CD40L Deficiency Ameliorates Adipose Tissue Inflammation and Metabolic Manifestations of Obesity in Mice


Objective—Obese adipose tissue shows hallmarks of chronic inflammation, which promotes the development of metabolic disorders. The mechanisms by which immune cells interact with each other or with metabolism-associated cell types, and the players involved, are still unclear. The CD40-CD40L costimulatory dyad plays a pivotal role in immune responses and in diseases such as atherosclerosis and may therefore be a mediator of obesity. Here we investigated whether CD40L is involved in adipose tissue inflammation and its associated metabolic changes.

Methods and Results—To assess a putative role of CD40L in obesity in vivo, we evaluated metabolic and inflammatory consequences of 18 weeks of high-fat feeding in CD40L+/+ and CD40L−/− mice. In addition, C57Bl6 mice were injected with neutralizing anti-CD40L (αCD40L) antibody for 12 weeks while being fed a high-fat diet. Genetic deficiency of CD40L attenuated the development of diet-induced obesity, hepatic steatosis, and increased systemic insulin sensitivity. In adipose tissue, it impaired obesity-induced immune cell infiltration and the associated deterioration of glucose and lipid metabolism. Accordingly, αCD40L treatment improved systemic insulin sensitivity, glucose tolerance, and CD4+ T-cell infiltration in adipose tissue with limited effects on adipose tissue weight.

Conclusion—CD40L plays a crucial role in the development of obesity-induced inflammation and metabolic complications. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: obese system ■ insulin resistance ■ leukocytes ■ metabolism ■ obesity

In recent years, it has become clear that obesity is associated with a chronic, low-grade inflammatory state, considered responsible for the development of insulin resistance, type 2 diabetes, and atherosclerosis. Obese adipose tissue shows hallmarks of chronic inflammation, with the involvement of T-cell subsets such as Th1, Th2, Th17, regulatory T cells, macrophage subsets, and also of other immune cells such as mast cells. These immune cells, their cell-cell interactions, and their interactions with adipocytes result in an increased expression of leukocyte adhesion molecules, chemokines, and cytokines in adipose tissue, thereby enhancing the inflammatory response and affecting insulin signaling. Although extensive research is dedicated to the effects of inflammation on obesity-related complications, little is known about the immunologic pathways and factors that initiate, regulate, and amplify the inflammatory cascade during obesity.

The CD40-CD40L dyad, known as a costimulatory receptor-ligand pair, plays an important role in enhancing and regulating immune responses, as well as inflammation, and contributes to a plethora of chronic inflammatory diseases, such as colitis, Crohn’s disease, allergic encephalitis, and multiple sclerosis. Disruption of the CD40L gene or inhibition of CD40L with different antibodies in ApoE−/− and LDL-R−/− mice abrogates atherosclerosis and results in plaques that are extremely low in inflammation and high in fibrosis, the equivalent of a clinically preferable, stable atherosclerotic plaque in humans. CD40L is predominantly expressed by CD4+ T cells and activated platelets and displays a variable expression in several other cell types, such as macrophages, mast cells, endothelial cells, and vascular smooth muscle cells.

Interestingly, 2 studies have shown that the CD40-CD40L dyad is involved in obesity-associated inflammatory processes. We have previously reported that CD40 mRNA levels in adipose tissue were correlated with body mass index and that CD40 was not only expressed in the stromal adipose fraction containing immune cells, but also on the adipocyte itself. Stimulation of human adipocytes by recombinant sCD40L or activated T lymphocytes elicited the production of proinflammatory adipokines and in addition decreased the expression of insulin signaling molecules such as GLUT4 and IRS-1. This phenomenon required activation of mitogen-activated protein kinase (MAPK) and IκB nuclear factor-κB.

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The study by Missiou et al confirmed these results and showed that supernatants of CD40L-stimulated adipocytes induced the activation of endothelial cells. Interestingly, patients afflicted by obesity, type 2 diabetes, and the metabolic syndrome exhibit elevated serum sCD40L levels that can be modulated by antidiabetic treatments.

