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

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Abstract

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

Keywords

Adipose tissue inflammation, CD40, CD8+ T cell, hepatic steatosis, insulin resistance
**Introduction**

Obesity is often associated with type 2 diabetes (14, 18), hepatic steatosis, non-alcoholic steatohepatitis (NASH) (4), cardiovascular complications (21, 42) and certain cancers (1, 44). 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 (22, 38). 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 (8, 10, 15, 30, 31). 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 (48), liver (3) and muscle (17), 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 (37, 50), 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 (2). 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 (13). Recent evidence indicates that the CD40-CD40L complex is also a potential mediator of chronic inflammation in obesity and its related metabolic disorders (23, 27, 33, 36), suggesting a broader role of CD40 in cell biology. Obese and diabetic individuals have higher levels of active soluble CD40L (sCD40L) in the circulation compared to lean healthy subjects (40) and CD40 mRNA levels in white adipose tissue were found to positively correlate with BMI (33). 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 (25). Currently, targeting CD40-CD40L signaling is considered to be a promising strategy for effecting plaque stabilization in the treatment of atherosclerosis (5).

Based upon the above considerations, we anticipated that suppression of the CD40-CD40L signaling cascade would also reduce the deleterious inflammation and metabolic effects associated with obesity. In fact, the CD40-CD40L axis has recently been implicated in the pathogenic complications of obesity (32, 43, 46). 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 (43,46) while another study indicated that the absence of CD40L attenuated diet-induced steatosis (32). Furthermore, in one study, CD40L deficiency improved insulin resistance (32), while in another study; lack of CD40L in mice did not ameliorate high fat diet-induced insulin resistance (46). 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 (51). We therefore designed the present studies to specifically evaluate the role of the CD40-CD40L dyad in adipose tissue inflammation, insulin resistance and hepatic steatosis associated with diet-
induced obesity. Here we evaluated the effects of depletion of CD40 itself in mice, which has not been yet reported. Surprisingly, we found that CD40 knockout (CD40−/−) mice paradoxically displayed significantly higher levels of adipose tissue inflammation, impaired glucose tolerance and remarkable hepatic steatosis without liver inflammation. These results suggest an unexpected primary role for CD40 in attenuating immune cell recruitment to the visceral adipose tissue during the progression of diet-induced obesity. This in turn reduces adipocyte lipolysis, thereby protecting the liver from increased influx of lipid from adipose tissue and enhancing whole-body glucose tolerance.
MATERIALS AND METHODS

Animal studies

Male CD40+/− (B6.129P2-Cd40^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 Jackson Laboratory via sibling mating (34 generations as of Nov, 2008). Since the C57BL/6Ncr mouse line from NIH have been separated from the B6.129P2-Cd40^tm1Kik/J mice for many generations, we therefore created littermate control animals for this study to avoid potential genetic drift from various C57BL/6Ncr substrains or colonies. We bred B6.129P2-Cd40^tm1Kik/J mice with C57BL/6J mice and then their F1 hybrids were used to generate knockout and wild-type littermates. All animals were fed standard chow diet (LabDiet PicoLab 5053, Purina Mills, St. Louis, MO) until 8 weeks of age and then divided into two groups; one was fed chow diet and the other group fed high fat diet (TD.93075, 55/Fat, Harlan Teklad, Madison, WI, fatty acid profile as % total fat: 28% saturated, 30% trans, 28% monounsaturated cis, 14 % polyunsaturated cis). Animals were housed in the University of Massachusetts (UMass) Medical School Animal Medicine facility with a 12-hour light/dark cycle and given ad libitum access to food and water. Mice and food were weighed weekly over the duration of the study.

