From hematopoietic stem and progenitor cells to mature immune cells in inflammatory diseases

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Blocking CD40-TRAF6 signaling is a novel therapeutic target in obesity-associated insulin resistance

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Abstract

The immune system plays an instrumental role in obesity and insulin resistance. Here we unravel the role of the co-stimulatory molecule, CD40, and its signalling intermediates, TNF-Receptor-Associated-Factors (TRAFs), in diet-induced obesity (DIO). Although not exhibiting increased weight gain, male CD40−/− mice in DIO displayed worsened insulin resistance, as compared to wild type mice. This was associated with excessive inflammation of adipose tissue (AT), characterized by increased accumulation of CD8+ T-cells and M1 macrophages, and enhanced hepatosteatosis. Mice with deficient CD40-TRAF2/3/5 signaling in MHCII+ cells exhibited a similar phenotype in DIO as CD40−/− mice. In contrast, mice with deficient CD40-TRAF6 signaling in MHCII+ cells displayed no insulin resistance, and showed a reduction in both AT inflammation and hepatosteatosis in DIO. To prove the therapeutic potential of inhibition of CD40-TRAF6 in obesity, DIO mice were treated with a small-molecule inhibitor that we designed to specifically block CD40-TRAF6 interactions; this improved insulin sensitivity, reduced AT inflammation and decreased hepatosteatosis. Our study reveals that the CD40-TRAF2/3/5 signaling pathway in MHCII+ cells protects against AT inflammation and metabolic complications associated with obesity, whereas CD40-TRAF6 interactions in MHCII+ cells aggravate these complications. Inhibition of CD40-TRAF6 signaling by our newly developed compound may provide a novel therapeutic option in obesity-associated insulin resistance.
Introduction

Emerging evidence points to inflammation as a critical contributor to the pathogenesis of metabolic disorders associated with obesity. Obese adipose tissue (AT) shows hallmarks of chronic low-grade inflammation, which is believed to facilitate the development of insulin resistance (IR) (1–3). Macrophages, especially proinflammatory M1-polarized macrophages, as well as different T-cell subsets and other immune cells, play a major role (1–5). Cytokines derived from immune cells in the AT microenvironment can directly interfere with insulin signaling (2, 3, 6). In addition, the actions carried out by these immune cells through cell–cell contact or paracrine cross-talk with adipocytes increase the expression of proinflammatory molecules such as chemokines and cytokines (7), which, in turn, further enhance accumulation of leukocytes in the AT.

The costimulatory receptor ligand pair, CD40-CD40L, is crucial in the initiation and progression of inflammatory diseases by enhancing inflammation (8). CD40-CD40L interactions are also implicated in obesity-related inflammation. Elevated levels of sCD40L are found in obese individuals (9). Moreover, CD40 is expressed on adipocytes, and stimulation of adipocytes with CD40L results in a reduction of IRS-1 and GLUT4 and induction of adipokines (10) whereas the medium of CD40L-stimulated adipocytes activates endothelial cells (11). Importantly, we and others recently found that genetic ablation or pharmacologic inhibition of CD40L ameliorated AT inflammation, IR, and hepatic steatosis in a mouse model of diet-induced obesity (DIO) (12, 13).

Because inhibition of CD40L by antibodies results in thromboembolic complications, which precludes its clinical use (14), targeting of CD40, the receptor for CD40L, or the CD40-associated signaling intermediates, specifically, the TNF receptor-associated factors (TRAFs), has become an interesting opportunity in inflammatory diseases.