Considering the important role of CD40-CD40L in inflammation, its effects on chronic inflammatory diseases, and our previous data showing involvement of CD40L in adipocyte stimulation, we hypothesize that CD40L plays a pivotal role in initiating and amplifying the chronic low-grade inflammatory status in adipose tissue, thereby inducing obesity-related metabolic complications. Our study reveals that genetic CD40L deficiency and neutralizing CD40L antibody treatment impair the development of inflammation in adipose tissue and obesity-induced metabolic disturbances, indicating a role for CD40L in the pathogenesis of metabolic disorders.
intraperitoneally injected with neutralizing anti-CD40L (for 18 weeks starting at 6 to 12 weeks of age; and (2) C57Bl6 mice fed the standard-fat diet or HFD (35% kcal fat, 20% kcal protein, 45% kcal carbohydrate). Two experimental studies were completed: (1) CD40L−/− and CD40L+/+ mice fed the standard-fat diet or HFD for 18 weeks starting at 6 to 12 weeks of age; and (2) C57Bl6 mice intraperitoneally injected with neutralizing anti-CD40L (aCD40L, clone MR-1) or control IgG antibodies (200 μg twice a week) and fed the HFD for 12 weeks over the same period beginning at 8 weeks of age.

Biochemical Measurements and Glucose/Insulin Tolerance Tests
A glucose tolerance test and an insulin tolerance test were performed, and fasting insulin levels were measured. The homeostasis model assessment of insulin resistance was calculated. Cholesterol, IP-10, interleukin (IL)-6, and leptin levels were determined in plasma.

Flow Cytometric Analysis
Stromal-vascular cells were isolated from epiAT. Fluorescence-activated cell sorting for CD3, CD4, CD8, CD25, FoxP3, Ly6G, B220, CD11c, and CD19 was performed on stromal-vascular cells and splenocytes.27,28

Morphometry and Immunohistochemistry
Immunohistochemistry for CD3 and macrophages was performed on epiAT and scAT. Liver sections were stained with hematoxylin-eosin, Sirius Red, and Oil Red O and used to determine the degree of steatosis and nonalcoholic steatohepatitis (NASH) as indicated by the NASH clinical research network.29

Real-Time Polymerase Chain Reaction
Primer sequences are available on request.

Statistical Analysis
Results are mean±SEM. A Mann–Whitney U test or a 2-way ANOVA was used. Significance was set at P<0.05.

Results
CD40L−/− Mice Are Protected From Weight Gain
On the standard-fat diet, a slight but significant decrease in body weight gain was observed in CD40L−/− mice (Figure 1A). However, scAT, epiAT, BAT, and liver weights were not affected by CD40L deficiency (Table). Food intake was also not different, but feeding efficiency was significantly less in CD40L−/− mice, suggesting a higher energy expenditure in absence of CD40L signaling.

In HFD-fed obese CD40L−/− mice, body weight gain, as well as food intake and feeding efficiency, was strongly reduced compared with obese wild-type (WT) mice (Figure 1A and Table). In addition, scAT and liver weights tended to decrease (Table). This indicates that during obesity, absence of CD40L reduces food intake and feeding efficiency and that fat pads and other fat-accumulating tissues, such as liver, tend to weigh less, thereby contributing to the reduction in total body weight.

Table. Characteristics of Wild-Type (WT) and CD40L−/− Mice Subjected to a Standard-Fat Diet (SFD) or a High-Fat Diet (HFD) for 18 wk

<table>
<thead>
<tr>
<th></th>
<th>WT-SFD</th>
<th>CD40L−/− SFD</th>
<th>WT-HFD</th>
<th>CD40L−/− HFD</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>30.17±0.26</td>
<td>29.97±1.71</td>
<td>45.10±1.90</td>
<td>38.73±0.76</td>
</tr>
<tr>
<td>Food intake, g/wk</td>
<td>18.22±0.26</td>
<td>18.69±0.31</td>
<td>27.36±1.4*</td>
<td>18.76±1.24§</td>
</tr>
<tr>
<td>Feeding efficiency, g/100 kcal</td>
<td>0.65±0.03</td>
<td>0.28±0.05§</td>
<td>1.21±0.14*</td>
<td>1.052±0.07§</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.09±0.03</td>
<td>1.00±0.15</td>
<td>1.50±0.12†</td>
<td>1.14±0.06</td>
</tr>
</tbody>
</table>

AT characteristics

<table>
<thead>
<tr>
<th></th>
<th>Epididymal adipose tissue, g</th>
<th>Epididymal adipocyte diameter, μm</th>
<th>Subcutaneous adipose tissue, g</th>
<th>Subcutaneous adipocyte diameter, μm</th>
<th>Brown adipose tissue, g</th>
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<tr>
<td></td>
<td>0.72±0.06</td>
<td>0.26±5.5</td>
<td>0.45±0.05</td>
<td>0.12±0.01</td>
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</tr>
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Data are mean±SEM. IL indicates interleukin; HOMA-IR, homeostasis model assessment of insulin resistance; ND, not determined.

