. LD, lipid droplet; M, mitochondria; EC, endothelial cell; RBC, red blood cell. Magnification: ×7900; scale bar = 2 µm.
Microarray analysis confirms PVAT has a characteristic brown adipose gene expression signature.

To validate and expand the above findings, DNA microarrays were used to contrast global differences in gene expression between adipose depots from mice under control and high fat feeding conditions. Total RNA was hybridized to Affymetrix Mouse Gene 1.0 ST chips, each of which contains probes targeting 28,853 genes. Analysis of the full genome set of gene expression differences among the four adipose depots showed high similarity of PVAT to BAT, and a much greater divergence of gene expression when compared to white adipose depots (SAT, VAT) (Figure 3.4, Table 3.1). When comparing PVAT to BAT in normal diet conditions, only 228 genes demonstrated significantly different expression (Figure 3.4). With regard to the specific genes, which were differentially expressed, in comparison to BAT, PVAT had greater expression of immunoglobulin genes (IgK, lgh-6), complement (Cfh, C7), and mast cell specific genes (Mcpt4) (not shown). In contrast, the vast majority of genes whose expression was greater in BAT than PVAT were related to skeletal muscle differentiation and function. Interestingly, additional genes expressed to a greater extent in BAT than PVAT were highlighted by the mesodermal developmental genes Tbx15 (3.8 fold), Myf6 (3.2 fold), and Zic1 (2.1 fold) (not shown).
Next, we refined this analysis by comparing gene expression differences in PVAT relative to BAT and SAT from mice fed ND with respect to selected specific gene categories (Table 3.1). Strikingly, there were no significant differences between PVAT and BAT in levels of expression of any genes known to be highly expressed in brown adipocytes (Table 3.1). Nor were there any differences between PVAT and BAT under ND conditions in the expression of the majority of specific genes examined, with the exception of \textit{Fads1} (Table 3.1). In contrast to these virtually identical gene expression profiles between PVAT and BAT, highly significant decreases in expression were displayed by brown adipocyte enriched genes in SAT and VAT compared to PVAT (Table 3.1). As expected, expression of white adipose enriched genes was significantly greater in SAT and VAT compared to PVAT (Table 3.1). In other gene categories, expression of the adipokines \textit{Lep}, \textit{Retn}, \textit{Rbp4}, and \textit{Adipoq} was higher in SAT and VAT, whereas the receptors for \textit{Adipoq} (\textit{Adipor1}/\textit{Adipor2}) were more highly expressed in PVAT (Table 3.1). In regards to lipid metabolism genes, those involved in synthesis or storage (\textit{Fads1}, \textit{Fads3}, \textit{Cidec}) were expressed to a greater extent in SAT and VAT, whereas those functioning in lipid oxidation (\textit{Gpam}, \textit{Acss1}, \textit{Acsl5}) were more highly expressed in PVAT and BAT (Table 3.1). Surprisingly, expression of immune cell enriched genes, such as chemokines (\textit{Ccl5}, \textit{Ccl8}), T cell receptors (\textit{Cd3g}, \textit{Cd3d}), and macrophage markers (\textit{Cd68}, \textit{Emr1}) was significantly greater in SAT and VAT compared to PVAT and BAT even under ND conditions (Table 3.1). Given this dramatic difference in
expression of immune cell genes in white compared to brown adipose depots, we compared the effects of high fat feeding on changes in expression of immune cell enriched genes in the four adipose depots.