Intraperitoneal glucose tolerance test (GTT), insulin tolerance test (ITT) and pyruvate tolerance test (PTT) was performed as previously described (49). Composition of total fat and lean mass was assessed by 1H-MRS-based body composition analysis (EchoMRI-3in1™, EchoMRI, Houston, TX). Measurements of energy expenditure, respiratory exchange ratio, indirect calorimetry and physical activity using metabolic cages (TSE Systems, Bad Homburg, Germany) were done by the UMass Mouse Metabolic Phenotyping Center. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees (IACUC) at UMass Medical School.
Hyperinsulinemic-euglycemic clamp studies

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

Hepatic triglyceride analyses

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

Histology

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

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

Western Blotting

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

Ex-vivo Lipolysis Assay in epididymal adipose tissue- explants

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

Flow cytometry

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

ELISA assay

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

Statistical Analysis

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

**CD40–/- mice 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 high fat diet (HFD) or a normal diet (ND). The CD40 null mice were found to be approximately 2 grams lighter than control mice on either diet throughout most of a 14-week feeding period (Figure 1A). These data are consistent with a previous study on CD40L–/- mice (32), suggesting a common role for CD40 and CD40L in the regulation of energy homeostasis. Food intake of CD40–/- mice was significantly lower on HFD (Figure 1B), potentially explaining in part the decreased body weight (Figure 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 1C). However, respiratory exchange ratio (RER) and energy expenditure were not significantly different between the two groups of mice (data not shown).

**CD40 deficiency exacerbates diet-induced insulin resistance**

Decreased weight, food intake and activity in CD40–/- mice suggested a role for CD40 in whole-body metabolism. To characterize this role, we did a series of metabolic analyses on these mice including a hyperinsulinemic-euglycemic glucose clamp study. CD40–/- mice on HFD showed a reduced steady-state glucose infusion rate (GIR) (Figure 1D), suggesting that absence of CD40 aggravates systemic insulin resistance in obesity. We also observed decreased 14C-2-deoxy-glucose uptake in the brown adipose tissue (BAT) of CD40–/- mice with no changes of glucose uptake in the white adipose tissue or muscle (Figure 1E). The morphology of pancreatic islets, fasting serum insulin, serum sCD40L, serum triglyceride (TG) and free fatty acids (FFA) were similar in both CD40–/- and WT mice on HFD (data not shown). To further analyze the metabolic
phenotype of the CD40\(^{-/-}\) mice, we subjected them to a glucose tolerance test (GTT) as well as an insulin tolerance test (ITT). Despite the reduced body weight of CD40\(^{-/-}\) mice on HFD, they surprisingly exhibited glucose intolerance (Figure 2A and 2E) and insulin resistance (Figure 2B and 2F) compared to WT controls. To assure these phenotypes we observed are due to CD40 deficiency and not due to the potential differences in mouse colonies or substrains, we created CD40\(^{-/-}\) mice and WT littermate control mice by breeding the F1 hybrids of CD40\(^{-/-}\) (B6.129P2-Cd40tm1Kik/J) and C57BL6/J parental mice. These cohorts were also subjected to GTT (Figure 2C and 2G) and ITT tests (Figure 2D and 2H). Similar to our original findings, the CD40\(^{-/-}\) mice were significantly more glucose intolerant and insulin resistant than littermate control animals.

**CD40\(^{-/-}\) mice develop hepatic steatosis and increased de novo lipogenesis in the liver.**

Recent literatures have implicated CD40L as a protective factor against hepatic steatosis (43, 46), which has a strong association with insulin resistance (29). Since we found impaired systemic insulin sensitivity in CD40\(^{-/-}\) mice, we evaluated the hepatic steatosis and hepatic insulin sensitivity in these mice. No difference was observed in lipid content of livers of CD40\(^{-/-}\) mice fed ND (Figure 3C) compared to WT controls on the same diet (Figure 3A). However, CD40\(^{-/-}\) mice on HFD displayed clearly steatotic livers, as observed both macroscopically and microscopically by H&E or Oil-red O staining (Figure 3D vs 3B). To quantify this increased steatosis, we measured total hepatic triglycerides (TG) levels and found a 59% increase in CD40\(^{-/-}\) mice (Figure 3E). Elevated expression of two lipid droplet proteins, cell death-inducing DFFA-like effector A (CIDEA) and Fat Specific Protein 27 (FSP27) (34, 35), accompanied the increase in total TGs in the liver (Figure 3F). These lipid droplet proteins are specifically expressed in steatotic livers and absent in lean livers (Figure 3F). Increased hepatic steatosis could be mediated by different pathways including increased dietary lipid absorption, increased de novo lipogenesis,
increased influx of non-esterified free fatty acids (FFA) from hypertrophied adipose tissue or decreased very low density lipoprotein (VLDL) secretion and fat oxidation (7, 9). As no difference in plasma FFAs, respiratory exchange ratio (RER) and heat production was observed between CD40 null and WT mice (data not shown), a role for increased dietary fat absorption or impaired fat oxidation is unlikely the cause for 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 3G). Acetyl-CoA carboxylase 2 (ACC2), Long-chain-fatty-acid-CoA ligase 1 (ACLS1) are genes both involved in de novo lipogenesis and were both increased in CD40−/− mice (Figure 3H). These data suggest that the hepatic steatosis observed in CD40−/− mice is probably due to an increased de novo lipogenesis.