In the present study, we investigated the effect of genetic CD40 deficiency on DIO. Surprisingly, and in contrast to CD40L deficiency, we found that CD40 deficiency was not protective but rather aggravated IR and obesity-associated liver and inflammation of AT. To understand this unexpected result, we explored the involvement of CD40-TRAF signaling cascades. Whereas loss of CD40-TRAF2/3/5 signaling mimicked the phenotype of CD40 deficiency, inactivation of CD40-TRAF6 signaling conversely protected against weight gain, AT inflammation, and metabolic complications. This finding suggested that specific blockade of the CD40-TRAF6 pathway could be used to prevent IR due to obesity. Indeed, we developed a compound specifically targeting the CD40-TRAF6 interaction, which improved insulin sensitivity, decreased M1 macrophage numbers in the AT, and reduced hepatosteatosis in mice with DIO. Thus, CD40-TRAF6 signaling inhibition may provide a therapeutic opportunity in obesity-associated IR.
Materials and Methods

Animals

CD40+/+ , CD40−/− mice (C57BL6 background) (15) as well as CD40-Twt, CD40-T2/3/5−/− and CD40-T6−/− mice (16) were fed SFD or HFD diets (SFD, 70% kcal carbohydrate, 10% kcal fat, 20% kcal protein, SDS Special Diets Services, Witham, UK or Research Diets, NJ, USA; HFD, 35% kcal carbohydrate, 45% kcal fat, 20% kcal protein from SDS Special Diets Services or 20% kcal carbohydrate, 60% kcal fat, 20% kcal protein from Research Diets) for different time points up to 30 weeks (CD40+/+ and CD40−/− mice, males; 60% kcal-HFD), or 20 weeks (CD40-TRAF mice, males; 45% kcal-HFD) starting at the age of 6-8 weeks. Body weights were measured weekly. After the experimental period, animals were euthanized, blood was collected and organs were dissected or stored at -80°C for further analysis. Studies were approved by the animal experimental commissions of the University of Maastricht, Amsterdam and Leiden and the Landesdirektion Dresden.

Biochemical measurements and Insulin tolerance test

An insulin tolerance test (ITT) was performed, and fasting insulin levels were measured. For the ITT, mice fasted for 5h were injected i.p. with insulin (0.75-2mU/g, Actrapid, Novo Nordisk, Bagsvaerd, Denmark or Huminsulin, Lilly, Bad Homburg, Germany). Glucose levels were measured in whole blood using a glucometer (Roche Diagnostics, Basel, Switzerland) or a glucose meter device (Accu-Chek, Roche, Mannheim, Germany). Fasting insulin levels were measured in plasma by enzyme-linked immunoabsorbent assay (Mercodia, Uppsala, Sweden). Cholesterol levels were measured using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany) and triglycerides, by enzymatic assay (Wako, Neuss, Germany).

Body composition analysis

Body composition was measured using 1H NMR spectroscopy (EchoMRI 3-in-1, Echo Medical Systems LTD, Houston, TX, USA). Data were analyzed using the manufacturer’s software.

Indirect calorimetry/metabolic cage analysis

CD40-Twt, CD40-T2/3/5−/−, CD40-T6−/− and CD40-T2/3/5/6−/− mice were subjected to indirect calorimetry/metabolic cage analysis (Phenomaster, TSE Systems, Bad Homburg, Germany). A period of at least 48 hours of acclimatization was included prior to initiation of the experiment. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were determined at 20-minute intervals. Respiratory exchange ratio (RER) was calculated as the ratio between VCO2 and VO2. Energy expenditure (EE), fat oxidation (FAox) rate and carbohydrate oxidation (CHox) rate were calculated as previously reported (17). Food intake (EI) and activity levels were monitored at 1 minute intervals.

Flow cytometric analysis

The stromal-vascular fraction (SVF) was isolated from gonadal AT using collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands or Invitrogen, Darmstadt, Germany). The samples were incubated at 37°C with agitation until complete digestion, passed through a cell strainer.
Blocking CD40-TRAF6 signaling improves obesity-associated IR

CD40 deficiency induces insulin resistance in DIO

CD40-deficient male mice were subjected to the DIO model. CD40-deficient (15) and CD40-sufficient mice were fed a high-fat diet (HFD) for up to 30 wk. CD40 deficiency in mice did not result in increased total body weight (Fig. 1A) but did lead to worsened IR after 30 wk of HFD (Fig. 1B). Although the weights of s.c. AT (scAT) and gonadal AT (gonAT) were similar or decreased, liver weights increased slightly in CD40 deficiency (Fig. 1C). CD40−/− mice exhibited significant liver abnormalities related to obesity with pronounced hepatosteatosis, compared with CD40-sufficient mice (Fig. 1D). Accordingly, hepatic genes associated with steatosis (PPARγ, PAI-1, and CHREBP), as well as genes involved in the regulation of glycolysis and lipid uptake (GK, LPK, and CD36), showed enhanced mRNA expression due to CD40 deficiency (Fig. 1E). On a standard-fat diet (SFD), CD40−/− mice did not develop any metabolic abnormalities (Fig. 1).
CD40 deficiency induces severe AT inflammation