Shown in Table 3.2 are the fold changes in expression for a series of selected immune cell enriched genes when comparing ND to HFD conditions in PVAT, BAT, SAT and VAT depots. VAT demonstrated the greatest fold increase in expression of immune cell genes after high fat feeding (Table 3.2). Although the other depots exhibited some trends for changes in gene expression between ND and HFD fed mice, none of them met statistical significance (Table 3.2). Taken together, the data in Figure 3.4 and Tables 3.1 and 3.2 reveal a remarkably similar global gene expression profile between PVAT and BAT. These two adipose depots show no significant differences in expression in the majority of genes previously shown to be enriched in brown adipocytes, white adipocytes or immune cells. In addition, the data in Table 3.2 is particularly revealing in that the levels of expression of immune cell genes, as reflected by their probe set signals, is uniformly higher in both SAT and VAT compared to PVAT and BAT in both ND and HFD conditions. These data imply that the PVAT and BAT depots are relatively resistant to inflammation induced by high fat feeding compared to WAT depots.
Figure 3.4: Microarray analysis reveals that PVAT is more similar to BAT than SAT or VAT. Microarray Computational Environment (MACE) database was queried in ND and HFD conditions for genes with a >2.0 fold change in expression at $P < 0.05$ level of significance between adipose depots. In ND conditions, PVAT was very similar to BAT, with differential expression of only 228 genes (0.79% of genes); in contrast, expression of 1,229 genes was differentially regulated when comparing PVAT and SAT (4.2% of genes). After high-fat feeding, PVAT became more similar to white adipose, as the number of genes differentially regulated between PVAT and SAT was reduced to 855 (2.9% of genes). White arrows, upregulated; black arrows, downregulated.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>PVAT vs. SAT</th>
<th>PVAT vs. VAT</th>
<th>PVAT vs. BAT</th>
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<td></td>
<td>FC</td>
<td>P value</td>
<td>FC</td>
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<td>29.3</td>
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<td>Mest</td>
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<td>&lt;0.01</td>
<td>-14.2</td>
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Table 3.1: Depot-specific expression of select gene categories in normal diet conditions. The eight most highly up-regulated genes in PVAT vs. SAT in normal diet conditions were ranked in descending order. Expression levels of the same genes in PVAT vs. VAT and PVAT vs. BAT in normal diet conditions were then queried, and are included in the last two columns for comparison. Expression of genes from select categories was also queried in normal diet conditions, and ranked in descending order according to expression in SAT. Genes with significantly increased expression in PVAT are in bold and genes with significantly decreased expression in PVAT are italicized. NS = not-significant.

<table>
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<td>Cd3g</td>
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</tr>
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<td>Ccr7</td>
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</tr>
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</tr>
<tr>
<td>Cd68</td>
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</tr>
<tr>
<td>Emr1</td>
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<table>
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<tr>
<td>Fads1</td>
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<tr>
<td>Cidec</td>
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</tr>
<tr>
<td>Scd1</td>
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</tr>
<tr>
<td>Fasn</td>
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<tr>
<td>Gpam</td>
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<tr>
<td>Acss1</td>
<td>3.2</td>
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<tr>
<td>Acsl5</td>
<td>3.7</td>
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<table>
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<tr>
<td>Retn</td>
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<td>IL6st</td>
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<td>Adipoq</td>
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<td>Tnfaip6</td>
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<td>Adipor1</td>
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<td>Adipor2</td>
<td>1.4</td>
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</table>
Table 3.2: Comparative expression of immune cell enriched genes in VAT, SAT, PVAT and BAT in normal and high fat diet conditions.

| Symbol | SAT | | | VAT | | | | | PVAT | | | | | BAT | | |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|        | ND  | HFD | FC  | P   | ND  | HFD | FC  | P   | ND  | HFD | FC  | P   | ND  | HFD | FC  | P   |
| Il7r   | 2038| 1299| -1.5| NS  | 268 | 2779| 10.4| <0.001| 178 | 350 | 1.9 | NS  | 62  | 164 | 2.6 | NS  |
| Tlr13  | 564 | 531 | -1.1| NS  | 397 | 2404| 6.0 | <0.001| 133 | 343 | 2.6 | NS  | 151 | 243 | 1.6 | NS  |
| Il1r   | 444 | 499 | 1.1 | NS  | 367 | 1923| 5.2 | <0.001| 220 | 403 | 1.8 | NS  | 208 | 269 | 1.2 | NS  |
| Cd84   | 665 | 574 | -1.2| NS  | 426 | 2079| 4.9 | <0.001| 128 | 281 | 2.2 | NS  | 92  | 171 | 1.9 | NS  |
| Mpeg1  | 1527| 1292| -1.2| NS  | 713 | 3311 | 4.6 | <0.001| 240 | 555 | 2.3 | NS  | 233 | 520 | 2.2 | NS  |
| Cd68   | 2434| 2160| -1.1| NS  | 2097| 6895| 3.3 | <0.001| 586 | 1451| 2.5 | NS  | 500 | 914 | 1.8 | NS  |
| Ccl3   | 303 | 329 | 1.1 | NS  | 207 | 662 | 3.2 | <0.001| 111 | 194 | 1.7 | NS  | 116 | 182 | 1.6 | NS  |
| Emr1   | 1247| 1177| -1.1| NS  | 1276| 3800| 3.0 | <0.001| 283 | 604 | 2.1 | NS  | 242 | 475 | 2.0 | NS  |
| Ccl2   | 536 | 556 | 1.0 | NS  | 546 | 1174| 2.1 | <0.05  | 158 | 225 | 1.4 | NS  | 138 | 185 | 1.3 | NS  |
| Ccl9   | 1457| 1691| 1.1 | NS  | 1529| 3262| 2.1 | <0.01  | 347 | 780 | 2.2 | NS  | 249 | 467 | 1.8 | NS  |
| Tnfrsf1b | 911 | 735 | -1.2| NS  | 682 | 1183| 1.7 | <0.01  | 226 | 304 | 1.3 | NS  | 184 | 256 | 1.4 | NS  |
Thoracic PVAT and interscapular BAT are relatively resistant to macrophage infiltration under HFD conditions.

