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

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The immune system plays an instrumental role in obesity and insulin resistance. Here, we unravel the role of the costimulatory molecule CD40 and its signaling 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, compared with wild-type mice. This worsening 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 compound 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 compound may provide a therapeutic option in obesity-associated insulin resistance.

metabolism | type 2 diabetes | immunity

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

Significance

Inflammation is a critical contributor to the pathogenesis of metabolic disorders associated with obesity. A group of molecules crucial in regulating the immune system are costimulatory molecules, including CD40. Our current study shows that CD40 acts as a double-edged sword in the metabolic syndrome through the initiation of differential signaling cascades. The CD40-TNF receptor-associated factor (TRAF) 2/3/5 signaling pathway protects against metabolic dysfunction and inflammation associated with obesity; conversely, the CD40-TRAF6 pathway contributes to the detrimental consequences of obesity. In the present study, we therefore designed, validated, and used a small-molecule inhibitor that blocks CD40-TRAF6 interactions. The improvement of insulin resistance by this specific CD40-TRAF6 inhibitor could represent a therapeutic breakthrough in the field of immunometabolism.

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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.

Results

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. 1*A*) but did lead to worsened IR after 30 wk of HFD (Fig. 1*B*). Although the weights of s.c. AT (sqAT) and gonadal AT (gonAT) were similar or decreased, liver weights increased slightly in CD40 deficiency (Fig. 1*C*). *CD40*$^{-/-}$ mice exhibited significant liver abnormalities related to obesity with pronounced hepatosteatosis, compared with CD40-sufficient mice (Fig. 1*D*). 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. 1*E*). On a standard-fat diet (SFD), *CD40*$^{-/-}$ mice did not develop any metabolic abnormalities (Fig. 1).

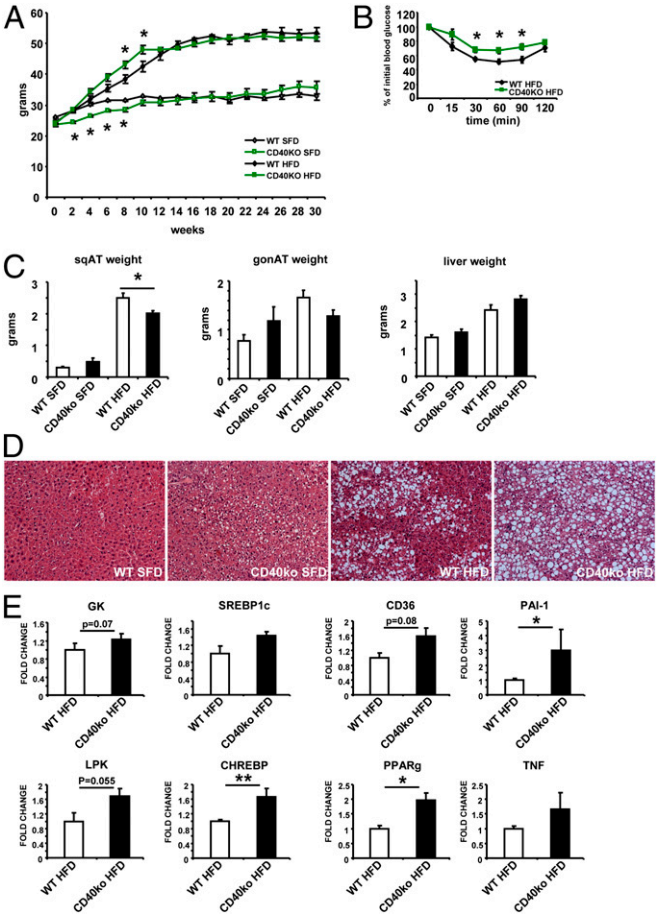

Fig. 1. *CD40*$^{-/-}$ mice on HFD display aggravated metabolic dysregulation. Wild-type (WT) and *CD40*$^{-/-}$ male mice were fed with SFD or HFD for 30 wk. (*A*) Body weight of WT and *CD40*$^{-/-}$ mice on SFD ($n = 5$) or HFD ($n = 6$–7). (*B*) Insulin tolerance test (ITT) in 5-h fasted WT and *CD40*$^{-/-}$ 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.

