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Genetic Deficiency of CD40 in Mice Exacerbates Metabolic Manifestations of Diet-induced Obesity: A Dissertation

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GENETIC DEFICIENCY OF CD40 IN MICE EXACERBATES METABOLIC MANIFESTATIONS OF DIET-INDUCED OBESITY

A Dissertation Presented

By

Chang-An Guo

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Interdisciplinary Graduate Program

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I dedicate this dissertation with love to my wife and my parents

in China, who supported me each step of the way.

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Abstract

The past two decades have seen an explosive increase of obesity rates worldwide, with more than one billion adults overweight and 300 million of them obese. Obesity and its associated complications have become leading causes of morbidity and mortality in the United States and major contributing factors to the rising costs of national health care.

The pathophysiology of obesity and type 2 diabetes in rodents and humans is characterized by low-grade inflammation and chronic activation of immune pathways 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. A clear understanding of how CD40 and its ligand communicate to regulate and sustain the inflammatory environment of obesity is lacking. 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 with their age-matched wild-type controls. Hyperinsulinemic-euglycemic clamp studies also demonstrated insulin resistance in glucose utilization by the CD40-null mice compared with wild-type mice. In contrast to liver, visceral 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-null 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 the chronic inflammation associated with obesity, thereby protecting against hepatic steatosis.

Keywords

Adipose tissue inflammation, CD40, $CD8^+$ T cell, hepatic steatosis, insulin resistance

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Abbreviations

Abbreviation	Term
ATM	Adipose Tissue Macrophage
BMI	Body Mass Index
LD	Lipid Droplet
TNFα	Tumor Necrosis Factor alpha
MCP-1	Monocyte Chemotactic Protein-1
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
IFNγ	Interferon γ
IR	Insulin Receptor
IRS-1/2	Insulin Receptor Substrate 1/2
PI3K	Phosphatidylinositol 3-Kinase
MAPK	Mitogen Activated Protein Kinase
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
FFAs	Free Fatty Acids
ERK1/2	Extracellular-signal Regulated Kinases 1 and 2
JNK1/2	cJun NH2-terminal Kinase 1 and 2
GSK3	Glycogen Synthase Kinase 3
p38 SAPK	p38 Stress Activated Protein Kinase

mTOR	mammalian Target Of Rapamycin
IKKβ	Inhibitor of nuclear factor Kappa-B Kinase subunit Beta
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells

Chapter I

Obesity and Metabolic Syndrome: Inflammation is the Link

Chapter Contents

1.1 Historical views on obesity

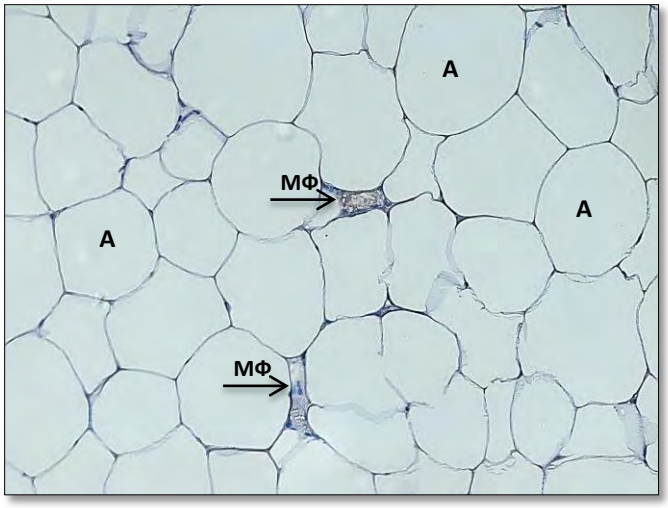
1.1.1 Fat is selected as a major energy reservoir in evolution

Fats and oils are universally used as reserved forms of energy in living organisms. Small lipid droplets (LDs) containing triglycerides (also called neutral fats or triacylglycerols) can be found frequently in certain groups of prokaryotes (e.g., *Mycobacteria*, *Rhodococcus*, *Streptomyces*, and *Nocardia*) [1]. In most eukaryotic cells, triglycerides form oily droplets in the aqueous environment of the cytosol, serving as depots of metabolic fuel. In vertebrates, specialized cells called adipocytes, or fat cells, store large amounts of triglycerides in the form of LDs that occupy most of the cell volume [2] (Fig. 1.1A). Triglycerides are also stored as oils in the seeds of many types of plants, providing energy and biosynthetic materials during seed germination. Adipocytes and germinating seeds contain lipases**,** the crucial enzymes that catalyze the hydrolysis of stored triglycerides, releasing fatty acids for export to sites where they are required as fuel.

There are two significant advantages to using triglycerides as stored fuels, rather than polysaccharides such as glycogen or starch:

1). Since the carbon atoms of fatty acids are more reduced than those of sugars (Fig 1.1B), fatty acids possess a higher calorific value. The yield from the

A



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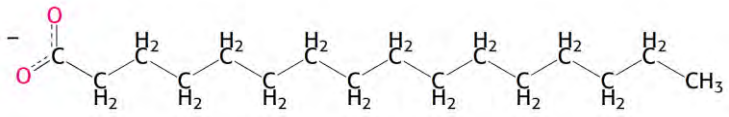
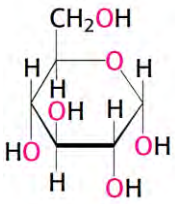


Fatty acids



Glucose

Figure 1.1 (A) Light micrograph of adipose tissue (200x)

A: adipocyte; MΦ: macrophages stained with F4/80 antibodies

(B) Molecular structure of fatty acids and glucose

complete oxidation of fatty acids is about 9 kcal g^{-1}, in contrast with about 4 kcal g^{-1} for carbohydrates and proteins.

2). Since triglycerides are hydrophobic, therefore unhydrated, organisms that carry fat as fuel do not have to carry the extra weight of water of hydration that is associated with stored polysaccharides (2 g per gram of polysaccharide). Consequently, a gram of nearly anhydrous fat stores nearly 7 times as much energy as a gram of hydrated glycogen, which is most likely the reason why triglycerides rather than glycogen were selected in evolution as the major energy reservoir. Soluble carbohydrates such as glucose and glycogen do offer certain advantages as quick sources of metabolic energy. However, the human body can only store less than a day's energy supply in the form of glycogen. In contrast, moderately obese people with 15 to 20 kg of triglycerides deposited in their adipocytes could meet their energy needs for months.

Virtually all animal species, from *Caenorhabditis elegans* to *Homo sapiens*, have found a way to store excess energy in the form of fat for future needs (Figure 1.2) [3]. *C. elegans* store fat in the intestinal epithelium [4] , while sharks store fat in the liver [5]. In most species, fat storage occurs in white adipose tissue (WAT). WAT locations vary among species. For invertebrates, amphibians, and many reptiles, the largest fat storage sites are intra-abdominal; in seals and whales, most fat is subcutaneous, whereas most of the mammals and birds have

Species	 *Caenorhabditis elegans*	 *Drosophila melanogaster*	 *Carcharodon carcharias*	 *Cyprinus carpio*
Fat storage	Stored in intestinal cells	Stored in the "fat body"	Stored in liver	Stored in WAT
Species	 *Xenopus laevis*	 *Gallus gallus domesticus*	 *Mus musculus*	 *Homo sapiens*
Fat storage	Intra-abdominal WAT (no sub-cutaneous WAT)	Subcutaneous and internal WAT	Subcutaneous and internal WAT	Subcutaneous and internal WAT

Figure 1.2 Evolution of Adipose Tissue Depots

Adipose tissue distribution and fat storage have evolved dramatically. In the worm *C. elegans,* fat is stored in the intestine, whereas *Drosophila* have a defined fat body. Sharks use their liver to store fat. Intra-abdominal white adipose tissue (WAT) becomes apparent in carp (*Cyprinus carpio*), amphibians and reptiles. Differentiation of subcutaneous and internal WAT occurs in higher species, such as birds and mammals, exemplified here by the chicken (*Gallus gallus* domesticus), mouse (*Mus musculus*), and human (*Homo sapiens*).

Adapted from *Cell. 2007 Oct 19;131(2):242-56.*

both intra-abdominal and subcutaneous WAT. Stored triglycerides can serve not only as energy stores but also as insulation against low temperatures. For example, hibernating animals like bears, reserve huge fat under the skin before hibernation. This seasonal fat storage serves the dual purposes of insulation and energy storage.

1.1.2 A long look at human obesity

In the natural world, starvation, infection, predation and harsh environments (e.g., cold) have long been the major killers of animals as well as humans [6]. Of all the above-mentioned adversities, starvation accounts for a major part of overall mortality. Nearly every continent in the world has experienced a period of famine throughout history. Some areas in sub-Saharan Africa continue to have extreme cases of famine today.

Evolutionarily, natural selection rewarded the “energy-thrifty” [7, 8] genotypes of those who could store the greatest amount of fat from the least amount of the then intermittently available foods. This ability to store surplus fat from the least possible amount of food intake may have made the difference between life and death, not only for the individual but also for the species. Those who could store fat efficiently had an evolutionary advantage in the harsh environment of early hunters, fishers, and gatherers [9]. However, today, in the presence of a continuous nutrition surplus, the once advantageous genetic trait sets a stage for

excess adiposity, which leads to overweight and obesity. What Arizona's Pima Indians have experienced over the past century becomes a living proof of the "thrifty gene" theory. These athletic hunters and assiduous desert farmers, once adopted a typical western lifestyle, soon exhibited the highest prevalence of obesity and type 2 diabetes of any ethnic group [10, 11].

The word *Obesity* comes from the Latin *obesitas*, which means "stout, fat, or plump." It was widely perceived as a symbol of wealth, prosperity, and fertility at times in history, and still is as such in some parts of the world. Sculptural representations of the human body, such as *Venus of Willendorf* (20,000-35,000 years ago) and figurines from Çatalhöyük in Turkey (around 7500 BC) (Fig 1.3A and B), depict obese females, which probably reflects of the time the aesthetic value and cultural significance attached to the obesity. Increased "flesh" was desirable is also reflected in ancient eastern arts, such as imperial paintings often seen in the Tang Dynasty of China (Figure 1.3 C).

Although, obesity in early history was rare among the general population, the health hazards associated with obesity were recognized by physicians from many diverse cultures. For example, Greek physician Hippocrates (460 BC-370 BC) noted the adverse effects of excess adiposity by stating that "corpulence is not only a disease itself, but the harbinger of others" and "sudden death is more common in those who are naturally fat than in the lean" [12]. 500 years after Hippocrates, the leading Roman physician Galen recounted one of the earliest

case studies of treatment for obesity: “I reduced a huge fat fellow to a moderate size in a short time, by making him run every morning until he fell into a profuse sweat; I then had him rubbed hard, and put into a warm bath…Some hours after, I permitted him to eat freely of food, which afforded but little nourishment; and lastly, set him to some work [12]”.

Obesity was also familiar to Abu Ali Ibn Sina (c. 980–1037) (Avicenna in Latinized version), one of the most prominent Arabic medical figures. In his medical encyclopedia, written in the early 11th century, Avicenna described the sweet taste of diabetic urine, and also referred to obesity and its dangers to health. In the eastern world, the Hindu physicians, Sushrut and Charak (500-400 BC) are credited with early recognition of the sugary taste of diabetic urine, and observed that the disease often affected overweight people who ate excessively, especially sweet and fatty foods. Chinese and Japanese words for diabetes are based on the same ideographs (糖尿病) which mean "sugar urine disease".

Despite such accounts, it was only much later in the18th century that the modern history of obesity emerged. There have been numerous attempts to quantify excess weight in ways that are appropriate for clinical practice, research and epidemiology. The Belgian astronomer and statistician Adolphe Quetelet (1796-1874) was one of the pioneers in developing mathematical measures of obesity. Quetelet suggested that the ratio of the subject’s weight divided by

A.

B.

C.

Figure 1.3 Cultural elaborations and aesthetic value of obesity

(A) The Venus of Willendorf, Naturhistorisches Museum, Vienna, Austria

(B) Seated Woman of Çatal Hüyük, Ankara Museum, Turkey,

(C) Du Qiu Figure by Zhou Lang, 1271-1368, the Palace Museum, Beijing, China.

the square of the height could be used as a measure of fatness that corrected for differences in height (see below). This unit, the Body Mass Index (BMI), has been shown to correlate with body fat content, and to predict risk for several of the comorbidities of obesity.

$$\mathbf{BMI} = \frac{\mathbf{Mass\ (kg)}}{\mathbf{Height\ (m^2)}}$$

1.2 Obesity has become a worldwide epidemic

Industrialized agriculture, hygiene and technology advances have made remarkable progress in alleviating our ancient challenges of starvation, infection and predation. However, novel threats are taking place, such as cardiovascular disease, type 2 diabetes, and cancer, which rapidly represent an increasing share of human morbidity and mortality. In recent years, obesity has emerged as the driving force behind these disturbing chronic diseases. According to the World Health Organization (WHO) reports in 2008, 44% of diabetes burden, 23% of ischemic heart disease burden and between 7% and 41% of certain cancer burdens are attributable to increased body weight and obesity.

The WHO definition of overweight and obesity is as illustrated in Fig 1.4:

- BMI greater than or equal to 25 is overweight
- BMI greater than or equal to 30 is obesity.

The Centers for Disease Control and Prevention (CDC) recorded the historical data of obesity prevalence in the United States from 1985 through 2011 via its Behavioral Risk Factor Surveillance System (BRFSS) (Fig 1.5), which is a cross-sectional telephone survey conducted by state health departments. In 1990, among states participating in the BRFSS, no state had obesity prevalence equal to or greater than 15%. In 2010, no state had a prevalence of obesity less than 20%. Thirty-six states had a prevalence equal to or greater than 25%; 12 of these states (Alabama, Arkansas, Kentucky, Louisiana, Michigan, Mississippi , Missouri, Oklahoma, South Carolina, Tennessee, Texas, and West Virginia) had a prevalence equal to or greater than 30% [13-18]. The most recent data from National Health and Nutrition Examination Survey (NHANES) in 2009–2010 reported that **35.7%** of U.S. adults were obese (Figure 1.6), defined as a BMI ≥ 30kg/m^2.

Worldwide, from 1980 to 2008, the number of obese individuals doubled to more than half a billion people, eclipsing the number of underweight individuals for the first time in human history and driving the obesity-attributable death rate to ~3 million per year [19]. Overall, more than 10% of the world's adult population are now obese. Once considered a problem only in high-income countries,

BMI classification	
Underweight	< 18.5
Normal range	18.5 - 24.9
Overweight	⩾ 25.0
Preobese	25.0 - 29.9
Obese	⩾ 30.0
Obese class I	30.0 - 34.9
Obese class II	35.0 - 39.9
Obese class III	⩾ 40.0

Figure 1.4 Body Mass Index (BMI) classifications

from World Health Organization

overweight and obesity are now dramatically on the rise in low and middle-income countries. For example, China, the largest and most populous developing country in the world, used to have one of the leanest populations [20]; is now rapidly catching up with the West not only in the economic growth but also the prevalence of obesity. Roughly 25% of Chinese adults are currently overweight or obese. China is now home to the world's largest diabetic population, with 92.4 million adults having diabetes and 148.2 million adults that are prediabetic in the year of 2008 [21].

There is no doubt that energy imbalance between calories consumed and calories expended causes obesity, but the question is what is producing the positive energy balance that promotes the increasing prevalence of obesity? Many causes exist for the obesity epidemic. Heredity and prenatal or early life influences certainly contribute to obesity, however, the most important causes of overweight and obesity are probably related to the overconsumption of highly processed, energy-dense foods and the reduction of physical activities of the modern sedentary lifestyle. We have unintentionally created an environment that heavily favors metabolic overloads. Combatting the rise of obesity rates worldwide will require addressing both individual behavior and this "obesogenic" environment [22].

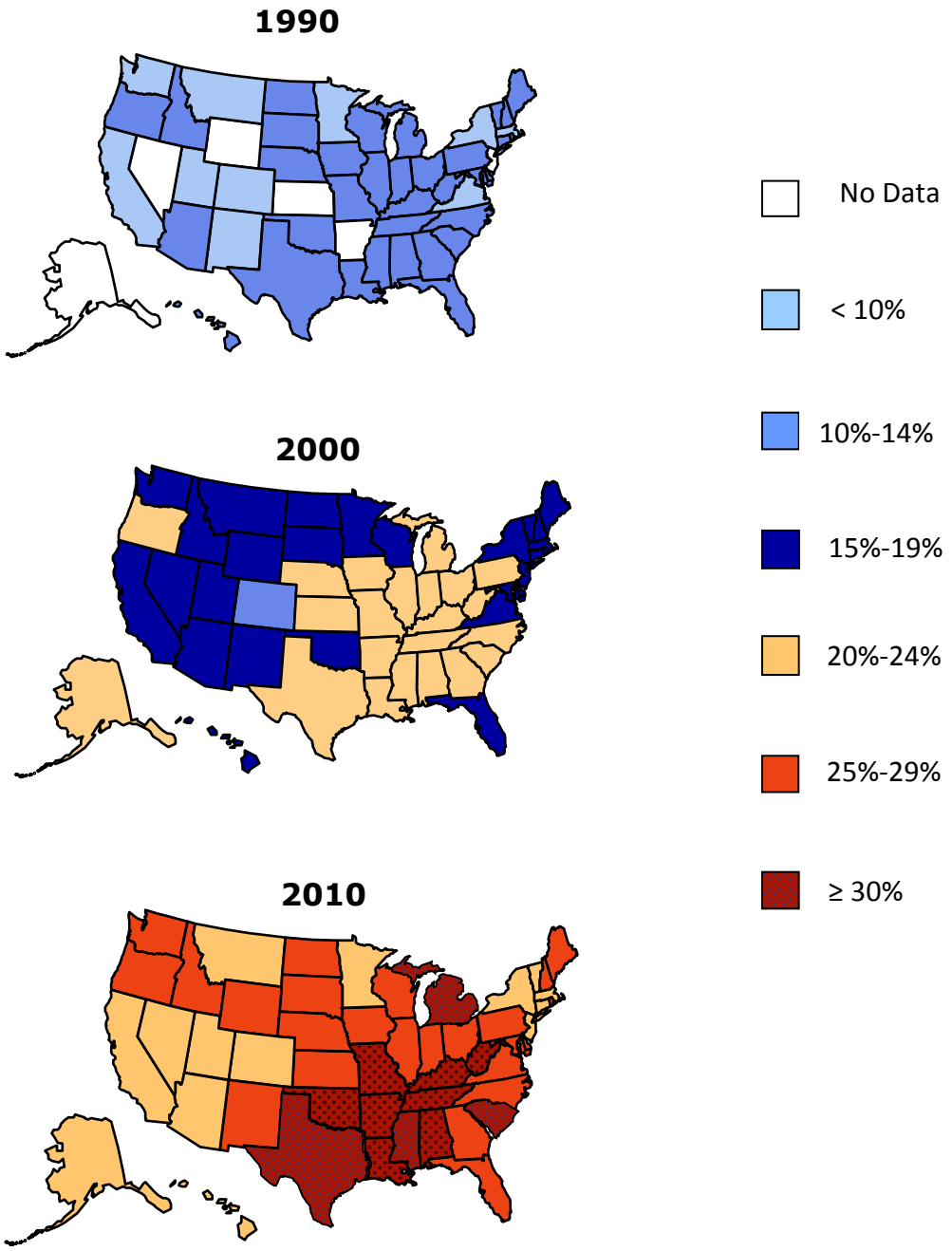


Figure 1.5 Obesity Trends Among U.S. Adults

BRFSS, 1990, 2000, 2010

Adapted from

the CDC website (http://www.cdc.gov/obesity/data/index.html)

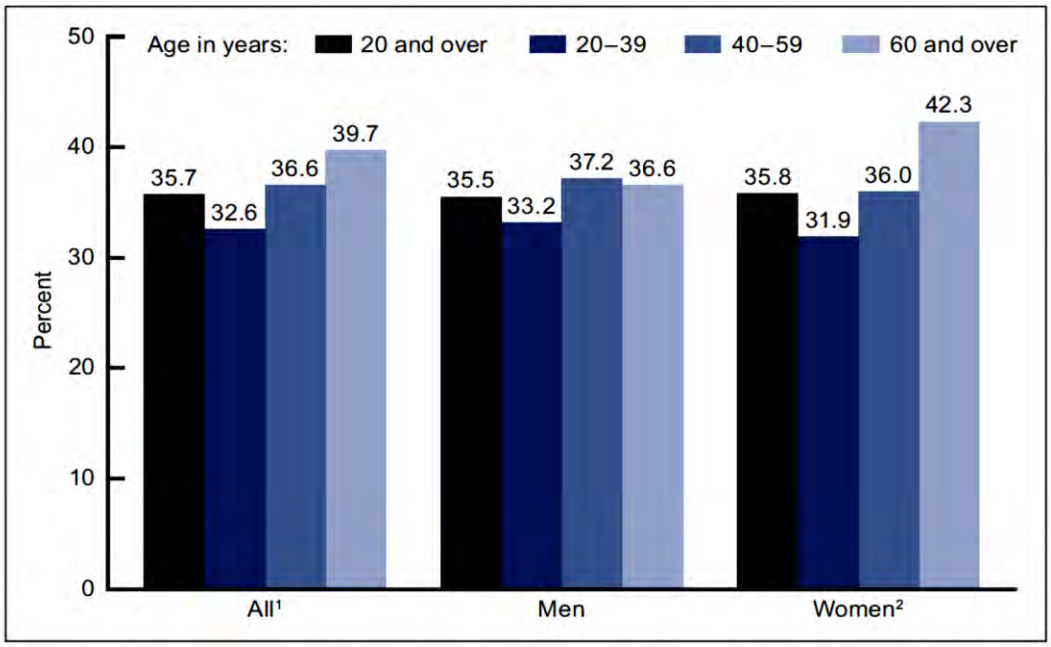


Figure 1.6 Prevalence of obesity among adults aged 20 and over, by sex and age: United States, 2009–2010

SOURCE: CDC/NCHS, NHANES.

1.3 Health risks of obesity

Overweight and obesity diminish almost every aspect of health. The rise in human obesity is closely associated with an alarmingly elevated incidence of diseases such as type 2 diabetes, fatty liver disease, cardiovascular disease, certain cancers, lung and musculoskeletal disorders, infertility, and other medical conditions including hyperglycemia, hyperinsulinemia, dyslipidemia and endothelial dysfunction [23] . Obesity causes these comorbidities by a variety of pathways, some as straightforward as the mechanical stress of carrying extra pounds and some involving complex changes in hormones and metabolism. Obesity decreases quality of life as well as life expectancy, and increases individual, national, and global healthcare burden. According to a recent study [24], the annual cost of managing obesity in the United States alone has climbed to approximately $190.2 billion per year, or 20.6% of national health expenditures. Obesity and its related disease clusters are now truly leading global public health problems.