Given the importance of AT inflammation for the development of IR and the well-established role of CD40 in inflammation, we then continued to assess the role of CD40 deficiency in inflammation of AT.

Flow-cytometric analysis of the stromal vascular fraction (SVF) of the gonAT of the HFD group revealed that CD40-deficient mice had increased numbers of CD45+ cells (Fig. 2A) and CD8+ T-cells (Fig. 2A). In addition, a significant increase in the number of CD11b+F4/80+ macrophages was observed (Fig. 2A). Further subtyping showed that the fraction of the proinflammatory classically activated M1-polarized macrophages, characterized by expression of CD11c and absence of CD206, was higher in CD40 deficiency (Fig. 2A).

Quantitative PCR analysis revealed increased expression of IL-6, IL-12, TNF, MCP1, ICAM1, and the macrophage and T-cell specific markers CD68, CD3, and CD8 in the gonAT of CD40−/− mice (Fig. 2B). Adiponectin, leptin, GLUT4, and PPARγ did not differ on HFD (Fig. 2B). On SFD, no differences in accumulation of immune cells and expression of inflammatory genes in the AT were observed due to CD40 deficiency. Although T-cell populations in the gonAT were similar between CD40−/− and wild-type mice on SFD, CD40−/− mice had reduced numbers of CD4+ T-cells and regulatory T-cells (Tregs) in the spleen (Fig. S1). Together, CD40 deficiency leads to an aggravation of AT inflammation and development of IR in DIO. These data were unexpected given the phenotype of the CD40L−/− mouse (12).

CD40 lacks intrinsic signaling capacity and requires adaptor molecules, the TRAFs, to elicit and steer the distinct CD40 downstream signaling pathways. To identify which CD40-TRAF signaling pathway is involved in metabolic regulation and AT inflammation in vivo, we used male CD40-deficient mice that contained a CD40 transgene under the control of the MHCII promoter, in which the TRAF2/3/5 or the TRAF6 binding sites on the CD40 C-terminal tail were mutated, leading to CD40-T2/3/5−/− and CD40-T6−/− mice (16). As a control, we engaged CD40-Twt mice carrying the CD40 transgene without any mutations. These mice were subjected to SFD or HFD.
Figure 1. CD40−/− mice on HFD display aggravated metabolic dysregulation. Wildtype (WT) and CD40−/− male mice were fed with SFD or HFD for 30 wk. (A) Body weight of WT and CD40−/− mice on SFD (n = 5) or HFD (n = 6–7). (B) Insulin tolerance test (ITT) in 5-h fasted WT and CD40−/− mice fed an HFD for 30 wk (n = 6–7). (C) Weights of sqAT, gonAT, and liver of WT and CD40−/− mice after 30 wk on SFD (n = 5) or HFD (n = 6–7). (D) Representative H&E-stained sections from liver of WT and CD40−/− mice on SFD or HFD for 30 wk. (E) Liver gene expression of WT and CD40−/− mice on HFD for 30 wk. The mRNA expression was normalized against 18S, and the gene expression of livers from WT HFD was set as 1 (n = 6–7). *P < 0.05 for comparison between WT and CD40−/− mice fed the same diet.
Figure 2. CD40 deficiency aggravates AT inflammation. (A) SVF cells were isolated from gonAT of WT or CD40−/− male mice fed a SFD or HFD for 18 weeks. CD45+ leukocytes, CD4+ or CD8+ lymphocytes, total macrophages (characterized as CD11b+F4/80+) and M1-macrophages (defined as F4/80+CD11b+CD11c+ or F4/80+CD11c+CD206+) or M2-macrophages (defined as F4/80+CD11c−CD206+) were analyzed by flow cytometry. (B) Gene expression in the gonAT of male WT and CD40−/− mice on SFD or HFD for 18 weeks. The mRNA expression was normalized against 18S and the gene expression of adipose tissue from WT SFD was set as 1. n = 4 or more/group for SFD groups and n = 7 or more/group for HFD groups. *p<0.05 for comparison between WT and CD40−/− mice fed the same diet.
Deficiency of CD40-TRAF2/3/5 signaling, but not CD40-TRAF6 signaling, exacerbates DIO