*P<0.001, †P<0.05, ‡P<0.01 for comparison between obese and lean mice within each genotype.

§P<0.05 for comparison between genotypes after the same diet.

Methods
For expanded methods, see the supplemental materials, available online at http://atvb.ahajournals.org.
Figure 2.

A. CD3+ T cell accumulation

B. CD3+ T cells (% of live cells)

C. CD3+ CLS (% of field)

D. Macrophage accumulation

E. Macrophage (% of live cells)

F. Macrophage CLS (% of field)

G. CD3+ T cells (% of live cells)

H. CD4+ T cells (% of live cells)

I. CD25+FoxP3+ (% of CD4+)

J. CD11c+high B220+

K. CD11c+high B220+

SFD vs HFD comparison.
Surprisingly, adipocyte size of epiAT but not that of scAT increased in obese CD40L−/− mice. In BAT, mRNA levels of UCP-1 were increased (Supplemental Figure I), indicating that deficiency of CD40L increases thermogenesis, thereby preventing body weight gain. Moreover, plasma leptin levels decreased in CD40L−/− mice (Table). This seems inconsistent with the reduction in food intake, but considering its role in regulating energy intake and expenditure, this is in accordance with the lower body weights in CD40L−/− mice.

CD40L Deficiency Improves Insulin Resistance
In lean mice, deficiency of CD40L did not affect fasting blood glucose, plasma insulin levels, or insulin sensitivity as measured by the homeostasis model assessment of insulin resistance (Table). However, in obese CD40L−/− mice, both plasma insulin levels and the homeostasis model assessment of insulin resistance were reduced (Table). Likewise, the insulin tolerance test displayed higher insulin sensitivity in CD40L−/− than in WT animals, as indicated by the lower levels of blood glucose after insulin injection (Figure 1B). However, CD40L−/− mice were not more glucose tolerant according to the glucose tolerance test (Figure 1C), which could be attributed to their lower baseline insulin level (Table). Absence of CD40L did not affect plasma cholesterol levels (Table). These results reveal that obese CD40L−/− mice are partially protected against the impaired insulin sensitivity associated with obesity.

CD40L Deficiency Attenuates Obesity-Induced Hepatic Steatosis
After the induction of diet-induced obesity, liver weights increase because of steatosis.30 Surprisingly, hepatic steatosis was only infrequently present in CD40L−/− mice (Table). Histological analysis revealed that 75% of WT mice developed NASH, whereas only 25% of CD40L−/− mice displayed some signs of NASH. Moreover, livers of CD40L−/− mice were protected against steatosis, foamy degeneration, ballooning, and lobular inflammation (Figure 1D and 1E).

Altered signaling in a number of biological pathways, including glycolysis, fatty acid synthesis, and fat uptake, is associated with liver steatosis.31 Indeed, expression of 2 major glycolytic genes, glucokinase and liver-specific pyruvate kinase, was reduced in livers of obese CD40L−/− mice (Figure 1F). Furthermore, livers of CD40L−/− mice tended to have a reduced expression of a number of genes involved in de novo lipid synthesis, such as the genes for ATP-citrate lyase, acetyl-coenzyme A carboxylase, and stearyl-coenzyme A desaturase (Figure 1G), and had a significantly reduced expression of the mRNA levels of the fatty acid transporter CD36 (Figure 1H). Consistent with decreased steatosis, the lipogenic transcription factor peroxisome proliferator-activated receptor-γ and sterol regulatory element-binding protein-1c were reduced in livers of CD40L−/− mice (Figure 1I).

These data indicate that CD40L deficiency attenuates obesity-induced deterioration of glucose and lipid homeostasis, as well as hepatic steatosis.