1.3.1 Obesity and insulin resistance

Insulin resistance commonly coexists with obesity and is a fundamental aspect of the etiology of type 2 diabetes. Insulin resistance is also linked to a wide array of other pathophysiologic consequences including hypertension, fatty

liver disease and atherosclerosis, which are often referred to as the “insulin resistance syndrome”, because resistance to insulin action in various tissue/organs/metabolic pathways is a common underlying feature of these complications [25].

Of note, insulin resistance, although widely considered as a precondition to the onset of the metabolic syndrome, as one intrinsic cellular mechanism, is an evolutionarily conserved adaptive response in many physiologic contexts such as infection and pregnancy, which are unassociated with obesity. Organisms have to maintain priority nutrient access for emerging metabolic requirements, such as fueling immune system activation or reserving metabolic resources for fetal development. Organisms meet these requirements by reducing systemic insulin sensitivity, decreasing nutrient uptake by non-priority cells and reserving glucose for priority cells and tissues [26, 27]. However, infection- and pregnancy-associated insulin resistance is relatively transient and recedes to normal levels after the completion of these physiological processes, demonstrating that transient insulin signaling inhibition can be advantageous. In contrast, insulin resistance seen in obesity is fundamentally different from the above-mentioned physiological conditions because it is a chronic and pathological condition that occurrs before other metabolic complications.

During the progression from lean to overweight and obesity, adipose tissue expands to store excess energy in the form of triglycerides. Insulin promotes

adipocyte triglyceride storage by a number of mechanisms, including fostering the differentiation of preadipocytes to adipocytes, stimulating glucose transport and triglyceride synthesis, and inhibiting lipolysis. The insulin signaling cascade that initiates these events is largely conserved throughout evolution from *C. elegans* to *Drosophila melanogaster* and humans (Fig. 1.7) [28]

Insulin-mediated reducations in blood glucose levels result from increased glucose uptake by insulin-responsive peripheral tissues such as muscle, liver and adipose tissue as well as hepatic glucose production suppression. Skeletal muscle has long been considered to be the major site of insulin-stimulated glucose uptake *in vivo*, whereas adipose tissue was thought to only account for a small fraction of total body glucose disposal [29]. However, some studies provided evidences implying a more important role of WAT in mediating systemic glucose homeostasis than was previously thought. For example, fat tissue specific overexpression of GLUT4 enhances whole body insulin sensitivity [30], and the increase of glucose uptake selectively in fat with β3 adrenergic receptor agonists improves whole body insulin-stimulated glucose disposal [31].

Far from being merely an inert energy storage depot, in recent two decades, adipose tissue has been recognized as a major endocrine organ with wide-reaching effects on other organs and tissues [32]. Adipose tissue modulates metabolism by releasing non-esterified fatty acids (NEFAs), peptide hormones,

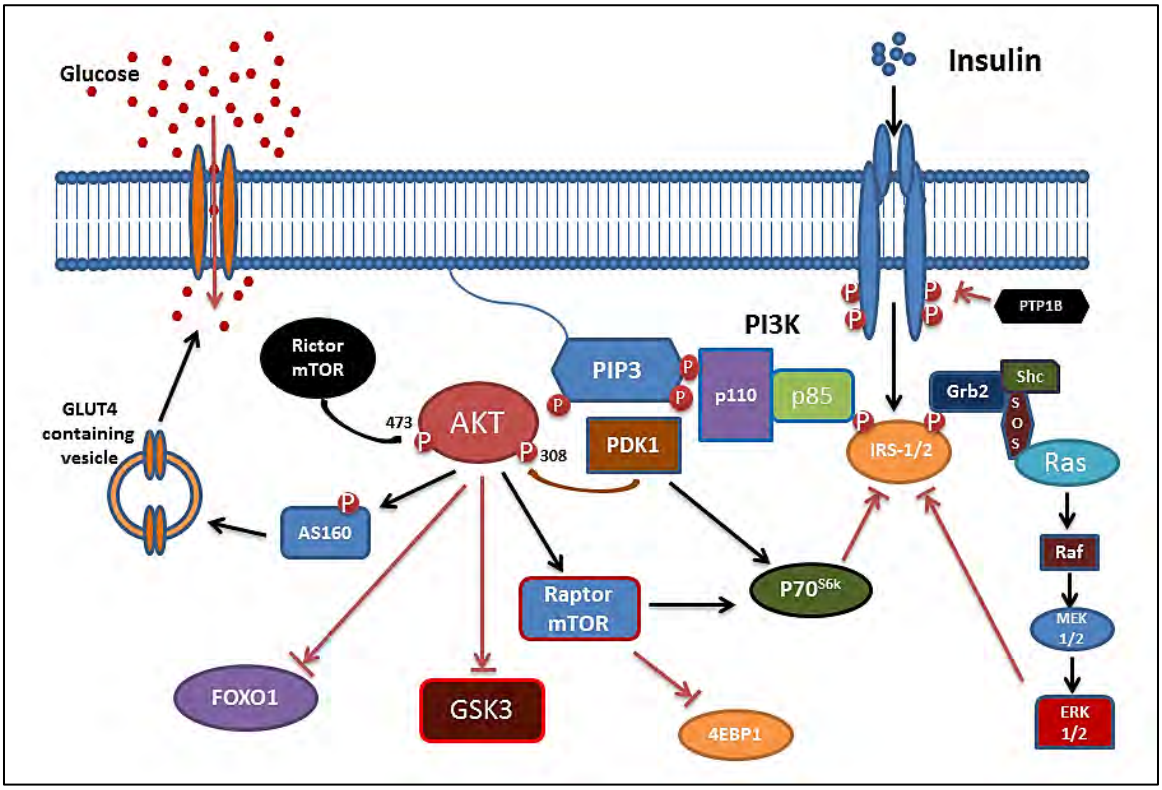


Figure 1.7 Signal transduction in insulin action

The insulin receptor tyrosine kinase undergoes autophosphorylation when bound to insulin on the outer surface of the plasma membrane, which transduces its signal to IRS proteins. The cytoplasmic signaling starts with tyrosine phosphorylation of IRS, which leads to a diverse series of signaling pathways including the activation of PI3K, MAP kinase and AKT. These pathways act in a coordinated fashion to regulate vesicle trafficking, protein synthesis, and gene expression.

and proinflammatory cytokines [33, 34]. In obesity, the production of many of these products is up-regulated, showing signs of dysfunction in the adipose tissue.

Among the secretory factors that are released by adipose tissue, NEFAs may be one of the most critical factors in modulating insulin sensitivity, which also explains the paradox that either absence (such as lipodystropic conditions [35]) or excess of adipose tissue causes insulin resistance; because in both circumstances, circulating serum NEFAs levels are elevated. The increased serum NEFAs may be because of defective adipose tissue in the case of lipodystrophy or expanded adipose mass in the case of chronic over-nutrition. The deficit in the capacity of adipose tissue to store excess energy results in lipid accumulation at undesired sites such as liver, skeletal muscle and heart, a phenomenon that has been described as ectopic fat deposition [36]. As a result, both professional metabolic and bystander tissues are exposed to supra-physiologic levels of metabolic substrates.

NEFA circulating in the plasma binds to albumin. Insulin resistance develops within hours of an acute increase in plasma NEFA levels produced by lipid emulsion infusion in humans [37]. On the other hand, insulin-mediated glucose uptake and glucose tolerance improve with an acute decrease in NEFA levels after treatment with acipimox, a nicotinic acid analogue and a potent inhibitor of adipose tissue lipolysis [38]. It has been proposed that increased NEFA delivery or decreased mitochondrial fatty acid oxidation results in an increase in

the intracellular fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA), and ceramides, which, in turn, activate a kinase cascade leading to the serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), and a reduced ability of these molecules to activate PI(3)K [39]. Subsequently, events downstream of insulin-receptor signaling are diminished (Fig 1.7).

Simple obesity is typically associated with insulin resistance; however, individuals with similar BMI and adiposity could display remarkably various levels of insulin sensitivity [40]. Part of this differences is because of alterations in body fat distribution. Individuals with a more peripheral distribution of fat are more insulin sensitive than individuals who have their fat distributed centrally-that is, in the abdominal areas [41]. Hypertrophied intra-abdominal or visceral adipocytes are characterized by a hyperlipolytic state that is resistant to the antilipolytic effect of insulin [42, 43]. The resulting NEFA flux travels directly to the liver via the hepatic blood supply (portal circulation), which may impair liver metabolism and cause hepatic insulin resistance, apolipoprotein B-containing lipoprotein overproduction, and increased hepatic glucose production. In addition to altered NEFA metabolism, expanded visceral adipose also leads to an altered proinflammatory profile including elevated plasma C-reactive protein (CRP), IL-6, TNFα and reduced adiponectin concentrations [44].

1.3.2 Obesity and nonalcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in both adults and children and is characterized by the pathological accumulation of hepatic lipids without significant alcohol consumption. NAFLD is strongly linked to obesity, insulin resistance and other metabolic complications and is a robust, independent marker of metabolic dysfunction [45, 46]. Obese persons who have normal liver lipid content tend to be resistant to developing obesity-related metabolic complications [47]. As a spectrum of diseases, NAFLD can progress from vesicular lipid accumulation in hepatocytes (simple steatosis) to steatohepatitis (NASH; steatosis with the presence of inflammatory infiltrate possibly with some fibrosis) to advanced fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma (Fig 1.8) [48].

Evidence from liver biopsies and imaging (ultrasound, computed tomography, MRI and proton magnetic resonance spectroscopy) studies [49] have demonstrated NAFLD in 20-35% of populations worldwide, with 10% of these cases being NASH. NAFLD prevalence is much higher among obese patients [50, 51] and type 2 diabetic patients [52]. A disturbance in the balance between the processes of lipid accumulation and lipid disposal causes hepatic steatosis. The potential sources of fatty acids that contribute to fatty liver development include dietary fatty acids arising from intestinal chylomicron uptake, newly synthesized fatty acids within the liver and circulating NEFA.

Hepatic lipids are disposed mainly through VLDL secretion or via mitochondrial β-oxidation. Defects in either of these pathways are associated with NAFLD. Stable isotope studies in rodents and humans have revealed that the excessive triglyceride accumulation as observed in hepatic steatosis occurs mainly because of increased hepatic lipogenesis and increased FFA influx from hypertrophied adipose tissue, whereas lipid disposal through β-oxidation or export has only minor effects [53].

NAFLD is often associated with adipose tissue and hepatic insulin resistance [54]. Insulin resistance, in turn, affects both hepatic gluconeogenesis [55] and glycogenolysis [56]. Therefore, NAFLD patients have been documented to have increased gluconeogenesis [57, 58]. Excess fatty acids not only induce hepatic insulin resistance but also impair insulin clearance *in vitro* and *in vivo* [59, 60], which contributes to the typical hyperinsulinemia of patients with NAFLD [61]. Hyperinsuliemia, in the context of elevated hepatic fatty acid flux as observed in obesity and NAFLD, stimulates hepatic sterol regulatory element binding protein 1c (SREBP-1c) activity and is associated with increased hepatic *de novo* lipogenesis [62]. However, although *de novo* lipogenesis is up-regulated in NAFLD, it contributes less to the total hepatic triglyceride pool compared with adipose tissue-derived fatty acids. Donnelly et al. reported that in patients with NAFLD, 59% of the triglycerides labeled in the liver arose from circulating fatty acid flux (mainly from adipose tissue lipolysis), 26% from *de novo* lipogenesis and 15% from the diet [62].

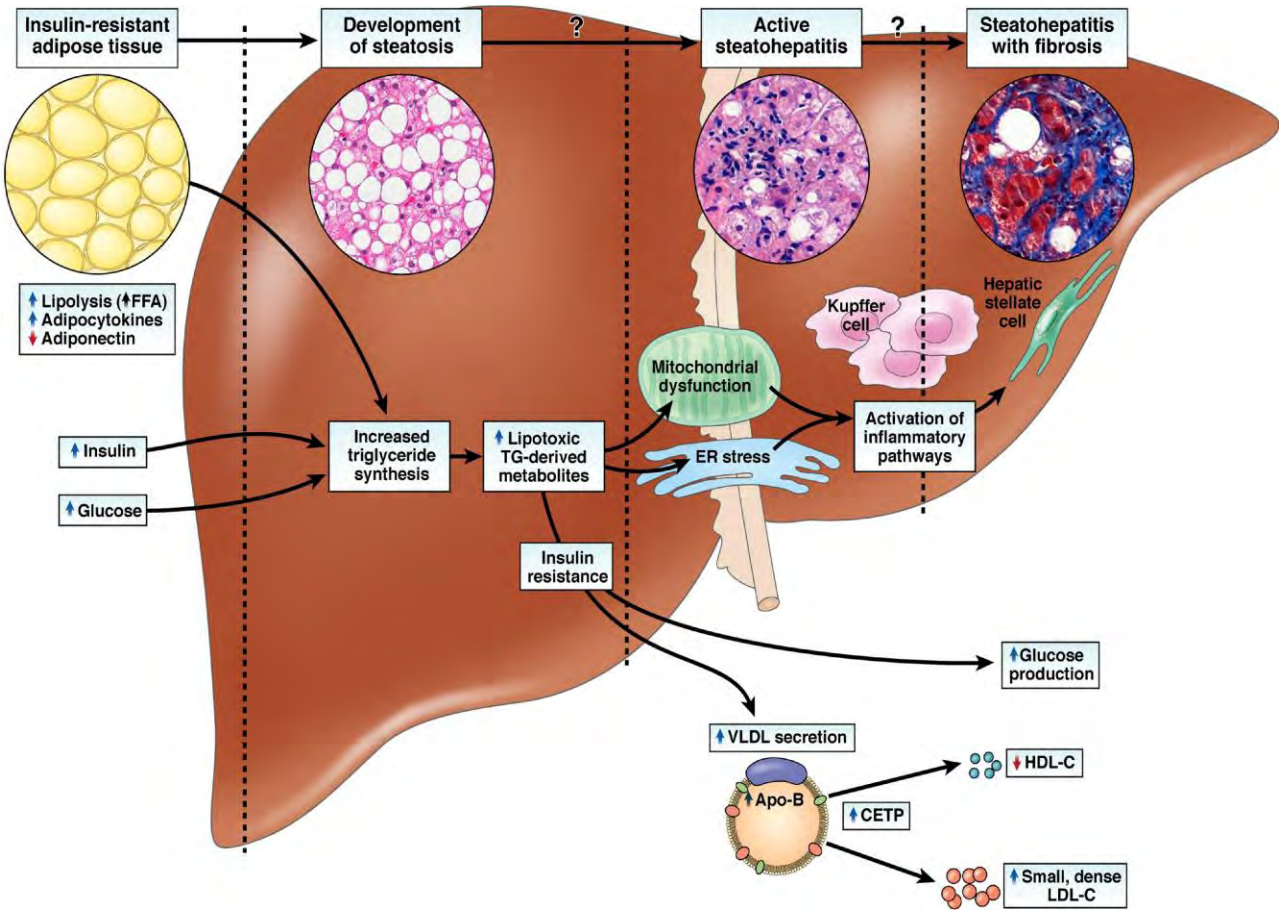


Figure 1.8 Schematic representation of the pathophysiology of NASH

Adapted from *Gastroenterology. 2012 Apr;142(4):711-72*

The most widely accepted model to describe NAFLD development and the progression from simple steatosis to NASH is the “two-hit hypothesis” [63]. The “first hit” is the aforementioned accumulation of lipids in the hepatocytes with insulin resistance as the key underlying pathogenic factor. The “second hit” leads to hepatocyte injury, inflammation and fibrosis. Factors initiating the second hit are oxidative stress and subsequent lipid peroxidation, mitochondrial dysfunction, and DNA damage. In addition, products of lipid peroxidation increase the expression of proinflammatory cytokines and recruit inflammatory cells into the liver. Inflammation plus hepatic stellate cells activation leads to collagen production and the initiation of fibrosis [64].

1.3.3 Obesity and Cardiovascular Disease

Body weight is directly associated with various cardiovascular (CV) risk factors, especially in individuals with a central deposition of adipose tissue [65]. As BMI increases, so do blood pressure, low-density lipoprotein (LDL), cholesterol, triglyceride, blood sugar, coagulation, fibrinolysis and inflammation. All of these changes translate into increased risk for coronary heart disease (CHD), stroke, and CV death. There are numerous adverse effects of obesity in general and CV health in particular (Table 1.1) [66].

It is well known that cigarette smoking is a classical CV risk factor. Smoking affects cytokines by causing a dose-dependent increase in plasma intercellular

adhesion molecule-1 (ICAM-1) and decrease in adiponectin levels [67], thereby inducing endothelial dysfunction. Smoking increases levels of TNF-α, a powerful cytokine that decreases adiponectin levels and induces insulin resistance. Smoking also enhances oxidative stress owing to the direct effects of the free radicals that are present in smoke. As described earlier, obesity exhibits almost the entire spectrum of adverse effects associated with smoking, including elevated inflammatory cytokines, attenuated adiponectin levels and increased oxidative stress. In fact, when compared with non-obese smokers, obese smokers have a clear reduction of life expectancy [68].

Numerous studies have demonstrated a direct link between excess body weight and CHD. Investigators conducted a meta-analysis of 21 long-term studies that followed more than 300,000 participants for an average of 16 years. Study participants who were overweight had a 32% higher risk of developing CHD, compared with participants who were at a normal weight; those who were obese had an 81% higher risk [69].

Increased NEFA levels in obesity affect endothelial nitric oxide production, thereby impairing endothelium-dependent vasodilation. On the other hand, the dyslipidemia seen in obesity is characterized by an altered LDL particle composition and distribution, resulting in an increased concentration of small, dense LDL. LDL particles are enriched in triglycerides, which are rapidly lipolyzed by hepatic lipase (HL), leaving smaller, denser LDL particles. These particles are

Table 1.1	Adverse Effects of Obesity
A. Increases in insulin resistance 1) Glucose intolerance 2) Metabolic syndrome 3) Type 2 diabetes mellitus	**B. Hypertension**
C. Dyslipidemia 1) Elevated total cholesterol 2) Elevated triglycerides 3) Elevated apolipoprotein-B 4) Elevated small dense LDL particles 5) Decreased HDL cholesterol 6) Decreased apolipoprotein-A1	**D. Abnormal left ventricular geometry** 1) Concentric remodeling 2) Left ventricular hypertrophy
E. Endothelial dysfunction	**F. Increased systemic inflammation and prothrombotic state**
G. Systolic and diastolic dysfunction	**H. Heart failure**
I. Coronary heart disease	**J. Atrial fibrillation**
K. Obstructive sleep apnea/sleep-disordered breathing	**L. Albuminuria**
M. Osteoarthritis	**N. Cancers**

more prone to oxidation/glycation and are mostly taken up by macrophage scavenger receptors instead of the LDL receptor pathway, which induces atherosclerosis.

1.4 Inflammation as a link between obesity and metabolic syndrome

Metabolic syndrome, also known as syndrome X or insulin resistance syndrome is a combination of medical disorders that, when occurring together, increase the risk of developing cardiovascular disease, stroke, and type 2 diabetes. Reaven, in his 1988 Banting lecture, described "Syndrome X" as the association of insulin resistance, hyperglycemia, hyperinsulinemia, hypertension, low HDL cholesterol and elevated VLDL triglycerides [70]. However, he did not include obesity as a contributing factor leading to syndrome X. Today, obesity, especially abdominal obesity, is considered t be one of the most important criteria for the metabolic syndrome.

In the past two decades, the search for a potential unifying mechanism behind the pathogenesis of obesity-associated metabolic syndrome has revealed a close relationship between nutrient excess and an alternative form of inflammation, called "metabolic inflammation" [71], which is characterized by the chronic, low-grade inflammation that is observed in obesity.

Appreciation of the involvement of inflammation in insulin resistance or type 2 diabetes started with the use of salicylate. In 1876, Ebstein first demonstrated that high doses of sodium salicylate could diminish glycosuria in diabetic patients [72]. In 1901, Williamson again pointed out that "sodium salicylate had a definite influence in greatly diminishing the sugar excretion" [73]. Of course, the hypoglycemic actions of salicylates and the molecular target of salicylates, IκB kinase-β (IKKβ)/NF-κB axis, were not known at the time [74, 75].

The association between insulin resistance and inflammation was later recognized with the identification of insulin resistance in patients with sepsis [76]. We are now familiar of that many diseases with active inflammatory responses display insulin resistance as a feature, including hepatitis C, HIV, and arthritis [77-79]. Direct proof of the link between inflammation and metabolic responses came from the genetic studies in mice by Hotamisligil and colleagues. They found that obese adipose tissue secretes inflammatory cytokines (such as TNFα) and these cytokines themselves can inhibit insulin signaling [80, 81].

As described earlier in this chapter, the ability to withstand starvation and the ability to fight off infections are critical functions for species survival. It is now appreciated that immune response and metabolic regulation are highly intertwined and evolutionarily conserved processes, therefore, proper function of one is dependent on the other. One example that represents this active interaction is the *Drosophila* fat body, which incorporates the mammalian

homologs of the liver, adipose tissue, and the hematopoietic immune system into one functional unit [82, 83]. The fly's fat body carries out a crucial function in sensing energy availability. The fat body is also the site of coordination of pathogen responses with metabolic status [82]. It is very possible that, in the fly, common and overlapping molecular pathways regulate both metabolic and immune functions and in higher organisms, tissues or organs that are responsible for metabolism and immunity maintain their developmental heritage. As such, it is conceivable that nutrient surplus might be able to activate a pathogen-sensing system, such as the Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) [84] and metabolically induce inflammatory responses.

1.4.1 Classic inflammation vs. Inflammation in obesity

Inflammation is usually referred to as classic acute inflammation, an adaptive response to harmful stimuli, such as infection, tissue injury and irritants. Acute inflammation is a protective attempt by the organism to remove the injurious stimuli, followed by a resolution and repair phase to restore homeostasis. The classic response of acute inflammation is a short-term and high-amplitude process, usually appearing within a few minutes or hours and resolving upon the removal of the injurious stimulus. Acute inflammation is characterized by five cardinal signs: pain, heat, redness, swelling and loss of function [85].