When fed an HFD for 20 wk, male CD40-T2/3/5/ mice initially gained more weight compared with their CD40-Twt controls; the difference in weight gain was significant during the first weeks of HFD feeding (Fig. 3A). CD40-T6/ mice experienced a milder weight gain (Fig. 3A) and a delay in reaching their maximal weight. This delay may be caused by a more active brown adipose tissue (BAT), as reflected by increased uncoupling protein (UCP)-1 mRNA levels in the BAT of CD40-T6/ mice (Fig. S2). Body composition was studied using 1HMRI analysis after 5 wk of HFD. Fat mass was significantly higher in CD40-T2/3/5/ mice and significantly lower in CD40-T6/ mice, both compared with CD40-Twt mice (Fig. 3B).

CD40-T2/3/5/ mice exhibited IR after 20 wk of HFD (Fig. 3C) but had similar blood-insulin levels as the CD40-Twt mice (Table S1). In addition, CD40-T2/3/5/ mice had increased plasma cholesterol and plasma triglyceride levels (Table S1), showing the metabolic complications of obesity. CD40-T6/ mice did not develop IR, and they displayed no increase in baseline glucose levels (Fig. 3C). Moreover, these mice showed no aberrations in plasma cholesterol or triglyceride levels (Table S1). No differences between the genotypes were observed on SFD.

During the first week of HFD feeding, indirect calorimetry metabolic cage analysis was performed in a group of mice separate from the long-term experimental groups. Both body weight and food intake were significantly higher in CD40-T2/3/5/ and lower in CD40-T6/ mice, compared with CD40-Twt mice (Table S1). Energy-expenditure levels did not differ between groups, nor did ambulatory physical activity levels. Absolute fat oxidation rates were somewhat lower in CD40-T2/3/5/ mice, compared with CD40-Twt mice, but did not differ in CD40-T6/ mice. In contrast, absolute carbohydrate oxidation values were significantly higher in CD40-T2/3/5/ mice, compared with CD40-Twt, but were similar in CD40-T6/ mice, compared with CD40-Twt mice (Table S1).

These data show that CD40-T2/3/5/ mice are prone to metabolic complications related to obesity, thereby resembling CD40-deficient mice, whereas CD40-T6/ mice seem protected from obesity-associated complications.

CD40-T2/3/5/ mice develop steatosis

CD40-T2/3/5/ mice, but not CD40-T6/ mice, had an increase in liver weight, associated with pronounced steatosis, after 20 wk of HFD (Fig. 3 D and E). Histologic analysis revealed that all genotypes developed steatosis on an HFD. The severest phenotype was found in CD40-T2/3/5/ mice where 87.5% of the mice developed grade 3 steatosis, compared with only 62.5% of the CD40-Twt mice (Fig. S3A). Deficiency of CD40-TRAF6 interactions resulted in a milder form of steatosis. CD40-T6/ mice mostly developed grade 1 or 2 steatosis with limited expansion (Fig. S3A). Steatosis extended from the central vein to the periportal vein in 87.5% of the CD40-T2/3/5/ mice, but in only 37.5% of the CD40-Twt mice (Fig. S3A). The liver parenchyma showed grade 1 lobular inflammation in all genotypes although 37.5% of the CD40-T6/ mice developed less than grade 1 inflammation (Fig. S3A). Ballooning of
hepatocytes was a frequent observation in all genotypes (87.5% of CD40-Twt and CD40-T2/3/5− mice) but was less prominent in CD40-T6− mice (62.5%) (Fig. S3A).