In order to directly test the extent of macrophage infiltration into BAT and PVAT, we immunostained sections of SAT, VAT, BAT and PVAT from the thoracic aorta for the macrophage marker F4/80 (Figure 3.5). Feeding mice a HFD for 13 weeks resulted in significant macrophage infiltration in VAT and SAT, as expected (Figure 3.5 B, D). In contrast, little or no F4/80 staining was detected in BAT or PVAT from these same mice (Figure 3.5 F, H). In order to test whether a longer duration of HFD would result in inflammation of BAT or PVAT, we continued HFD for 20 weeks in a second cohort of animals (Fig. 6). Again, visceral fat showed abundant macrophage infiltration (Figure 3.6 D), but despite marked enlargement and coalescence of lipid droplets in BAT and PVAT, macrophages were absent (Figure 3.6 F, H). The same results were obtained after staining BAT and VAT from animals fed a HFD for 20 weeks with a second macrophage marker, CD68 (Data not shown).
Figure 3.5: PVAT and BAT are resistant to inflammation after 13 wk of HFD. SAT (A and B), VAT (C and D), BAT (E and F), and PVAT (G and H) from the aortic arch was harvested from lean and obese mice (n = 3 per group). Samples were fixed in 4% formalin, sectioned, and stained with a rat anti-mouse F4/80 primary antibody (ABd Serotec). Staining was visualized with a horseradish-peroxidase-linked rabbit anti-rat secondary antibody. Abundant macrophages
were seen predominantly in VAT, but also SAT, forming crown-like structures (arrowheads). No macrophages were seen in BAT or PVAT (magnification: ×25).
Figure 3.6: Perivascular and brown adipose tissue are resistant to inflammation after 20 wk of HFD. A second cohort of mice was continued on HFD for 20 wk. SAT (A and B), VAT (C and D), BAT (E and F), and PVAT (G and H) from the aortic arch were harvested from lean and obese mice (n = 3 per group). Samples were fixed in 4% formalin, sectioned, and stained with a rat anti-mouse F4/80 primary antibody (ABd Serotec). Staining was visualized with a HRP linked rabbit anti-rat secondary antibody. Again, abundant macrophages were seen in VAT (D). No macrophages were seen in BAT (F) or PVAT (H) despite distortion and enlargement of lipid droplet morphology (magnification: x25).
**FACS analysis confirms prolonged HFD in mice results in little macrophage infiltration of BAT compared to VAT.**