In contrast, inflammation induced by metabolic surplus is distinctive and is not caused by the classic instigators of inflammation: infection or injury. Instead, this altered form of inflammation is associated with the malfunction of tissues that are not directly related to host defense or tissue repair.

First, the trigger of inflammation in obesity is different. In obesity-related metabolic syndrome, metabolic signals emerging from metabolic cells (such as adipocytes) induce inflammatory responses and deteriorate metabolic homeostasis. An array of inflammatory cytokines are increased in obese adipose tissue (and in adipocytes themselves), including TNFα, IL-6, IL-1β, CCL2, MCP-1, SAA and many others [34]. In addition to adipose tissue, the liver [86], pancreas [87], brain [88] and muscle [89] all possibly contribute to the elevated systemic inflammation in the obese state. Importantly, the obesity-associated inflammation discussed here, though significant, is often modest when compared with that of an infection or trauma.

Second, the duration of inflammation that occurs in obesity is vastly different from the duration of acute inflammation. Contrary to classic acute inflammation, in obesity, the increased inflammatory cytokine expression that occurs in various metabolic tissues and organs appears to happen gradually and remains unresolved over time. Several studies carefully examined the timing of obesity-induced inflammation in mice and one reported that increases in inflammatory and macrophage-specific gene expression in adipose tissue occurred after 3-

week of high-fat diet (HFD) feeding, with a more dramatic increase at 16 and 26 weeks on the same diet [90]. Thus, it seems that the inflammation induced by nutritional overload is not dramatic enough as acute inflammation to mount a full resolution program, and therefore the low-grade inflammation is maintained in a chronic manner for years. This lack of resolution may be due to the lack of evolutionary selection to develop such a response to chronic energy surplus.

1.4.2 Molecular pathways that link inflammation and insulin resistance

Obesity-associated tissue inflammation is now recognized as a major cause of decreased insulin sensitivity. How inflammatory signals disrupt insulin action in obesity has been an intensive area of research.

As demonstrated in Figure 1.7 and Figure 1.9, insulin receptors acquire docking proteins, insulin receptor substrates (IRS), to propagate downstream signaling cascades [91]. The tyrosine phosphorylation of IRS is a crucial step in insulin signaling and is inhibited by counter-regulatory serine/threonine phosphorylation [92]. IRS tyrosine phosphorylation is often defective in many cases of insulin resistance [33]. IRS-1 can be phosphorylated at inhibitory serine residues by a variety of kinases, such as JNK, IKKβ, S6 Kinase, mTOR and protein kinase C (PKC) and this serine phosphorylation on IRS-1 blunts insulin signaling [93-95]. In response to various metabolic stresses that occur in obesity,

JNK [93], IKK [96] and PKC [97] are often activated, which inhibits insulin action (Fig 1.9).

More specifically, JNK activity increases dramatically in critical metabolic sites of obesity [98], such as adipose tissue, liver, and hypothalamus [99]. Upon exposure to cytokines, free fatty acids, reactive oxygen species, or endoplasmic reticulum (ER) stress, JNK is acutely activated [93, 100], which contributes to obesity-induced insulin resistance. These observations were validated by the JNK-1 genetic deletion in mice. JNK-1 deficiency protected mice from IRS-1 serine phosphorylation, and improved systemic glucose homeostasis and insulin sensitivity [101].

IKKβ is another inflammatory kinase that is critical in the development of insulin resistance. As mentioned earlier, high dose salicylates administration improves insulin action by inhibiting IKKβ kinase activity [75]. Experimental activation of IKKβ in the liver is sufficient to cause systemic insulin resistance [86].

Protein kinase C has various isoforms and is important for the interaction between inflammatory and metabolic pathways. PKC is thought to be downstream of lipid signals [102, 103]. Intermediate metabolites of fatty acids, such as fatty acyl-CoA, DAG, and ceramides may activate PKC-θ in muscle and PKC-δ in the liver, which eventually inhibits insulin action [104]. PKC-θ knockout mice are protected from obesity-induced insulin resistance, confirming the contribution of this kinase to metabolic regulation *in vivo* [103].

In addition to IRS-1 serine phosphorylation, the inflammatory kinases mentioned above exert their effects through regulation of gene expression, including inducing inflammatory gene expression via activation of transcription factors, such as activator protein-1 (AP-1) and NF-κB [105]. When NF-κB pathways are activated, more inflammatory cytokines and chemokines, such as TNFα, IL-6, IL-1β and MCP-1 are produced, which further activates JNK, IKKβ, and PKC and establishes a stable, feed-forward signaling loop. Other mechanisms that link inflammation with insulin resistance may include suppressor of cytokine signaling (SOCS) proteins, which are involved in the degradation of IRS-1 [106], as well as organelle dysfunction [107].

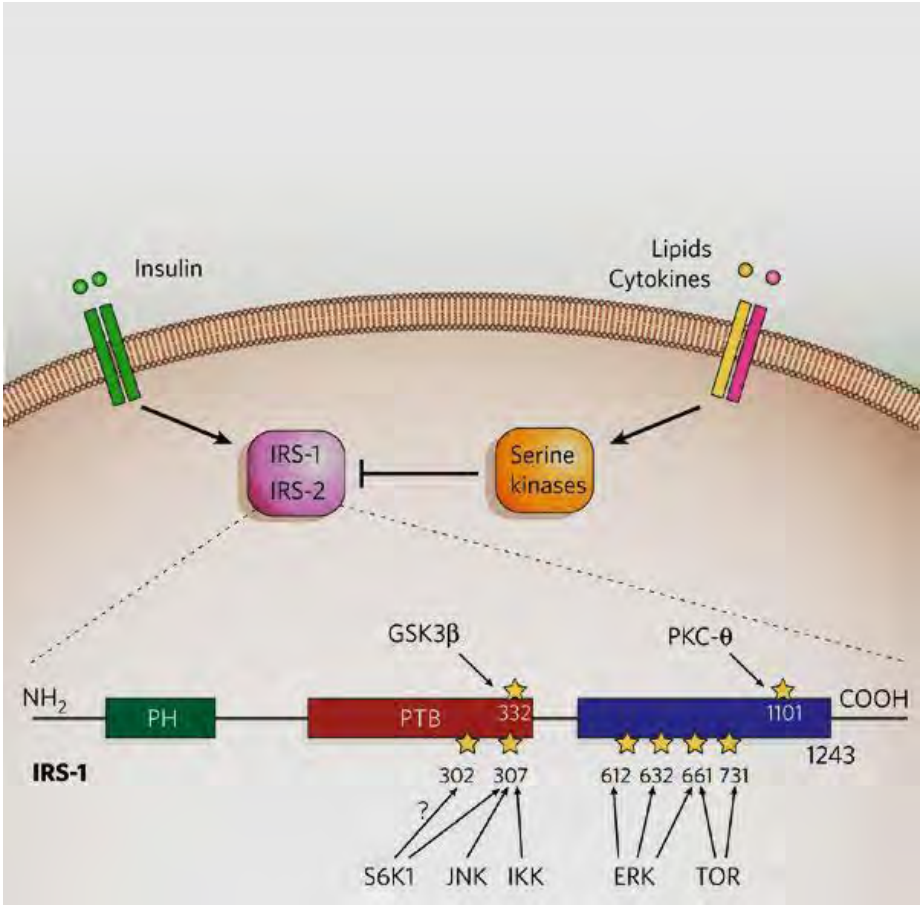


Figure 1.9 Insulin-receptor signaling interfaces with inflammatory signaling at the level of insulin-receptor substrates via activation of serine kinases

Serine kinases (ERK, extracellular regulated mitogen-activated protein kinase; GSK3, glycogen synthase kinase 3; S6K1, ribosomal protein S6 kinase polymerase 1; TOR, target of rapamycin) respond to both lipids and cytokines. IRS contains a number of serine residues (indicated by stars) that are targeted by various kinases.

Adapted from *Nature. 2006 Dec 14;444(7121):860-7*.

1.4.3 Resident and infiltrated immune cells in adipose tissue

Among one of the first organs that influenced by nutritional excess is the adipose tissue, which can communicate with other peripheral tissues through the release of adipokines, cytokines and fatty acids; thereby having effects on systemic inflammation and insulin sensitivity. Genetic manipulations within adipocytes that enhance local insulin sensitivity often improve systemic insulin sensitivity and glucose homeostasis [108-111], indicating a primary role of adipose tissue in whole body metabolism.

One hallmark of the inflammatory state of obesity is increased immune cell infiltration into metabolic tissues, especially, WAT. Several types of stresses such as endoplasmic reticulum (ER) stress, oxidative stress and hypoxia are associated with adipose tissue expansion [107]. All of these cellular stresses result in the production of chemokines and proinflammatory cytokines, such as MCP-1, IL-6 and TNFα. The proinflammatory environment of obese adipose tissue activates not only resident leukocytes but also creates a chemotactic gradient that further attracts various inflammatory cells [112-115].

1.4.3.1 Macrophages

Macrophages, the sentinels of innate immunity, reside in almost every tissue in the body, where they participate in various homeostatic functions in

addition to host defense. An important discovery that helped to elucidate the cause of tissue inflammation was that adipose tissues from obese animals and humans are infiltrated by large numbers of macrophages [115]. These cells display remarkable heterogeneity in their cell surface markers. By flow cytometry, macrophages are defined as $F4/80^{+}/CD11b^{+}$ cells. In the progression of weight gain from lean to obese, macrophage content increase from approximately 10% of all adipose tissue cells to over 50%.

Adipose tissue macrophages (ATMs) in lean and obese animals have different inflammatory potential along with distinct surface markers and cytokine signatures. In lean adipose tissue, macrophages are mostly alternatively activated M2-polarized macrophages, which are characterized by the presence of CD206 (mannose receptor) and macrophage galactose-type C-type lectin 1 (MGL1), and can be further subdivided into M2a, M2b and M2c cells [116, 117]. M2 macrophages in lean adipose tissue produce anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1Ra) [118, 119]. However, obesity swaps macrophage population from an immunoregulatory M2 phenotype towards a proinflammatory M1 bias. In obese adipose tissue, M1-polarized macrophages known as classically activated macrophages express CD11c (integrin alpha X chain protein), with diminished CD206 and MGL1 expression. M1 cells also express high levels of iNOS and proinflammatory cytokines (TNFα, IL-1β and IL-6). These cytokines can act locally in a paracrine manner, or they can leak out of the adipose tissue and have systemic effects, thus decreasing whole body insulin

sensitivity [120-122]. Selective depletion of $CD11c^+$ immune cells by utilizing conditional cell ablation system (based on transgenic expression of the diphtheria toxin receptor under the control of the CD11c promoter) resulted in reduced macrophage numbers as well as adipose tissue inflammation and improved insulin sensitivity in the diet-induced obese mice [123]. It is important to note that tissue macrophages respond to perturbations of energy homeostasis rapidly by constantly altering their transcriptional programs, thus, ATMs may actually represent a phenotypic spectrum. The M1/M2 classification described here is very likely an oversimplified view of more dynamic macrophage populations that are present in the adipose tissue.

In general, most adipose macrophages are thought to originate from bone marrow-derived monocytes, which infiltrate adipose tissue from circulation. However, recent data from our laboratory has shown that macrophages can undergo significant cell division within visceral and subcutaneous adipose tissues of obese mice, suggesting that *in situ* proliferation might also be an important mechanism by which ATMs accumulate (Amano et al. unpublished data).

1.4.3.2 Lymphocytes

Other than macrophages, recent studies have revealed a growing list of immune cells that infiltrate obese adipose tissue.

T cells, as one subset of lymphocytes have been shown to infiltrate adipose tissue during obesity. In 2009, three independent studies demonstrated the critical role of T cells in obese adipose tissue for the regulation of insulin sensitivity in genetic or diet-induced obese mouse models [124-126]. Within the adipose microenvironment, the direct or indirect interactions between T cells and adipocytes may be multifaceted. Multiple cytokines and chemokines may regulate lymphocyte recruitment to the WAT. One such protein known as regulated upon activation, normal T cell expressed and secreted (RANTES), which is secreted by murine and human adipocytes as well as stromal vascular fraction (SVF) cells [127], may promote the accumulation of lymphocytes into WAT via binding to their receptor CCR5. Similar to RANTES, the interaction between IFNγ inducible protein-10 (IP10), which is expressed by adipocytes [128], and its receptor CXCR3, which is present on T cells, can alter the balance between effector T cells and regulatory T cells (Tregs) [129] and contribute to T cells recruitment into adipose tissue [130]. Furthermore, lymphocytes may modulate adipocyte cytokine secretion via the activation of the CD40–CD40L dyad and vice versa [131]. This possibility will be discussed in much detail later.

Distinct T cell subtypes play different roles in the course of obesity-related adipose tissue inflammation. T cells that express the surface marker CD8, referred to as effector, or cytotoxic T cells secrete proinflammatory cytokines. A study by Nishimura and colleagues has demonstrated that $CD8^+$ T cell infiltration precedes that of macrophages during HFD feeding, and this T cell subset

promotes the recruitment and activation of ATMs [124]. In addition, overweight individuals contain a higher proportion of $CD8^+$ cells in visceral adipose tissue compared with subcutaneous adipose tissue, and the percentage of $CD8^+$ cells is positively correlated with the activity of caspase-1, which is a component of the NLR pyrindomain-containing 3 (NLRP3) inflammasome [132].

T helper (T_H) cells express the surface marker CD4 and can be divided into three subpopulations: T_H1 cells, which produce proinflammatory cytokines (such as IFNγ); T_H2 cells, which produce anti-inflammatory cytokines (such as IL-4, IL-10 and IL-13) [133] and the recently identified IL-17-producing T_H17 subpopulation [134]. In obesity, adipose tissue-associated lymphocytes undergo reorganization with the transition of small T_H2 cell dominated repertoire (associated with lean phenotype) giving way to a much larger and more inflammatory T_H1 and $CD8^+$ cell dominated population [125]. This change leads to a progressively proinflammatory environment promoting insulin resistance. Compared with wild-type mice, increased body weight and increased insulin resistance in $RAG1^{-/-}$ mice, which lack T lymphocytes, were reversed after adoptive transfer of $CD4^+$, but not $CD8^+$ T cells [125]. These results suggest that metabolic dysfunction caused by obesity is at least partially modulated by $CD4^+$ cells, and that the imbalance between T_H1 and T_H2 cells in the WAT may represent a pathophysiological component of obesity and insulin resistance.

Another $CD4^+$ T cell population, Tregs, express forkhead-winged-helix transcription factor (Foxp3) and are characterized as $CD4^+/CD25^+/Foxp3^+$ cells. Tregs secrete anti-inflammatory signals that inhibit macrophage migration and induce M2-like macrophage differentiation. These cells are considered to be important players in obesity-related adipose tissue inflammation. The number of adipose tissue Tregs decreases with obesity [126, 135, 136], whereas adoptive transfer of Tregs improves WAT inflammation and ameliorated insulin resistance in *ob/ob* mice [136, 137].

Natural killer T (NKT) **cells** represent a heterogeneous T cell subpopulation with innate-like characteristics and are capable of producing both T_H1 and T_H2 cytokines [138]. The role of NKT cells in obesity-related inflammation is not entirely clear. On one hand, adoptive transfer of NKT cells improves nonalcoholic steatohepatitis and glucose intolerance in *ob/ob* mice [139]; on the other hand, NKT-deficient mice on HFD also display the same trend with reduced macrophage accumulation and better glucose tolerance compared with control mice [140]. Further investigations are required to elucidate the role of NKT cells in obesity-associated inflammation.

B cells can also accumulate in the WAT of HFD-fed obese mice [141]. Some studies have reported that B cells infiltrate into the WAT earlier than T cells or macrophages and prior to the appearance of insulin resistance [142]. B cell-mediated effects on glucose metabolism are linked to proinflammatory

macrophages and T cell activation [143]. Furthermore, B cells can cause systemic insulin resistance through the production of pathogenic IgG autoantibodies [141].

1.4.3.3 Mast cells and Eosinophils

Mast cells are involved in early leukocyte recruitment and adaptive immunity via activation of antigen presenting dendritic cells. In response to different signals, mast cells can secrete a wide spectrum of biologically active products with either pro- or anti-inflammatory properties [144]. In 1963, Hellman and colleagues found that there were increased numbers of mast cells in both epididymal and subcutaneous adipose tissue of obese, diabetic mice, suggesting a potential role for mast cells in obesity [145]. This role of mast cells was confirmed by a recent study that described that genetic depletion or pharmacological stabilization of mast cells in mice reduced weight gain and ameliorated glucose homeostasis [114].

Eosinophils are associated with parasite immunity or allergy and can regulate obesity-associated adipose tissue inflammation by modulating macrophage polarization. This particular cell type was recently found to assist in glucose homeostasis by maintaining anti-inflammatory M2 macrophage content in WAT [146]. In this study, eosinophils within WAT were identified as the main source of IL-4 and IL-13, which contribute to the anti-inflammatory phenotype of macrophages. In the absence of eosinophils, M2 macrophages content was

greatly attenuated [146]. Interestingly, helminth-induced adipose tissue eosinophilia enhances glucose tolerance in HFD-fed obese mice [146].

1.4.4 Anti-inflammatory therapies for metabolic diseases

Since chronic inflammation in obesity causes adverse metabolic effects in various tissues, it is natural to raise the question of whether therapeutic interventions that modulate inflammatory signaling cascades could have beneficial effects on metabolism. Several previous and ongoing studies have targeted inflammation at different points of the inflammatory pathway in obesity.

As described above, salsalate, a salicylate prodrug, suppresses IKKβ/NFκB activity and improves glycemic control as well as lipid parameters in type 2 diabetic patients [147, 148]. Studies involving JNK antagonists, tested in mice, yielded positive results in glucose metabolism [149]. Inflammatory cytokines blockade also showed promising results on metabolic disease. Although anti-TNF-α trials yielded conflicting results [150, 151], IL-1 receptor antagonists (anakinra) improved hyperglycemia and β-cell function and decreased systemic inflammatory markers [152]. Cell-based immunotherapy targeting obesity induced-inflammation has recently attracted attention. Several animal studies demonstrated significant metabolic benefits because of depletion or stimulation of specific cell populations. For example, as discussed above, elimination of

$CD11c^+$ immune cells or mast cells in obese mice yielded beneficial metabolic effects [114, 123].

These studies provide proof-of-principle that damping the inflammatory component of obesity results in improved insulin sensitivity in mice and humans.

1.5 CD40-CD40L: a novel therapeutic target in obesity-induced adipose tissue inflammation?

Although the association between inflammation and obesity is extensively described in the literature as discussed earlier in this chapter, the nature and source of the early signals that initiate the proinflammatory phenotype in obese adipose tissue remain unknown. The exact immune cells that mediate adipose tissue inflammation and the spatial-temporal interplay between those different cell-types are unclear.

Two recent studies reported that major histocompatibility complex (MHC) class II expression in adipose tissue increased during the course of diet-induced obesity [153, 154]. One study indicated that ATMs can function as antigen presenting cells (APCs) [153], whereas the other study suggested that adipocyte MHC II is functional, therefore, adipocytes themselves can perform APC functions and induce antigen-specific $CD4^+$ T cell activation via MHC II pathways [154].

1.5.1 T cell activation

T cells within adipose tissue are increasingly recognized to modulate adipose tissue inflammation and systemic inflammation [155]. It is generally assumed that the full activation of naïve T cells, including $CD4^+$ T helper cells as well as $CD8^+$ cytotoxic T cells, requires 3 signals. These signals involve both contact-dependent interactions through surface molecules and soluble factors like cytokines (Fig 1.10).

- **Signal 1**, the initial signal, is the recognition by the T-cell receptor (TCR) of antigenic peptides presented by MHC II molecules. This signal alone often leads to T cell inactivation by anergy or deletion.

- **Signal 2** or co-stimulation is mainly provided by the interaction between co-stimulatory molecules that are expressed as receptors and ligands on the membrane of APC (B7/CD40) and the T cell (CD28/CD40L). Along with signal 1, signal 2 drives the T cell towards an activation state, which is characterized by sustained proliferation and the up-regulation of surface markers.

- **Signal 3** or polarization is transmitted by APCs and various inflammatory cytokines, which further enhance, modify, and skew T cell differentiation into different effector phenotypes.

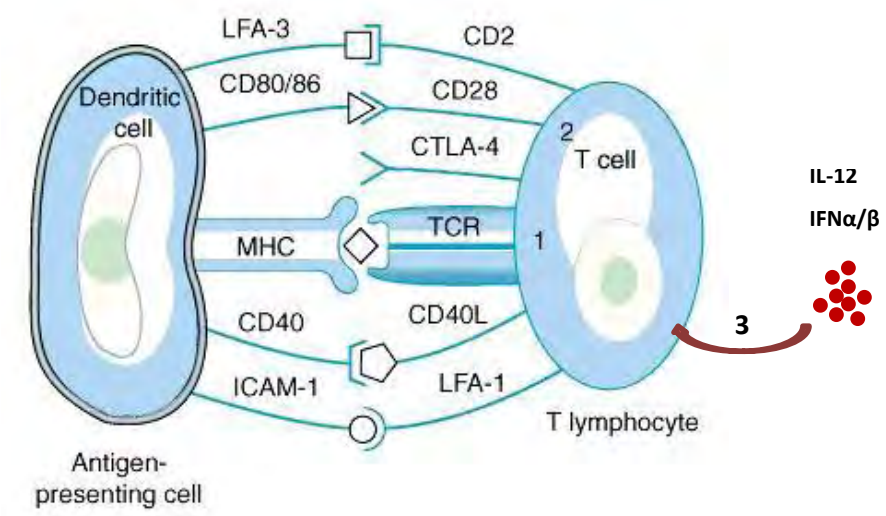


Figure 1.10 Three signals for T cell activation

The first signal involves T-cell receptor (TCR) triggering by donor antigen on APCs (Dendritic cells, macrophages etc.). The second signal or “costimulation signal” is delivered when CD80/86/40 on the surface of APCs engage CD28/CD40L on T-cells.The third signal involves various cytokines resulting in T cell differentiation.

1.5.2 The CD40-CD40L signaling pathway

Poggi et al. in 2009 [131] and Missiou et al. in 2010 [156], independently reported that the inflammatory receptor CD40 is expressed on the surface of both human and mouse adipocytes and that CD40 mRNA levels in adipose tissue were positively correlated with BMI [131]. The data presented in these two studies suggest that adipocytes do not passively but rather actively interact with lymphocytes, and that CD40 contributes to this crosstalk through direct cell-cell contact. These results have significant implications and suggest that CD40-CD40L signaling may play a critical role and may be among the early signals that ignite inflammation in obesity.