Consistent with these results, we found genes associated with metabolism to be altered in CD40-T2/3/5− mice. Genes involved in glycolysis, such as liver glucokinase (GK) and liver pyruvate kinase (LPK), but not glucose transporter 2 (GLUT2), were elevated (Fig. S3B). Furthermore, we detected higher mRNA levels of the fatty acid transporter CD36, which stimulates glycolysis and lipogenesis (Fig. S3B). These findings imply an important function for CD40-TRAF2/3/5 signaling in liver metabolism in DIO.

**Disruption of CD40-TRAF2/3/5 signaling, but not of CD40-TRAF6 signaling, exacerbates AT inflammation in DIO**

Flow-cytometric analysis of the SVF of the gonAT revealed an increased F4/80high CD11b+ macrophage fraction in mice lacking CD40-TRAF2/3/5 signaling, compared with CD40-Twt mice (Fig. 4A). In keeping with these findings, CD68 mRNA was also increased in CD40-T2/3/5− mice (Fig. S4A). Analysis of cytokines revealed elevated levels of TNF and IL-1α (Fig. S4A), suggesting an M1 macrophage-biased response.

The percentage and number of CD3+ T-cells were slightly elevated in CD40-T2/3/5− mice (2.1 ± 0.11 × 10^5 CD3+ cells; P = 0.08) compared with CD40-Twt mice (1.92 ± 0.31 × 10^5 CD3+ cells) whereas the percentage and numbers of CD3+ T-cells in CD40-T6− mice (1.89 ± 0.48 × 10^5 CD3+ cells) equalled the levels in CD40-Twt mice. Remarkably, the increased percentage of CD3+ T-cells was accompanied by an increase in the CD8+ T cell fraction and a decrease in CD4+ T cells in the AT of CD40-T2/3/5− mice (Fig. 4B). Accordingly, mRNA levels of CD3 as well as IL-2 were elevated in the gonAT of these mice (Fig. S4B). In CD40-T2/3/5− mice, the fraction and number of Treg cells (CD40-Twt, 4.2 ± 0.6 × 10^5 vs. CD40-T2/3/5−, 1.7 ± 0.2 × 10^4 Tregs; P < 0.05) in the gonAT were decreased (Fig. 4C). In the spleen, CD40-T6− mice had an increased Treg fraction (6.8 ± 0.3% in CD40-Twt vs. 9.4 ± 0.2% in CD40-T6− mice) whereas total splenic CD3+ and CD4+ T-cell numbers were unchanged. The CD8+ T-cell fractions in gonAT of CD40-T2/3/5− mice displayed an increase in CD44highCD62Llow effector cells, with a concomitant decrease in CD44lowCD62Lhigh naïve T-cells (Fig. 4D). This T-cell profile is indicative of a more vigorous (CD8+) T-cell response, and a migratory potential, thereby likely resulting in aggravation of AT inflammation.
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**Figure 3.** Deficiency of CD40-TRAF2/3/5 signalling aggravates obesity and promotes metabolic dysfunction and hepatosteatosis. (A) Body weight of CD40-Twt, CD40-T2/3/5−/− and CD40-T6−/− mice on SFD or HFD for 20 weeks (N = 12-15). (B) Fat mass as determined by 1H NMR spectroscopy (N = 8 per group). (C) ITT in 5-hr fasted CD40-Twt, CD40-T2/3/5−/−, CD40-T6−/− mice fed a HFD for 18 weeks (N = 8 per group). (D) Liver weight and (E) Oil red O stained liver cryosections of CD40-Twt, CD40-T2/3/5−/− and CD40-T6−/− mice fed a HFD for 20 wks. Scale bar = 100μm. Values are mean +/− SEM. *p<0.05 for comparison with CD40-Twt mice.