CD40L Deficiency Alters Obesity-Associated Inflammation
Surprisingly, the obesity-associated increase in immune cells was reduced in CD40L−/− mice compared with WT mice. CD3+ T-cell numbers, as well as the number of T-cell crown-like structures (CLSs), were lower in epiAT of obese CD40L−/− mice than in obese WT mice (Figure 2A to 2C), as were the number of macrophages and the number of macrophage CLSs (Figure 2D to 2F). In contrast to the epiAT, scAT contained fewer immune cells that were more dispersed and rarely seen in clusters. However, a similar reduction in T-cell and macrophage content was also detected in scAT of obese CD40L−/− mice (CD3+ T-cell infiltration in WT versus CD40L−/−: 20.7±3.9 versus 12.9±2.0 CD3+ per 100 adipocytes, macrophage infiltration in WT versus CD40L−/−: 12.0±2.9 versus 3.9±1.9 per 100 adipocytes).

Fluorescence-activated cell sorting analysis of the SVF revealed that in epiAT, induction of obesity resulted in an increase of the CD3+ T-cell fraction (Figure 2G). Within this CD3+ T-cell fraction, clear populations of CD8+, CD4+, and CD4+CD8− T cells could be identified. On induction of obesity, the fraction of CD8+ cells significantly increased in WT epiAT, whereas CD40L deficiency prevented this increase (Figure 2G and 2H). As reported before,6–8 we detected a greater subtraction of CD4+CD25+FoxP3+ regulatory T cells in epiAT compared with lymphoid organs, such as spleen and lymph nodes (Supplemental Figure II). Interestingly, the amount of regulatory T cells was even higher in epiAT of CD40L-deficient mice (Figure 2J). This suggests that deficiency of CD40L induces immune modulation in obese adipose tissue. Likewise, conventional dendritic cells (CD11c+highB220− gated on non-CD3+CD19− cells) were abundantly present in adipose tissue of obese WT mice, but not in obese CD40L−/− mice (Figure 2K).

In concordance with the flow cytometry data and immunohistochemistry data, epiAT of CD40L−/− mice showed a reduced expression of inflammatory mediators, such as monocyte chemotactic protein-1 and IL-6 (Figure 3A). In addition, CD40L deficiency reduced the expression of the Th1 cytokines interferon-γ, RANTES, tumor necrosis factor-α, and IL-12; reduced the expression of the macro-

Figure 2. (Continued) Immune cell accumulation in epididymal adipose tissue. A to F, Immunohistochemical analysis of CD3+ T cells (arrows) and macrophages (arrows) in epiAT from lean and obese wild-type (WT) and CD40L−/− mice after 18 weeks of diet. Graphs in B, C, E, and F represent the number of positive cells per 100 adipocytes and the numbers of crown-like structures/field in each group. *P<0.05, **P<0.01 for comparison between obese and lean mice within each genotype. †P<0.05 for comparison between genotypes mice after the same diet. SFD indicates standard-fat diet; HFD, high-fat diet. G to K, Fluorescence-activated cell sorting analysis of the stromal vascular fraction from the epiAT of WT (white bars) and CD40L−/− mice (black bars) fed an SFD or HFD for 18 weeks. The fractions of CD3+ T cells, CD8+, CD4+ T cells, Tregs (CD4+CD25+FoxP3+), and conventional dendritic cells (CD11c+highB220− gated on non-CD3+CD19− cells) was determined in the immune cell gate of the SVF. n=7 and 8 for SFD and HFD-fed WT mice, respectively; n=5 to 6 for CD40L−/− mice.
phage markers F4/80, CD68, and CSF-R-1; and induced the expression of the antiinflammatory M2 macrophage marker CD163 (Figure 3A and 3B).