CD40 was first discovered as an antigen expressed on human bladder carcinomas [157] and later in B lymphocytes [158]; however, subsequent studies demonstrated that CD40 is more ubiquitously expressed than what was initially thought. A list of cells expressing CD40 and the ligand of CD40, CD40L, is described in Table 1.2. The wide expression pattern of this co-stimulatory dyad indicates its broad roles in different physiological processes.

CD40 is a 48 kDa type I transmembrane glycoprotein cell surface receptor that belongs to the tumor necrosis factor receptor (TNFR) superfamily. Human CD40 contains a 193 amino acid (aa) extracellular domain, a 21-aa leader sequence, a 22-aa transmembrane domain, and a 62-aa intracellular domain [159]. CD40L, also known as CD154, gp39 or TNFSF5, is a member of the TNF

superfamily and is a type II transmembrane protein, with variable molecular weight between 32 and 39kDa because of post-translational modifications [159]. A soluble form of CD40L, sCD40L, which is mainly derived from activated platelets and activated T cells retains activities similar to its membrane-bound form [160].

Unlike the insulin receptor described earlier in this chapter, the CD40 cytoplasmic domain does not have intrinsic kinase activity. Similar to other members of the TNF receptor family, CD40 signaling is mediated in large part by a complex series of downstream events. The engagement of CD40 by CD40L leads to trimeric clustering of CD40 and the recruitment of adapter proteins known as TNFR-associated factors (TRAFs) to the cytoplasmic tail of CD40 [161]. The cytoplasmic domain of CD40 contains a proximal binding site for TRAF6, and two distal sites that bind TRAF1, TRAF2, TRAF3 and, indirectly, TRAF5 (Fig 1.11). Binding of the TRAFs results in the formation of a signaling complex that activates different pathways including the canonical (p50 dependent) and non-canonical (p52 dependent) NFκB signaling pathways [162], p38/mitogen-activated protein kinases (MAPKs), c-Jun-NH2-kinase (JNK) as well as phosphoinositide 3-kinase (PI3K) signaling pathways [161, 163]. Independent of theTRAF-dependent signaling shown here, CD40 can also bind directly to Janus kinases 3 (JAK3). JAK3 binding induces the activators of a transcriptional program, for example, via the phosphorylation of signal transducer and activator of transcription 5 (STAT5) [164, 165].

Table 1.2 Expression of CD40 and CD40L in various cell types

Cells	CD40	CD40L	Refs
B cells	+	+	[166-168]
T cells	+	+	[169-172]
Monocytes and macrophages	+	+	[173, 174]
Dendritic cells	+	+	[167, 175, 176]
Neutrophils	+	-	[177, 178]
Mast cells	+	+	[179]
Platelets	+	+	[180-182]
Endothelial cells	+	+	[183, 184]
Fibroblast	+	-	[167, 185]
Smooth muscle cells	+	+	[184, 186]
Adipocytes	+	-	[131, 156, 187]

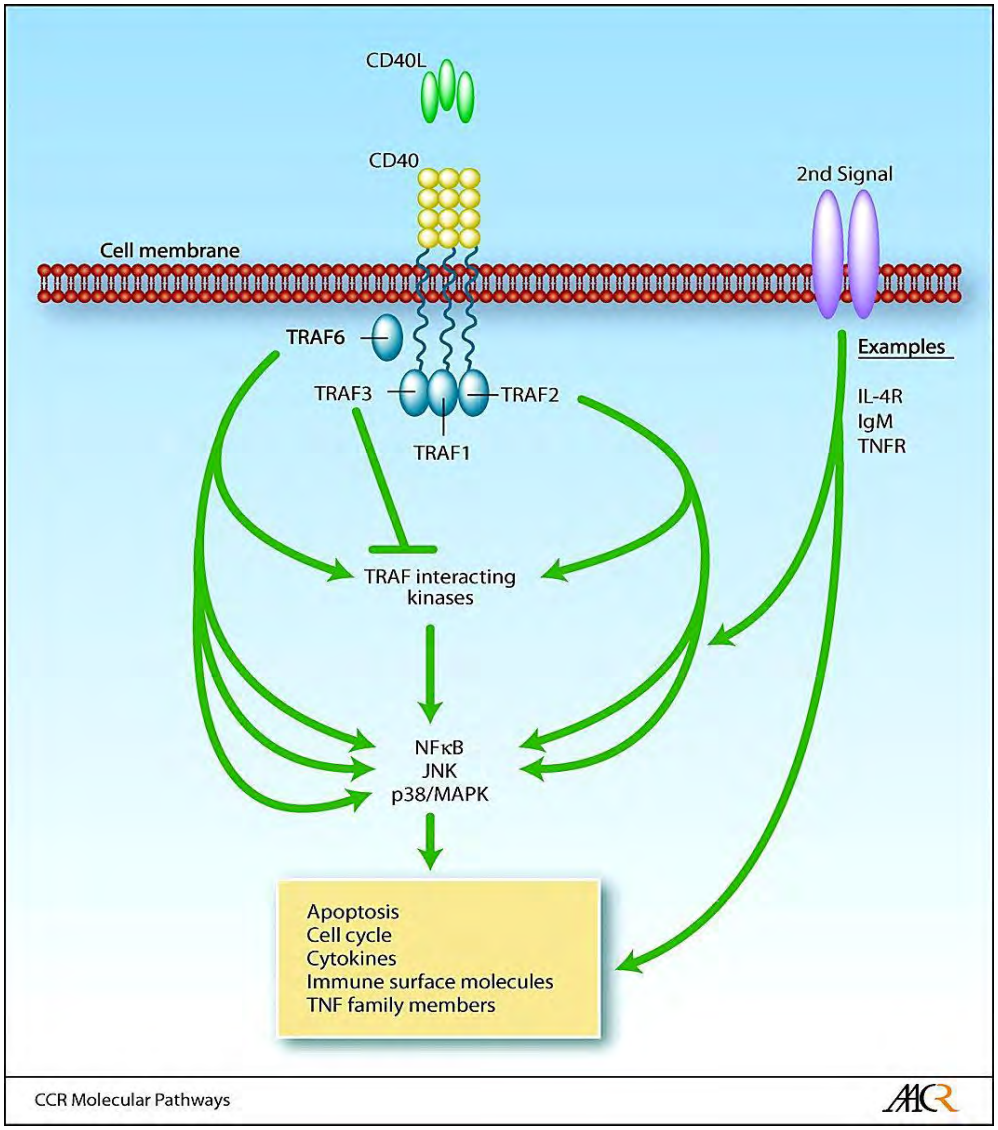


Figure 1.11 The CD40 signaling pathway

The interaction between CD40 and CD40L causes a conformational change that recruits adapter molecules known as TNF receptor associated factors (TRAF) to the binding sites on the CD40 cytoplasmic tail. TRAFs then recruit TRAF-interacting kinases and together influence a number of signal transduction pathways, including the nuclear factor-κB, p38/mitogen-activated protein kinase (MAPK), and c-Jun-NH2-kinase (JNK) pathways. Target genes of CD40 signaling regulate apoptosis, cell cycle progression, cytokine production, expression of cell surface immune modulators, and TNF family members and other pathways

Adapted from *Clin Cancer Res. 2007 Feb 15;13(4):1083-8*

Taken together, these complex pathways elicit essential signals that are mediated through CD40 to regulate diverse cellular processes including apoptosis, cell cycle progression, inflammatory chemokine and cytokine production, dendritic cell survival and prolonged MHC/antigen complex presentation.

It is now clear that CD40-CD40L signaling regulates antibody production and T cell function through processes that involve T helper cells, APCs, and circulating B cells. The *in vivo* critical role of CD40-CD40L in regulating immunity is emphasized by the finding that mutations in either CD40L or CD40 gene cause hyper IgM syndrome (HIGM) in patients, termed HIGM1 and HIGM3, respectively [188]. HIGM is characterized by increased susceptibility to opportunistic pathogens, such as *Pneumocystis carinii* and *Cryptosporidium* [189, 190] and by elevated concentrations of serum IgM with very low levels of IgG, IgA and IgE [191, 192]. Similar deficiencies in mounting immune responses were observed in genetically modified $CD40L^{-/-}$ [193, 194] and $CD40^{-/-}$ mice [195, 196].

To date, much is known about the consequences that defective CD40-CD40L interactions have on the immune system. In contrast, very little is known about the exact role of CD40 signaling in non-hematopoietic cell types. Increasing evidence from experimental and clinical studies has suggested a complex but not fully elucidated role of CD40-CD40L signaling in regulating the pathogenesis of obesity, type 2 diabetes and vascular complications. For example, the interaction between CD40 and CD40L triggers a cascade of events

that are involved in early and late stages of atherosclerosis [197] and sCD40L levels may predict risk for major cardiovascular events, including acute myocardial infarction and sudden cardiac death [198, 199]. In addition, Lutgens and colleagues indicate that absence of CD40L-CD40-TRAF6 signaling abolished atherosclerosis by skewing the immune response towards an anti-inflammatory profile [200]. Furthermore, Poggi et al. demonstrated that CD40 mRNA levels in adipose tissue and plasma levels of CD40L positively correlated with BMI [131]. CD40 is expressed on adipocytes as well as immune cells in adipose tissue SVF and CD40L exposure reduces insulin mediated glucose uptake by adipocytes. These data suggest that resident or infiltrated T cells in obese adipose tissue may directly induce adipocyte inflammation and impair adipose tissue insulin sensitivity, thereby contributing to systemic inflammation [131].

The therapeutic potential of targeting the CD40-CD40L signaling axis to reduce the deleterious effects of inflammation has been extensively investigated in multiple diseases involving acute inflammation, such as atherosclerosis, inflammatory bowel disease, psoriasis, rheumatoid arthritis, allograft rejection and type I diabetes [201]. However, whether this strategy can be applied to the treatment of complications like chronic low-grade inflammation in obesity and type 2 diabetes remains unanswered. Importantly, because costimulation blockade interrupts an effective immune response, long-term CD40-CD40L signaling interventions will not be without risk of immune-suppression, therefore it is crucial to understand the detailed molecular mechanism of CD40-CD40L

signaling in the pathophysiology of obesity to fine tune this signaling pathway meticulously.

Chapter II

CD40 Deficiency Exacerbates Obesity-induced Adipose Tissue Inflammation, Hepatic Steatosis, and Insulin Resistance in Mice

Chapter contents

2.1 Introduction

Obesity is often associated with type 2 diabetes [202, 203], hepatic steatosis, NASH [204], cardiovascular complications [205, 206] and certain cancers [207, 208]. 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 [209, 210]. 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 [124, 126, 140, 142, 211]. 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 [90], liver [86], and muscle [212], 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 [74, 213], 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 [214]. 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 [215]. Recent evidence indicates that the CD40-CD40L complex is also a potential mediator of chronic inflammation in obesity and its related metabolic disorders [131, 156, 187, 216], suggesting a broader role of CD40 in cell biology. Obese and diabetic individuals have higher levels of active sCD40L in the circulation compared to lean healthy subjects [217] and CD40 mRNA levels in WAT were found to positively correlate with BMI [131]. 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 [218]. Currently, targeting CD40-CD40L signaling is considered to be a promising strategy for effecting plaque stabilization in the treatment of atherosclerosis [219].

On the basis of 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 [220-222]. 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 [221, 222], whereas another study indicated that the absence of CD40L attenuated diet-induced steatosis [220]. Furthermore, in one study, CD40L deficiency improved insulin resistance [220], whereas in another study lack of CD40L in mice did not ameliorate HFD induced insulin resistance [222]. 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 [223]. 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 the increased influx of lipid from adipose tissue and enhancing whole body glucose tolerance.

2.2 Materials and Methods

2.2.1 Animal studies

Male CD40$^{-/-}$ (B6.129P2-Cd40^{tm1Kik}/J) and control C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The background strain of B6.129P2-Cd40^{tm1Kik}/J mice in this study is C57BL/6Ncr, which is maintained by the Jackson Laboratory via sibling mating (34 generations as of Nov, 2008). Since the C57BL/6Ncr mouse line from NIH has been separated from the B6.129P2-Cd40^{tm1Kik}/J mice for many generations, we created littermate control animals for this study to avoid potential genetic drift from various C57BL/6Ncr substrains or colonies. We bred B6.129P2-Cd40$^{tm1K\ k}$/J mice with C57BL/6J mice and then their F1 hybrids were used to generate knockout and wild-type littermates. All animals were fed a standard chow diet (LabDiet PicoLab 5053; Purina Mills, St. Louis, MO) until 8 weeks of age and then were divided into two groups; one was fed chow diet and the other group a HFD (TD.93075, 55/Fat, Harlan Teklad, Madison, WI, fatty acid profile as % total fat: 28% saturated, 30% trans, 28% monounsaturated cis, 14 % polyunsaturated cis). Animals were housed in the University of Massachusetts (UMass) Medical School Animal

Medicine facility with a 12:12-h 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 [224]. Briefly, after a 16-hour overnight fast, GTTs or PTTs were performed with intraperitoneal (i.p.) injection of 10% w/v D-glucose in sterile water (1 g glucose/kg body weight). Whole blood glucose values were measured using Ascencia Breeze glucose monitors (Bayer Healthcare Diabetes Care Division, Tarrytown, NJ) before and after the indicated times post challenge/injection. ITTs were similarly performed with i.p. injection of recombinant human insulin (1 U insulin/kg body weight; Novolin R, Novo Nordisk Inc, Princeton, NJ) following a shorter fast period of 4-hour and blood glucose measurements were done at the indicated times before and post challenge/injection. Composition of total fat and lean mass was assessed by 1H-MRS-based body composition analysis (EchoMRI-3in1TM, 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.

2.2.2 Hyperinsulinemic-euglycemic clamp studies

The clamp study was performed at the UMass Mouse Metabolic Phenotyping Center. Mice fed HFD for 16 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 [225]. Whole body glucose turnover was assessed with a continuous infusion of [3-^{3}H] glucose and 2-deoxy-D-[1-^{14}C] 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.

2.2.3 Hepatic triglyceride analyses

Hepatic triglyceride content measurement was performed as previously described [226]. 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).

2.2.4 Histology

Liver, pancreas, and epididymal WAT 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 (7 μm) were stained with 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.

2.2.5 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 listed in Table 2.1. Fold change in mRNA expression was determined using the $2^{-\Delta\Delta C_T}$ method, with all genes normalized to the ribosomal mRNA 36B4.

2.2.6 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 a BCA protein assay kit (Thermo Scientific, Waltham, MA), resolved on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat 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 (Ser^{473}) and total Akt (Cell Signaling, Danvers, MA).

2.2.7 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 of DMEM (Life Technologies) containing 2% fatty acid-free serum albumin (Sigma-Aldrich). Subsequently, fat pads were incubated in 250 µl of KRH buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2.6 mM $MgSO_4$, 5 mM HEPES, pH 7.2) plus 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.

2.2.8 Flow cytometry

Epididymal adipose tissue was isolated from mice and treated with 2 mg/mL collagenase (Sigma) for 45 minutes at 37^{o}C. Digested tissue was filtered through a 200 μM nylon mesh and then centrifuged at 1000 rpm for 10 min. Pelleted SVF of adipose tissue was stained with fluorescently conjugated primary antibodies according to manufacturers' instructions. Primary antibodies used were CD4-FITC (BD Pharmingen cat. no. 553046), CD3-APC-Cy7 (BD Pharmingen cat. no.1452C11), CD25-APC (eBiosciences cat. no. 17-0251-81), CD8a-PerCP-Cy5.5 (BD Pharmingen cat. no. 551162), CD90-PE-Cy7 (BD Pharmingen cat. no. 561558), CD11b-PerCP-Cy5.5 (BD Pharmingen cat. no. 562127), F4/80-APC (ABD Serotec), NK1.1-APC (eBiosciences cat. no. 17-5941), CD19-Alexa 405 (BD Pharmingen cat. no. 560375), and FoxP3-PE (eBiosciences cat. no. 12-4774). Samples were run on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed in FlowJo (TreeStar).

2.2.9 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.

2.2.10 Vascular permeability assay

Evans blue dye (5 mg/ml in PBS; Sigma-Aldrich) was injected into the tail vein of mice at the dose of 25 mg/kg. After one hour of complete circulation of the dye, the mice were euthanized by CO_2 inhalation and were perfused with 30 ml warm PBS via the left ventricle to remove the Evans blue dye from the vasculature. Visceral adipose tissues were surgically removed, blotted dry, and weighed. The Evans blue dye was extracted from the fat tissue with 1 ml of formamide overnight at 55°C and measured spectrophotometrically at 620 nm. The optical density (OD) value was transformed into the concentration according to a standard curve of Evans blue dye.

2.2.11 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 were considered significant.

Table 2.1 Primers for Real-Time Quantitative RT-PCR

36B4	Forward: TCCAGGCTTTGGGCATCA Reverse: CTTTATCAGCTGCACATCACTCAGA
C/EBPα	Forward: CAAGAACAGCAACGAGTACCG Reverse: GTCACTGGTCAACTCCAGCAC
C/EBPβ	Forward: CTGAGCGACGAGTACAAGAT Reverse: CTGCTTGAACAAGTTCCG
PPARγ	Forward: TCGCTGATGCACTGCCTATG Reverse: GAGAGGTCCACAGAGCTGATT
SREBP-1c	Forward: CATCAACAACCAAGACAGTC Reverse: CCAGAGAAGCAGAAGAGAAG
ChREBP	Forward: AGATGGAGAACCGACGTATCA Reverse: ACTGAGCGTGCTGACAAGTC
LXRα	Forward: CTCAATGCCTGATGTTTCTCCT Reverse: TCCAACCCTATCCCTAAAGCAA
FASN	Forward: GGAGGTGGTGATAGCCGGTAT Reverse: TGGGTAATCCATAGAGCCCAG
ACC1	Forward: ATGGGCGGAATGGTCTCTTTC Reverse: TGGGGACCTTGTCTTCATCAT
SCD1	Forward: TTCTTGCGATACACTCTGGTGC Reverse: CGGGATTGAATGTTCTTGTCGT
ACSL1	Forward: TGCCAGAGCTGATTGACATTC Reverse: GGCATACCAGAAGGTGGTGAG
ACSL4	Forward: CTCACCATTATATTGCTGCCTGT Reverse: TCTCTTTGCCATAGCGTTTTTCT
ACCβ	Forward: CGCTCACCAACAGTAAGGTGG Reverse: GCTTGGCAGGGAGTTCCTC
ACOT3	Forward: GGAATTGGAAGTGGCCTTCTG Reverse: TCCATGTCCTTAGGGAGGTCC
DGAT1	Forward: TCCGTCCAGGGTGGTAGTG Reverse: TGAACAAAGAATCTTGCAGACGA
DGAT2	Forward: GCGCTACTTCCGAGACTACTT Reverse: GGGCCTTATGCCAGGAAACT
DYK	Forward: TGAACCTGAGGATTTGTCAGC Reverse: CCATGTGGAGTAACGGATTTCG
MTTP	Forward: CTCTTGGCAGTGCTTTTTCTCT Reverse: GAGCTTGTATAGCCGCTCATT
ICAM1	Forward: GTGATGCTCAGGTATCCATCCA Reverse: CACAGTTCTCAAAGCACAGCG
VCAM1	Forward: AGTTGGGGATTCGGTTGTTCT Reverse: CCCCTCATTCCTTACCACCC
NLRP3	Forward: ATTACCCGCCCGAGAAAGG Reverse: TCGCAGCAAAGATCCACACAG
F4/80	Forward: CCCCAGTGTCCTTACAGAGTG Reverse: GTGCCCAGAGTGGATGTCT
CD68	Forward: GGACCCACAACTGTCACTCA Reverse: AAGCCCCACTTTAGCTTTACC
CD11b	Forward: CCATGACCTTCCAAGAGAATGC Reverse: ACCGGCTTGTGCTGTAGTC
CD11c	Forward: CTGGATAGCCTTTCTTCTGCTG Reverse: GCACACTGTGTCCGAACTCA
MGL1	Forward: TGAGAAAGGCTTTAAGAACTGGG Reverse: GACCACCTGTAGTGATGTGGG

CD3γ	Forward: ACTGTAGCCCAGACAAATAAAGC Reverse: TGCCCAGATTCCATGTGTTTT
IFNγ	Forward: ATGAACGCTACACACTGCATC Reverse: CCATCCTTTTGCCAGTTCCTC
TNFα	Forward: TCAGCCGATTTGCTATCTCATA Reverse: AGTACTTGGGCAGATTGACCTC
IL-1β	Forward: GCAACTGTTCCTGAACTCAACT Reverse: ATCTTTTGGGGTCCGTCAACT
Cidea	Forward: ATCACAACTGGCCTGGTTACG Reverse: TACTACCCGGTGTCCATTTCT
FSP27	Forward: ATCAGAACAGCGCAAGAAGA Reverse: CAGCTTGTACAGGTCGAAGG
IL-6	Forward: TAGTCCTTCCTACCCCAATTTCC Reverse: TTGGTCCTTAGCCACTCCTTC
CD40	Forward: TTGTTGACAGCGGTCCATCTA Reverse: GCCATCGTGGAGGTACTGTTT
CD154	Forward: CCTTGCTGAACTGTGAGGAGA Reverse: CTTCGCTTACAACGTGTGCT
SAA2	Forward: TGGCTGGAAAGATGGAGACAA Reverse: AAAGCTCTCTCTTGCATCACTG
CD4	Forward: CTAGCTGTCACTCAAGGGAAGA Reverse: CGAAGGCGAACCTCCTCTAA
CD8α	Forward: CCGTTGACCCGCTTTCTGT Reverse: TTCGGCGTCCATTTTCTTTGG
vWF	Forward: CTTCTGTACGCCTCAGCTATG Reverse: GCCGTTGTAATTCCCACACAAG
CD31	Forward: CTGCCAGTCCGAAAATGGAAC Reverse: CTTCATCCACCGGGGCTATC

2.3 Results

2.3.1 CD40$^{-/-}$ mice display 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 HFD or a normal diet (ND). The CD40-null mice were found to be ~2 grams lighter than control mice on either diet throughout most of a 14-week feeding period (Figure 2.1A). These data are consistent with a previous study on CD40L$^{-/-}$ mice [220], 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) (Figure 2.2A) and energy expenditure (Figure 2.2B) were not significantly different between the two groups of mice.