**Figure 4.** CD40-TRAF2/3/5 deficiency results in increased numbers of inflammatory cells in gonadal AT. (A) FACS analysis of the number of F4/80highCD11b+ macrophages, (B) CD3+ T-cells, CD8+ T-cells and CD4+ T-cells, (C) CD4+CD25+FoxP3+ T-reg, (D) CD8+CD44lowCD62Lhigh naive T-cells and CD8+CD44highCD62Llow effector memory T-cells. Values are mean +/- SEM. *p<0.05 for comparison to CD40-Twt mice. N = 8-10 mice/group
CD40-TRAF2/3/5 deficiency changes inflammatory gene expression in DIO

Analysis of gene expression in the gonAT of obese mice revealed a signature of proinflammatory chemokine expression in CD40-T2/3/5−/− mice. Genes important in recruiting T-cells and macrophages, and genes involved in activating T- and B-cells, such as chemokine C-C motif ligand 3 (CCL3), CCL5, and chemokine C-X-C motif receptor 3 (Cxcr3) were found to be upregulated in CD40-T2/3/5−/− mice, compared with CD40-Twt mice (Fig. S4). Furthermore, we observed increased mRNA expression of the proinflammatory cytokines IL-1α and TNF in CD40-T2/3/5−/− mice (Fig. S4).

Remarkably, gonAT of CD40-T6−/− mice showed reduced inflammation. The expression of E-selectin, as well as of chemokine C-C motif receptor 7 (CCR7), was significantly reduced in CD40-T6−/− mice, suggesting less inflammatory cell recruitment. Moreover, the costimulatory molecule CD28 and its counter receptor CD86 were also decreased in gonAT of CD40-T6−/− mice (Fig. S4D).

No differences in immune-cell accumulation or levels of inflammatory genes in the AT or in the degree of hepatosteatosis were observed between the three genotypes on a SFD. These results showed aggravated metabolic dysregulation in CD40-T2/3/5−/− mice, which thus phenotypically resembled CD40−/− mice; in contrast, blocking the CD40-TRAF6 pathway ameliorated metabolic complications and slightly reduced AT inflammation in DIO.

Pharmacologic inhibition of the CD40-TRAF6 pathway ameliorated obesity-related metabolic complications

We next explored whether the CD40-TRAF6 pathway could represent a therapeutic target for metabolic dysfunction related to obesity. To this end, we developed a small-molecule inhibitor specifically targeting the CD40-TRAF6 interaction (Seijkens et al., in preparation; chapter 7).

To assess whether this inhibitor could interfere with obesity related metabolic abnormalities in a therapeutic setting (i.e., after initiation of DIO), C57BL/6 mice were fed an HFD for 6 wk and then received the small-molecule inhibitor 6877002 or vehicle for the next 6 wk. Treatment with compound 6877002 resulted in improved insulin sensitivity, compared with vehicle-treated mice (Fig. 5 A and B), whereas no alterations in weight were observed. Moreover, inflammation of gonAT was decreased after treatment with 6877002, with a remarkable reduction in CD11b+F4/80+CD11c+ (M1) macrophages (Fig. 5C). Interestingly, treatment with the CD40-TRAF6 inhibitor also reduced hepatosteatosis (Fig. 5D). These data indicate that the CD40-TRAF6 axis is a valuable therapeutic target in obesity, especially for ameliorating metabolic complications such as IR and hepatosteatosis.
Figure 5. CD40-TRAF6 interaction inhibitor improves metabolic dysregulation and AT inflammation. WT male mice were fed an HFD for a total of 12 wk, receiving a CD40-TRAF6 interaction inhibitor (6877002) (10 μmol/kg/day i.p.) or vehicle starting at week 6 of feeding. (A) Body weight of mice fed an HFD for 12 wk and treated with inhibitor or control. (B) ITT of mice fed an HFD for 12 wk and treated with inhibitor or control. (C) SVF cells from gonAT of control- or inhibitor-treated mice were analyzed by FACS. CD45+ leukocytes and total macrophages or M1 macrophages, characterized as CD11b+F4/80+ and F4/80+CD11b+CD11c+, respectively, are shown. (D) Representative H&E-stained sections from livers of control- or inhibitor-treated mice. *P < 0.05 for comparison with control-treated mice; n = 7-8 mice per group.
Discussion