Concomitant with an increased inflammatory state, obesity decreased the expression of genes required for lipid metabolism and insulin signaling in WT mice. This decrease was prevented in CD40L−/− mice, where mRNA levels of genes involved in adipocyte function, such as lipe (hormone-sensitive lipase) and gpam (glycerol-3-phosphate acyltransferase, mitochondria), were higher than in WT adipose tissue (Figure 3C). Also, adiponectin mRNA levels were higher in obese adipose tissue of CD40L−/− compared with that of WT mice (Figure 3C). A similar but less pronounced effect of CD40L deficiency was observed in scAT for genes involved in lipid metabolism, adiponectin, and other inflammatory cytokines, such as IL-6, tumor necrosis factor, and the macrophage marker CD68 (Supplemental Figure III). In addition to local inflammation in AT, obesity also increased plasma levels of leptin and the inflammatory cytokines IP-10 and IL-6. These levels were reduced in CD40L−/− mice (Table).

Overall, these data show that CD40L−/− mice are protected against obesity-induced immune cell accumulation and activation in adipose tissues and in the circulation.

**Anti-CD40L (αCD40L) Treatment Prevents Obesity-Associated Anomalies**

To examine the potential of therapeutic inhibition of CD40L during obesity, we examined the effects of a CD40L inhibiting antibody on the inflammatory and metabolic responses in our diet-induced obesity model. Antibody treatment was continued for only 12 weeks to prevent the formation of autoreactive antibodies. To confirm the effectiveness of αCD40L treatment, immune cell activation and composition of the spleen was analyzed by fluorescence-activated cell sorting. After 12 weeks of antibody treatment, we were able to show that CD3+ FoxP3+, CD4+ FoxP3+, and CD11c+ highB220− cell contents were reduced (Figure 4). After the 12-week treatment period, mice had developed intermediate obesity compared with the first study (Figure 5A). As in the CD40L−/− mouse study, total body weight and scAT, epiAT, and liver weights tended to be lower in αCD40L-treated mice (Figure 5A to 5D). As in CD40L-deficient mice, food intake was reduced on inhibition of CD40L (Figure 5E).

In intermediate obesity, treatment with the αCD40L antibody significantly increased glucose tolerance, as well as insulin sensitivity, and lowered plasma insulin levels (Figure 5F to 5H). In addition, IL-6 and leptin plasma levels tended to be diminished (IL-6, IgG-treated versus αCD40L-treated mice, 8.7±3.7 versus 2.8±0.9; leptin, IgG-treated versus αCD40L-treated mice, 14.6±4.2 versus 10.2±2.1).

Quantitative immunohistochemistry provided a tendency similar to that observed in CD40L−/− mice. The number of CD3+ cells and macrophages, as well as the number of CLSs, were reduced in αCD40L-treated mice (number of CD3+ T cells per 100 adipocytes: 30.0±6.0 versus 19.7±3.6, *P*<0.09; number of CD3+ CLSs/field: 0.5±0.2 versus 0.1±0.04, *P*<0.05; number of macrophages/100 adipocytes: 18.3±4.2 versus 10.6±2.7; number of macrophages CLSs/field: 0.5±0.2 versus 0.1±0.0, *P*<0.05 for control versus αCD40L-treateed mice).

Fluorescence-activated cell sorting analysis in epiAT of αCD40L-treated mice showed an intermediate activation state of the immune system compared with the CD40L−/− mouse study. First of all, the CD3+ T-cell fraction of the SVF was not altered, nor was the CD8+ T-cell fraction within the CD3+ gate. However, the CD4+ T-cell fraction had decreased, without affecting the regulatory T-cell fraction, suggesting that αCD40L treatment during intermediate obe-
sensitivity reduces the number of T-effector cells (Figure 5I to 5M). Furthermore, the expression of MHCII on antigen-presenting cells was reduced on treatment (Figure 5L), suggesting fewer TCR-MHCII interactions and thereby less activation of the immune system. As shown in our first experiment, immune regulation by regulatory T cells seems to be an event that occurs in more severe stages of obesity, whereas reduced immune cell activation occurs in moderate obesity.