2.3.2 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

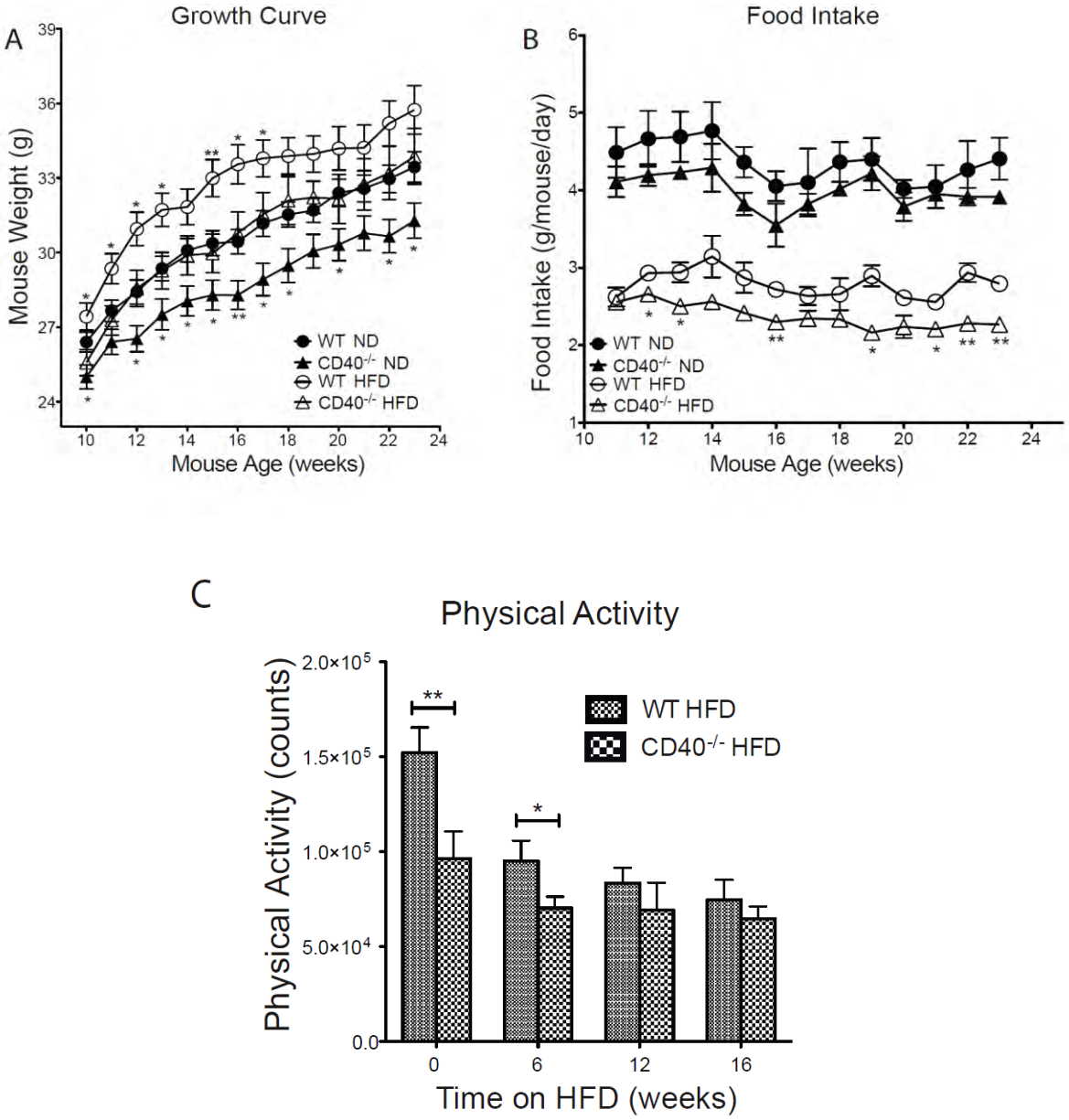


Figure 2.1 CD40 deficiency alters energy homeostasis

(A) Growth curve of mice fed either normal diet (ND) or high-fat diet (HFD) for a period of 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 a period of 13 weeks. (C) Ambulatory activity as estimated by infrared beam breaks over a 24-hour period for mice on HFD for 0, 6, 12 or 16 weeks (n = 6). Data presented are mean ± S.E.M. Statistically significant differences are indicated * $P<0.05$, ** $P<0.01$ vs. control.

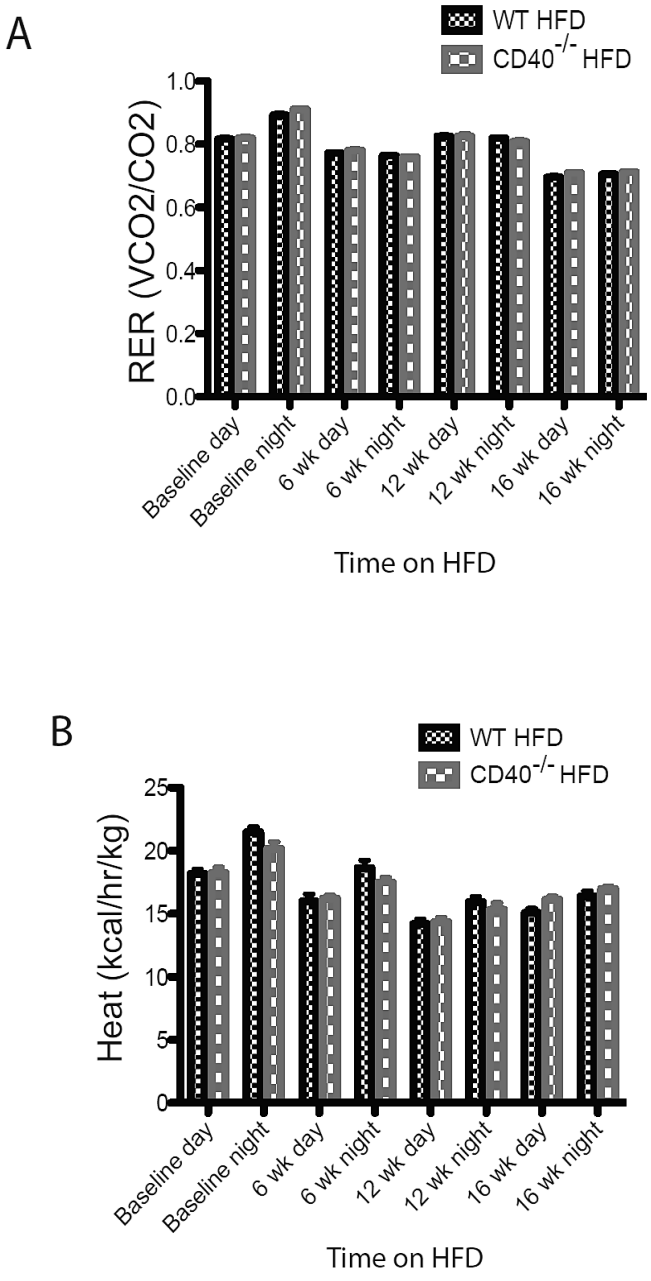


Figure 2.2 Respiratory exchange ratio and Heat production of mice on HFD

A) Respiratory exchange ratio as of 0,6,12, and 16 weeks on HFD
B) Heat production as of 0,6,12, and 16 weeks on HFD

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 2.3A), suggesting that the absence of CD40 aggravates systemic insulin resistance in obesity. We also observed decreased 2-deoxy-[^{14}C] glucose uptake in the brown adipose tissue (BAT) of $CD40^{-/-}$ mice with no changes of glucose uptake in the WAT or muscle (Figure 2.3B). The morphology of pancreatic islets (Figure 2.4), fasting serum insulin, serum sCD40L, serum triglycerides and free fatty acids (FFA) were similar in both $CD40^{-/-}$ and WT mice on HFD (Figure 2.5). To further analyze the metabolic phenotype of the $CD40^{-/-}$ mice, we subjected them to a GTT as well as an ITT. Despite the reduced body weight of $CD40^{-/-}$ mice on HFD, they exhibited glucose intolerance (Figure 2.6 A and E) and insulin resistance (Figure 2.6 B and 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 sub-strains, we created $CD40^{-/-}$ mice and WT littermate control mice by breeding the F1 hybrids of $CD40^{-/-}$ (B6.129P2-$Cd40^{tm1Kik}$/J) and C57BL6/J parental mice. These cohorts were also subjected to GTT (Figure 2.6 C and G) and ITT tests (Figure 2.6 D and H). Similar to our original findings, the $CD40^{-/-}$ mice were significantly more glucose intolerant and insulin resistant than littermate control animals.

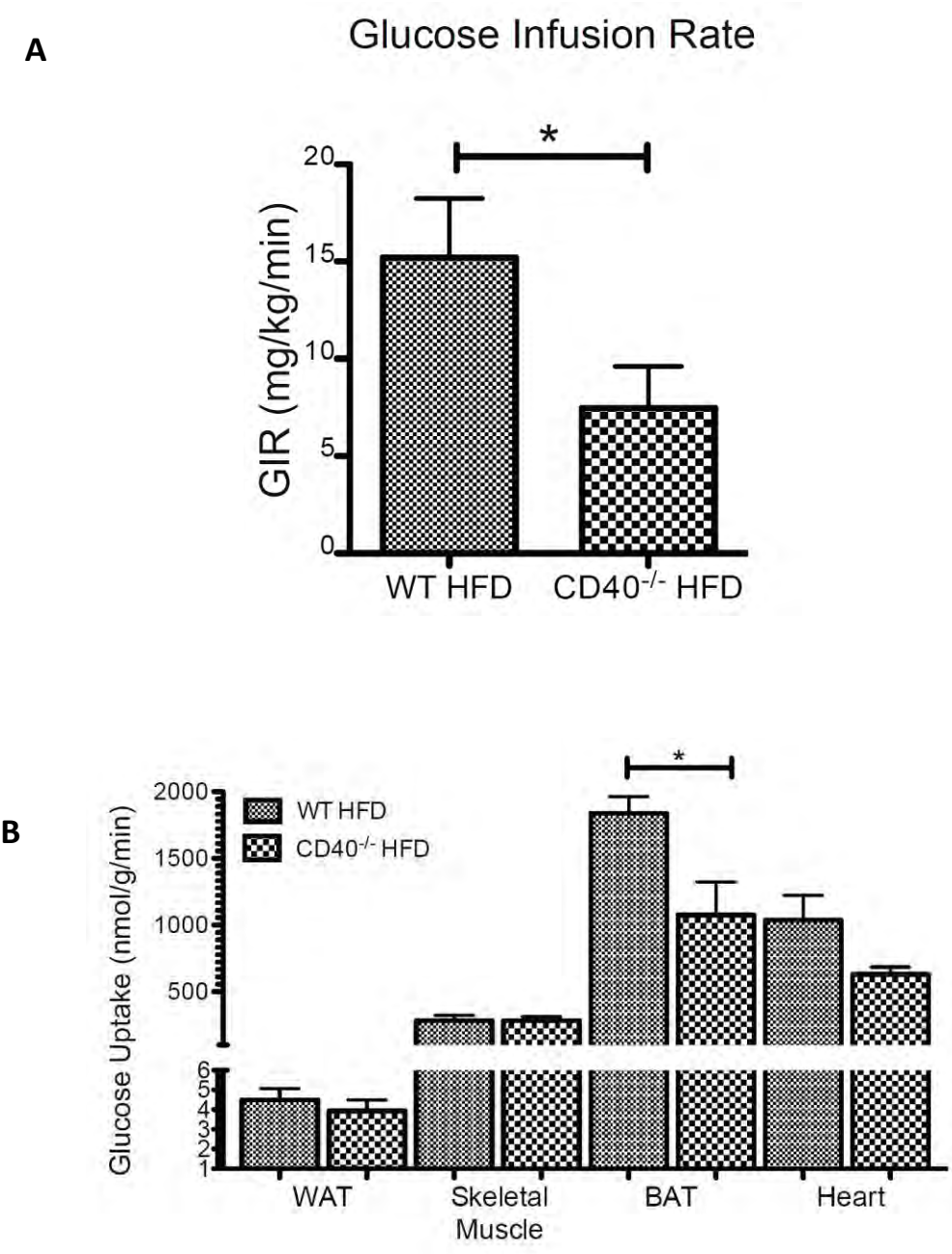


Figure 2.3 Hyperinsulinemic-euglycemic clamp analysis on mice fed HFD

(A) 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). (B) 2-deoxy-[^{14}C] glucose uptake in visceral WAT, gastrocnemius muscle (Skeletal Muscle), intrascapular brown adipose tissue (BAT), and heart. Data presented are mean ± S.E.M. Statistically significant differences are indicated * P<0.05, ** P<0.01 vs. control.

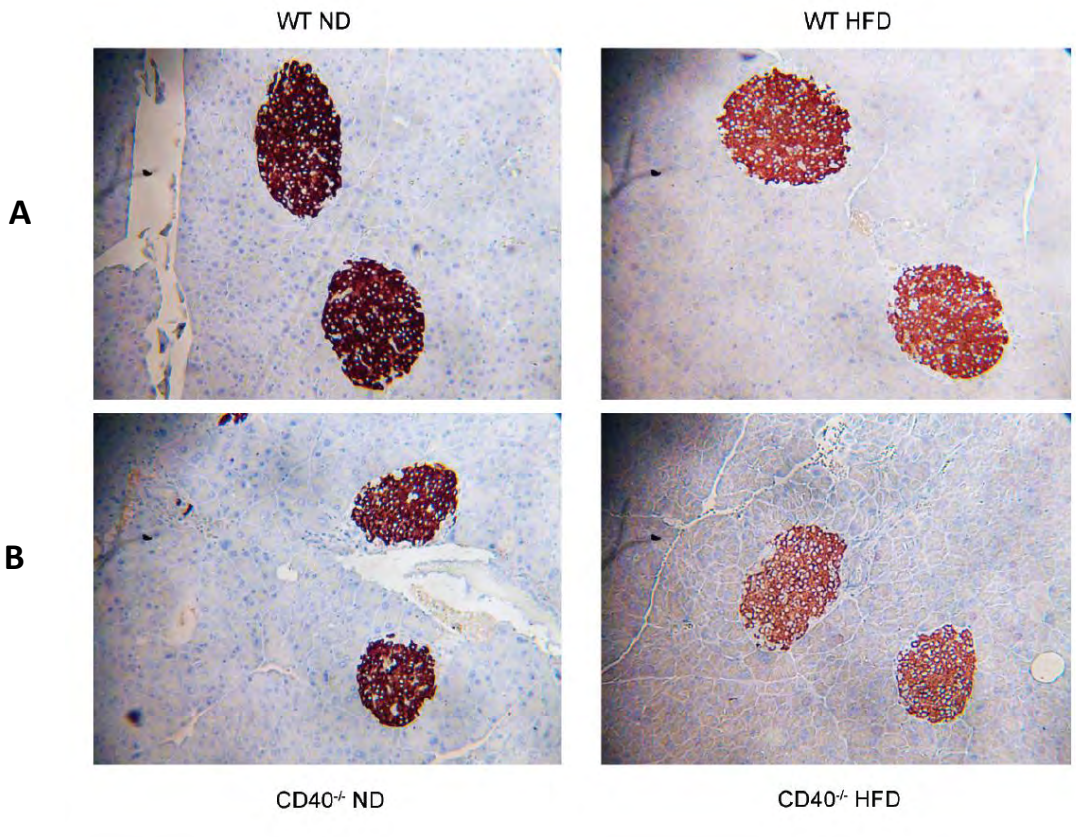


Figure 2.4 Morphology of pancreatic islets in CD40$^{-/-}$ and wild-type mice

Pancreatic islets were stained with insulin antibody (Brown)

(A) WT on ND and HFD

(B) CD40$^{-/-}$ on ND and HFD

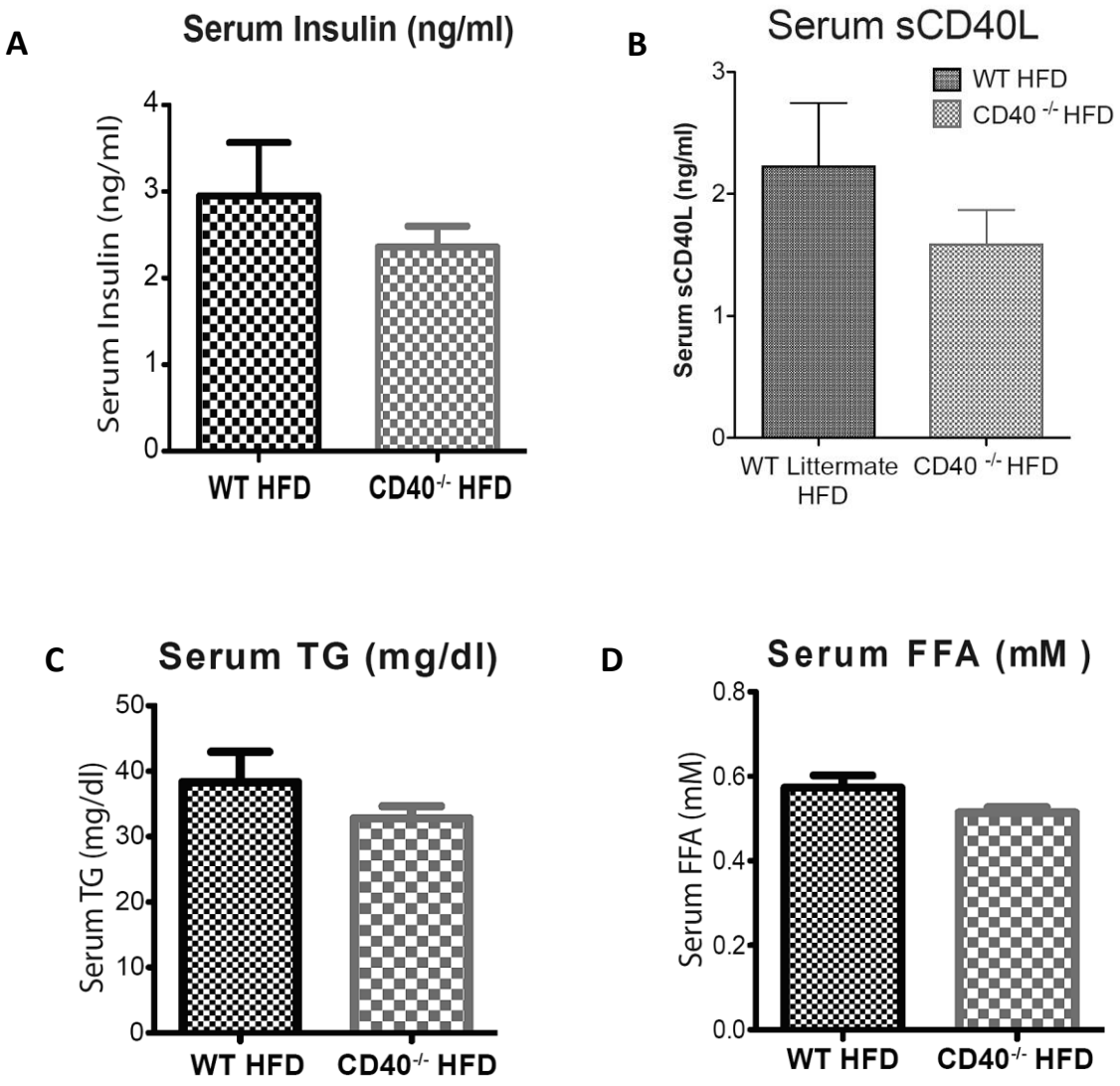


Figure 2.5 Fast serum insulin, sCD40, triglyceride (TG), and Free fatty acid (FFA) in HFD fed CD40$^{-/-}$ and wild-type mice

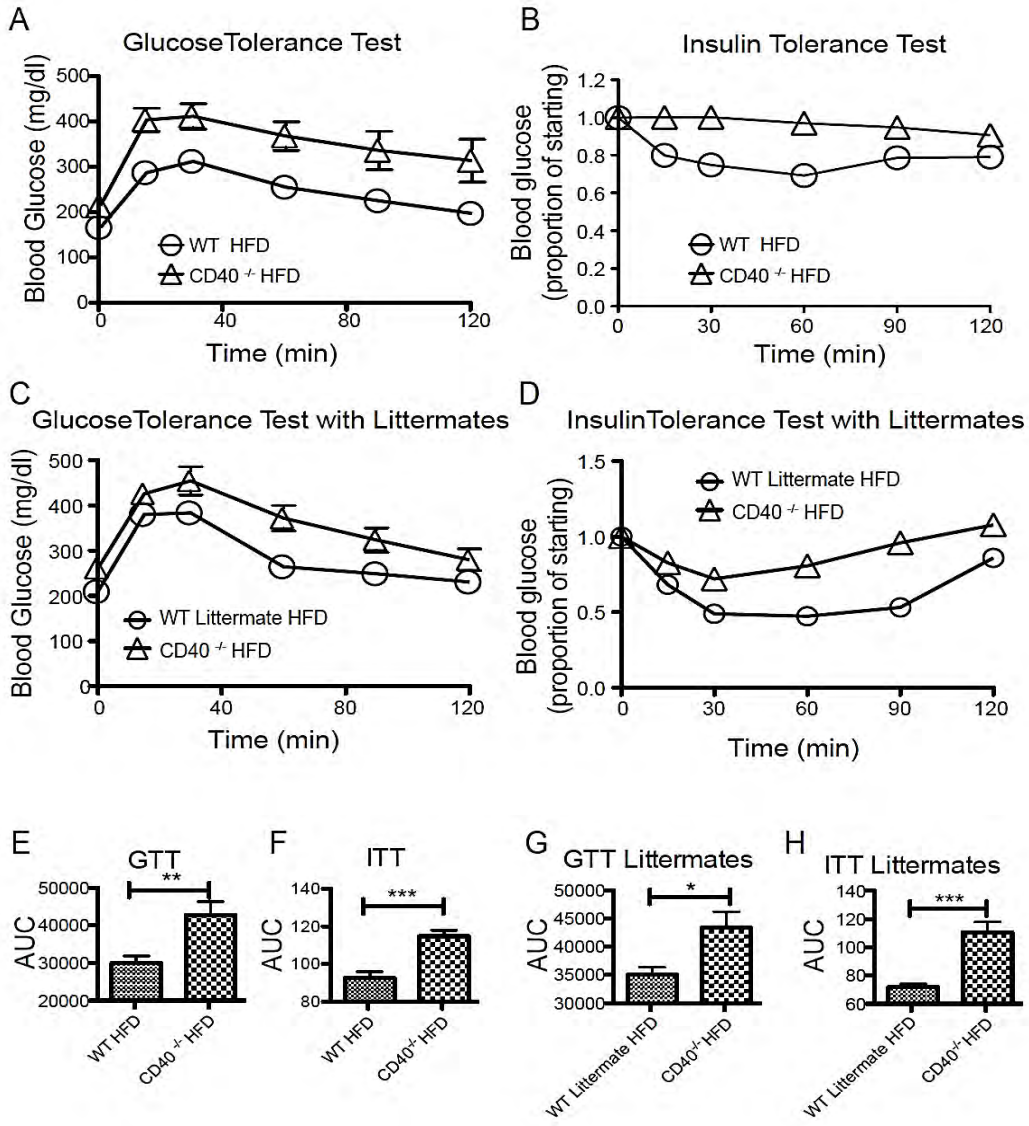


Figure 2.6 CD40$^{-/-}$ mice fed HFD are more glucose intolerant and insulin resistant

(A) Glucose tolerance test (GTT) for CD40$^{-/-}$ and C57BL/6J control mice (30-week 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-week 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)** GTT for CD40$^{-/-}$ and littermate control mice (18-week 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)** ITT; 18-week 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 WT HFD and CD40$^{-/-}$ HFD groups are indicated *P < 0.05, ** P< 0.01, ***P<0.001).

