CD40 deficiency in mice exacerbates obesity-induced adipose tissue inflammation, hepatic steatosis, and insulin resistance

Chang-An Guo,1*, Sophia Kogan,1* Shinya U. Amano,1 Mengxi Wang,1 Sezin Dagdeviren,1,2 Randall H. Friedline,1,2 Myriam Aouadi,1 Jason K. Kim,1,2 and Michael P. Czech1

1Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts; and 2Department of Medicine, Division of Endocrinology, Metabolism, and Diabetes, University of Massachusetts Medical School, Worcester, Massachusetts

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 pharmacological 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 than 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).

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 (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) whereas an-
other study indicated that the absence of CD40L attenuated diet-induced steatosis (32). Furthermore, in one study, CD40L deficiency improved insulin resistance (32), whereas 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 a 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 hepatic steatosis without liver inflammation. 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-Cd40tm1Kik/J) and control C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The background strain of B6.129P2-Cd40tm1Kik/J mice in this study is C57BL/6Ncr, which is maintained by Jackson Laboratory via sibling mating (34 generations as of November 2008). Since the C57BL/6Ncr mouse line from NIH has been separated from the B6.129P2-Cd40tm1Kik/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-Cd40tm1Kik/J mice with C57BL/6J mice, and then their F1 hybrids were used to generate knockout and wild-type (WT) littermates. All animals were fed a standard chow diet (LabDiet PicoLab 5053; Purina Mills, St. Louis, MO) until 8 wk of age and then were divided into two groups; one was fed the chow diet and the other group a high-fat diet (HFD; TD.93075, 55/Fat; Harlan Teklad, Madison, WI; fatty acid profile as %total fat: 28% saturated, 30% monounsaturated, 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.

Intraportal glucose tolerance (GTT), insulin tolerance (ITT) and pyruvate tolerance tests (PTT) were performed as previously described (49). Composition of total fat and lean mass was assessed by 1H-MRS-based body composition analysis (EchoMRI-3n1; 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 16 wk were subjected to an overnight fast (~15 h), and a 2-h hyperinsulinemic-euglycemic clamp was conducted in awake mice with a primed and continuous infusion of human insulin (150 mU/kg body wt priming followed by 2.5 mU·kg−1·min−1; 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 TG analyses.** Hepatic TG content measurement was performed as previously described (11). Mice were fasted for 4 h; 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 TG (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 h, 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.

**Real-time quantitative RT-PCR.** RNA was extracted from homogenized liver and adipose tissue using the TRIzol reagent according to the 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, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM benzamide, 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), and incubated with primary antibody overnight. The blots were washed with TBST, and horseradish peroxidase secondary antibody was applied. Proteins were visualized using Western Lightening Plus ECL (PerkinElmer). 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 (~100 mg, n = 4/mouse) were preincubated for 1 h 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 KRB buffer (125 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 2.6 mM MgSO4, 5 mM HEPES, pH 7.2) plus 2% BSA (fatty acid free) with or without the presence of isoproterenol (10 μM) for 2 h 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 min at 37°C. Digested tissue was filtered through a 200 μM nylon mesh and then centrifuged at 1,000 rpm for 10 min. Pelleted stromal vascular fraction of adipose tissue was stained with fluorescein-conjugated primary antibodies according to the manufacturer’s 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-
CD40-deficient mice display decreased weight, food intake, and physical activity. To investigate the role of CD40 in diet-induced obesity, CD40$^{−/−}$ mice and 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 g lighter than control mice on either diet throughout most of a 14-wk feeding period (Fig. 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 (Fig. 1B), potentially explaining in part the decreased body weight (Fig. 1A). Physical activity measurements on mice fed the HFD over a 24-h period indicated that CD40$^{−/−}$ mice were significantly less active than WT mice at 8 and 14 wk of age (0 and
6 wk on HFD) (Fig. 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) (Fig. 1D), suggesting that absence of CD40 aggravates systemic insulin resistance in obesity. We also observed decreased 2-deoxy-[14C]glucose uptake in the brown adipose tissue (BAT) of CD40−/− mice with no changes of glucose uptake in the white adipose tissue or muscle (Fig. 1E). The morphology of pancreatic islets, fasting serum insulin, serum sCD40L, serum 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 GTT as well as an ITT. Despite the reduced body weight of CD40−/− mice on HFD, they exhibited glucose intolerance (Fig. 2, A and E) and insulin resistance (Fig. 2, B and F) compared with WT controls. To ensure that these phenotypes were observed due to CD40 deficiency and not 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 (Fig. 2, C and G) and ITT tests (Fig. 2, D and H). 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 literature has 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 (Fig. 3C) compared with WT controls on the same diet (Fig. 3A). However, CD40−/− mice on HFD displayed clearly steatotic hepatic appearance (not shown).