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. 2*A*) and CD8$^+$ T cells (Fig. 2*A*). In addition, a significant increase in the number of CD11b$^+$F4/80$^+$ macrophages was observed (Fig. 2*A*). 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. 2*A*).

Quantitative PCR analysis revealed increased expression of *IL6*, *IL12*, *TNF*, *MCP1*, *ICAM1*, and the macrophage and T cell-specific markers *CD68*, *CD3*, and *CD8* in the gonAT of *CD40*$^{-/-}$ mice (Fig. 2*B*). *Adiponectin*, *leptin*, *GLUT4*, and *PPARγ* did not differ on HFD (Fig. 2*B*). 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.

Deficiency of CD40-TRAF2/3/5 Signaling, but Not CD40-TRAF6 Signaling, Exacerbates Diet-Induced Obesity. 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. 3*A*). *CD40-T6*$^{-/-}$ mice experienced a milder weight gain (Fig. 3*A*) and a delay in

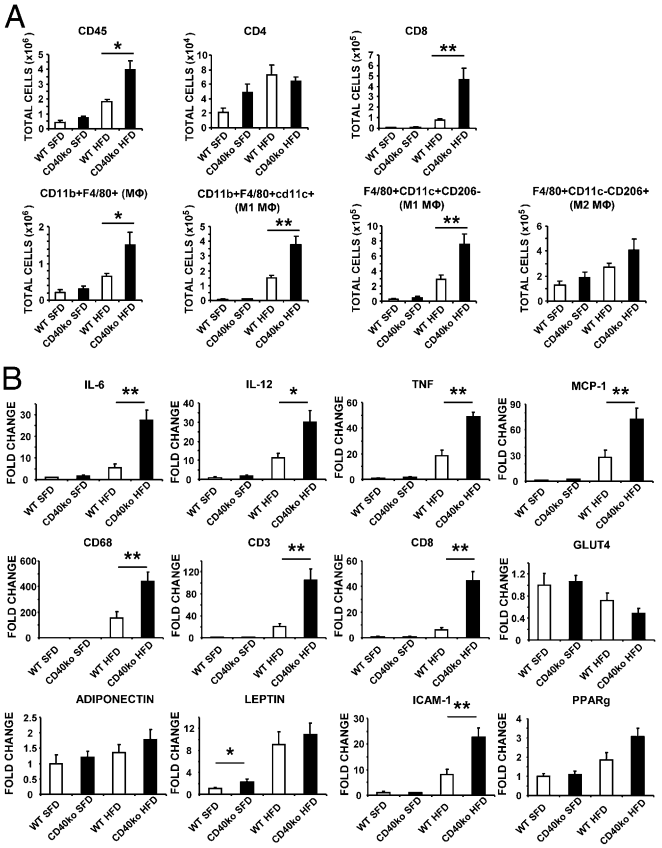

Fig. 2. CD40 deficiency aggravates AT inflammation. (*A*) SVF cells were isolated from gonAT of WT or *CD40*$^{-/-}$ male mice fed an SFD or HFD for 18 wk. CD45$^+$ leukocytes, CD4$^+$ or CD8$^+$ lymphocytes, total macrophages (characterized as CD11b$^+$F4/80$^+$), and M1 macrophages (defined as F4/80$^+$CD11b$^+$CD11c$^+$ 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 wk. 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 per group for SFD groups and $n = 7$ or more per group for HFD groups. *$P < 0.05$ for comparison between WT and *CD40*$^{-/-}$ mice fed the same diet.

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 ^{1}HMRI 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. 3*B*).

CD40-T2/3/5$^{-/-}$ mice exhibited IR after 20 wk of HFD (Fig. 3*C*) 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. 3*C*). 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*$^{-/-}$, 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. S3*A*). 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. S3*A*). 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. S3*A*). 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. S3*A*). 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. S3*A*).

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. S3*B*). Furthermore, we detected higher mRNA levels of the fatty acid transporter *CD36*, which stimulates glycolysis and lipogenesis (Fig. S3*B*). 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 F4/80^{high} CD11b$^+$ macrophage fraction in mice lacking CD40-TRAF2/3/5 signaling, compared with *CD40-Twt* mice (Fig. 4*A*). In keeping with these findings, *CD68* mRNA was also increased in *CD40-T2/3/5*$^{-/-}$ mice (Fig. S4*A*). Analysis of cytokines revealed elevated levels of *TNF* and *IL1α* (Fig. S4*A*), suggesting an M1 macrophage-biased response.