**CD40−/− mouse livers are insulin resistant despite decreased inflammation**

Since hepatic steatosis is correlated with hepatic insulin resistance in mouse and human obesity, we tested the hypothesis that CD40−/− mice on HFD had greater hepatic insulin resistance. We injected mice intraperitoneally with insulin (1mU/g) and measured Akt phosphorylation at Ser-473 as an indicator of hepatic insulin signaling. Compared to WT controls, CD40−/− mice on HFD for 24 weeks exhibited 45% lower Ser-473 phosphorylation on immunoblot densitometry analysis (Figure 4A and 4B). Hepatic insulin resistance often results in dysregulated hepatic gluconeogenesis that contributes to glucose overproduction and obesity-related hyperglycemia (9).

Indeed, we found that livers of CD40−/− mice expressed increased levels of the transcription factor forkhead box protein O1 (FOXO1) (Figure 5A). The expression of glucose-6-phosphatase (G6pc),
a key gluconeogenic enzyme, regulated by FOXO1/PGC-1α, was also upregulated in the livers of CD40<sup>−/−</sup> mice (Figure 5A). To test whether gluconeogenesis was increased in HFD-fed CD40<sup>−/−</sup> mice, a pyruvate tolerance test (PTT) was performed with 16-hour fasted CD40<sup>−/−</sup> and WT mice. We found that CD40<sup>−/−</sup> mice displayed significantly higher rate of hepatic glucose output by converting pyruvate into glucose (Figure 5B). These data suggest that hepatic insulin resistance in CD40<sup>−/−</sup> mice leads to elevated levels of hepatic gluconeogenesis. Liver inflammation has been proposed as a link between hepatic steatosis and insulin resistance (39). However, in testing liver inflammation in CD40<sup>−/−</sup> mice, we surprisingly found that nucleotide-binding domain and leucine-rich-repeat-containing protein-3 (NLRP3), interferon gamma (IFNγ), and interleukin-1 beta (IL-1β) were significantly decreased in CD40<sup>−/−</sup> mice fed HFD (Figure 5C). In accordance with the lower level of inflammatory cytokine secretion in the livers of CD40<sup>−/−</sup> mice, the expression of macrophage marker, F4/80, was downregulated (Figure 5C). These data indicate that the hepatic steatosis and insulin resistance observed in the CD40<sup>−/−</sup> mice on HFD occurs independently of liver inflammation.

CD40<sup>−/−</sup> mice exhibit increased basal lipolysis in the adipose tissue.

Lipolysis is a process that releases fatty acids from the adipose tissue through hydrolysis of triglyceride stores. Decreased adipose tissue mass and the associated lipolysis have been correlated with hepatic steatosis (7), thus we examined the adipose tissue mass and lipolytic rate of HFD-fed WT and CD40<sup>−/−</sup> mice. There was no statistically significant difference in the percent of lean (Figure 6A) or fat mass (Figure 6B) between CD40<sup>−/−</sup> mice and WT controls. However, when examined histologically (Figure 6C), epididymal adipose tissue from CD40<sup>−/−</sup> mice had increased numbers of crown-like structures (CLS). CLS are characterized by infiltrated immune cells, especially macrophages, surrounding necrotic adipocytes (28) and are a hallmark of adipose
tissue inflammation, which is associated with increased lipolysis. Indeed, higher basal lipolytic activity was observed in the epididymal adipose tissue from CD40<sup>−/−</sup> mice when compared with the same adipose tissue from WT mice (Figure 6D). These data suggest that increased adipose tissue lipolysis providing increased fatty acid flux to the liver could be a mechanism by which CD40<sup>−/−</sup> mice develop hepatic steatosis.