In order to better characterize the levels of macrophage accumulation in VAT versus BAT, we performed FACS analysis on the stromal vascular fraction (SVF) of collagenase digested adipose tissue from VAT and BAT in ND and HFD conditions. CD68 and F4/80 markers define total macrophage populations in adipose tissue, but do not differentiate between cells at various levels of activation [106, 194, 237]. Therefore, after exclusion of CD31+ endothelial cells from the SVF, we chose to doubly stain cells for CD11b and CD11c, the latter being a marker of classical (M1) macrophage differentiation [237]. Eleven weeks of HFD resulted in a striking and significant enrichment of CD11b+/CD11c+ macrophages in VAT compared to that observed for BAT (31.7% vs. 1.03%)(Fig. 7A). As we failed to observe macrophage recruitment into BAT with 11 weeks of HFD, we extended the duration of the HFD to 20 weeks (Figure 3.7B). This prolonged HFD duration resulted in a marked, significant increase in CD11b+/CD11c+ macrophages in VAT, while BAT showed no enrichment of this cell population. Unfortunately, we were unable to perform FACS on PVAT due to the small quantity of this tissue in the aortic arch. However, the results of FACS analysis confirm that VAT is enriched with M1 macrophages after 11 and 20 weeks of HFD, while BAT was resistant to this effect.
Figure 3.7: BAT is resistant to inflammation after 11 and 20 wk of HFD. A: after 11 wk of HFD, the stromal vascular fraction was isolated from VAT and BAT and stained with CD31-PE, CD11b-PerCp Cy5.5, and CD11c-PE Cy7. Samples were analyzed on a LSRII flow cytometer, gating for CD31-negative cells. High-fat feeding resulted in a significant increase in the percentage of CD11b- and CD11c-positive cells in the SVF of visceral but not brown adipose (31.7 vs. 1.03%). B: similar results were obtained after 20 wk of HFD. Results represent 3 independent experiments.
DISCUSSION

A major finding of the present studies is that PVAT from the murine thoracic aorta displays a gene expression pattern that is nearly identical to interscapular BAT (Fig. 3.4, Table 3.1). The similarity of gene expression between PVAT and BAT includes identical expression levels of the brown adipocyte genes *Cidea, Ucp-1, Dio2,* and *Prdm16.* Only 0.79% of genes represented on chip were differentially regulated between PVAT and BAT in normal diet conditions at the stringency level used. Interestingly, immunoglobulin genes were expressed to a greater extent in PVAT; this has also been demonstrated in human coronary PVAT [238]. We hypothesize that this due to the presence of vascular associated lymphoid tissue [239], although we have been unable to visualize such tissue using B cell specific markers (Data not shown).

In contrast to these subtle differences between PVAT and BAT, major differences between PVAT and WAT were observed in the expression of genes previously shown to be highly and selectively expressed in brown or white adipose tissues (Table 3.1). Our data thus clarify and definitively substantiate previous indications that mouse thoracic PVAT is similar to BAT[25, 26, 225]. Importantly, these previous studies demonstrate that PVAT can have characteristics of both BAT and WAT, but this dichotomy largely depends upon the anatomical context; that surrounding the abdominal aorta resembling WAT and that surrounding the thoracic aorta BAT [26, 225]. Our studies directly
comparing thoracic PVAT gene expression (Table 3.1, and Figure 3.4) and histological appearance (Figure 3.2) to mouse intrascapular BAT show unequivocally that these two adipose depots are virtually identical. This appears different from human coronary PVAT which demonstrates an intermediate phenotype between white and brown adipose [222].

After 13 weeks of high fat feeding, VAT displayed the expected dramatic increase in expression of many immune cell enriched genes, while thoracic PVAT and BAT did not. This was somewhat surprising because some previous reports indicated that increased infiltration of macrophages and T cells into both PVAT [220, 225, 226] and BAT [92, 240] could be detected in mouse models. Xu et al. reported that genetic and diet induced obesity resulted in chronic inflammation in WAT but not BAT[194]. Thus the extent of BAT inflammation may greatly depend upon the mouse strain and conditions used. Our data are consistent with the results of Xu et al. suggesting BAT is comparatively resistant to inflammation in C57BL6/J mice after HFD feeding.

In contrast to our present findings of very low inflammation of PVAT in mice after 13 weeks of HFD, some investigators have proposed that PVAT is an intrinsically pro-inflammatory depot [222, 224, 238]. For example, humans with established coronary artery disease have higher mRNA and protein levels of IL-1β, IL-6, MCP-1, and TNFα in epicardial adipose than in paired SAT samples. More recently, Chatterjee et al. published data regarding human coronary PVAT and murine thoracic PVAT, the same anatomic fat depot we have studied here.
In control mice, relative mRNA expression of Acrp30, Pparγ and Fabp4 was lower in PVAT than SAT. With two weeks of HFD feeding, expression of these adipocyte specific genes further declined, while expression of the pro-inflammatory genes Lep and Mip1α increased [222]. The authors concluded that in comparison to SAT, PVAT was poorly differentiated and intrinsically pro-inflammatory, and hence possibly an etiologic factor in the development of vascular disease [222].