2.3.3 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 [221, 222], which has a strong association with insulin resistance [227]. 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 2.7C) compared with WT controls on the same diet (Figure 2.7A). However, CD40$^{-/-}$ mice on HFD displayed clearly steatotic livers, as observed both macroscopically and microscopically by H&E or Oil-red O staining (Figure 2.7D vs 2.7B). To quantify this increased steatosis, we measured total hepatic triglyceride levels and found a 59% increase in CD40$^{-/-}$ mice (Figure 2.7E). Elevated expression of two lipid droplet proteins, cell death-inducing DFFA-like effector A (CIDEA) and Fat Specific Protein 27 (FSP27) [228, 229], accompanied the increase in total triglycerides in the liver (Figure 2.7F). These lipid droplet proteins are specifically expressed in steatotic livers and are absent in lean livers (Figure 2.7F). Increased hepatic steatosis could be mediated by different pathways including increased dietary lipid absorption, increased *de novo* lipogenesis, the increased influx of nonesterified FFAs from hypertrophied adipose tissue, or decreased very low density lipoprotein (VLDL) secretion and fat oxidation [62, 230]. As no difference in plasma FFAs, respiratory exchange ratio and heat production were observed between CD40-null and WT mice (Figure 2.2

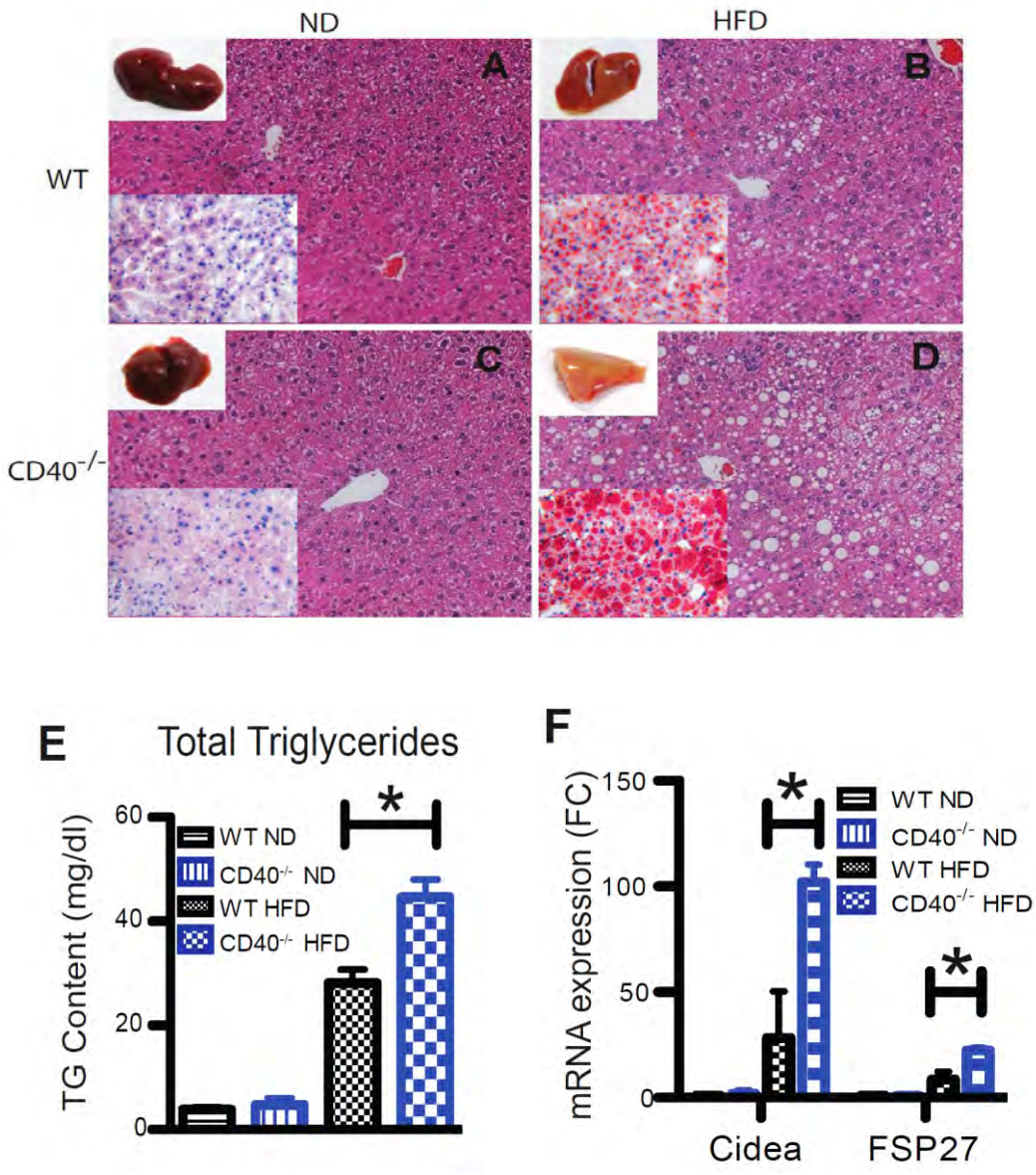


Figure 2.7 CD40 protects against diet-induced hepatic steatosis

(A-D) Macroscopic (top left insets) 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 insets) (Magnification: 200X). (E) Hepatic total triglycerides in WT and CD40$^{-/-}$ mice fasted for 6 hours (n=6). (F) Gene expression of lipid droplet proteins Cidea and FSP27 in the livers of WT and CD40$^{-/-}$ mice (n=6) was measured by qRT-PCR. Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40$^{-/-}$ HFD are indicated: *P < 0.05, ** P< 0.01.

and Figure 2.5), a role for increased dietary fat absorption or impaired fat oxidation is unlikely the cause of the hepatic steatosis in CD40$^{-/-}$ 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 (PPARγ) and sterol-regulatory element-binding protein-1c (SREBP1-c), two key regulators of hepatic lipogenesis, were both significantly elevated in the CD40$^{-/-}$ mice fed the HFD (Figure 2.8A). Acetyl-CoA carboxylase-2 (ACC2) and long-chain fatty-acid-CoA ligase-1 (ACLS1) are genes both involved in *de novo* lipogenesis, and were both increased in CD40$^{-/-}$ mice (Figure 2.8B). These data suggest that the hepatic steatosis observed in CD40$^{-/-}$ mice is probably due to an increased *de novo* lipogenesis.

2.3.4 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 with WT controls, CD40$^{-/-}$ mice on HFD for 24 weeks exhibited 45% lower Ser^{473} phosphorylation on immunoblot

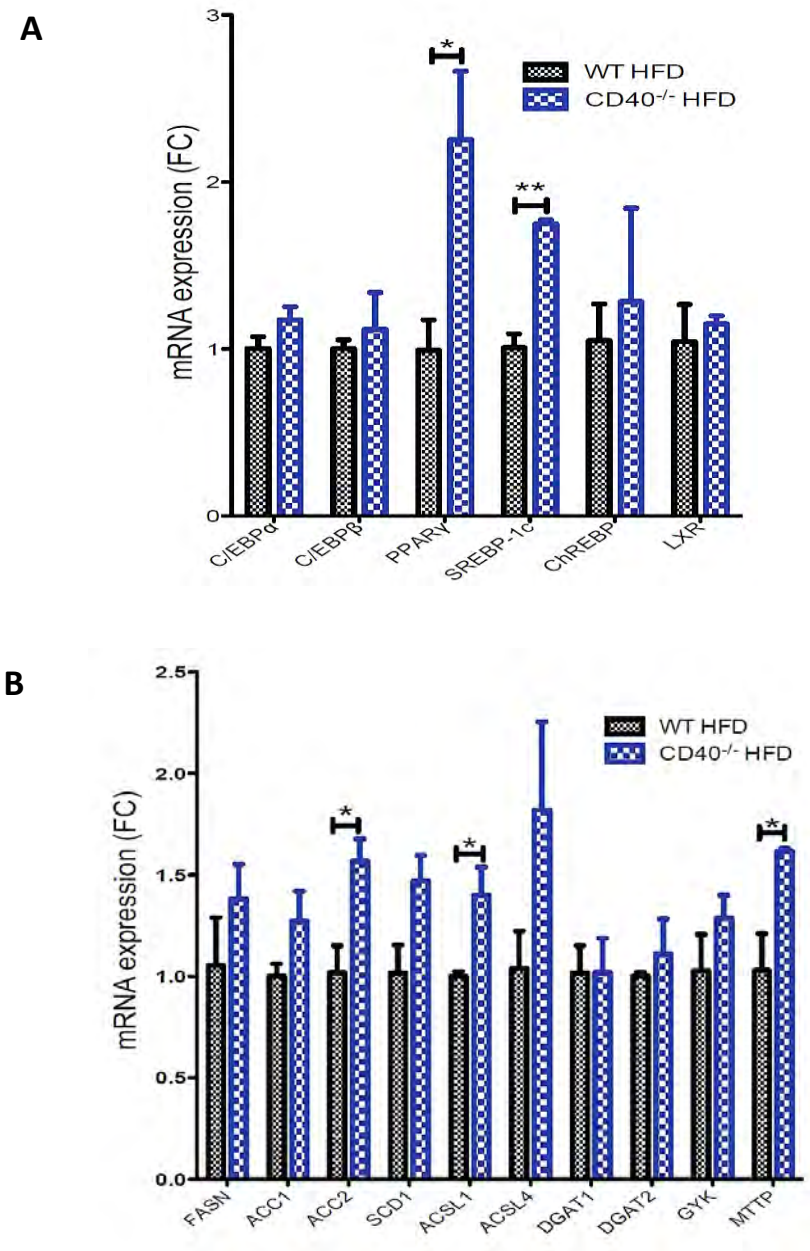


Figure 2.8 CD40 deficiency enhances hepatic lipogenesis

(A) Expression of genes that encode lipogenic transcription factors and coactivators (C/ebpα, C/ebpβ, Pparγ, Srebp1c, Chrebp and Lxr) in livers of HFD fed WT and CD40 $^{-/-}$ mice (n=6). (B) Expression of genes that encode enzymes (Fasn, Acc1/2, SCD1, Acsl1/4, Dgat1/2, Gyk, Mttp) that promote lipogenesis in livers of HFD fed WT and CD40 $^{-/-}$ mice (n=6). Data are presented as mean ± S.E.M. Statistically significant differences between group WT HFD and CD40$^{-/-}$ HFD are indicated: *P < 0.05, ** P< 0.01).

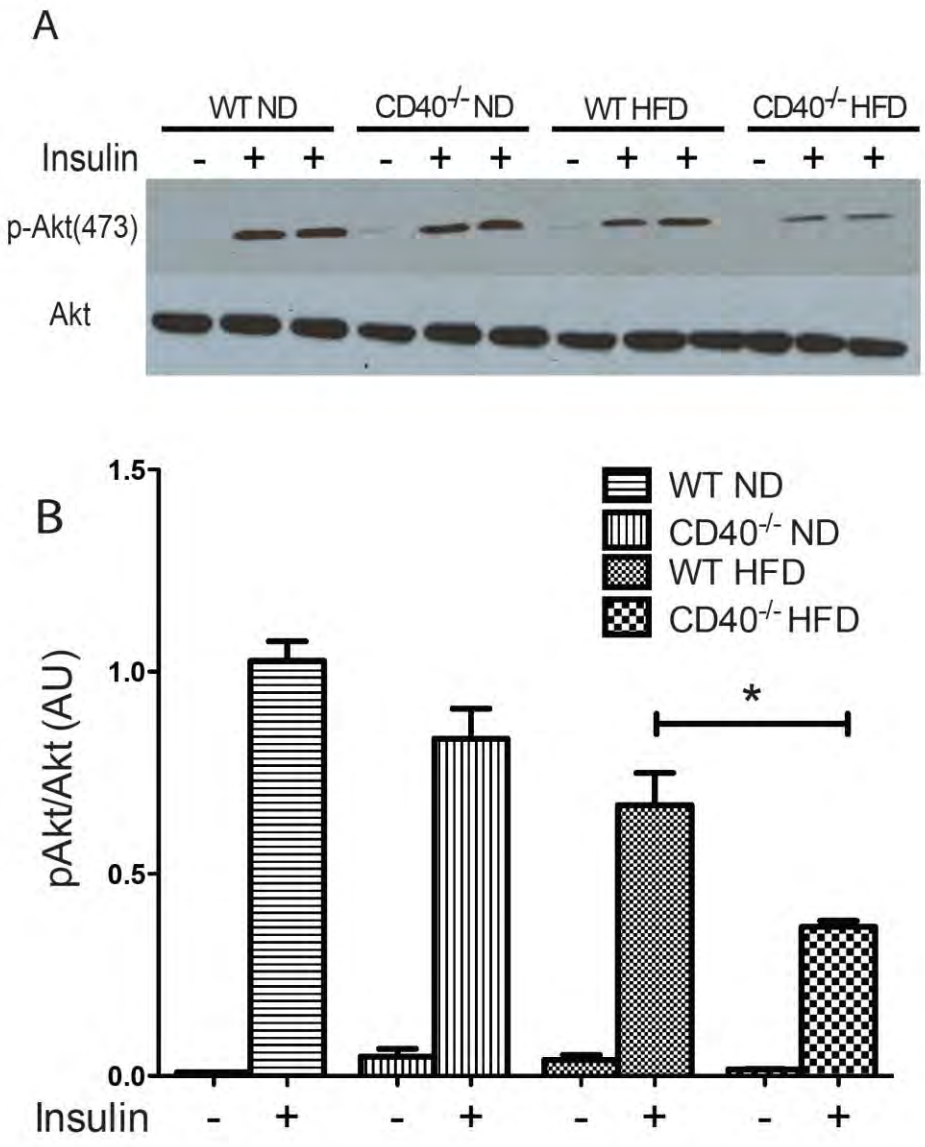


Figure 2.9 CD40 deficiency exacerbates hepatic insulin resistance.

(A) Hepatic Akt activation following acute insulin injection (1U/kg i.p., 10 min). Western blot analysis of liver extracts showing phosphor-(Ser^{473}) 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 ± S.E.M. Statistically significant differences between groups WT HFD and CD40$^{-/-}$ HFD are indicated *$P < 0.05$.

densitometry analysis (Figure 2.9A and 2.9B). Hepatic insulin resistance often results in dysregulated hepatic gluconeogenesis that contributes to glucose overproduction and obesity-related hyperglycemia [230]. Indeed, we found that the livers of $CD40^{-/-}$ mice expressed increased levels of the transcription factor forkhead box protein O1 (FOXO1) (Figure 2.10A). The expression of glucose-6-phosphatase (G6Pase), a key gluconeogenic enzyme, regulated by FOXO1/PPARγ coactivator 1α (PGC-1α), was also upregulated in the livers of $CD40^{-/-}$ mice (Figure 2.10A). 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 2.10B). 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 [231]. However, in testing liver inflammation in $CD40^{-/-}$ mice, we surprisingly found that nucleotide-binding domain and leucine-rich-repeat-containing protein-3 (NLRP3), interferon-γ (IFNγ), and interleukin-1β (IL-1β) were significantly decreased in $CD40^{-/-}$ mice fed HFD (Figure 2.11). 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 2.11). These data indicate that the hepatic steatosis and insulin resistance observed in the $CD40^{-/-}$ mice on HFD occurs independently of liver inflammation.

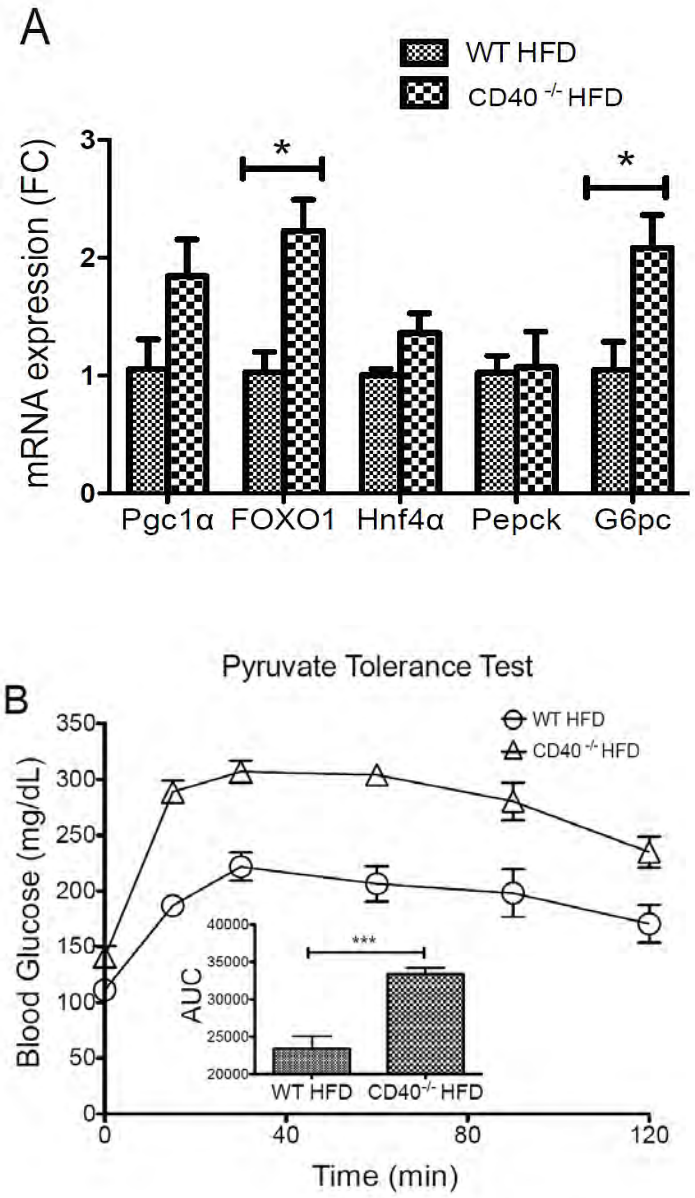


Figure 2.10 CD40$^{-/-}$ mice display elevated hepatic gluconeogenesis

(A) Expression of genes that encode gluconeogenic transcription factors and coactivators (Pgc1α, FOXO1, Hnf4α, Pepck, G6pc) in 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. Data are presented as mean ± S.E.M. Statistically significant differences between group WT HFD and CD40$^{-/-}$ HFD are indicated: *P < 0.05, ** P< 0.01, ***P<0.001.

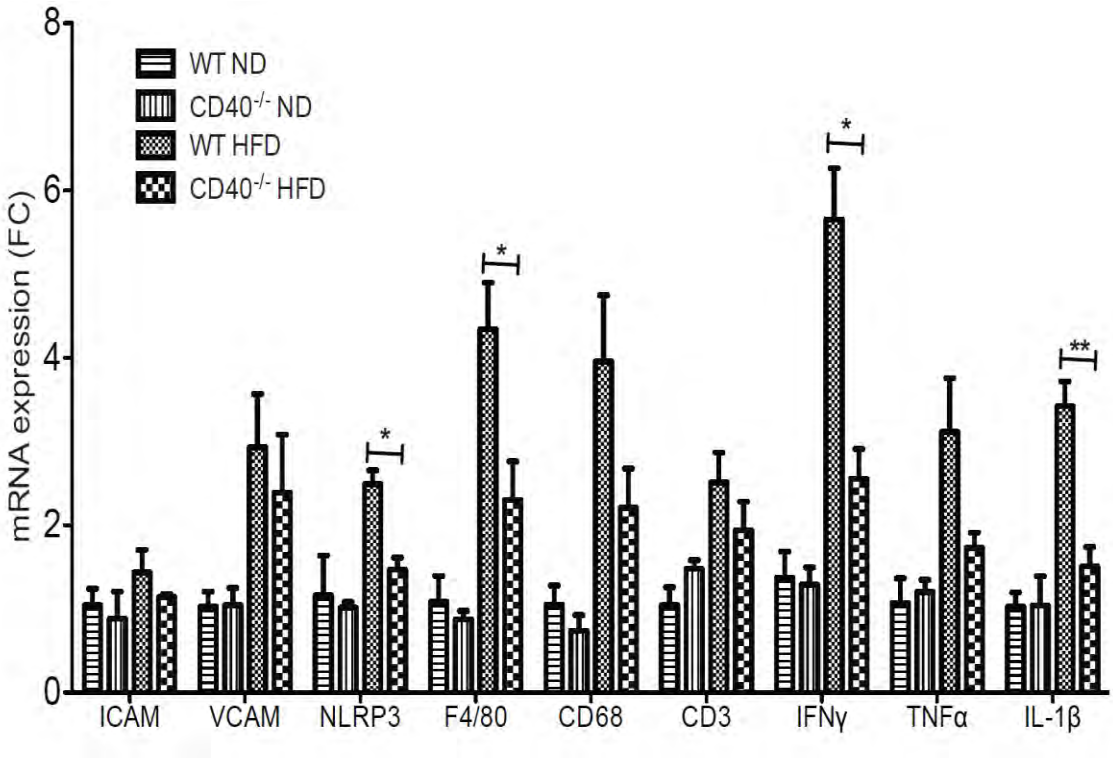


Figure 2.11 CD40$^{-/-}$ mice display decreased hepatic inflammation.