**CD40 depletion increases adipose tissue inflammation.**

Since increased lipolysis and CLS in adipose tissue are often associated with increased infiltration of macrophages and other proinflammatory cells, we analyzed epididymal fat pads from HFD-fed mice for the presence of inflammation. First, we measured the presence of cells expressing macrophage surface markers F4/80 and CD11b in the stromal vascular fraction (SVF) of epididymal fat by flow cytometry and did not detect a difference (Figure 7A). 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<sup>+</sup> CD11b<sup>+</sup>, 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<sup>−/−</sup> mice (Figure 7B). The macrophage population in the epididymal adipose tissue of CD40<sup>−/−</sup> mice tends to have higher expression of the proinflammatory integrin CD11c (Figure 7B), suggesting an enhanced M1 macrophage polarization in this fat depot (24). Consistently, macrophage infiltration was also increased in the epididymal adipose tissue of CD40<sup>−/−</sup> mice compared to their WT littermate controls (data not shown).

Since T cells can also infiltrate adipose tissue in HFD-induced obesity and contribute to metabolic dysfunction, we measured T cell content in the adipose tissue of CD40<sup>−/−</sup> mice. We
observed a 57% increase of CD3⁺CD90⁺ cells in the epididymal SVF of HFD fed CD40⁻/- mice by
flow cytometry (Figure 7C). This increase was confirmed by measuring CD3 expression levels by
qRT-PCR (Figure 7D). We also analyzed the mRNA expression of inflammatory cytokines to
confirm the elevated inflammation in the epididymal fat depot of CD40⁻/- mice. Tumor necrosis
factor alpha (TNFα) and interferon gamma (IFNγ), two inflammatory cytokines expressed by
activated macrophages and T cells, were both significantly increased (Figure 7E). 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 7F). These results suggest that CD40⁻/- mice have increased
adipose tissue inflammation characterized by increased macrophage and T cell content and
inflammatory cytokine expression.

CD40⁻/- mice have increased cytotoxic T cell content in the adipose tissue

Many different sub-populations constitute the normal repertoire of T cells in the adipose tissue of
obese mice and humans. A previous study suggested that T cells contribute to the recruitment of
adipose tissue macrophages and increased CD8⁺ effector T cells precede the accumulation of
macrophages (30). Therefore, we investigated the proportion of these different T cells populations
in the epididymal adipose tissue of CD40⁻/- and WT mice by flow cytometry. Of all CD3⁺CD90⁺
T cells, the percentage of CD4⁺ cells (T helper cells) was decreased by 29% and the percentage of
CD8⁺ cells (cytotoxic T cell) in adipose tissue was about doubled in the CD40⁻/- mice (Figure 8A,
8B and 8D). This increase in CD8⁺ cells was also observed when we used WT littermates as the
control group (data not shown). The percentage of CD25⁺FoxP3⁺ regulatory T cells (Tregs), a
subgroup of CD4⁺ T cells, was increased by 82% in the CD40⁻/- mice (Figure 8C), an increase that
was also observed when WT littermates were used as the control group (data not shown). In
summary, the absence of CD40 increased overall T cell abundance in adipose tissue and skewed the T cell population of adipose tissue towards increased CD8$^+$ cells and decreased CD4$^+$ cells (Figure 8D).

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

The roles of macrophages and T cells in adipose tissue inflammation are well described and recently more literatures have shown that other immune cell types are also present in obese adipose tissue (8, 31, 47). As such, we analyzed epididymal adipose tissue for the presence of eosinophils (Figure 9A), B cells (Figure 9B), NK and NKT cells (Figure 9C) and found that all were increased in CD40$^{-/-}$ mice fed HFD. NK cells were also increased in the epididymal adipose tissue of CD40$^{-/-}$ mice when WT littermates were used as the control group (data not shown).

Discussion

Contrary to the generally accepted concept that disrupting the CD40-CD40L signaling cascade alleviates inflammation (5, 13), 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 7, Figure 8 and Figure 9). Elevated immune cell infiltration into this tissue in CD40$^{-/-}$ mice creates a highly inflamed adipose depot characterized by increased levels of inflammatory cytokines (Figure 7E) and significantly higher basal lipolysis (Figure 6D). 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 3D), in the face of the decreased liver inflammation,
expected when CD40 is deficient (Figure 5C). With combined adipose tissue inflammation and steatotic liver, CD40⁻/⁻ mice exhibit dramatic glucose intolerance and systemic insulin resistance (Figure 1D and Figure 2).