---

Fig. 2. CD40−/− mice fed HFD are more glucose intolerant and insulin resistant. A: glucose tolerance test (GTT) for CD40−/− and C57BL/6J control mice (30 wk old) on HFD for 22 wk (n = 8). Animals were fasted overnight, and glucose was injected at a dose of 1 g/kg body wt ip. B: insulin tolerance test (ITT); 31-wk-old CD40−/− and C57BL/6J control mice on HFD for 23 wk (n = 8) were fasted for 4 h and injected with insulin at a dose of 1 U/kg. C: GTT for CD40−/− and littermate control mice (18 wk old) on HFD for 10 wk (n = 4). Animals were fasted overnight, and glucose was injected at a dose of 1 g/kg body wt ip. D: ITT; 18-wk-old CD40−/− and littermate control mice on HFD for 10 wk (n = 4) were fasted for 4 h and injected with 1 U/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 means ± SE. Statistically significant differences between WT HFD and CD40−/− HFD groups are indicated: *P < 0.05, **P < 0.01, ***P < 0.001.
livers, as observed both macroscopically and microscopically by H&E or Oil-red O staining (Fig. 3, D vs. B). To quantify this increased steatosis, we measured total hepatic TG levels and found a 59% increase in CD40<sup>−/−</sup>/H11002<sup>−/−</sup>/H11002<sup>−/−</sup> mice (Fig. 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 (Fig. 3F). These lipid droplet proteins are specifically expressed in steatotic livers and are absent in lean livers (Fig. 3F). Increased hepatic steatosis could be mediated by different pathways, including increased dietary lipid absorption, increased de novo lipogenesis, increased influx of nonesterified FFA from hypertrophied adipose tissue, or decreased very-low-density lipoprotein (VLDL) secretion and fat oxidation (7, 9). As no difference in plasma FFAs, 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 of the hepatic steatosis in CD40<sup>−/−</sup>/H11002<sup>−/−</sup>/H11002<sup>−/−</sup> mice. Therefore, we measured the expression of transcription factors that promote hepatic lipogenesis as well as genes that encode enzymes contributing to lipogenesis. Peroxisome proliferator-activated receptor-γ (PPARγ) and sterol-regulatory element-binding protein-1c (SREBP1-c), two key regulators of hepatic lipogenesis, were both significantly elevated in the CD40<sup>−/−</sup>/H11002<sup>−/−</sup>/H11002<sup>−/−</sup> mice fed the HFD (Fig. 3G). 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 (Fig. 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 (1 mU/g) and measured Akt phosphorylation at Ser473 as an indicator of hepatic insulin signaling. Compared with WT controls, CD40−/− mice on HFD for 24 wk exhibited 45% lower Ser473 phosphorylation on immunoblot densitometry analysis (Fig. 4, A and B). Hepatic insulin resistance often results in dysregulated hepatic gluconeogenesis that contributes to glucose overproduction and obesity-related hyperglycemia (9). Indeed, we found that livers of CD40−/− mice expressed increased levels of the transcription factor forkhead box protein O1 (FOXO1) (Fig. 5A). 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 (Fig. 5A). To test whether gluconeogenesis was increased in HFD-fed CD40−/− mice, a pyruvate tolerance test (PTT) was performed with 16-h fasted CD40−/− and WT mice. We found that CD40−/− mice displayed significantly higher rate of hepatic glucose output by converting pyruvate into glucose (Fig. 5B). These data suggest that hepatic insulin resistance in CD40−/− mice leads to elevated levels of hepatic gluconeogenesis. Liver inflammation has been proposed as a link between hepatic steatosis and insulin resistance (39). However, in testing liver inflammation in CD40−/− mice, we surprisingly found that nucleotide-binding domain and leucine-rich-repeat-containing protein-3 (NLRP3), interferon-γ (IFNγ), and interleukin-1β (IL-1β) were significantly decreased in CD40−/− mice fed HFD (Fig. 5C). 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 (Fig. 5C). These data indicate that the hepatic steatosis and insulin resistance observed in the CD40−/− mice on HFD occurs independently of liver inflammation.

CD40−/− mice exhibit increased basal lipolysis in adipose tissue. Lipolysis is a process that releases fatty acids from the adipose tissue through hydrolysis of TG 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−/− mice. There was no statistically significant difference in the percentage of lean (Fig. 6A) or fat mass (Fig. 6B) between CD40−/− mice and WT controls. However, when examined histologically (Fig. 6C), epididymal adipose tissue from CD40−/− 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−/− mice compared with the same adipose tissue from WT mice (Fig. 6D). 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.

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 (Fig. 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+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 (Fig. 7B). The macrophage population in the epididymal adipose tissue of CD40−/− mice tends to have higher expression of the proinflammatory integrin CD11c (Fig. 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−/− mice compared with their WT littermate controls (data not shown).