**Discussion**

The communication between cells of the innate32 and adaptive6,7 immune system is key in the pathogenesis of obesity, a disease in which the immune system not only mediates obesity-induced (tissue) inflammation but also affects metabolic processes. The main communicators of the immune system are costimulatory molecules, implying a profound role for this family in the pathogenesis of obesity.28,33 In the present study, we identified the costimulatory molecule CD40L as an important player in intermediate and severe forms of diet-induced obesity. In general, deficiency of CD40L reduced the amount of immune cell infiltration in adipose tissue. In a model of intermediate obesity (12 weeks of HFD), a clear reduction in the CD4+ effector T-cell fraction, associated with an improved insulin resistance,6 as well as a decrease in the antigen presenting capacity of the DCs were observed after inhibition of CD40L. In a severe obesity model (18 weeks of HFD), deficiency of CD40L decreased the CD8+ T-cell fraction, the T-cell subtype associated with adipose tissue inflammation and activation of adipose tissue macrophages,6 and strongly increased the fraction of regulatory T cells. We can therefore conclude that during the progression of obesity, the effect of CD40L inhibition switches from mere downregulation of T-cell activation to a mode of strong immune modulation.

Besides mediating (AT) inflammation, T cells and macrophages have recently been shown to contribute to metabolic dysfunction, such as insulin resistance and fat distribution, such as occurs in diet-induced obesity. Genetic and immunologic depletion of CD8+ T cells improves insulin resistance in obesity, whereas CD8+ T-cell adoptive transfer aggravates metabolic dysfunction.6 The balance between CD4+ effector T cells and regulatory T cells in AT affects metabolic functions, with a clear beneficial role for regulatory T cells.7 Interestingly, a reduction of macrophages and CLSs in AT, as well as a reduction in classically activated macrophages, not only reduces AT inflammation but also ameliorates hepatic steatosis and improves glucose homeostasis and insulin sensitivity.10,34 Consistent with these studies, the attenuated CD8+ T cell response and the increase in Tregs in our CD40L−/− mice with severe obesity, the decrease in CD4+ effector T cells in AT of αCD40L antibody-treated mice with intermediate obesity, the reduction in macrophage accumulation, and the switch to alternatively activated macrophages coincided with a reduction in weight gain, decreased insulin plasma levels, and an improved systemic insulin resistance.

### Figure 4.

Anti-CD40L treatment: immune cell composition of the spleen. Fluorescence-activated cell sorting analysis of the spleen cells of mice fed a high-fat diet (HFD) and treated with IgG or αCD40L antibody for 12 weeks. CD3+ (A), CD4+ (B), CD8+ (C), Tregs (CD4+CD25+FoxP3+) (D), MHCII expression (E), and conventional DCs (CD11c+ gated non-CD3+CD19+ cells) (G) were analyzed.

*P<0.05, **P<0.01, ***P<0.001, n=9 for IgG-treated mice and n=11 for αCD40L antibody-treated mice.
Steatosis and NASH are strongly associated with obesity and insulin resistance and are now recognized as being part of the metabolic syndrome. We found that in CD40L/H11002 mice, liver steatosis and NASH were strongly reduced, as were the expression levels of genes involved in glycolysis, fatty acid synthesis, and fat uptake. Contrary to our results, Villeneuve et al found that CD40L was protective against the development of steatosis after administration of olive oil. However, the different dietary stresses that are used in the 2 models may be accountable for this difference.

Both genetic deficiency of CD40L and inhibition of CD40L by antibody treatment reduced food intake, thereby preventing body weight gain. Remarkably, leptin levels were lower. Because decreased leptin levels are expected to decrease satiety and increase food intake, other mechanisms may play a role. It is very possible that the absence of CD40L protects against the development of leptin resistance, which is usually associated with obesity. On the other hand, leptin is not the only factor associated with the regulation of appetite and energy expenditure. Reduced food intake could for example be caused by alterations of neuropeptide Y or proopiomelacortin levels in the central nervous system.