We found that genetic deficiency of CD40 reduced food intake, which attenuated weight gain (Figure 1B and 1A), observations that were also noted in CD40L deficient animals (32). 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 and 1D). Of note, age matched C57BL/6J mice were used as wildtype controls for part of this study. These mice are known to be susceptible to diet-induced obesity and display glucose intolerance when fed HFD. The genetic locus underlying this phenotype was mapped to nicotinamide nucleotide transhydrogenase (Nnt) (12, 26). 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 (12). Importantly, the CD40⁻/⁻ mice used in this study, which are on the C57BL/6Ncr background, having intact Nnt gene, are more insulin resistant than the C57BL/6J controls, an effect that is in the opposite direction of that expected from the genetic background effect, if any exists, suggesting that CD40 plays a critical role in regulating mouse whole body glucose hemostasis.

The decreased steady-state GIR in clamp studies on CD40⁻/⁻ mice could be caused by reduced efficiency of glucose disposal in tissues (muscle, adipose tissue, liver, heart and brain) as well as elevated hepatic glucose production. Indeed, reduced glucose uptake by brown adipose tissue is at least partly responsible for the overall decreased GIR in CD40⁻/⁻ mice (Figure 1E). Hepatic insulin resistance (Figure 4) in CD40⁻/⁻ mice accounts for part of their overall systemic insulin resistance phenotype. We observed increased gene expression of major regulators and enzymes (Figure 5A
and 5C) in the gluconeogenesis pathway and increased hepatic glucose production in CD40^{+/−} mice was confirmed by a pyruvate tolerance test (Figure 5B). We were unsuccessful to detect elevated hepatic glucose production in CD40^{+/−} mice during our glucose clamp study (data not shown), but our study compared two groups of mice that were on a high fat diet for 16 weeks, therefore even the control mice were already highly insulin resistant. Thus, suppression of hepatic glucose output by insulin was already inhibited in the control mice making it difficult to detect a further significant inhibition for CD40^{−/−} mice in the clamp study.

The infiltration of activated macrophages into obese adipose tissue is correlated with adipose tissue dysfunction and systemic insulin resistance. CD8^{+} T cells also infiltrate into obese adipose tissue and their depletion improves systemic insulin sensitivity while adoptive transfer to CD8 null mice aggravates adipose tissue inflammation (30). Despite reduced body weight, CD40 deficient animals on HFD exhibited increased adipose tissue inflammation and lipolysis (Figure 6C & Figure 6D). In the present study, we observed increased macrophages and T cells in the adipose tissue of CD40^{−/−} mice with T cells increasingly biased towards CD8^{+} cells (Figure 7 and Figure 8). Importantly, it was reported that CD40 deficiency did not affect hematopoietic development or differentiation; therefore no systemic abnormality in the number and ratio of T and B cells was detected in these CD40 null mice (19). 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 9). Interestingly, Tregs and eosinophils are typically correlated with decreased inflammation in obesity (10, 47). We believe their presence in the inflamed CD40^{+/−} adipose tissue may play an important compensatory role to neutralize the deleterious effect from the activated macrophage and CD8^{+} effector T cells. Previous studies on CD40L^{−/−} mice indicated that CD40L deficiency
attenuates HFD-induced adipose tissue inflammation (32, 46), which is opposite to what we
observed here in CD40^{−/−} mice. However, since CD40L can signal through a non-CD40 pathway
(51), differences between CD40^{−/−} and CD40L^{−/−} mouse models are not surprising.

The surprising elevation in the adipose tissue inflammation in CD40^{−/−} mice was accompanied by
an expected decrease in hepatic inflammation due to the CD40 deficiency (Figure 5C). The
remarkably elevated hepatic steatosis in CD40^{−/−} mice fed a HFD is consistent with two reports on
CD40L^{−/−} mice (43, 46) but inconsistent with another (32). 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 3F).