Expression of genes (Icam, Vcam, Nlrp3, F4/80, CD68, CD3, IFNγ, TNFα and IL-1β) associated with inflammation in livers of WT and CD40$^{-/-}$ mice (n=6). Data are presented as mean ± S.E.M. Statistically significant differences between group WT HFD and CD40$^{-/-}$ HFD are indicated: *P < 0.05, ** P< 0.01.

2.3.5 CD40$^{-/-}$ 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 [232]; thus, we examined the adipose tissue mass and lipolytic rate of HFD-fed WT and CD40$^{-/-}$ mice. There was no statistically significant difference in the percent of lean (Figure 2.12A) or fat mass (Figure 2.12B) between CD40$^{-/-}$ mice and WT controls. However, when examined histologically (Figure 2.12C), epididymal adipose tissue from CD40$^{-/-}$ mice had increased numbers of crown-like structures (CLS). CLS are characterized by infiltrating immune cells, especially macrophages, surrounding necrotic adipocytes [233] 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$^{-/-}$ mice compared with the same adipose tissue from WT mice (Figure 2.13). These data suggest that increased adipose tissue lipolysis providing increased fatty acid flux to the liver could be a mechanism by which CD40$^{-/-}$ mice develop hepatic steatosis.

2.3.6 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

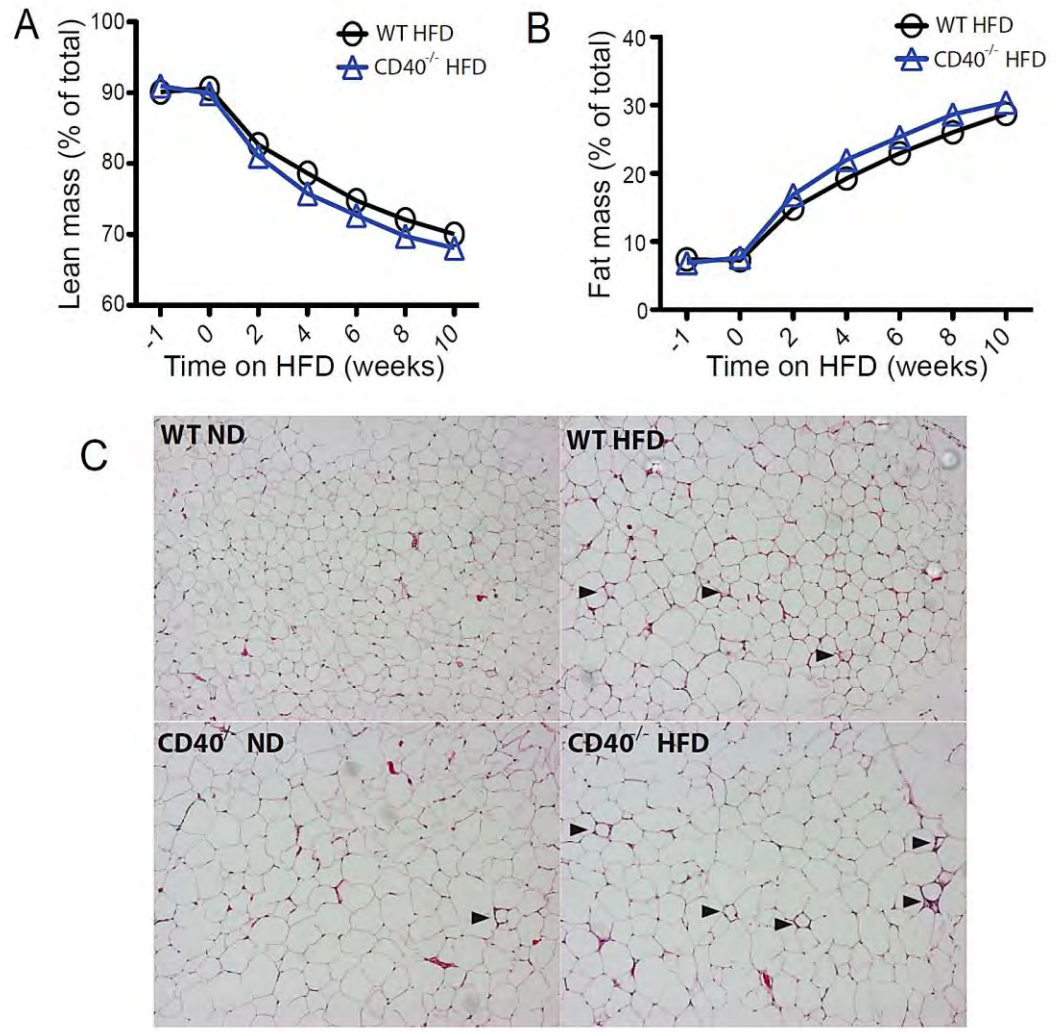


Figure 2.12 CD40$^{-/-}$ mice have similar lean/fat mass with wild-type mice but increased immune cell infiltration in the adipose tissue

(A) lean mass and (B) fat mass in WT and CD40$^{-/-}$ mice fed HFD was measured by 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$^{-/-}$ mice were stained with hematoxylin-eosin. Arrows indicate crown-like structures. (Magnification: 200X)

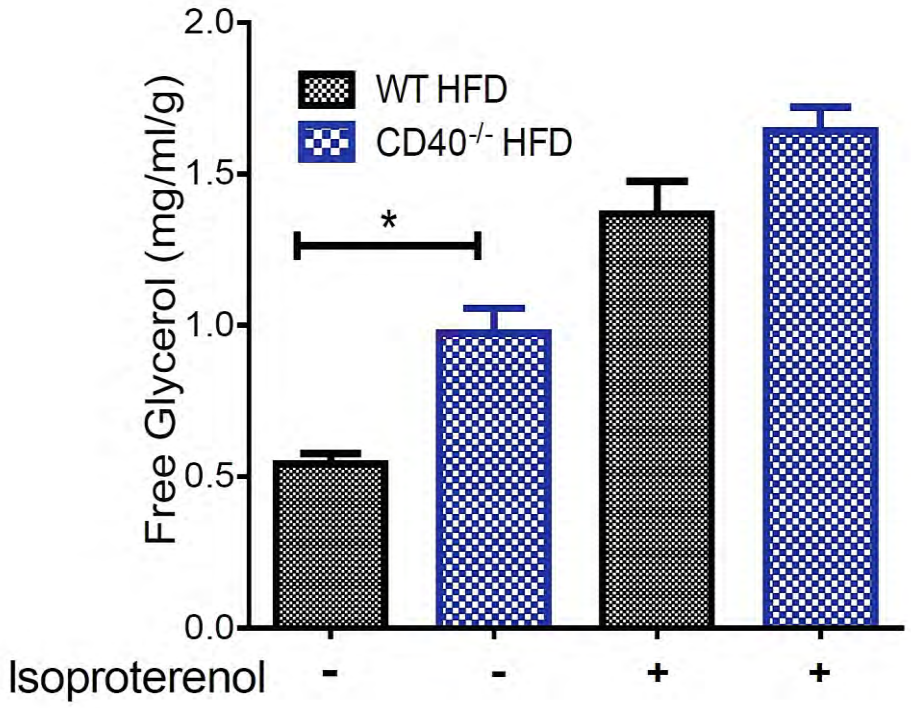


Figure 2.13 CD40$^{-/-}$ mice display elevated adipose tissue lipolysis.

ex vivo lipolysis assay on epididymal adipose tissue explants from HFD fed WT and CD40$^{-/-}$ 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 ± S.E.M. Statistically significant differences between mouse group WT HFD and CD40$^{-/-}$ HFD are indicated: *$P < 0.05$.

analyzed epididymal fat pads from the 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 SVF of epididymal fat by flow cytometry and did not detect a difference (Figure 2.14A). 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 2.14B). The macrophage population in the epididymal adipose tissue of $CD40^{-/-}$ mice tends to have higher expression of the proinflammatory integrin CD11c (Figure 2.14B), suggesting an enhanced M1 macrophage polarization in this fat depot [118]. Consistently, macrophage infiltration was also increased in the epididymal adipose tissue of $CD40^{-/-}$ mice compared with their WT littermate controls (Figure 2.14C).

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 2.15A). This

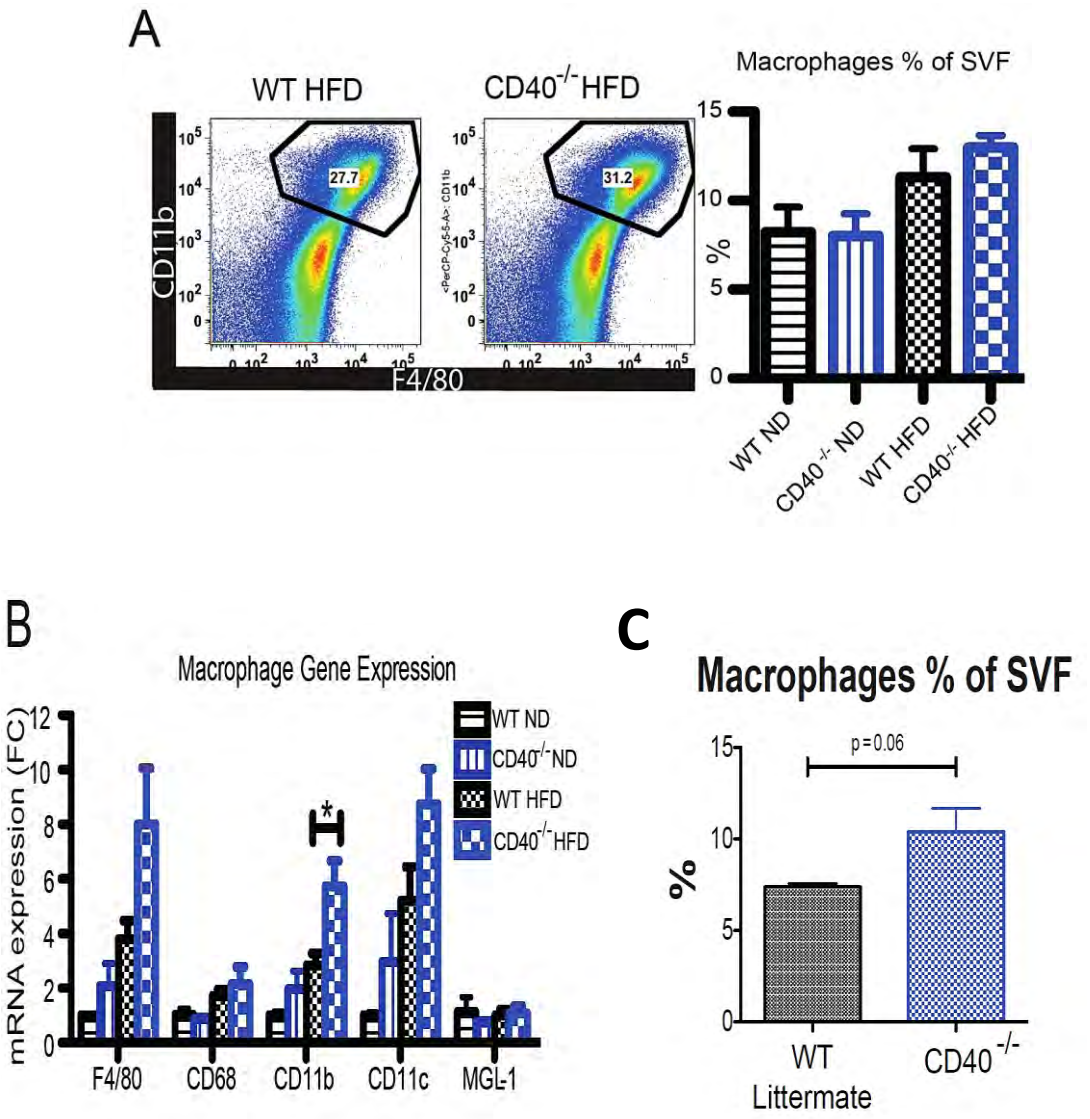


Figure 2.14 CD40 deficiency increases macrophage infiltration in visceral adipose tissue

(A) After 24 weeks on HFD, the 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)** Quantitative FACS analysis on macrophage population in epididymal adipose tissue of CD40-/- and WT littermate mice (18-week old) (n=4). Data are presented as mean ± SEM. Statistically significant differences are indicated: *P < 0.05 vs. control.

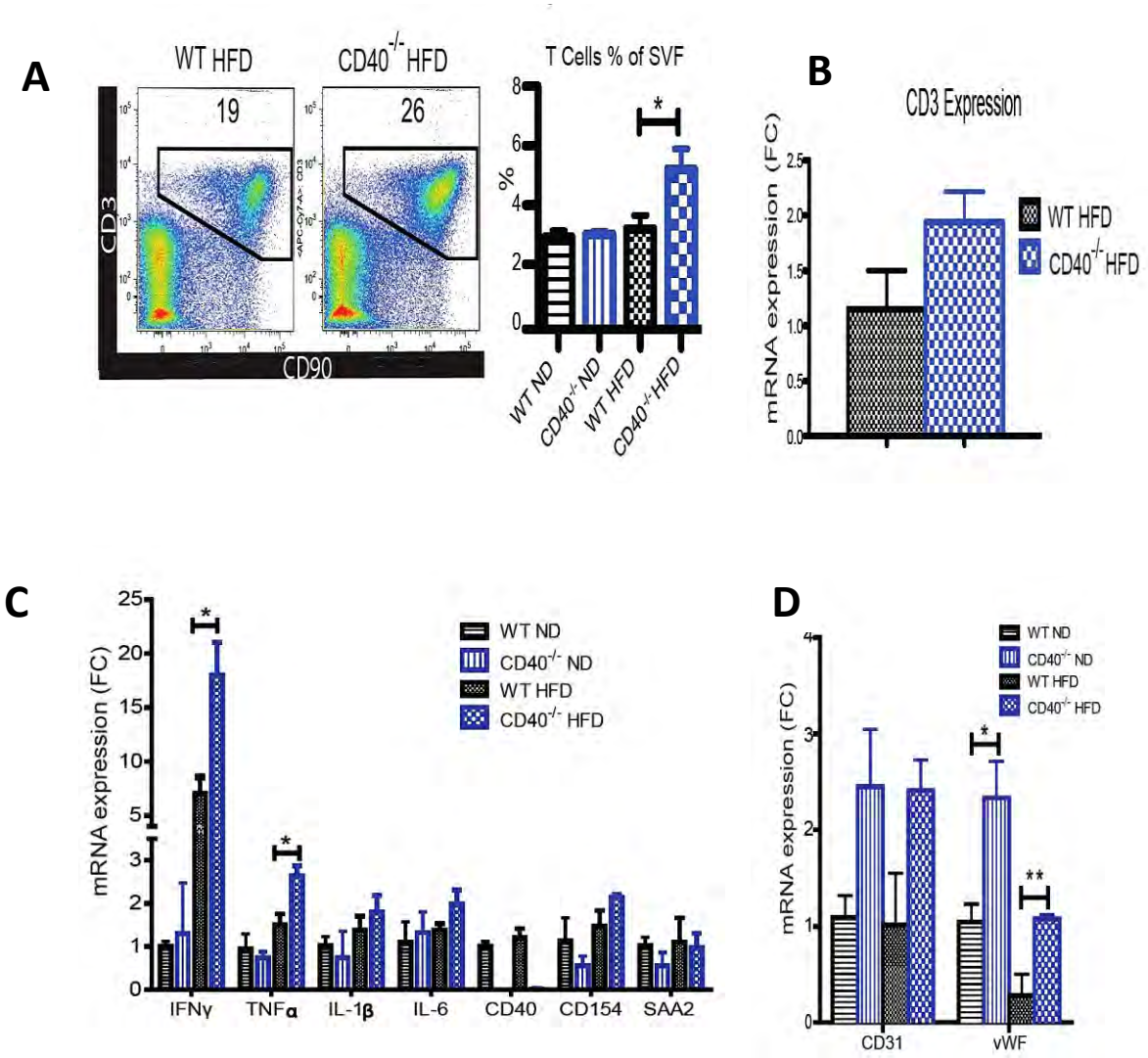


Figure 2.15 CD40 deficiency increases T cell infiltration and inflammation in visceral adipose tissue

(A) After 24 weeks on HFD, the SVF was isolated from epididymal adipose tissues of WT and CD40$^{-/-}$ mice and 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). **(B)** Gene expression of CD3 from whole epididymal adipose tissues (n=6). **(C)** Gene expression of inflammatory genes (IFNγ, TNFα, IL-1β, IL-6, CD40, CD154 and SAA2) from whole epididymal adipose tissues (n=6). **(D)** 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.

increase was confirmed by measuring CD3 expression levels by qRT-PCR (Figure 2.15B). We also analyzed the mRNA expression of inflammatory cytokines to confirm the elevated inflammation in the epididymal fat depot of $CD40^{-/-}$ mice. TNFα and IFNγ, two inflammatory cytokines expressed by activated macrophages and T cells, were both significantly increased (Figure 2.15C). 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 2.15D). These results suggest that $CD40^{-/-}$ mice have increased adipose tissue inflammation characterized by increased macrophage and T cell content and inflammatory cytokine expression.

2.3.7 $CD40^{-/-}$ mice have increased cytotoxic T cell content in the adipose tissue

Many different subpopulations constitute the normal repertoire of T cells in the adipose tissue of obese mice and humans. Nishimura and colleague's study suggested that T cells contribute to the recruitment of ATMs and increased $CD8^+$ effector T cells precede the accumulation of macrophages [124]. 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_H cells) was decreased by 29% and the

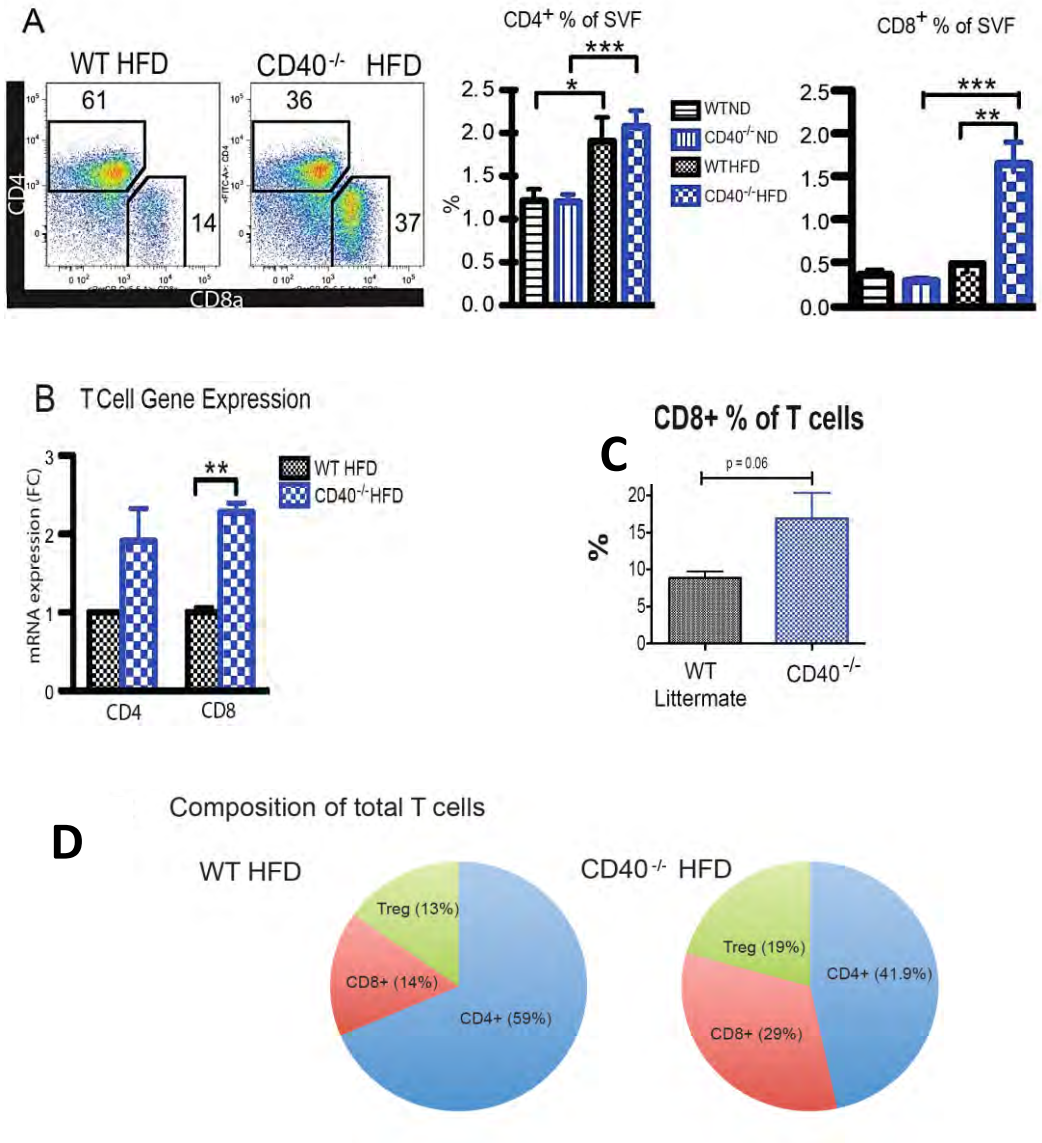


Figure 2.16 Deficiency of CD40 leads to increased CD8:CD4 ratio in visceral adipose tissue in obese mice

(A) After 24 weeks on HFD, 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)** Quantitative FACS analysis on $CD8^{+}$ T cell population in epididymal adipose tissue of CD40-/- and WT littermate mice (18-week old) (n=4) **(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 ± S.E.M. Statistical significances are indicated: $^{*}P < 0.05$. $^{**}P<0.01$. $^{***}P<0.001$ vs control.

