Corrections

**PERSPECTIVE**

The authors note that on page 582, left column, first paragraph, line 3, “10 metric tons (MT)” should instead appear as “10 million metric tons (MT).”

www.pnas.org/cgi/doi/10.1073/pnas.1402460111

**BIOPHYSICS AND COMPUTATIONAL BIOLOGY**

The authors note that, due to a PNAS error, the author contributions footnote appeared incorrectly. The corrected author contributions footnote appears below.

Author contributions: H.R. and E.P. designed research; H.R. performed research; H.R. and S.M. performed fluorescence measurements; S.M.I. and B.R. contributed the computational analysis; H.R., S.M.I., S.M., B.R., and E.P. analyzed data; and H.R. and E.P. wrote the paper.

www.pnas.org/cgi/doi/10.1073/pnas.1402205111

**MEDICAL SCIENCES**

The authors note that the author name Klaus Schulte should instead appear as Klaus-Martin Schulte. The corrected author line appears below. The online version has been corrected.


www.pnas.org/cgi/doi/10.1073/pnas.1403231111

The authors note the following: “Recent high throughput sequencing has indicated that an upstream region of the dio3 promoter sequence in our paper was the result of a PCR fusion error. The reverse primer located –140bp upstream of the start codon was not specific to Siberian hamsters. As a result, the transcription factor binding site analysis and sodium bisulfite-treated DNA sequence analyses in the original publication were incorrect. To correct this error, we have resequenced the dio3 proximal promoter and conducted replications of the transcription factor binding site analyses and sequencing of sodium bisulfite-treated DNA, using primer sequences with confirmed specificity to Siberian hamster DNA. The corrected dio3 promoter sequence exhibited greater homology with mice and human dio3 promoter (revised Fig. S1), a greater number of CpG sites and a higher CpG frequency (revised Fig. S2) than previously reported. Analysis of sodium bisulfite-treated DNA in the acute LD-SD (Fig. 1F) and photorefractory experiments (Fig. 3E) yielded results consistent with the originally-published report: dio3 promoter DNA methylation was reduced in SD (revised Fig. 1F) and increased in SD-R (revised Fig. 3E). Revised transcription factor binding site analyses have also been performed (revised Table S1). See corrected Table S2 for primers used on sodium bisulfite treated DNA. In addition, the reverse primers for the sequencing and MSRE PCR reactions (Table S2) were originally listed in the incorrect (3′→5′) orientation; the correct orientation (5′→3′) now appears in the revised version of Table S2.

“We thank Drs. Hugues Dardente and David Hazlerigg for their assistance in identifying these errors.”

As a result of this error, Figs. 1 and 3 and their legends appeared incorrectly. The figures and their corrected legends appear below. These errors do not affect the main conclusions of the article.

Fig. 1. Short photoperiods inhibit reproduction and activate hypothalamic mRNA expression via epigenetic mechanisms. Acute transfer from LD to SD photoperiods caused gonadal regression (A), increased hypothalamic dio3 mRNA expression (B), and decreased hypothalamic dnmt1 and dnmt3b mRNA expression (C) after 8 wk. (D) Immunocytochemical localization of DNMT3b (DNMT3b-ir) in the hamster medio basal hypothalamus (MBH). DNMT3b-ir was evident throughout the MBH and in the ependymal cell (EC) layer along the third ventricle (III). (E) Transfer from LD to SD reduced DNA methylation in the dio3 proximal promoter, as measured using an MSRE assay. (F) Proportion of LD and SD hamsters in which no unmethylated DNA was detected at each of 17 CpG sites in the dio3 proximal promoter, as assessed by direct sequencing of sodium bisulfite-treated DNA. The abscissa (not to scale) depicts the 17 CpG sites from –249 to the start codon of the dio3 proximal promoter. Averaging across the entire promoter region that was sequenced, evidence of unmethylation was evident on 15% of CpG sites in LD (i.e., on 85% of CpG sites examined in LD hamsters, no detectable C-to-T bisulfite conversion occurred), whereas in SD, evidence of unmethylated DNA was present on 42% of CpG sites ($\chi^2 = 18.4, P < 0.002$). All data in panels A–E are mean ± SEM. *P < 0.05, **P < 0.005 vs. LD value.
Fig. 3. Neuroendocrine refractoriness to SD reverses patterns of DNA methylation induced by acute SD exposure. (A) Acute (10 wk, SD) exposure to SD induced gonadal regression, whereas prolonged exposure (42 wk, SD-R) triggered neuroendocrine refractoriness and gonadal recrudescence. Refractoriness in SD-R hamsters was characterized by a complete reversal of hypothalamic dio3 and dnmt3b mRNA expression (B and C) and by remethylation of DNA in the dio3 proximal promoter (D). (E) Proportion of LD, SD, and SD-R hamsters in which no unmethylated DNA was detected in each of 17 CpG sites in the dio3 proximal promoter, as assessed by direct sequencing of sodium bisulfite DNA from the whole hypothalamus. The abscissa (not to scale) depicts the 17 CpG sites from −249 to the start codon of the dio3 proximal promoter. Averaging across the promoter region that was sequenced, evidence of unmethylation was evident on 25% of CpG sites in LD hamsters, whereas in SD hamsters, evidence of unmethylation was present on 39% of CpG sites (χ² = 5.08, P < 0.03). In SD-R hamsters, methylation patterns returned to LD-like values, and evidence of unmethylation was detected on 29% of CpG sites examined (χ² = 0.46, P > 0.40 vs. LD). Five of 17 sites (sites 16, 13, 12, 2, and 1) exhibited reversals in the pattern of methylation in SD-R hamsters. All data in panels A–D are mean ± SEM. *P < 0.05; ***P < 0.005 vs. LD value.
Blocking CD40-TRAF6 signaling is a therapeutic target in obesity-associated insulin resistance