Studies in rodents and humans have revealed that the accumulation of TG observed in hepatic
steatosis is mainly due to the increased availability of free fatty acids (FFAs) arising from the
visceral adipose tissue through unabated lipolysis as well as increased hepatic lipogenesis. These
two pathways appear to account for more than 80% of the fat storage in steatotic livers (38). 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 6D). Chronic exposure of tissues to elevated FFAs
is known to induce impaired responsiveness of Akt to insulin and decreased insulin signaling (6).
Indeed, we observed impaired insulin-mediated phosphorylation of Akt (Ser473) in the livers of
CD40^{−/−} mice upon acute insulin treatment (Figure 4).

How can the deficiency of a proinflammatory receptor protein that normally activates adaptive
immunity paradoxically cause elevated inflammation in the adipose tissue of mice? We found
increased immune cell infiltration in the adipose tissue of CD40^{−/−} mice but decreased
inflammation in the liver, suggesting that the phenotype observed is tissue specific and not due to
a global defect of the immune system. One possible mechanism could involve endothelial cells in
adipose tissue. Endothelial cells control leukocyte entry into tissues from the vasculature through
the expression of adhesion molecules involved in leukocyte rolling and extravasation. CD40 is
present on both epithelial and endothelial cells and different vascular beds express various levels
of CD40 (45). Previous studies demonstrated that the CD40-CD40L dyad affects endothelial cell
function and migration during angiogenesis (16, 41). In our studies presented here, increased
expression of endothelial cell markers von Willebrand factor and CD31 in the adipose tissue of
CD40−/− mice (Figure 7F) suggests an increased presence of endothelial cells. In contrast, CD40
expression in the vascular beds of the liver is undetectable (45). Future studies will be needed to
test whether adipose endothelial cell disruption of CD40 might lead to the phenotype we observed
in CD40 null mice.

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

The authors declare no conflicts of financial interest in the work described.

Author contributions

C.A.G. and S.K. designed and performed experiments, analyzed data, and wrote the manuscript. S.U.A., M.W., S.D. and R.H.F. performed experiments and analyzed data. J.K.K. analyzed data. M.A. and M.P.C. designed the studies, analyzed the data and wrote the manuscript.


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Figure Legends

Figure 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). (D) Steady-state glucose infusion rate (GIR) for WT and CD40−/− mice on HFD for 16 weeks to maintain euglycemia during hyperinsulinemic-euglycemic clamps (n = 10). (E) 14C-2-deoxy-glucose uptake in visceral white adipose tissue (WAT), gastrocnemius muscle (Skeletal Muscle), intrascapular brown adipose tissue (BAT) and heart. The data presented are mean ± S.E.M. Statistically significant differences are indicated (* p<0.05, ** p<0.01 vs. control).

Figure 2. CD40−/− mice fed a high fat diet are more glucose intolerant and insulin resistant.

(A) Glucose tolerance test (GTT) for CD40−/− and C57BL/6J control mice (30 weeks old) on HFD for 22 weeks (n=8). Animals were fasted overnight and glucose was injected intraperitoneally at a dose of 1g/kg of body weight. (B) Insulin tolerance test (ITT). 31 weeks old CD40−/− and C57BL/6J control mice on HFD for 23 weeks (n=8) were fasted for 4 hours and injected with insulin at a dose of 1U/kg. (C) Glucose tolerance test (GTT) for CD40−/− and littermate control mice (18 weeks old) on HFD for 10 weeks (n=4). Animals were fasted overnight and glucose was injected intraperitoneally at a dose of 1g/kg of body weight. (D) Insulin tolerance test (ITT). 18 weeks old CD40−/− and littermate control mice on HFD for 10 weeks (n=4) were fasted for 4 hours and injected with 1U/kg insulin. (E) Area under the curve (AUC) for A. (F) AUC for B. (G) AUC for C. (H) AUC for D. Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40−/− HFD are indicated (*p < 0.05, ** p< 0.01, ***p<0.001).
Figure 3. CD40 protects against diet-induced hepatic steatosis and decreases hepatic lipogenesis.