Another function of leptin is the activation of immune cells. For example, leptin polarizes T cells toward a Th1 phenotype, thereby inducing proatherogenic cytokine production. Moreover, in murine dendritic cells, leptin has been shown to induce CD40 expression and drive T-cell proliferation. In our study, the reduced leptin levels may have contributed to the observed antiinflammatory phenotype.
Although it is difficult to distinguish the primary effects of CD40L deficiency on metabolic parameters from secondary effects, such as reduced food intake and impaired body weight gain, we believe that CD40L directly affects metabolism, independently of the reduction in weight gain. For example, despite the strong reduction in body weight in CD40L−/− mice, their epiAT weight was virtually similar. Epididymal adipocyte size was even higher in CD40L−/− mice. Interestingly, expression of genes involved in lipid metabolism, such as *lpe* and *gpm* stayed elevated, whereas they are known to be downregulated in obese adipose tissue. This indicates that in CD40L−/− mice, lipogenesis and lipolysis do not shut down during obesity, which may be responsible for their protection against weight gain and fat accumulation in others regions of the body, such as the liver. Indeed, in obesity, counterintuitive lipogenesis can represent a decreased capacity to store the excess circulating triglycerides and induce ectopic fat accumulation. A high level of UCP-1, with its unique expression in brown adipose tissue, could further explain the protection against body-weight gain in CD40L−/− mice. BAT is specialized for thermogenic energy expenditure and provides a natural defense against cold and obesity. Moreover, the finding that neutralizing anti-CD40L antibody treatment did not affect body weight but improved systemic insulin action indicates an important metabolic role for the CD40L genotype independent of the weight. These data suggest a direct effect of CD40L on the insulin pathway independent of inflammatory reaction.

Collectively, we have identified a crucial role for the costimulatory molecule CD40L in obesity, obesity-induced activation of the immune system, and metabolic parameters such as insulin sensitivity in vivo. Our data increase the knowledge about the mechanisms underlying the inflammatory and inflammatory-induced metabolic changes in obese adipose tissue and raise the potential to identify new therapeutic targets in the CD40L pathway that may prevent or ameliorate obesity and obesity-related vascular comorbidities.

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**Disclosures**

None.

**References**


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Supplemental methods section

Animals

CD40L⁻/⁻ and CD40L⁺/⁺ mice (100% C57Bl6J background) and C57Bl6J mice were obtained from the Jackson laboratories (Bar Harbor, ME, USA) (n=45). All mice were male, maintained under 12-hour light-dark cycle and were allowed free access to food and water. Mice were fed with standard-fat diet (SFD, 70% kcal carbohydrate, 10% kcal fat, 20% kcal protein, 3.68 kcal/g, SDS Special Diets Services, Witham, UK) or high-fat diet (HFD, 35% kcal carbohydrate, 45% kcal fat, 20% kcal protein, 4.54 kcal/g, SDS Special Diets Services). Two experimental studies were completed: 1) CD40L⁻/⁻ and CD40L⁺/⁺mice fed with the SFD or HFD for 18 weeks starting at 6-12 weeks of age 2) C57Bl6 mice intraperitoneally (ip) injected with neutralizing anti-CD40L (αCD40L, clone: MR-1) or control IgG antibodies (200µg twice per week) and fed with the HFD for 12 weeks over the same period beginning at 8 weeks of age. Food intake was measured weekly. After the experimental period, overnight-fasted animals were euthanized, blood was collected and organs (subcutaneous [scAT] and epididymal white adipose tissue [epiAT], brown adipose tissue [BAT], liver, spleen) were dissected. Studies were approved by the animal ethics committee of Maastricht University and executed according the institutional guidelines.

Biochemical measurements and glucose/insulin tolerance tests

For the glucose tolerance test, overnight-fasted conscious mice were injected ip with glucose (1mg/g). For the insulin tolerance test, 5h-fasted conscious mice were injected ip with insulin (0.75mU/g, Actrapid, Novonordisk, Bagsvaerd, Denmark). Glucose levels were measured from whole blood using a glucometer (Roche Diagnostics, Basel, Switzerland) at times indicated on the figures. Fasting insulin (Mercodia, Uppsala, Sweden), IP-10 (RD systems, Minneapolis, MN), IL-6 (Invitrogen, Breda, Netherlands) and leptin (Crystal chem., Downers Grove, IL, USA)
levels were measured in plasma by enzyme-linked immunoabsorbent assay. The homeostasis model assessment of insulin resistance (HOMA-IR) was estimated using the following formula: fasting insulin (µ international unit/ml) x glucose /22.5. Cholesterol levels were measured using a colorimetric assay (CHOD-PA, Roche) and triglycerides by enzymatic assay (Wako, Neuss, Germany).