Antonios Chatzigeorgiou\textsuperscript{a,b,c,1}, Tom Seijkens\textsuperscript{d,1}, Barbara Zarzycka\textsuperscript{e,1}, David Engelf\textsuperscript{f,1}, Marjorie Poggii\textsuperscript{g,1}, Susan van den Berg\textsuperscript{h}, Sjoerd van den Berg\textsuperscript{h}, Oliver Soehlein\textsuperscript{i}, Holger Winkels\textsuperscript{i}, Linda Beckers\textsuperscript{d}, Dirk Lievens\textsuperscript{d,1} Ann Driessen\textsuperscript{p}, Pascal Kusters\textsuperscript{l}, Erik Biessen\textsuperscript{p}, Ruben Garcia-Martin\textsuperscript{n}, Anne Klotzsche-von Ameln\textsuperscript{o}, Marion Gijbels\textsuperscript{b,1}, Randolph Noelle\textsuperscript{m,n}, Louis Boon\textsuperscript{p}, Tilman Hackeng\textsuperscript{p}, Klaus-Martin Schulte\textsuperscript{p}, Aimin Xu\textsuperscript{l}, Gert Vriend\textsuperscript{d}, Sander Nabuurs\textsuperscript{r,s}, Kyoung-Jin Chung\textsuperscript{p}, Kuo Willems van den Dijk\textsuperscript{h,k}, Patrick C. N. Rensen\textsuperscript{e}, Norbert Gerdes\textsuperscript{d,1}, Menno de Winther\textsuperscript{d}, Norman L. Block\textsuperscript{v,w}, Andrew V. Schally\textsuperscript{v,w,2}, Christian Weber\textsuperscript{d,l,x}, Stefan R. Bornstein\textsuperscript{b,y}, Gerry Nicolaes\textsuperscript{o,3}, Triantafyllos Chavakis\textsuperscript{b,c,e,2,3}, and Esther Lutgens\textsuperscript{d,2,3}