The costimulatory CD40-CD40L dyad is a powerful mediator of inflammation and immunity (21). We previously reported that CD40L deficiency ameliorated AT inflammation and metabolic dysregulation in DIO, particularly by reducing the CD8+ T-cell fraction and increasing the Treg content in obese AT (12). Wolf et al. reported a similar observation; CD40L−/− mice displayed reduced AT inflammation (13). Despite the well-established proinflammatory role of CD40, the receptor for CD40L, in different disorders, such as atherosclerosis, Crohn disease, and multiple sclerosis (22), we unequivocally demonstrate here the unexpected finding that genetic loss of CD40 does not mirror the effects of CD40L deficiency in DIO. On the contrary, CD40 deficiency aggravated obesity-related AT inflammation and caused metabolic dysregulation.

The increase in classically activated (M1) macrophages in the AT of CD40−/− mice, accompanied by the increased CD8+ T-cell fraction, is likely the driving force underlying the exacerbated AT inflammation in CD40−/− mice. Classically activated M1 macrophages are abundantly present in obese AT and secrete a plethora of proinflammatory mediators (3, 6), thereby eliciting IR. CD8+ T-cell accumulation within the AT is associated with AT inflammation and activation of AT macrophages (4). Loss of CD8+ T-cells was also shown to diminish IR whereas adoptive transfer of CD8+ T-cells aggravated metabolic dysfunction (4). Thus, the aggravation of metabolic complications in DIO in CD40−/− mice could be attributed to the proinflammatory AT phenotype of these mice.

The likely explanation of why the phenotype of the CD40L−/− mouse does not mirror that of the CD40−/− mouse in DIO could be the differential involvement of the CD40-TRAF-signalling intermediates in AT inflammation and metabolic dysfunction associated with obesity. CD40 precisely modulates cellular inflammation via distinct signaling pathways, which can be initiated through binding to the different TRAF molecules (22). As demonstrated here, CD40-TRAF2/3/5 and CD40-TRAF6 signaling have opposite roles in obesity-associated metabolic dysregulation. Whereas loss of CD40-TRAF2/3/5 signaling resembled the phenotype of CD40 deficiency in DIO, deficiency of CD40-TRAF6 signaling ameliorated IR, hepatosteatosis, and inflammation of AT related to obesity. In other words, deficiency of CD40-TRAF6 signaling resembled the phenotype of CD40L deficiency. These versatile actions of the CD40-CD40L axis in DIO and IR development suggested that blocking the CD40-TRAF6 pathway specifically, rather than the CD40-CD40L interaction, could represent a promising therapy in metabolic dysfunction associated with DIO. To this end, we treated DIO mice with a compound designed to block CD40-TRAF6 signaling, and we could thereby partially reverse the IR and hepatosteatosis induced by DIO, which were accompanied by reduced numbers of M1-like inflammatory macrophages in the obese AT. M1 macrophages are crucial for development of IR and hepatosteatosis (2, 23, 24). Therefore, by promoting polarization of macrophages to the M1-like inflammatory phenotype (19), CD40-TRAF6 signaling contributes to development of IR and hepatosteatosis.

Previously, we demonstrated that specific deficiency in CD40-TRAF6 signaling, but not CD40-TRAF2/3/5 signaling, in MHCII+ cells prevented neointima formation (25), as well as atherosclerosis, and led to an anti-inflammatory immune profile (19). In atherosclerosis, inactivation of CD40-TRAF6 interactions reduced numbers of circulating Ly6Chigh monocytes...
and prevented monocytes from entering the arterial wall. Concurrently, deficiency of CD40-TRAF2/3/5 interactions in atherosclerosis resulted in an increase in CD4+ effector cells, which was compensated by an increase in Treg cells, thereby leaving plaque burden unaffected (19).