**Flow Cytometry**

Stroma-vascular cells (SVC) were isolated from epiAT using collagenase (Sigma-Aldrich, Zwijndrecht, Netherlands) as previously described. Briefly, the samples were incubated at 37°C with shaking until complete digestion, passed through a 100µm cell strainer (Falcon, distributed by BD biosciences, Breda, the Netherlands), washed and centrifuged to obtain the final SVC pellet. Splenocytes were obtained as previously described. Spleens were pressed through a 70µm cell strainer and washed after erythrocyte lysis. Fc-blocking (CD16/32 antibody) was performed prior to cell labelling. Cells were labelled with fluorescent antibodies against CD3, CD4, CD8, CD25, FoxP3, Ly6G, B220, CD11c and CD19. All the antibodies were purchased from e-Biosciences (San Diego, CA) or BD Pharmingen (distributed by BD biosciences).

**Morphometry and Immunohistochemistry (IHC)**

Deparaffinized 4 µm thick sections of epiAT and scAT were incubated with antibodies directed against CD3 (Dako, Glostrup, Denmark) or macrophages (AIA31240, SanBio, Uden, the Netherlands). After washing, slides were incubated with a biotinylated secondary antibody and with the avidin–peroxidase complex (Vectastain ABC System, Vector Laboratories, Burlingame, CA, USA). The signal was detected using diaminobenzidine or Vectastain Red substrate. As negative control, sections were incubated with corresponding control isotype-matched antibody. All morphometric parameters were determined using a microscope coupled to a
computerized morphometry system (Qwin, Leica, Rijswijk, The Netherlands). From each animal, images of 6-7 fields were acquired. Subsequently, the diameters of all adipocytes and the number of crown-like structures (CLS) and positively stained cells were determined in each field by an observer blinded to the conditions using Qwin3 software.

Four µm liver sections were stained with haematoxylin-eosin, sirius red or Oil red O and used for determining hepatic parameters as indicated by the Pathology committee of the non-alcoholic steato-hepatitis (NASH) clinical research network.³

Real-Time PCR

Total RNA was extracted using the Trizol method (Invitrogen). cDNA was synthesized using i-Script cDNA synthesis kit (BIO-RAD). PCRs were performed with a Bio-Rad instrument and software under standard conditions. The relative amounts of the different mRNAs were quantified by using the second derivative maximum method. The relative quantification for mRNA was corrected to 36B4 mRNA values used as an invariant control. Results were expressed relative to the control group (WT SFD), which was assigned a value of 1. Primer sequences are available upon request.

References


Supplemental Figure Legends

Supplemental figure I
mRNA levels of UCP-1 in brown adipose tissue of WT (white bars) and CD40L−/− brown adipose tissue mice (black bars) after a SFD or HFD. n=7 and 8 respectively for SFD and HFD-fed WT mice, n=5 for CD40L−/− mice.

Supplemental figure II:
FACS analysis of the spleen cells of mice fed with SFD and HFD for 18 weeks. CD3+ (A), CD8+ (B), CD4+ cells (C) and T regs (CD4+CD25+FoxP3+) (D) were analyzed. Values are mean±SEM *p<0.05, ***p<0.001 for comparison between obese and lean mice within each genotype. †p<0.05 for comparison between genotypes after the same diet. n=7 and 8 respectively for SFD and HFD-fed WT mice, n=5 for CD40L−/− mice.

Supplemental figure III
mRNA levels of inflammatory molecules (A), macrophage markers (B) and genes involved in metabolism (C) in scAT of WT (white bars) and CD40L−/− mice (black bars) after 18 weeks of HFD. *p<0.05, **p<0.01. n=7 and 8 respectively for SFD and HFD-fed WT mice, n=5 for CD40L−/− mice.
Supplemental figure I

![Graph showing UCP-1 mRNA levels for SFD and HFD groups. The graph indicates a significantly higher UCP-1 mRNA level in the HFD group compared to the SFD group, with a symbol indicating significance.]
Supplemental Figure II

**A**

CD3+ T cells (% of live cells)

**B**

CD8+ T cells (% of CD3+)

**C**

CD4+ T cells (% of CD3+)

**D**

CD4+CD25+FoxP3+ (% of CD4+)

- **A**: Comparison of CD3+ T cell percentages between Wt and Ko mice on SFD and HFD diets. 
- **B**: CD8+ T cell percentages on SFD and HFD diets. 
- **C**: CD4+ T cell percentages on SFD and HFD diets. 
- **D**: CD4+CD25+FoxP3+ cell percentages on SFD and HFD diets.