The percentage and number of CD3$^+$ T cells were slightly elevated in *CD40-T2/3/5*$^{-/-}$ mice ($2.1 \pm 0.11 \times 10^5$ CD3$^+$ cells; $P = 0.08$) compared with *CD40-Twt* mice ($1.92 \pm 0.31 \times 10^5$ CD3$^+$ cells) whereas the percentage and numbers of CD3$^+$ T cells in *CD40-T6*$^{-/-}$ mice ($1.89 \pm 0.48 \times 10^5$ CD3$^+$ cells) equaled 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. 4*B*). Accordingly, mRNA levels of CD3 as well as IL2 were elevated in the gonAT of these mice (Fig. S4*B*). In *CD40-T2/3/5*$^{-/-}$ mice, the fraction and number of Treg cells (CD40-Twt, $4.2 \pm 0.6 \times 10^4$ vs. CD40-T2/3/5$^{-/-}$, $1.7 \pm 0.2 \times 10^4$ Tregs; $P < 0.05$) in the gonAT were decreased (Fig. 4*C*). In the spleen, *CD40-T6*$^{-/-}$ mice had an increased Treg fraction ($6.8 \pm 0.3\%$ in *CD40-Twt* vs. $9.4 \pm 0.2\%$ 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 CD44^{high}CD62L^{low} effector cells, with a concomitant decrease in CD44^{low}CD62L^{high} naïve T cells (Fig. 4*D*). 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.