Our findings are in contrast to the above [222] in that expression of Acrp30 and Pparγ2 was not different in thoracic PVAT compared to other fat depots. A possible explanation for the difference in expression of Acrp30 and Pparγ2 between the two studies is that there may be diet or age dependent changes in the expression of these two genes; Chatterjee et al. used a shorter duration HFD and younger mice [222]. For example, it may be that younger and older mice than those studied herein (21 weeks) are prone to inflammation of PVAT. Two studies which have reported obesity associated inflammation of abdominal and femoral PVAT, used 40 week and 22 week old mice respectively[225, 226]. Therefore it is likely that many factors including age, strain, diet, and specific anatomic location influence whether or not PVAT becomes inflamed. Finally, although they did not report expression of BAT genes in their mouse model, their failure to observe significant expression of immune cell specific genes Cd68 and Cd3 by qPCR does concur with our results [222].
Importantly, both we and Chatterjee et al. have studied PVAT from the thoracic aortic arch, as this a segment of the aorta prone to atherosclerosis both in humans and mice [222]. Although PVAT was originally hypothesized to signal in a “vasocrine” fashion to influence vascular tone [23], it has subsequently been implicated in adventitial inflammation which might promote atherosclerosis [23, 220, 222, 224, 238]; our study was designed to test these latter hypotheses.

The overall evidence from the literature combined with our present work suggests that PVAT can have different characteristics depending upon its anatomical location. Thus BAT-like adipose surrounds the thoracic aorta and WAT surrounds the abdominal aorta [26, 225]. As such, we suggest that PVAT surrounding the abdominal aorta, like its visceral fat counterpart, is prone to the dysregulated adipocyte biology of obesity and subsequent inflammation. This idea has been verified by Takaoka et al., who demonstrated that PVAT surrounding the femoral artery is WAT that has beneficial properties in lean conditions which are mediated by the paracrine effects of adiponectin (Acrp30)[226]. In obese states, inflammation and macrophage infiltration of PVAT surrounding the femoral artery results in decreased adiponectin secretion and increased Tnfa expression, both of which facilitate pathological neointimal hyperplasia in response to vessel injury [226]. In humans, neointimal hyperplasia contributes to the pathophysiology of coronary artery disease. For example, diabetes and obesity are associated with increased rates of in-stent restenosis following percutaneous intervention (PCI) due to neointimal hyperplasia [241].
A scenario in which white PVAT is beneficial in lean conditions, but becomes dysfunctional in obese conditions, is analogous to the current model of obesity induced insulin resistance [8]. These and other reports support the concept that PVAT with white adipose features conforms to this model [223, 225, 226, 242]. The important contribution of our work is that brown PVAT surrounding the thoracic aorta appears to be resistant to obesity induced inflammation, and hence may offer protection from the associated changes to the arterial adventitia.

The finding that PVAT and BAT display little or macrophage infiltration under HFD conditions, while WAT does, raises the interesting question: what is unique to WAT that causes infiltration of macrophages? A recent paper by Kosteli et al. may shed insight into this important question [80]. Using caloric restriction of previously HFD fed mice, the authors showed that rates of adipocyte lipolysis correlate with macrophage infiltration. These studies suggest that the chronic elevation in WAT lipolysis observed in obesity causes increased release of fatty acids, which serve to stimulate macrophage infiltration [80]. This hypothesis offers a potential explanation for why BAT and BAT-like PVAT fail to attract immune cells. The unique function of BAT and PVAT to rapidly metabolize fatty acids via high capacity for beta-oxidation likely results in relatively low rates of local free fatty acid release from the cells in comparison to WAT [243]. A second possibility, is that the fatty acid species released by BAT and PVAT may also afford an anti-inflammatory effect. The ratio of saturated to polyunsaturated fatty acids has been shown to increase in the VAT and abdominal PVAT of high
fructose fed rats [244]. Thus under these conditions WAT may release relatively higher levels of saturated fatty acids, which may be more active in stimulating macrophage chemotaxis [79, 245]. Finally, abdominal or white PVAT may secrete pro-inflammatory adipokines or cytokines. We have found that although white adipose (SAT, VAT) has greater expression of adiponectin, which has anti-inflammatory effects, it also has greater mRNA expression of adipokines which correlate with vascular disease (Rbp4, Resistin)(Table 3.1)[246, 247]. Furthermore, abdominal PVAT has been shown to secrete greater amounts of the chemokine MCP-1 than thoracic PVAT; our data also confirm that Mcp-1(Ccl-2) mRNA is increased in VAT but not thoracic PVAT after high fat feeding (Table 3.2)[225]. The comparatively greater diameter of white adipocytes in VAT and abdominal PVAT may be one factor responsible for increased Mcp-1 transcription [248, 249].