The intriguing discrepancy between the opposite phenotypes observed in mice with CD40 and CD40L deficiency might also be explained by the fact that CD40L can engage functionally different receptors than CD40: for example, Mac-1 integrin. CD40 deletion in an atherosclerotic mouse model did not result in smaller lesions whereas binding of CD40L to Mac-1-integrin induced Mac-1-dependent adhesion and migration of leukocytes (26, 27). However, Mac-1−/− mice displayed an obesity phenotype (28). Thus, CD40L-Mac-1 interactions are an unlikely explanation for the discrepancy between the phenotypes of CD40 and CD40L deficiency. Recently Guo et al. (29) also reported that CD40−/− mice have increased inflammation of AT but did not describe the underlying signaling mechanisms involved.

In conclusion, CD40-TRAF pathways in MHCII+ cells potently regulate obesity-associated inflammation and metabolic dysfunction in mice. The differential regulation of metabolism by CD40-TRAF pathways opens possibilities for potential therapeutic strategies to combat obesity. Currently, agonistic CD40 antibodies are being evaluated in cancer patients and could reduce the tumor load in pancreatic cancer (30). However, continuous activation of the entire CD40 pathway, as would be required for the chronic inflammatory nature of obesity, is therapeutically not feasible because long-term immune activation may result in substantial side effects. Therefore, targeting only parts of the CD40-signaling pathway, while leaving the rest of CD40-mediated immune actions intact, may be preferable. In the present paper, we have provided evidence that specific targeting of the CD40-TRAF6 pathway represents a promising therapeutic mechanism in obesity-associated metabolic dysregulation as a small CD40-TRAF6 inhibitory compound counteracted the metabolic and inflammatory complications of DIO, such as IR and hepatosteatosis. The approach based on the small compound is promising because only one of the CD40-TRAF pathways is blocked (i.e., CD40-TRAF6) whereas the other pathway (i.e., CD40-TRAF2/3/5) remains functional. The immune system therefore is less compromised, and treatment is less likely to cause severe immune-suppressive side effects. However, the effects of such selective targeting strategies will have to be meticulously scrutinized before being translated into a clinical setting.
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References


Supplemental figures

Fig. S1. T cells in spleens of WT and CD40−/− mice fed a standard-fat diet (SFD) for 30 wk. CD4 and CD8 T cells are expressed as percentage of splenic cells whereas CD4+CD25+Foxp3+ regulatory T cells (Tregs) are expressed as percentage of CD4-positive cells in the spleen. Data are mean ± SEM, *P < 0.05 (n = 5 per group).

Fig. S2. Brown adipose tissue (BAT) activity is increased in CD40−T6−/− mice. Uncoupling protein (UCP)-1 mRNA expression in the BAT of CD40-Twt and CD40-T6−/− mice fed an SFD or a high-fat diet (HFD). Values are mean ± SEM. *P < 0.05 for comparison with CD40-Twt mice (n = 6 per group).
Fig. S3. CD40-TRAF2/3/5 deficiency aggravates diet-induced obesity (DIO)-associated hepatosteatosis. (A) Hepatic steatosis was graded from 0 to 3 (severe). Steatosis was classified as follows: minimal steatosis, steatosis in the central vein area, centro-medial, or central vein-portal. The percentage of mice exhibiting hepatocyte injury, characterized by ballooning, and of mice exhibiting lobular inflammation was also determined. (B) Hepatic mRNA levels of genes involved in glycolysis, lipolysis, and lipogenesis (values represent mRNA expression of the gene of interest/mRNA expression of housekeeping genes). Values are mean ±SEM. *P < 0.05 for comparison with CD40-Twt mice (n = 8–12 mice per group).
Fig. S4. CD40-TRAF2/3/5 deficiency results in increased inflammatory gene expression in gonadal AT in DIO. (A) mRNA levels of macrophage markers and genes involved in macrophage activation (CD68, TNF, IL1α). (B) T lymphocyte markers and activators (CD3, IL2). (C) Chemokines and chemokine receptors (CCR7, CCL3, CCL5, and CXCR3). (D) Genes down-regulated in CD40 T6−/− mice (E-selectin, CD28, and CD86). Values are mRNA expression of the gene of interest/mRNA expression of housekeeping genes. *P < 0.05, for comparison with CD40-Twt mice (n = 6 mice per group).