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

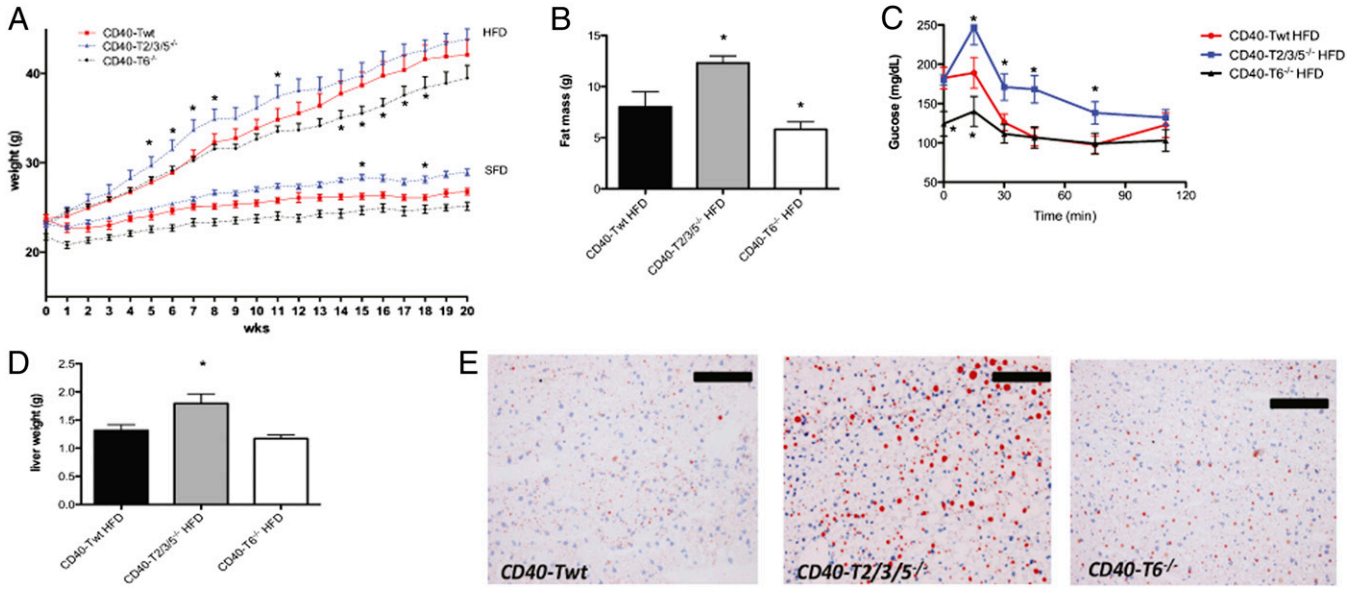

Fig. 3. Deficiency of CD40-TRAF2/3/5 signaling aggravates obesity and promotes metabolic dysfunction and hepatosteatosis. (*A*) Body weight of *CD40-Twt*, *CD40-T2/3/5*$^{-/-}$, and *CD40-T6*$^{-/-}$ mice on SFD or HFD for 20 wk (n = 12–15). (*B*) Fat mass as determined by ^{1}H NMR spectroscopy (n = 8 per group). (*C*) ITT in 5-h fasted *CD40-Twt*, *CD40-T2/3/5*$^{-/-}$, and *CD40-T6*$^{-/-}$ mice fed an HFD for 18 wk (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 an HFD for 20 wk. (Scale bar: 100 μm.) Values are mean ± SEM. *P < 0.05 for comparison with *CD40-Twt* mice.

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 *IL1α* 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. S4*D*).

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.

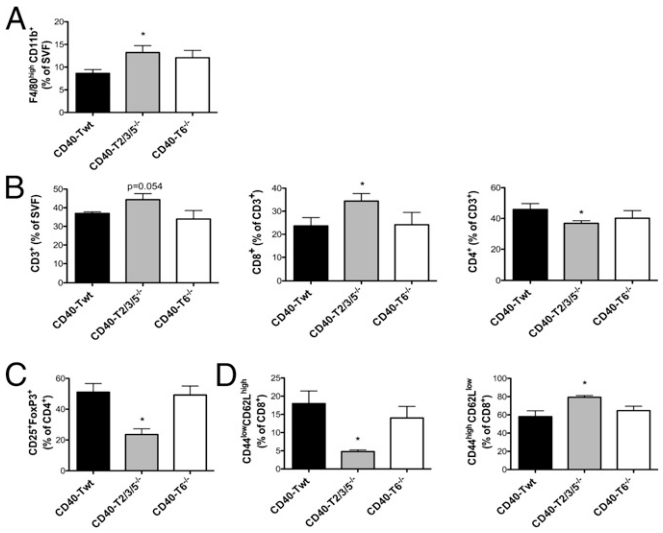

Fig. 4. CD40-TRAF2/3/5 deficiency results in increased numbers of inflammatory cells in gonadal AT in DIO. (*A*) FACS analysis for F4/80^{high}CD11b^{+} macrophages, (*B*) CD3^{+} T cells, CD8^{+} T cells, and CD4^{+} T cells, (*C*) CD4^{+}CD25^{+}FoxP3^{+} Tregs, and (*D*) CD8^{+}CD44^{low}CD62L^{high} naïve T cells and CD8^{+}CD44^{high}CD62L^{low} effector T cells. Values are mean ± SEM. *P < 0.05 for comparison with *CD40-Twt* mice. n = 8–10 mice per group.

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.

To identify drug-like molecules that can inhibit the CD40-TRAF6 interaction, we used an approach for virtual ligand screening (VLS) based on *in silico* structures. A detailed description of the results and the validation of the compound can be found in *SI Text* and Figs. S5–S7.

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. 5*C*). Interestingly, treatment with the CD40-TRAF6 inhibitor also reduced hepatosteatosis (Fig. 5*D*). 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.

Discussion

The costimulatory CD40-CD40L dyad is a powerful mediator of inflammation and immunity (17). We previously reported that