In summary, results from this present study demonstrate that PVAT surrounding the thoracic aorta is effectively BAT, as shown by light and electron microscopy, and full genome expression analysis. Thoracic PVAT and interscapular BAT are resistant to inflammation induced by 13 and 20 weeks of HFD, as shown by reduced expression of immune cell enriched genes, immunohistochemistry with macrophage markers, and FACS analysis for activated macrophages. This work provides an important mandate to study expression of BAT genes in human PVAT from patients with and without vascular disease. In light of the recent discovery of functional BAT in adult humans, and
given the known beneficial metabolic and herein described anti-inflammatory properties of BAT, promotion of a BAT phenotype in the perivascular niche may have important effects in preventing vascular diseases such as hypertension and atherosclerosis.
CHAPTER IV: DISCUSSION

The correlation between obesity and inflammation has been well-described. Obese adipose tissue is characterized by local inflammation and obese individuals have elevated serum markers of inflammation [90, 95, 101, 110, 115, 120, 174, 195, 218, 250-255]. Numerous molecules are secreted from obese adipose tissue, and these molecules modulate local and systemic insulin resistance. Locally, these molecules decrease adipocyte insulin response and increase FFA release into circulation [256]. Systemically, these molecules and the increased FFAs are able to impair insulin responsiveness in other insulin target tissues such as the liver and skeletal muscle. Attempts to delineate the individual roles of these inflammatory molecules have been made and numerous knockout mice have been created and challenged with a HFD.

In the second chapter of this thesis, we characterized one such molecule, CD40, and its role in adipose tissue inflammation and insulin resistance. We found that CD40-/- mice were smaller, had decreased physical activity and food intake on HFD. Surprisingly, despite their decreased size, knockout mice were more insulin resistant as measured by glucose tolerance test (GTT) and insulin tolerance test (ITT) and a hyperinsulinemic-euglycemic clamp study found decreased glucose uptake in BAT with no changes to glucose uptake in WAT or muscle. Additionally, we found that CD40-deficient mice had increased hepatic steatosis on HFD likely due to increased lipolysis from WAT and increased de novo lipogenesis in the liver. These steatotic livers were more insulin resistant as
measured by Akt phosphorylation following intraperitoneal insulin injection and had increased gluconeogenesis as determined by rtPCR and PTT despite decreased inflammation by rtPCR. Increased lipolysis in visceral fat was likely due to increased inflammation in epididymal adipose tissue as defined by macrophages, inflammatory T cells, B cells, NK, NKT cells and eosinophils as well as rtPCR. The CD40 knockout mouse is therefore an interesting model of hepatic steatosis and insulin resistance in the absence of hepatic inflammation.

In the third chapter of this thesis we established that PVAT is similar to BAT in gross morphology, gene expression and in terms of resistance to inflammation in diet-induced obesity. Inflammation was defined by the presence of F480/CD11c+ macrophages and the expression of inflammatory genes by rtPCR and microarray. We hypothesize that the lack of inflammation in BAT and PVAT is likely due to decreased FFA and/or chemokine release by these tissues compared to WAT.