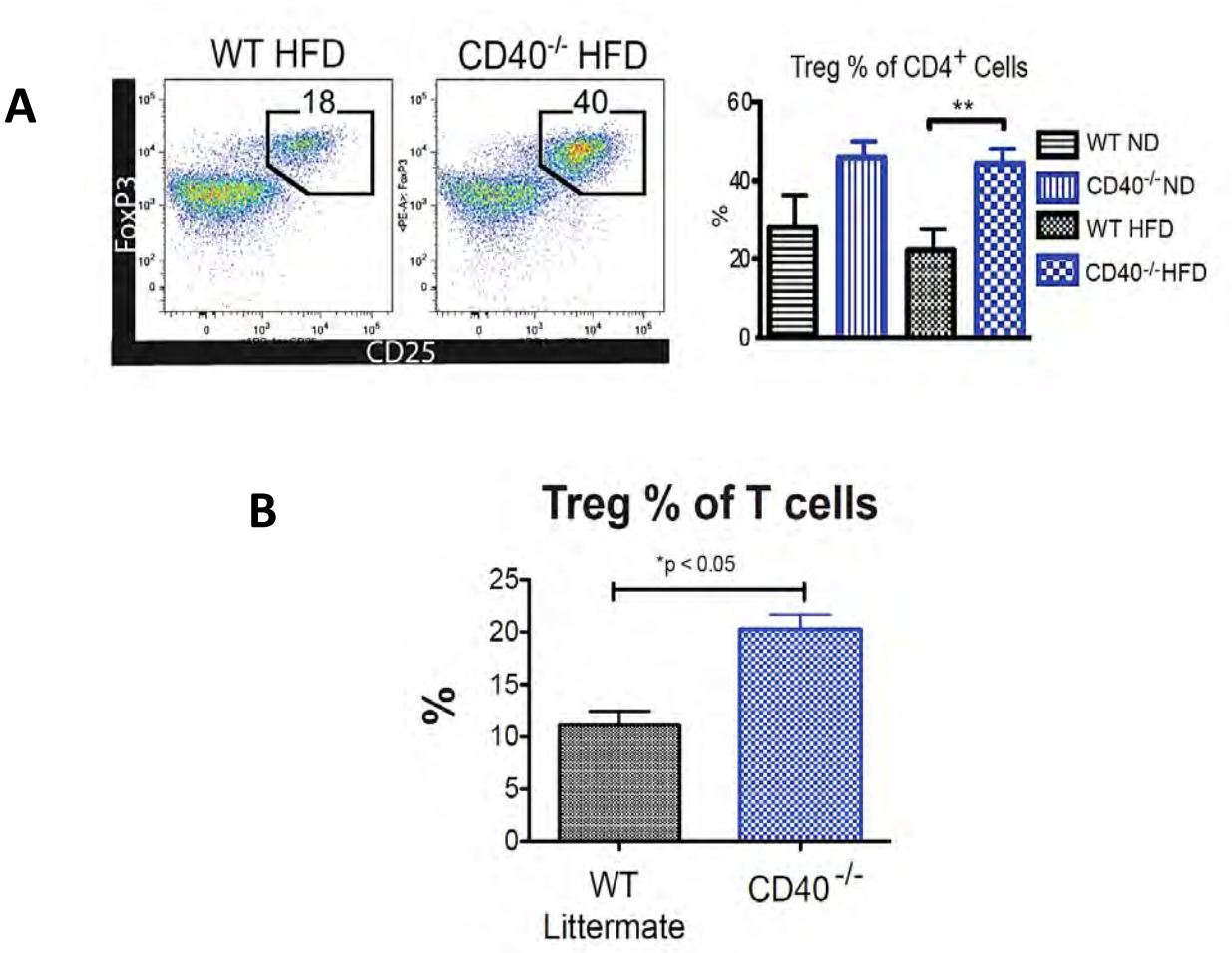


Figure 2.17 Deficiency of CD40 leads to increased Tregs in visceral adipose tissue in obese mice

(A) 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). **(B)** Quantitative FACS analysis on $CD4^{+}CD25^{+}FoxP3^{+}$ T cell population in epididymal adipose tissue of $CD40^{-/-}$ and WT littermate mice (18-week old) (n=4) Data are presented as mean ± S.E.M. Statistical significances are indicated: **P* < 0.05. ***P*<0.01. vs control.

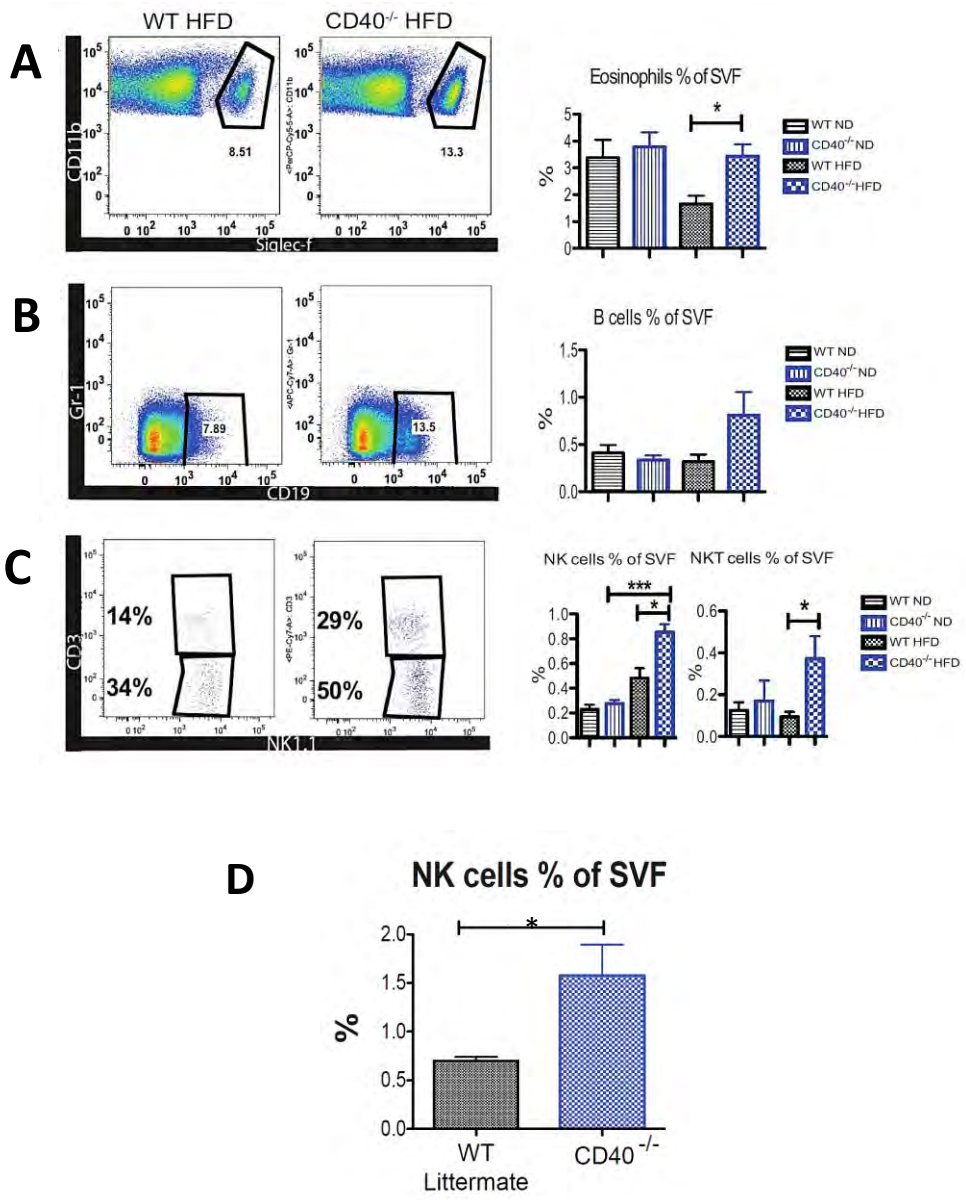


Figure 2.18 CD40 deficiency leads to increased eosinophil, B cell and NK/NKT cell recruitment into visceral adipose tissue in obese mice.

After 24 weeks on HFD, SVF was isolated from epididymal adipose tissue of WT and CD40$^{-/-}$ mice and analyzed for expression of CD11b, siglec-f **(A)**, Gr-1, CD19 **(B)**, CD3, and NK1.1 **(C).** 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^{+}. **(D)** Quantitative FACS analysis on NK cell population in epididymal adipose tissue of CD40$^{-/-}$ and WT littermate mice (18-week old) (n=4) Data are presented as mean ± S.E.M. Statistical significances are indicated: *P < 0.05. ***P<0.001 vs control.

percentage of $CD8^+$ cells (cytotoxic T cell) in adipose tissue was about doubled in the $CD40^{-/-}$ mice (Figure 2.16A, 2.16B and 2.16D). This increase in $CD8^+$ cells was also observed when we used WT littermates as the control group (Figure 2.16C). The percentage of $CD25^+FoxP3^+$ Tregs, a subgroup of $CD4^+$ T cells,was increased by 82% in the $CD40^{-/-}$ mice (Figure 2.17A), an increase that was also observed when WT littermates were used as the control group (Figure 2.17B). In summary, the absence of CD40 increased overall T cell abundance in adipose tissue and skewed the T cell population of adipose tissue toward increased $CD8^+$ cells and decreased $CD4^+$ cells (Figure 2.16D).

2.3.8 *$CD40^{-/-}$ mice have increased eosinophil, B cell, NK/NKT cell infiltration in adipose tissue*

The roles of macrophages and T cells in adipose tissue inflammation are well described and recently more literatures has shown that other immune cell types are also present in obese adipose tissue [140, 142, 146]. As such, we analyzed epididymal adipose tissue for the presence of eosinophils (Figure 2.18A), B cells (Figure 2.18B), NK and NKT cells (Figure 2.18C) 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 (Figure 2.18D).

Chapter III

Discussion and Final Thoughts

Discussion

The tightly controlled interplay between metabolism and inflammation is crucial for the development and maintenance of systemic insulin sensitivity [71]. The CD40-CD40L dyad is at the interface of innate and adaptive immunity and plays a key role in mediating communication between different cell types in the obese adipose tissues [216].

Contrary to the generally accepted concept that disrupting the CD40-CD40L signaling cascade alleviates inflammation [215, 219], 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 2.14, Figure 2.15, Figure 2.16, Figure 2.17 and Figure 2.18). Elevated immune cell infiltration into this tissue in $CD40^{-/-}$ mice creates a highly inflamed adipose depot characterized by increased levels of inflammatory cytokines (Figure 2.15C) and significantly higher basal lipolysis (Figure 2.13). 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 2.7D), in the face of the decreased liver inflammation, expected when CD40 is deficient (Figure 2.11). With combined adipose tissue inflammation and steatotic

liver, CD40$^{-/-}$ mice exhibit dramatic glucose intolerance and systemic insulin resistance (Figure 2.3A and Figure 2.6).

We found that genetic deficiency of CD40 reduced food intake, which attenuated weight gain (Figure 2.1B and Figure 2.1A), observations that were also noted in CD40L deficient animals [220]. 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 2.6 and Figure 2.3A). Of note, age-matched C57BL/6J mice were used as wild-type 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) [234, 235]. 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 [234]. 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 homeostasis.

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 (BAT) is at least partly responsible for the overall decreased GIR in CD40$^{-/-}$ mice (Figure 2.3B). BAT is a highly energetic organ that contains large amounts of mitochondria to dissipate chemical energy, such as glucose and fatty acids [236]. Our clamp data suggested a potential link between receptor CD40 and the activity of BAT. However, more future work is needed to elucidate the molecular mechanism involved.

Hepatic insulin resistance (Figure 2.9) in CD40$^{-/-}$ mice accounts for part of their overall systemic insulin resistance phenotype. We observed increased gene expression of major regulators and enzymes (Figure 2.10A) in the gluconeogenesis pathway and increased hepatic glucose production in CD40$^{-/-}$ mice was confirmed by a pyruvate tolerance test (Figure 2.10B). We were unsuccessful in detecting elevated hepatic glucose production in CD40$^{-/-}$ mice during our glucose clamp study, but our study compared two groups of mice that had been 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 technically 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, whereas adoptive transfer to CD8-null mice aggravates adipose tissue inflammation [124]. Despite reduced body weight, CD40-deficient animals on HFD exhibited increased adipose tissue inflammation and lipolysis (Figure 2.15C & Figure 2.13). In the present study, we observed increased macrophages and T cells in the adipose tissue of $CD40^{-/-}$ mice with T cells increasingly biased toward $CD8^+$ cells (Figure 2.14, Figure 2.15, and Figure 2.16). 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 [195]. 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 2.18). Interestingly, Tregs and eosinophils are typical anti-inflammatory immune cells and commonly correlated with decreased inflammation in obesity [126, 146]. 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 attenuated HFD-induced adipose tissue inflammation [220, 222], which is opposite to what we observed here in CD40$^{-/-}$ mice. However, since CD40L can signal through a non-CD40 pathway [223], differences between CD40$^{-/-}$ and CD40L$^{-/-}$ mouse models are not surprising.

The surprising elevation in the adipose tissue inflammation (Figure 2.15C) in CD40$^{-/-}$ mice was accompanied by an expected decrease in hepatic inflammation due to the CD40 deficiency (Figure 2.11). The remarkably elevated hepatic steatosis in CD40$^{-/-}$ mice fed a HFD is consistent with two reports on CD40L$^{-/-}$ mice [221, 222] but inconsistent with another [220]. 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 2.7F).

Studies in rodents and humans have revealed that the accumulation of triglycerides observed in hepatic steatosis is mainly due to the increased availability of 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 [62]. 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 2.13). In addition to the FFAs overflow, enhanced *de novo* lipogenesis also contributes to the formation of steatotic liver in $CD40^{-/-}$ mice (Figure 2.8). Chronic exposure of tissues to elevated FFAs is known to induce impaired responsiveness of Akt to insulin and decreased insulin signaling [237]. Indeed, we observed impaired insulin-mediated phosphorylation of Akt (Ser^{473}) in the livers of $CD40^{-/-}$ mice upon acute insulin treatment (Figure 2.9).

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 system through the expression of adhesion molecules involved in leukocyte rolling and extravasation.

Recently, Evans and colleagues' study shed light on the relationship between vasculature network and the regulation of metabolism. They reported the role of fibroblast growth factor 1 (FGF1) on adaptive adipose remodeling and metabolic homeostasis [238]. Interestingly, $FGF1^{-/-}$ mice exhibit many metabolic phenotypes that are very similar to the phenotypes of $CD40^{-/-}$ mice. They showed

that FGF1$^{-/-}$ mice displayed no metabolic or histological abnormalities when mice were fed a normal chow diet, and when placed on a HFD, FGF1$^{-/-}$ and wild-type cohorts showed equivalent changes in serum adipokines, cytokines (leptin, resistin, interleukin-6 (IL-6), TNFa, total and high-molecular-weight adiponectin) and serum lipids (cholesterol, free fatty acids and triglycerides). These are exactly what we observed in the CD40$^{-/-}$ mice compared with age-matched wild-type cohorts. However, FGF1 is highly induced in adipose tissue in response to HFD and that mice lacking FGF1 develop an exaggerated diabetic phenotype which is characterized by severe insulin resistance, enlarged steatotic livers, dramatically increased ATM infiltration and accentuated inflammatory response. Again, these phenotypes are strikingly similar to what we have seen in the HFD-fed CD40$^{-/-}$ mice. Evans and colleagues suggest that FGF1 mediates the proper coupling of nutrient storage to adaptive remodeling of adipose tissue and the defects in adipose tissue vasculature are likely responsible for the systemic metabolic dysfunction in HFD-fed FGF1$^{-/-}$ mice.

CD40 is present on both epithelial and endothelial cells. Different vascular beds express various levels of CD40 [239]. Previous studies demonstrated that sCD40L reduced the viability and proliferative capacity of endothelial progenitors [240] and ligation of CD40 with CD40L prevented endothelial cell migration during angiogenesis [241]. In our studies presented here, increased expression of endothelial cell markers von Willebrand factor and CD31 in the adipose tissue of

$CD40^{-/-}$ mice (Figure 2.15D) suggests an increased presence of endothelial cells. In contrast, CD40 expression in the vascular beds of the liver is undetectable [239].

During the progression of obesity, the accumulation of proinflammatory immune cells in adipose tissue, such as M1 macrophages, T_H1, and $CD8^+$ effector T cells, normally accompanies with a gradual diminishment of anti-inflammatory immune cells, such as Tregs and eosinophils. However, in the present study, we observed a general increase of a full spectrum of immune cells that infiltrate into the visceral fat of $CD40^{-/-}$ mice (Figure 2.18), suggesting that, in the absence of CD40, a loss of precise control over the interaction between the vascular system and immune cells. It is highly possible that CD40 plays an important role in regulating endothelial cell function in the vasculature of adipose tissue.

To test whether CD40 deficiency affects the permeability of the vascular system in the obese visceral fat, thereby alters immune cell infiltration, we performed a vascular permeability assay *in vivo* by injecting Evans Blue Dye via tail vein of $CD40^{-/-}$ mice and wild-type controls. Indeed, we found a trend toward higher permeability of adipose vasculature in $CD40^{-/-}$ mice on both ND and HFD (Figure 3.1). These data suggest a potential leakiness of vasculature in $CD40^{-/-}$ adipose tissue and provide a possible explanation for why CD40 deficiency leads to higher immune cell infiltration in the adipose tissue. This information also

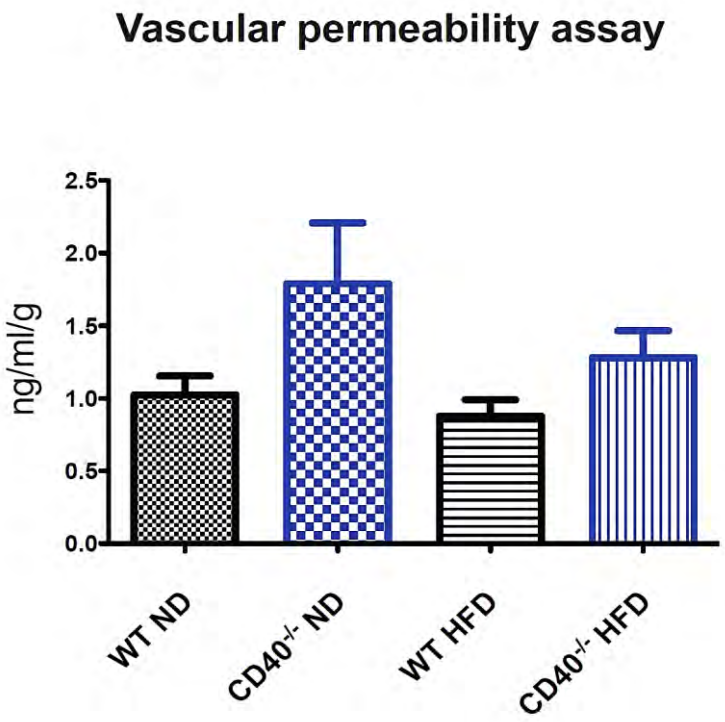


Figure 3.1 Vascular permeability in adipose tissue of $CD40^{-/-}$ and wild-type mice

Evans blue dye (25 mg/kg) was injected into the tail vein of ND or HFD fed $CD40^{-/-}$ and wild-type mice (n=6). After one hour, the mice were euthanized by CO_2 inhalation and were perfused with 30 ml warm PBS via the left ventricle to remove the Evans blue dye from the vasculature. The Evans blue dye was extracted from the fat tissue with 1 ml of formamide overnight at 55°C and measured at 620 nm. The optical density (OD) value was transformed into the concentration according to a standard curve of Evans blue dye.

provides us clues critical to understanding how adipose tissue inflammation is initiated and propagated in diet-induced obesity.

One limitation of our study is that the CD40 deficient mice used here are whole body CD40 knockout mice. Further studies are needed to elucidate whether tissue-specific knockout of CD40 in endothelial cells might lead to the same phenotypes we observed in the current study. How does CD40 affect the permeability of adipose vasculature network is another important question for future investigations.

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 diseases and glucose intolerance.

Final thoughts

Gut microbiota and CD40$^{-/-}$ mice metabolism

In the past few years, the functional relationship between gut microbiota and host metabolism is increasingly recognized owing to technology advances, such as next-generation sequencing, high-throughput transcriptomics / metabolomics approaches, and the use of germ-free animals [242, 243].

Gut microbiota refers to the trillions of microorganisms residing in our intestine, whose cell count vastly outnumbers our own [244]. These microbial inhabitants of the gut have recently been proposed as an environmental factor responsible for the altered energy metabolism that accompanies obesity. For example, genetically obese *ob/ob* mice or humans who are obese have more Firmicutes and fewer Bacteroidetes in their gut compared with lean healthy subjects [245, 246]. Gut microbiota affects host metabolism by ways including, (1) the innate immune response to the structural components of bacteria (e.g., LPS) resulting in inflammation; and (2) bacterial metabolites of dietary compounds (e.g., short-chain fatty acid, SCFA), which have biological activities.

It is apparent that the intestinal immune system faces unique challenges, as it has to continuously deal with enormous amount of microbial load. As such, one essential function of the intestinal immune system is to control the exposure of bacteria to host tissues. Various mechanisms are adapted by the host to

achieve this goal. For example, intestinal goblet cells create layers of mucus coating on the intestinal epithelial cell surface, which serves as a physical barrier to block direct contact [247]. Intestinal epithelium also secretes antibacterial proteins, such as RegIIIγ and α-defensin, which limit bacterial penetration [248, 249].

Several studies have found the essential role of secreted immunoglobulin A (IgA) in preventing intestinal bacteria from breaking the epithelial barrier [250, 251]. IgA specific for intestinal bacteria is produced with the help of intestinal dendritic cells. These bacteria-laden dendritic cells interact with B and T cells in the Peyer's patches, inducing B cells to produce IgA against intestinal bacteria. Mice engineered to lack IgA show priming of serum IgG responses against commensals, indicating that these bacteria have been exposed to the systemic immune system [250].

Importantly, $CD40^{-/-}$ mice display typical Hyper IgM syndrome, which is characterized by normal or elevated concentrations of serum IgM but profound reduction of serum IgG, IgE and IgA [195]. Whether this reduced level of IgA in $CD40^{-/-}$ mice affects certain gut bacteria growth is not known.

The host immune system can control the composition of gut microbiota. Studies have shown that mice with immune deficiencies have altered microbial communities in the gut. For example, $Tbx21^{-/-}/Rag2^{-/-}$ mice lacking adaptive immunity developed ulcerative colitis in a microbiota-dependent manner [252]. Similarly, mice deficient in TLR2 exhibited an increase in LPS absorption,

subclinical inflammation, insulin resistance, glucose intolerance, and later, obesity [253].

Due to limitations of time and the availability of animals, in this study we did not perform a metagenomic screen on the gut microbiota of $CD40^{-/-}$ mice and the littermate controls, but the potential differences in gut microbiota, if any exist, can certainly play a part for the altered metabolic phenotypes in the $CD40^{-/-}$ mice, in addition to what we have reported.

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