\textsuperscript{1}Department of Clinical Pathobiology, Technische Universität Dresden, 01307 Dresden, Germany; \textsuperscript{2}Department of Medicine, Technische Universität Dresden, 01307 Dresden, Germany; \textsuperscript{3}Paul-Langerhans Institute Dresden, German Center for Diabetes Research, 01307 Dresden, Germany; \textsuperscript{4}Department of Medical Biochemistry, Subdivision of Experimental Vascular Biology, Academic Medical Center, University of Amsterdam, 1105 AZ, Amsterdam, The Netherlands; \textsuperscript{5}Department of Biochemistry, University of Maastricht, 6229 ER, Maastricht, The Netherlands; \textsuperscript{6}Department of Pathology, University of Maastricht, 6229 ER, Maastricht, The Netherlands; \textsuperscript{7}Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 1062, and Faculté de Médecine, Aix-Marseille Université, F-13385 Marseille, France; \textsuperscript{8}Department of Human Genetics, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands; \textsuperscript{9}Department of Pathology, Academic Medical Center, University of Amsterdam, 1105 AZ, Amsterdam, The Netherlands; \textsuperscript{10}Institute for Cardiovascular Prevention, Ludwig Maximilians University, 80336 Munich, Germany; \textsuperscript{11}Department of Pathology, University of Antwerp, 2650 Antwerp, Belgium; \textsuperscript{12}Cardiovascular Research Institute Maastricht, Maastricht University, 6229 ER, Maastricht, The Netherlands; \textsuperscript{13}Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Lebanon, NH 03766; \textsuperscript{14}Medical Research Council Centre of Transplantation, Guy’s Hospital, King’s College London, London SE1 9RT, United Kingdom; \textsuperscript{15}Bioceros BV, 3884 CM, Utrecht, The Netherlands; \textsuperscript{16}Department of Endocrine Surgery, King’s College Hospital, Denmark Hill, London SE5 9RS, United Kingdom; \textsuperscript{17}Department of Medicine, University of Hong Kong, Hong Kong, China; \textsuperscript{18}Centre for Molecular and Biomolecular Informatics, Radboud University Medical Center, 6295 EN, Nijmegen, The Netherlands; \textsuperscript{19}Lead Pharma Medicine, 6525 EN, Nijmegen, The Netherlands; \textsuperscript{20}Einhoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands; \textsuperscript{21}Department of Endocrinology, Leiden University Medical Center, 2333 ZA, Leiden, The Netherlands; \textsuperscript{22}Division of Endocrine and Metabolismology, Departments of Pathology and Medicine, University of Miami Miller School of Medicine, Miami, FL 33136; \textsuperscript{23}Veterans Affairs Medical Center, Miami, FL 33125; \textsuperscript{24}German Centre for Cardiovascular Research, Munich, 80336, Germany; \textsuperscript{25}Diabetes and Nutritional Sciences Division, King’s College London, Denmark Hill, London SE5 9NU, United Kingdom; and \textsuperscript{26}Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, 01307 Dresden, Germany

Contributed by Andrew V. Schally, January 9, 2014 (sent for review December 13, 2013)

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\textsuperscript{−/−} mice in DIO displayed worsened insulin resistance, compared with wild-type mice. This worsening was associated with increased inflammation of adipose tissue (AT), characterized by increased accumulation of CD8\textsuperscript{T} T cells and M1 macrophages, and enhanced hepatosteatosis. Mice with deficient CD40-TRAF2/3/5 signaling in MHCII\textsuperscript{+} cells exhibited a similar phenotype in DIO as CD40\textsuperscript{−/−} mice. In contrast, mice with deficient CD40-TRAF6 signaling in MHCII\textsuperscript{+} 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\textsuperscript{+} cells protects against AT inflammation and metabolic complications associated with obesity whereas CD40-TRAF6 interactions in MHCII\textsuperscript{+} cells aggravate these complications. Inhibition of CD40-TRAF6 signaling by our compound may provide a therapeutic option in obesity-associated insulin resistance.

**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.

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 metabolism | type 2 diabetes | immunity


1A.C., T.S., B.Z., D.E., and M.P. contributed equally to this work.
2To whom correspondence may be addressed. E-mail: andrew.schally@va.gov, Triantafyllos.Chavakis@uniklinikum-dresden.de, or e.lutgens@amc.uva.nl.
3G.N., T.C., and E.L. contributed equally to this work.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400419111/-/DCSupplemental.
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 (15). These findings 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 (−/−) in 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 (sqAT) 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 glucose and lipid uptake (GK, LKP, and CD36), showed increased 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+ (SVF) of the gonAT of the HFD group revealed that CD40-deficient mice had increased numbers of CD45+ macrophages was observed (Fig. 2A). Flow cytometry characterized by expression of CD11c and absence of CD206, was inflammatory 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 IL6, IL12, 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 were mutated (Fig. 2C). In addition, a significant increase in the number of CD11b+F4/80+ macrophages was observed (Fig. 2D). 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. 2E).