(A-D) Macroscopic (upper left inlays) and microscopic examination of WT and CD40\(^{+/−}\) mouse livers on ND or 24-week HFD. Representative sections are stained with hematoxylin-eosin or Oil red O (bottom left inlays) (Magnification: 200X). (E) Hepatic total triglycerides in WT and CD40\(^{−/−}\) mice fasted for 6 hours (n=6). (F) The gene expression of lipid droplet proteins Cidea and FSP27 in the livers of WT and CD40\(^{−/−}\) mice (n=6) was measured by qRT-PCR. (G) The expression of genes that encode lipogenic transcription factors and coactivators (C/ebp\(_{α}\), C/ebp\(_{β}\), Ppar\(_γ\), Srebp1c, Chrebp and Lxr) in the livers of HFD fed WT and CD40\(^{−/−}\) mice (n=6). (H) The expression of genes that encode enzymes (Fasn, Acc1/2, SCD1, Acsl1/4, Dgat1/2, Gyk, Mttp) that promote lipogenesis in the livers of HFD fed WT and CD40\(^{−/−}\) mice (n=6). Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40\(^{−/−}\) HFD are indicated (*p < 0.05, ** p< 0.01).

Figure 4. CD40 deficiency exacerbates hepatic insulin resistance.

(A) Hepatic Akt activation following acute intraperitoneal insulin injection (1U/kg, 10 minutes). Western blot analysis of liver extracts showing phospho (Ser473) AKT (pAkt) levels under control (PBS, indicated as “−”) and insulin (“+”) treated conditions. (B) Densitometric quantitation of pAkt/Akt ratios (n=6). Data are presented as mean ± SEM. Statistically significant differences between groups WT HFD and CD40\(^{−/−}\) HFD are indicated (*p < 0.05, ** p< 0.01).

Figure 5. CD40\(^{−/−}\) mice display elevated gluconeogenesis and decreased hepatic inflammation.

(A) The expression of genes that encode gluconeogenic transcription factors and coactivators (Pgc1α, FOXO1, Hnf4α, Pepck, G6pc) in the livers of HFD fed WT and CD40\(^{−/−}\) mice (n=6). (B) Pyruvate tolerance test (PTT) for mice (32-week old) on HFD for 24 weeks (n=8). Animals were fasted overnight and sodium pyruvate was injected intraperitoneally at a dose of 2g/kg of body weight. (C) The expression of genes (Icam, Vcam, Nlrrp3, F4/80, CD68, CD3, IFN\(_γ\), TNF\(_α\) and IL-1\(_β\)) associated with inflammation in the livers of WT and CD40\(^{−/−}\) mice (n=6). Data are presented as mean ± SEM. Statistically significant differences between group WT HFD and CD40\(^{−/−}\) HFD are indicated (*p < 0.05, ** p< 0.01, ***p<0.001).
Figure 6. CD40−/− mice have increased immune cell infiltration in adipose tissue as well as elevated adipose tissue lipolysis.

(A) lean mass and (B) fat mass in WT and CD40−/− mice fed HFD was measured by magnetic resonance imaging (MRI) analysis as a percentage of total mass over an 11-week period (n=10). (C) Representative sections of the epididymal adipose tissue from ND and HFD fed WT and CD40−/− mice were stained with hematoxylin-eosin. Arrows indicate crown-like structures. (Magnification: 200X) (D) 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 ± SEM. Statistically significant differences between mouse group WT HFD and CD40−/− HFD are indicated (*p < 0.05).

Figure 7. CD40 protects against adipose tissue inflammation in obese mice.

(A) After 24 weeks on HFD, the stromal vascular fraction (SVF) was isolated from epididymal adipose tissues of WT and CD40−/− mice and analyzed for CD11b and F4/80 expression by flow cytometry. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). (B) Gene expression of macrophage markers (F4/80, CD68, CD11b, CD11c and MGL-1) from whole epididymal adipose tissues (n=6). (C) SVF from epididymal adipose tissue of WT and CD40−/− mice was analyzed for CD3 and CD90 expression by flow cytometry. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). (D). Gene expression of CD3 from whole epididymal adipose tissues (n=6). (E) Gene expression of inflammatory genes (IFNγ, TNFα, IL-1β, IL-6, CD40, CD154 and SAA2) from whole epididymal adipose tissues (n=6). (F) Gene expression of CD31 and Von Willebrand factor (vWF) from whole epididymal adipose tissue (n=6). Data are presented as mean ± SEM. Statistically significant differences are indicated p < 0.05, **p<0.01 vs control.