The above findings lead to new questions and directions for further work. Which cell type or types are involved in CD40 action in the adipose and liver phenotypes observed in knockout animals? How can the absence of CD40 lead to increased inflammation in adipose tissue and decreased inflammation in liver? Which factor(s) are involved in BAT and PVAT's resistance to inflammation and how does this inform our knowledge of WAT pathophysiology? Is inflammation in PVAT involved in vascular disease, like atherosclerosis?
The role of CD40 in diet-induced obesity

Our studies suggest that CD40 plays a protective role in metabolic disease by attenuating inflammation in diet-induced obesity. Immune cells infiltrate obese adipose tissue and contribute to an increasingly pro-inflammatory environment. CD40 functions to decrease adipose tissue inflammation and thus protects against local and systemic insulin resistance (Figure 4.1). The CD40 knockout mouse that we used in our studies lacks CD40 on all cells types. However, CD40 is expressed on many cell types, and its activation can have a myriad of effects depending on the cell and tissue type (Table 1.3).
Figure 4.1: Proposed effect of CD40 on adipose tissue inflammation and insulin sensitivity. CD40 decreases adipose tissue inflammation thereby protecting against systemic insulin resistance in diet-induced obesity.
Increasing adipose tissue inflammation is observed with increasing obesity and numerous immune cells have been implicated in the process (Table 1.2). It would be interesting to sort the immune cells involved in normal obesity-induced adipose tissue inflammation and measure CD40 expression. The cells that highly express CD40 in obese adipose tissue would be targets for further study. B cells, monocytes/macrophages, T cells, eosinophils and endothelial cells all express functional CD40 and are involved in adipose tissue inflammation. We could compare cells from CD40 knockout and WT animals in terms of their activation state and response to proinflammatory stimuli.

The major shortcoming of these techniques is that, like our study, they are observational. They do not distinguish between primary defects in cells caused by CD40 deficiency and secondary defects caused by interaction with other CD40-deficient cells. The way to determine the cell-specific role of CD40 in DIO is to create a conditional knockout mouse that lacks CD40 specifically in one cell type using the Cre-lox system. The Cre recombinase is a 38 kDa protein that can catalyze the deletion of a DNA segment that is flanked by loxP sites [257]. A conditional knockout mouse could be obtained by the crossing of a mouse expressing CD40 flanked by loxP sites with a Cre transgenic mouse that is specific for the cell of interest. Mice expressing the cre recombinase under the control of promoters that are specific for each of these cells are commercially available from Jackson Laboratory [258-262]. If cell-specific CD40 is responsible for the exacerbated insulin resistance of our knockout mice, then I would expect
that conditional knockout mice specific for the cell(s) involved will have a phenotype similar to that of our global knockout. These mice would also be useful in studying the mechanisms of atherosclerosis and autoimmune diseases.
The role of BAT and PVAT in diet-induced obesity

Chapter III of this dissertation established that PVAT is similar to BAT in morphology and gene expression. The contributions of different fat depots to insulin resistance or insulin sensitivity in the context of obesity have been extensively characterized. The contrast between SAT and VAT in their role in insulin sensitivity in obesity have motivated increased interest in characterizing other fat depots that could play a role in metabolic disease. Future studies should examine the function and significance of this tissue in obesity-related vascular disorders such as atherosclerosis.

A major limitation of our study was its observational nature. Unfortunately, conditional knockout or transgenic mice targeting the PVAT do not exist because PVAT-specific genes have not been identified and there is no current known method to induce inflammation specifically in PVAT. Several studies have linked PVAT inflammation with vascular disease by transplantation of VAT to the area adjacent to major vessels [263]. To further investigate the role of PVAT in atherosclerosis, we could utilize the Apoe-/- mouse [264, 265]. The Apoe-/- mouse has extremely high plasma cholesterol due to inability to take up chylomicron remnants and VLDL [266]. If PVAT inflammation plays a role in atherosclerosis progression, I hypothesize that Apoe-/- mice would have PVAT inflammation adjacent to atherosclerotic areas of vessels. If this is the case, we could investigate which genes are associated with initiating PVAT inflammation by looking at gene expression in PVAT over a time course of HFD.
Conclusion

Obesity is associated with increased local and systemic inflammation, which is associated with abnormalities in insulin signaling. We identify CD40 as a novel mediator of adipose tissue inflammation that attenuates inflammation in adipose tissue during diet-induced obesity. Additionally, in Chapter III of this dissertation we report that PVAT is similar to BAT in its morphology and resistance to inflammation in obesity. We believe that the work presented here provides impetus to further study the role of CD40 in adipose tissue and the functional role of PVAT in atherosclerosis in the context of diet-induced obesity.