Deficiency of CD40-TRAF2/3 Signaling, but Not CD40-TRAF6 Signaling, Exacerbates Diet-Induced Obesity. When fed an HFD for 20 wk, male CD40−/− 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-Twt−/− 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-T2/3/5−/− mice (Fig. S2). Body composition was studied using 1H-MRI 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−/−, but not CD40-T6−/− mice, had an increase in liver weight, associated with pronounced steatosis, after 20 wk of HFD (Fig. 3D 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). In addition, accumulation 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 IL1α (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 vs. 103 CD3+ cells; P = 0.08) compared with CD40-Twt mice (1.92 ± 0.31 × 105 CD3+ cells) whereas the percentage and numbers of CD3+ T cells in CD40-T6−/− mice (1.89 ± 0.48 × 105 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. 4B). Accordingly, mRNA levels of CD3 as well as IL2 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 × 106 vs. CD40-T2/3/5−/−, 1.7 ± 0.2 × 106 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 CD44hiCD26low effector cells, with a concomitant decrease in CD44hiCD26hi 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.
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. 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.

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. 5A 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.

Discussion

The costimulatory CD40-CD40L dyad is a powerful mediator of inflammation and immunity (17). 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 (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 dysregulation 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 and CD40-TRAF6 signaling have opposite roles in obesity-associated metabolic dysregulation. Whereas loss of CD40-TRAF2/3 signaling resembled the phenotype of CD40 deficiency in DIO, deficiency of CD40-TRAF6 signaling ameliorated IR and hepatosteatosis, and inflammation of AT related to obesity. In other words, deficiency of CD40-TRAF2/3 signaling resembling the phenotype of CD40L deficiency in 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 M2-like anti-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 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+ monocytes and prevented monocytes from entering the arterial wall. Concurrently, deficiency of CD40-TRAF2/3 signaling 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, concurrently, deficiency of CD40-TRAF2/3 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).
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 hepatoestesia. 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 tolerance testing, body composition analysis, indirect calorimetry/metabolic analysis, including biochemical measurements, insulin tolerance testing, body composition analysis, indirect calorimetry/metabolic cage analysis, flow cytometric analysis, real-time PCR, and histochomistry—are also found in SI Methods.

CD40 and CD40L mice (C57BL/6 background) (15), as well as CD40-Twt, CD40-T2/3/5/6−, and CD40-76− mice (16), were fed SFO or HFD diets (SFO, 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 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 μmol·kg−1·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.

ACKNOWLEDGMENTS. We acknowledge support from the Netherlands Cardiovascular Research Initiative (the Dutch Heart Foundation, the Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development, and the Royal Netherlands Academy of Sciences) for the project “Generating the best evidence-based pharmaceutical targets for atherosclerosis” (CVON2011-19). This work was supported by the Humboldt Foundation [Soﬁa Kovaleskaja grant (to E.L.), the Netherlands Organization for Scientiﬁc Research (vici and vidi grants) (to E.L.), a vidi grant (to O.S.), a vidi grant (to C.W.), a meed in investment grant (to G.N.)], the Netherlands Heart Foundation [Dr. E. Dekker grant (to T.S.) and an established investigator grant (to E.L., M.D.W., and P.C.N.R.)], the Rembrandt foundation (Susan van den Berg, M.D.W., E.L., and P.C.N.R.), the German-Australian Institute for Translational Medicine (S.R.B.), the Deutsche Forschungsgemeinschaft [DFG FOR809: LU1643/1-2, 808/6-3, WE1913/11-2, SFB1194-808, and SFB 1054-804 and -808 (to E.L., N.G., O.S., and C.W.), CH2795/1-2 (to T.C.)], the European Research Council Grants 281296 (to T.C.) and ERC Adg 249929 (to C.W.), and by a grant from the German Federal Ministry of Education and Research to the German Center for Diabetes Research (to T.C.) and the German Center for Cardiovascular Research, Munich Heart Alliance VD1 (to C.W.), the Cardiovascular Research Institute Maastricht (to G.N.), the European Union [Grant KBBE-2011-5 289530 (to G.V.), the Transnational University Limburg (to G.N.), and Cyttorl II [FP7/08 (to G.N. and T.H.)]. Further support was received from the Medical Research Service of the Veterans Affairs Department, the Miller Medical School, University of Miami, Departments of Pathology and Medicine, Divisions of Hematology/Oncology and Endocrinology, and the South Florida Veterans Affairs Foundation for Research and Education (all to A.V.S.), and by the L. Austin Weeks Endowment for Urologic Research (to N.L.B.).