Figure 8. Deficiency of CD40 leads to increased CD8:CD4 ratio and increased Tregs in visceral adipose tissue in obese mice.

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

Figure 9. CD40 deficiency leads to increased eosinophil, B cell and NK/NKT cell recruitment into visceral adipose tissue in obese mice.

After 24 weeks on HFD, the SVF was isolated from epididymal adipose tissue of WT and CD40−/− mice and analyzed for expression of (A) CD11b, siglec-f, (B) Gr-1, CD19, (C) CD3 and NK1.1. Representative dot plots are shown on the left and summarized in bar graph on the right (n=8). Eosinophils were defined as CD11b+Siglec-f+. B cells were defined as Gr-1−CD19+. NK cells were defined as CD3−NK1.1+. NKT cells were defined as CD3−NK1.1+. Data are presented as mean ± SEM. Statistical significances are indicated *p < 0.05. ***p<0.001 vs control.
Figure 1

A. Growth Curve

B. Food Intake

C. Physical Activity

D. Glucose Infusion Rate

E. Glucose Uptake

Mouse Weight (g)

Food Intake (g/mouse/day)

Glucose Infusion Rate (mg/kg/min)

Food Intake (g/mouse/day)

Glucose Uptake (nmol/g/min)

WT ND

CD40−/− ND

WT HFD

CD40−/− HFD

WT ND

CD40−/− ND

WT HFD

CD40−/− HFD

WT HFD

CD40−/− HFD

WAT Skeletal Muscle BAT Heart
Figure 2
Figure 3
Figure 4

A

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Insulin</th>
<th>p-Akt(473)</th>
<th>Akt</th>
</tr>
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<tr>
<td>WT ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD40⁻/⁻ ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WT HFD</td>
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<td>-</td>
</tr>
<tr>
<td>CD40⁻/⁻ HFD</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

B

![Graph showing pAkt/Akt (AU) levels with different treatments and insulin conditions.](chart)

Legend:
- WT ND
- CD40⁻/⁻ ND
- WT HFD
- CD40⁻/⁻ HFD

*significant difference
Figure 6

(A) Lean mass (% of total) vs. Time on HFD (weeks)
(B) Fat mass (% of total) vs. Time on HFD (weeks)
(C) Images showing muscle tissue with arrows indicating differences between conditions.
(D) Bar graph showing Free Glycerol (mg/ml/g) with and without Isoproterenol, comparing WT HFD and CD40^{-/-} HFD.
**Figure 7**

(A) Flow cytometry analysis of Macrophages % of SVF between WT HFD and CD40\(^{-/-}\) HFD.

(B) Bar graph showing Macrophage Gene Expression levels for WT ND, WT HFD, CD40\(^{-/-}\) ND, and CD40\(^{-/-}\) HFD.

(C) Flow cytometry analysis of T Cells % of SVF between WT HFD and CD40\(^{-/-}\) HFD.

(D) Bar graph showing CD3 Expression levels for WT HFD and CD40\(^{-/-}\) HFD.

(E) Graph depicting mRNA expression (FC) for IFN\(\gamma\), TNF\(\alpha\), IL-1\(\beta\), IL-6, CD40, CD154, and SAA2 across different groups.

(F) Graph showing mRNA expression (FC) for CD31 and vWF across different groups.
**Figure 8**

**A**

WT HFD vs. CD40^{-/-} HFD

- **CD4**
  - WT HFD: 61
  - CD40^{-/-} HFD: 36

- **CD8**
  - WT HFD: 14
  - CD40^{-/-} HFD: 37

**B**

TCell Gene Expression

- **CD4**
  - mRNA expression (FC)
  - WT HFD
  - CD40^{-/-} HFD

- **CD8**
  - mRNA expression (FC)
  - WT HFD
  - CD40^{-/-} HFD

**C**

WT HFD vs. CD40^{-/-} HFD

- **FoxP3**
  - WT HFD: 18
  - CD40^{-/-} HFD: 40

- **CD25**

**D**

Composition of total T cells

- **WT HFD**
  - CD4+ (59%)
  - CD8+ (14%)
  - Treg (13%)

- **CD40^{-/-} HFD**
  - CD4+ (41.9%)
  - CD8+ (19%)
  - Treg (19%)

**Figure 8**
Figure 9