Since T cells can also infiltrate adipose tissue in HFD-induced obesity and contribute to metabolic dysfunction, we measured T cell content in the adipose tissue of CD40−/−
mice. We observed a 57% increase of CD3+CD90+ cells in the epididymal SVF of HFD fed CD40−/− mice by flow cytometry (Fig. 7C). This increase was confirmed by measuring CD3 expression levels by qRT-PCR (Fig. 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-α (TNFα) and IFNγ, two inflammatory cytokines expressed by activated macrophages and T cells, were both significantly increased (Fig. 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 (Fig. 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 subpopulations 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 cell 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 (Fig. 8, A, B, and D). 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 (Fig. 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 toward increased CD8+ cells and decreased CD4+ cells (Fig. 8D).

**CD40−/− mice have increased eosinophil, B cell, and 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 (8, 31, 47). As such, we analyzed epididymal adipose tissue for the presence of eosinophils (Fig. 9A), B cells (Fig. 9B), and NK and NKT cells (Fig. 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 unexpect-
edly aggravates adipose tissue inflammation in obesity. This enhanced adipose inflammation in CD40-null mice includes increased recruitment of macrophages, CD8<sup>+</sup> effector T cells, and other immune cells, including B cells, eosinophils, NK cells, NKT cells, and Tregs (Figs. 7, 8 and 9). Elevated immune cell infiltration into this tissue in CD40<sup>−/−</sup> mice creates a highly inflamed adipose depot characterized by increased levels of inflammatory cytokines (Fig. 7E) and significantly higher basal lipolysis (Fig. 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<sup>−/−</sup> mice fed HFD (Fig. 3D) in the face of the decreased liver inflammation, expected when CD40 is deficient (Fig. 5C). With combined adipose tissue inflammation and steatotic liver, CD40<sup>−/−</sup> mice exhibit dramatic glucose intolerance and systemic insulin resistance (Figs. 1D and 2).

We found that genetic deficiency of CD40 reduced food intake, which attenuated weight gain (Fig. 1, B and A),
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 (Figs. 2 and 1D). Of note, age-matched C57BL/6J mice were used as WT 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 an 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.

Fig. 7. CD40 protects against adipose tissue inflammation in obese mice. A: after 24 wk on HFD, stromal vascular fraction (SVF) was isolated from epididymal adipose tissue 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 means ± SE. Statistically significant differences are indicated: *P < 0.05, **P < 0.01 vs. control.
The decreased steady-state GIR in clamp studies on CD40<sup>−/−</sup> 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<sup>−/−</sup> mice (Fig. 1E). Hepatic insulin resistance (Fig. 4) in CD40<sup>−/−</sup> mice accounts for part of their overall systemic insulin resistance phenotype. We observed increased gene expression of major regulators and enzymes (Fig. 5, A and C) in the gluconeogenesis pathway, and increased hepatic glucose production in CD40<sup>−/−</sup> mice was confirmed by PTT (Fig. 5B). We were unsuccessful in detecting elevated hepatic glucose production in CD40<sup>−/−</sup> mice during our glucose clamp study (data not shown), but our study compared two groups of mice that were on HFD for 16 wk; 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<sup>−/−</sup> 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<sup>+</sup> 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 (30). Despite reduced body weight, CD40-deficient animals on HFD exhibited increased adipose tissue inflammation and lipolysis (Fig. 6, C and D). In the present study, we observed increased macrophages and T cells in the adipose tissue of CD40<sup>−/−</sup> mice, with T cells increasingly biased toward CD8<sup>+</sup> cells (Figs. 7 and 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 (Fig. 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 (Fig. 5C). The remarkably elevated hepatic steatosis in CD40−/− mice fed a HFD is consistent with two reports on CD40L−/− mice (43, 46) but is 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 (Fig. 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 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 (Fig. 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 (Fig. 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 (Fig. 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 costimulatory signaling CD40-CD40L dyad as an important feature controlling adipose tissue inflammation and its connection to metabolic disease and glucose intolerance.

ACKNOWLEDGMENTS

We thank Dr. Yu Liu in the Morphology Core Facility of the Diabetes Research Center at UMass Medical School for assistance with histology analysis. We thank Pranitha Vangala for excellent technical support in mouse handling. We acknowledge Drs. Adilson Guilherme, Joseph Virbasius, and Rachel Roth Flach for insightful discussion of the data.

GRANTS

This study was supported by grants to M. P. Czech from the National Institutes of Health (National Institutes of Health) (DK-085753, AI-046629), a grant from the International Research Alliance at Novo Nordisk Foundation Center for Metabolic Research, and a grant to J. K. Kim (UMass Mouse Metabolic Phenotyping Center funded by NIH U24-DK-093000).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