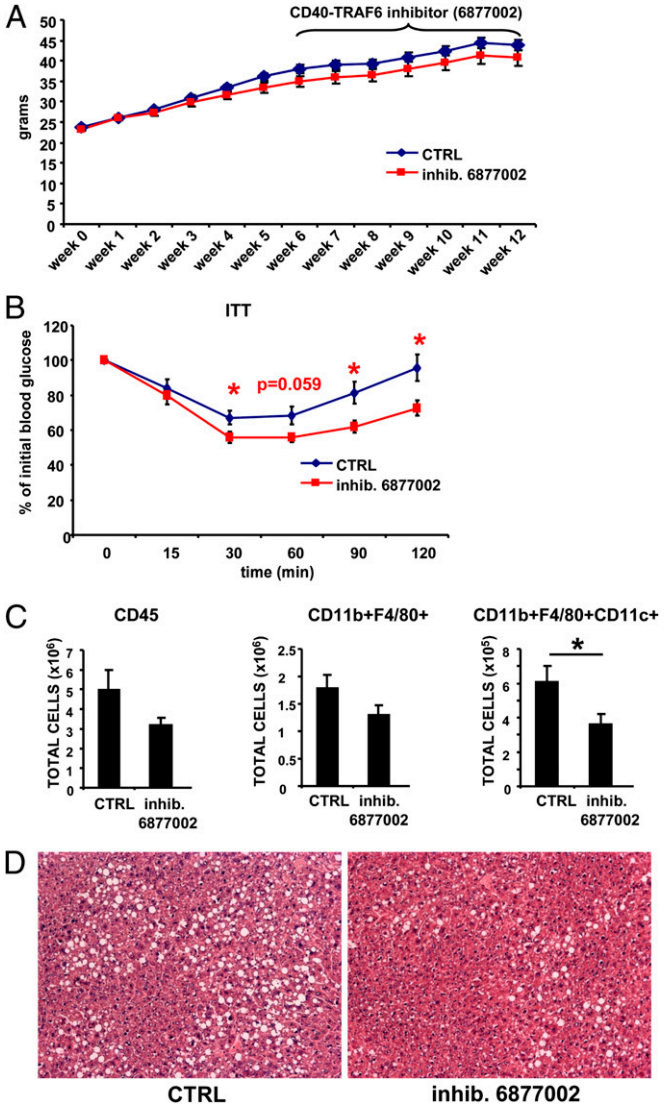

Fig. 5. CD40-TRAF6 interaction inhibitor improves metabolic dysregulation and AT inflammation. WT male mice were fed an HFD for a total of 12 wk, receiving a CD40-TRAF6 interaction inhibitor (6877002) (10 μmol·kg^{-1}·d^{-1} i.p.) or vehicle starting at week 6 of feeding. (*A*) Body weight of mice fed 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.

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 (18), 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–signaling 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 (18). 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, 19, 20). Therefore, by promoting polarization of macrophages to the M1-like inflammatory phenotype (21), 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, (22) as well as atherosclerosis, and led to an anti-inflammatory immune profile (21). In atherosclerosis, inactivation of CD40-TRAF6 interactions reduced numbers of circulating Ly6C^{high} 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 (21).

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 (23, 24). However, Mac-1$^{-/-}$ mice displayed an obesity phenotype (25). Thus, CD40L–Mac-1 interactions are an unlikely explanation for the discrepancy between the phenotypes of CD40 and CD40L deficiency. Recently Guo et al. (26) 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 (27). 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.

Methods

Full details regarding methods are included in *SI Methods*. More specifically, virtual ligand screening (VLS), validation of the compound, TRAF6 C-domain expression, purification and binding analyses, in vitro screening, and in vitro macrophage culture are found in *SI Methods*. Moreover, methods pertinent to the animal experiments–including biochemical measurements, insulin tolerance testing, body composition analysis, indirect calorimetry/metabolic cage analysis, flow cytometric analysis, real-time PCR, and histochemistry–are also found in *SI Methods*.

CD40$^{+/+}$and *CD40*$^{-/-}$ mice (C57BL/6 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 or Research Diets); HFD, 35% kcal carbohydrate, 45% kcal fat, 20% kcal protein (SDS Special Diets Services) or 20% kcal carbohydrate, 60% kcal fat, 20% kcal protein (Research Diets)] for different time points up to 30 wk (CD40$^{+/+}$ and *CD40*$^{-/-}$ mice, males; 60% kcal-HFD) or 20 wk (*CD40-TRAF* mice, males; 45% kcal-HFD), starting at the age of 6–8 wk. In the CD40-TRAF6 small-molecule inhibitor experiment, male C57BL/6 mice (Janvier) were fed the 60% kcal-HFD for 12 wk, receiving compound 6877002 (10 $\mu mol \cdot kg^{-1} \cdot d^{-1}$) or vehicle control for 6 wk i.p.

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 Universities of Maastricht, Amsterdam, and Leiden and the Landesdirektion Dresden.

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